

Biomolecular mechanisms of staphylococcal biofilm formation

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1	Biomolecular Pathogenesis of Staphylococcal Biofilm Formation
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3	Garry Laverty*, Sean P. Gorman, Brendan F. Gilmore
4	Biomaterials Research Group, School of Pharmacy, Queens University of Belfast,
5	Medical Biology Centre, 97 Lisburn Road, Belfast, BT9 7BL, UK
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8	
9	
10	
11	
12	
13	*Author for Correspondence
14	Dr Garry Laverty,
15	School of Pharmacy
16	Queen's University of Belfast,
17	Medical Biology Centre,
18	97 Lisburn Road,
19	Belfast BT9 7BL, UK
20	Tel: +44 (0) 28 90 972 047
21	Fax: +44 (0) 28 90 247 794
22	Email: <u>garry.laverty@qub.ac.uk</u>
23	Keywords: Quorum sensing, adhesion, biomaterial, ica operon, adhesins
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25	

26 Abstract

27 The multitude of biomolecular and regulatory factors involved in staphylococcal 28 adhesion and biofilm formation owes much to their ability to colonise surface and 29 become the preferential bacterial phenotype. Judging on total number, biomass and 30 variety of environments colonised, bacteria can be categorised as the most successful 31 life form on earth. This is due to the ability of bacteria and other microorganisms to 32 respond phenotypically via biomolecular processes to the stresses of their surrounding 33 environment. This review focuses on the specific pathways involved in the adhesion 34 of the Gram-positive bacteria Staphylococcus epidermidis and Staphylococcus aureus 35 with reference to the role of specific cell surface adhesins, the *ica* operon, 36 accumulation associated proteins and quorum sensing systems and their significance 37 in medical device related infection. 38 39 Main text 40 Introduction 41 Microorganisms have been implicated in a variety of problems within the food, oil, 42 paper and medical industries [1]. The ability of microorganisms to attach to surfaces 43 provides an evolutionary advantage allowing maturation, increased survival and 44 symbiotic relationships to be established within the biofilm environment. 45 Upregulation of specific genes allow and the associated molecular processes enable 46 planktonic free-flowing cells to attach to surfaces, aggregate and form a hydrated 47 extracellular polymeric matrix which is phenotypically advantageous for survival [2]. 48 Gram-positive microorganisms such as Staphylococcus epidermidis and 49 Staphylococcus aureus are present on the skin of humans as part of their resident

50 microflora [3]. In healthy individual they confer a mutualistic benefit with their host

51 by preventing colonisation of the skin surface by transient pathogenic 52 microorganisms. However in circumstances were the host's immunity becomes 53 impaired, such as trauma associated with medical device implantation, resident 54 bacteria can become opportunistic attaching to the biomaterial surface and forming 55 resistant biofilms. The purpose of this review is to explore the differences and 56 similarities in the molecular processes involved in Gram-positive biofilm formation, 57 with particular relevance to staphylococci. Understanding these processes may 58 provide a means whereby the biofilm's properties of increased resistance to shear 59 stress, superior utilisation of nutrients, energy and increased antimicrobial resistance 60 may be overcome.

61

62 Processes involved in Gram-positive biofilm formation: *Staphylococcus*

63 epidermidis and Staphylococcus aureus

Staphylococcus epidermidis is the most prevalent biofilm forming coagulase negative
staphylococci [4]. Numerous research has been conducted to characterize the various
stages, genes and pathways involved in biofilm formation, the majority of these

67 factors are outlined in Figure 1.

68

69 Adhesion in staphylococci

70 Cell surface hydrophobicity and cell surface adhesins

71 The primary or nonspecific adhesion of staphylococci is due mainly to the cell and

cell surface hydrophobicity [5]. In terms of adherence to smooth, abiotic surfaces,

such as those present on many biomaterial surfaces, the galactose and glucosamine

rich capsular polysaccharide-adhesin is reported to have an important role [6].

75 Capsular polysaccharide-adhesin is composed of a high molecular weight (28 kDa)

76 polymer of β -1,6-linked *N*-acetylglucosamine residues with O-linked phosphate, 77 succinate and acetate substituents on the amino groups. These groupings confer 78 further hydrophobic character to the *Staphylococcus* bacterial capsule [7]. Another 79 role of capsular polysaccharide-adhesin in staphylococci is to offer protection against 80 the host's immune response, for example complement-mediated antibody-independent 81 opsonic killing, through the physical formation of the slimy bacterial capsule that acts 82 as a barrier to phagocytosis [8]. The glucose rich extracellular slime associated 83 antigen was discovered by Christensen *et al* [9]. Antigenically different to capsular 84 polysaccharide-adhesin, slime associated antigen is also heat and protease stable. It 85 was observed, through characterisation of capsular polysaccharide-adhesin positive 86 and slime associated antigen positive and negative strains, that capsular 87 polysaccharide-adhesin was responsible for the process of surface attachment whereas 88 slime associated antigen is linked to accumulation and biofilm maturation at the 89 surface. Research has shown slime associated antigen to be chemically identical to 90 polysaccharide intercellular adhesin [10]. Both polysaccharide intercellular adhesin and capsular polysaccharide-adhesin share a β -1,6-linked-polyglucosamine backbone, 91 92 with differences occurring in the primary substituent present on the amino groups. 93 They are both synthesized from the proteins encoded by the *ica* operon [11]. 94 95 The discovery of a Tn917 insertion mutant of *Staphylococcus epidermidis* by 96 Heilmann et al confirmed the importance of hydrophobicity, particularly in relation to 97 plastics [12]. They observed that this mutant was significantly less hydrophobic than 98 a wild type strain (O-47) and thus was unable to adhere to a polystyrene surface. 99 Another Tn917 mutant was also lacking in four important cell surface adhesins,

100 required for secondary adhesion, but the genetic restoration of one of these adhesins

101 (of molecular mass 60 kDa) fully restored adherence capabilities and showed the 102 importance of surface bound adhesins in *Staphylococcus* adhesion. The secondary 103 attachment of Staphylococcus epidermidis is improved by the presence of the cell 104 adhesion autolysin E, which binds to plasma proteins such as vitronectin present in the conditioning layer formed on implanted biomaterials [13]. The 60-kDa adhesion 105 106 analysed by Heilmann et al was shown to be a proteolytic fragment of autolysin E 107 [14]. Heilmann *et al* are also responsible for the characterisation of a novel autolysin-108 adhesin in Staphylococcus epidermidis [15]. This surface bound novel autolysin-109 adhesin was shown to be 35kDa in molecular mass and possess bacteriolytic 110 properties, with saturable dose dependent adhesion to fibronectin, fibrinogen and vitronectin also shown in vitro. Biofilm formation in Staphylococcus epidermidis is 111 112 not reliant on autolysin and autolysin-adhesin expression alone and it is still unknown 113 whether autolysin E mediates attachment directly or helps to expose alternative 114 adhesins [16].

115

116 There are several surface bound proteins in Staphylococcus epidermidis that are responsible for binding specifically to collagen, vitronectin, fibronectin and 117 118 fibrinogen and other proteins present in the extracellular matrix. Included in these 119 proteins together with autolysin and autolysin-adhesin are; the collagen binding 120 extracellular lipase GehD [17]; the large (1 MDa) fibronectin binding protein Embp 121 [18] and the fibrinogen binding proteins and SdrG [19]. Both fibrinogen binding 122 protein and SdrG are members of the same staphylococcal surface protein gene 123 family, sharing similar dipeptide serine-aspartate repeats, sortase cleavage sites, 124 hydrophobic and cationic domains [20]. The gene encoding for fibrinogen binding 125 protein (*fbe*) has been isolated in the majority of *Staphylococcus epidermidis* strains, 126 with an incidence of 95% in clinical isolates [21]. Fibrinogen binding protein is the 127 only true microbial surface components recognizing adhesive matrix molecule 128 (MSCRAMM) found in *Staphylococcus epidermidis* and although it is present in 129 Staphylococcus aureus it also has similar structural and functional properties to 130 clumping factor (ClfA) found in some strains of Staphylococcus aureus [22]. 131 Clumping factor A (ClfA) is a cell wall-associated adhesin that mediates binding to 132 fibrinogen and platelets, and although staphylococci share many adhesive properties 133 and mechanisms it has only been isolated in *Staphylococcus aureus* [23]. Similarly 134 the cell-wall protein clumping factor B (ClfB) of Staphylococcus aureus aids 135 adhesion and colonisation to squamous epithelial cells present in nasal passages [24]. 136 MSCRAMMs are more prevalent in Staphylococcus aureus, including clumping 137 factors A and B (ClfA and ClfB), collagen binding protein and fibronectin binding 138 factors A and B [25]. Binding to fibrinogen by these isolates varies however, leading 139 to the hypothesis that fibrinogen binding protein and other surface adhesins are 140 expressed to different degrees when comparing multiple isolates. Factors such as 141 protease activity, sortase cleavage of the Leu-Pro-Xaa-Thr-Gly (LPXTG) amino acid 142 sequence motif, insufficient length of Shine-Dalgarno repeat region and capsular 143 formation may determine the extent to which adhesins are exposed [26].

144

The action of sortase, namely sortase A, in staphylococci and other Gram-positives is of importance in the covalent anchoring of surface adhesins to peptidoglycan in the cell wall allowing them to be readily available for attachment [27]. MSCRAMMs such as fibrinogen binding protein are composed of three distinct regions namely; a hydrophobic portion; a charged tail and most importantly a LPXTG motif, where X represents any amino acid [28]. By cleavage of this motif between the threonine and

151	glycine residues	an acyl-enzym	ne intermediate	is formed	within the so	rtase active site,
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152 with nucleophilic attack of the amino groups present in the cell wall crosslinks

allowing binding of MSCRAMMs to peptidoglycan in the cell wall [29].

154

155 The role of teichoic acids

156 Cell wall teichoic acids are the highest source of polyanionic charge on the 157 staphylococcal bacterial cell envelope [30]. Research has also shown that increased 158 cationic charge provided by incorporation of D-alanine into teichoic acids, an 159 important component of the staphylococcal extracellular matrix, is a determinant in 160 the successful attachment of staphylococci to biomaterials [31][32][33]. The 161 production of teichoic acids is controlled by the *dlt* gene operon; it is this gene 162 sequence that is responsible for D-alanine incorporation [30]. Gross et al showed 163 gene mutants of *dlt*, namely *dltA*, that did not incorporate D-alanine were teichoic 164 acid negative and failed to adhere to glass and polystyrene [33]. They concluded that 165 despite other adherence factors being present, including the *ica* operon and 166 polysaccharide intercellular adhesin production, the electrostatic repulsive forces 167 induced by increased cell negativity of staphylococci lead to prevention of bacterial 168 adhesion to polystyrene and glass. Although these results may show some correlation 169 between cell surface charge and electrostatic forces in biofilm formation, there is no 170 conclusive evidence for the activity of *dltA* staphylococcal mutants in other polymers 171 such as Teflon. Research performed by Vergara-Irigaray and colleagues showed cell 172 wall absent teichoic acid mutants to have similar levels of poly-N-acetylglucosamine 173 production; a higher degree of cell aggregation but reduced capacity to form biofilms 174 compared with wild type [34]. Attachment with Biofilm formation itself has been 175 shown to be restored with the addition of magnesium but not calcium ions, showing

that the balance of charge at the surface of Gram-positive bacteria is important in determining adhesion and ultimately biofilm formation, with the cationic charge of magnesium ions acting as a direct replacement for that of D-alanine [31][35]. Mutant *dltA* staphylococci have also been shown to be more sensitive to vancomycin and host defence peptides [36].

181

182 Accumulation and the *ica* operon in staphylococci

183 The accumulation of cellular aggregates at the surface of the biomaterial is a key stage 184 in the adhesion of biofilm forming microorganisms in medical device related 185 infection. Approximately 85% of Staphylococcus epidermidis strains from infective 186 blood cultures have been shown to possess the *ica* gene cluster [37]. Polysaccharide 187 intercellular adhesin is localized to the cell surface and is the key component for the 188 intercellular adhesion of Staphylococcus epidermidis. Together with capsular 189 polysaccharide-adhesin, polysaccharide intercellular adhesin is a product of the *ica* 190 gene operon, the most understood biofilm mediating pathway in staphylococci [38]. 191 Sharing the same linear β -1,6-linked-polyglucosamine backbone as capsular 192 polysaccharide-adhesin, polysaccharide intercellular adhesin can exist as one of two 193 polysaccharides termed polysaccharide intercellular adhesin I or polysaccharide 194 intercellular adhesin II with an average chain length of 130 residues [39]. Deacylated 195 N-acetylglucosamine accounts for 15-20% of polysaccharide intercellular adhesin and 196 is essential for its functional properties including the ability to colonize, form biofilms 197 and resist phagocytosis by neutrophils and antibacterial peptides [40]. The *ica* gene 198 operon codes for the proteins and enzymes responsible for polysaccharide 199 intercellular adhesin production.

200

201 This *ica* gene cluster can be further differentiated to the *icaA*, *icaD*, *icaB* and *icaC* 202 loci each responsible for relevant pathogenic and virulent factors involved in 203 polysaccharide intercellular adhesin synthesis [40][38][41]. The transcription of the 204 *ica*ADBC gene operon is negatively regulated by an adjacent five nucleotide base 205 *icaR* gene sequence, that itself codes for a transcriptional regulator that binds to the 206 *ica*ADBC promoter [42][35]. Evidence for the role of *icaR* has been verified through 207 deletion of the *icaR* gene corresponding to increased polysaccharide intercellular 208 adhesin production [43]. The proteins transcribed, icaA, icaD, icaB and icaC have 209 separate but correlating functions in polysaccharide intercellular adhesin synthesis 210 (Figure 2). IcaA is a transmembrane protein similar to N-acetyl-211 glucosaminyltransferases and works in tandem with icaD, also positioned on the 212 cytoplasmic membrane, to form N-acetyl-glucosamine oligomers with UDP-N-213 acetylglucosamine as a substrate [35]. When both proteins are transcribed oligomers 214 may form to a maximum of 20 residues in length. The presence of the integral 215 membrane protein icaC increases both the length of N-acetyl-glucosamine oligomers 216 and allows for the translocation of the polysaccharide through the cytoplasmic 217 membrane to the cell surface [41]. The expression of *icaA*, *icaD* and *icaC* are a 218 necessary requirement for the production of polysaccharide intercellular adhesin, with 219 the deacetylase-like icaB conferring significant functional virulence and cationic 220 charge by deacetylation of the poly-*N*-acetylglucosamine sequence [41][40]. It is 221 likely that an uncharged fully acetylated N-acetylglucosamine primary product is 222 produced, with a second icaB protein mediated deacetylation step leading to 223 positively charged N-glucosamine oligomers.

224

225 This hypothesis has developed from the observation that in *in vitro* synthesis 226 pathways no virulence dependent deacetylated residues have been isolated. There has 227 been much debate as to the location of icaB as many papers hypothesize it to be 228 secreted into the surrounding medium acting as a peptide signal molecule [38][35]. 229 More recently Vuong et al obtained results to indicate that icaB interacts with the 230 staphylococcal cell surface through non-covalent means, with its location likely to be in the cell surface matrix [40]. The role of the *ica* gene operon in regulating biofilm 231 232 formation, adhesion and virulence has been proven by the introduction of the 233 *icaRADBC* sequence into strains of *Staphylococcus epidermidis* that were previously 234 *icaADBC* negative and biofilm negative [13]. The presence of the *icaRADBC* gene 235 cluster allows the production of polysaccharide intercellular adhesin leading to 236 increased biofilm formation when sufficient IcaB protein allows for 237 deacetylation[44][40].

238

239 Regulation of *icaR* transcription in *Staphylococcus epidermidis* is controlled by the alternative sigma factor σ^{B} which itself is positively regulated by the protein RsbU via 240 241 activation of environmental stresses for example heat, acid, salt or ethanol shock [45]. 242 Also included in this regulatory cascade are; the anti-sigma factor RsbW, the anti-anti 243 sigma factor RsbV, with RsbU acting as a RsbV-specific phosphatase as outlined in 244 Figure 3. This mechanism is true for *Staphylococcus epidermidis* but not 245 Staphylococcus aureus [46]. The production of an uncharacterized intermediate protein molecule, σ^{B} indirectly represses the transcription of the *icaR* operon and its 246 247 expression is especially important in the stability of Staphylococcus epidermidis 248 biofilm under environmental stresses, such as lack of nutrients [47]. Knobloch et al 249 proved alterations in the gene responsible for RsbU transcription (*RsbU*), via the use

250 of a Tn917 insertion mutant, results in a *Staphylococcus epidermidis* strain that cannot express σ^{B} . It was observed in this class III mutant, labelled M15, that the *icaADBC* 251 operon was not transcribed suggesting σ^{B} expression is essential for *icaADBC* activity 252 in *Staphylococcus epidermidis* [45]. Both ethanol and high osmolarity (both 253 environmental stresses) have been shown to be inducers of σ^{B} . Knobloch *et al* also 254 255 observed that the presence of ethanol could result in the restoration of biofilm 256 formation in mutant M15 but the presence of sodium chloride (NaCl) salt would not 257 restore biofilm formation. However it has also been proposed by Conlon et al 258 that *icaADBC* operon activation by ethanol is only *icaR* dependent whereas for NaCl to activate *icaADBC* expression both *icaR* and σ^{B} activity are required [48]. With 259 260 these theories in mind two regulatory pathways could exist in Staphylococcus 261 epidermidis to control biofilm formation with the ethanol mediated pathway acting 262 independently of σ^{B} [49]. This alternative ethanol induced pathway could involve activation of σ^{B} by RsbU substitutes or the formation of polysaccharide intercellular 263 adhesin by a completely different pathway independent of σ^{B} , as Conlon *et al* suggest 264 265 [48]. This mechanism may follow that of other biofilm forming staphylococcal species [50]. It is still unclear how responsible σ^{B} is for the control of *icaADBC* 266 operon transcription as no identifiable σ^{B} binding site has been identified close to 267 *icaADBC* [51]. One explanation of σ^{B} control of the *icaADBC* is through the presence 268 269 of genes that code for staphylococcal accessory regulator, a global regulator that is commonly associated with *Staphylococcus aureus* biofilm development, where σ^{B} is 270 271 only essential in a minority of strains [52][53][46].

272

273 SarA is an essential element in the synthesis of polysaccharide intercellular adhesin

and biofilm development in *Staphylococcus aureus* through the *icaADBC* operon with

275 environmental signals such as ethanol, salt stress and iron limitation important [54]. 276 For *Staphylococcus aureus* in particular, the staphylococcal accessory regulator protein A has been shown to be positively regulated by σ^{B} [51]. Although further 277 research by Valle *et al* has shown σ^{B} negative *Staphylococcus aureus* to still have 278 279 biofilm forming potential suggesting the production of staphylococcal accessory 280 regulator has still to be characterized fully [46]. 84% of the staphylococcal accessory 281 regulator protein present in *Staphylococcus epidermidis* corresponds to that of 282 Staphylococcus aureus, however the regulation of staphylococcal accessory regulator 283 varies due to the differing organisation of staphylococcal accessory regulator 284 promoters at a nucleotide level [55]. Staphylococcal accessory regulator binds to and 285 positively regulates the *icaADBC* operon with high affinity through an *icaR* 286 independent mechanism [56]. The staphylococcal accessory regulator gene has been 287 implicated in the agr quorum sensing system of staphylococci but mediates biofilm 288 formation via an *agr* independent pathway [57]. Purine biosynthesis is also 289 associated with *ica* expression and biofilm formation in Gram-positive 290 microorganisms and although no direct binding site for purines or preceding genes 291 that code for purines exist on the *icaADBC* operon, purines may play an indirect role 292 in *icaADBC* regulation [58].

293

294 The accumulation associated proteins in staphylococci

The importance of biofilm formation for the survival of *Staphylococcus epidermidis* and staphylococci generally means that the *ica* operon itself is not a necessity for biofilm formation. A number of *ica* independent mechanisms exist as shown by strains of *Staphylococcus epidermidis* lacking *icaADBC* but still forming biofilms [37][59][60]. Accumulation associated protein has been shown to be involved in the 300 accumulation of Staphylococcus epidermidis independently of polysaccharide 301 intercellular adhesin. Past research had deemed accumulation associated protein to be 302 a cell wall receptor for polysaccharide intercellular adhesin [61]. In *Staphylococcus* 303 aureus the surface protein G is homologous to the accumulation associated protein of 304 Staphylococcus epidermidis, however although it has been linked to intranasal 305 adhesion of Staphylococcus aureus its in vivo activity is less characterized than 306 accumulation associated protein [62]. Rohde *et al* proved that limited proteolysis of 307 accumulation associated protein by endogenous serine and metalloproteases and 308 exogenous trypsin, elastase and cathepsin G induced biofilm formation [63]. 309 Proteolytic processing of accumulation associated protein leads to the removal of the 310 N-terminal domain resulting in the exposure of N-acetylglucosamine binding 311 domains, also termed G5 domains due to the prominence of glycine residues [64]. 312 Protease production itself is controlled via quorum sensing pathways such as the agr 313 and sarA in staphylococci, thus biofilm formation via accumulation associated protein 314 is linked to virulence [65]. 315

- 316 Quorum sensing in staphylococci:
- 317 I. The accessory gene regulator system (*agr*)

Symbiosis, antibiotic production, biofilm formation and virulence are defined by two quorum sensing systems in staphylococci. These are the accessory gene regulator system (*agr*) and the *luxS* system [66][67][68]. The accessory gene regulator system (*agr*) consists of two units RNA-II and RNA-III whose transcription is dependent on the activation of their respective P2 and P3 *agr* promoters [69]. RNA-II consists of four genes *agrB*, *agrD*, *agrC* and *agrA* [70]. The autoinducing peptide backbone is synthesized via transcription of the *agrD* gene. The product of *agrB* transcription is a 325 protease that cleavages portions of the agrD product to form a thiolactone ring 326 structure (lactone ring in one case) of approximately 8 amino acids in length, 327 otherwise known as autoinducing peptide [71]. AgrC is the sensory kinase of the agr 328 quorum sensing system with the binding of a threshold concentration of autoinducing 329 peptide to this transmembrane protein resulting in activation of AgrA via 330 phosphorylation or dephosphorylation (Figure 4). This autoinductive pathway results 331 in RNA-II and RNA-III (the effector molecule of the *agr* system) transcription via the 332 activation of the promoters P2 and P3 by activated AgrA aided by SarA [58].

333

334 The activation of the *agr* system correlates to the mid to end point of exponential 335 growth and entry into the stationary phase of growth with the down regulation of cell 336 surface protein related genes but an upregulation in virulence factors [72]. This leads 337 to the production of the regulatory RNA-III molecule that initiates the transcription of 338 genes coding for a variety of virulent proteins (toxins) including enterotoxin B also 339 known as *Staphylococcus aureus* exoprotein expression regulator and *Staphylococcus* 340 serine proteases and *Staphylococcus* proteases (spr) and controls the downregulation 341 of genes encoding cell surface proteins and adhesion, for example Staphylococcus 342 protein A and fibronectin-binding [73][74]. The overall picture is not as simplistic 343 however, as research conducted by Vuong *et al* has shown the genes coding the 344 adhesin autolysin E (altE) are upregulated by agr quorum sensing pathways in 345 Staphylococcus epidermidis and sarA appears upregulated similarly in Staphylococcus 346 *aureus* thereby increasing biofilm formation [72]. However as stated previously 347 staphylococcal accessory regulator gene has been implicated in the agr quorum 348 sensing system of staphylococci but mediates biofilm formation via an agr 349 independent pathway [57]. The possibility still remains that *agr* may mediate

350 adhesion in *Staphylococcus epidermidis* strains particularly in reference to

biomaterials [13]. As intercellular adhesion in staphylococci is influenced by

352 polysaccharide intercellular adhesin production, it has been shown that the *luxS*

- 353 quorum sensing system, not *agr*, has a role in down-regulating this process [67].
- 354

355 The importance of *agr* to the biofilm process is greatest at the detachment phase of growth [75][76]. Wild type staphylococci that utilize *agr* have biofilms that are less 356 357 thick than *agr* negative mutants due to an ability to detach from the matured biofilm, 358 rather than decreased microbial growth [77]. Detachment in both Staphylococcus 359 epidermidis and Staphylococcus aureus occurs due to the production of short 360 amphipathic peptides known as phenol-soluble modulins, such as δ -toxin, encoded by 361 regulatory RNA-III molecule and mediated by the *agr* regulatory system. These 362 peptides themselves have no autoinducing or regulatory affect on the agr system [76]. 363

364 The ability of microorganisms to coordinate a range of actions and phenotypic traits, 365 via a process such as quorum sensing, shows that this mechanisms itself may be a specific target in reducing biofilm formation and virulence associated with medical 366 367 device related pathogens [78][79]. Research by Balaban et al have shown that RNA-368 III inhibiting peptide has significant activity in preventing *Staphylococcus* 369 epidermidis and Staphylococcus aureus biofilm formation using an in vivo rat Dacron 370 graft model [80]. RNA-III inhibiting peptide targets RNA-III activating protein, to 371 prevent the phosphorylation of the protein target of RNA-III activating protein. The 372 release of RNA-III activating protein and phosphorylation of the protein target of 373 RNA-III activating protein is itself a quorum sensing process leading to the formation 374 of numerous surface adhesion proteins, together with the autoinducing expression of

the *agr* operon controlling biofilm formation in staphylococci (Figures 5 and 6) [81].

376 RNA-III inhibiting peptide itself is a heptapeptide of structure of amide form,

377 YSPWTYNF-NH₂, is non-pathogenic as it inhibits cell to cell communication via

378 competing for binding sites on the protein target of RNA-III activating protein but it is

not bactericidal [82].

380

381 II. Quorum sensing in staphylococci: the *luxS* system

382 Whereas the *agr* system has no effect on the *icaADBC* gene operon and

383 polysaccharide intercellular adhesin formation the presence of an alternative quorum

384 sensing *luxS* has been linked to preventing the production of polysaccharide

385 intercellular adhesin in staphylococci via downregulation of *icaADBC* [83]. Present

in both Gram-positive and Gram-negative bacteria the *luxS* quorum sensing system

results in the formation of autoinducing peptide-II [84][85][86]. LuxS and agr absent

388 mutants both shared the common properties of forming thicker but less virulent

389 biofilms than wild type strains of *Staphylococcus epidermidis* [67]. This research by

390 Xu *et al* claimed that thinner biofilm growth in *luxS* positive strains was due to a

391 downregulation in the *icaADBC* operon rather than cellular metabolic processes as

392 there were no noticeable differences in the growth patterns of *luxS* negative and

393 positive strains. This contrast to the theory put forward by Vendeville *et al* who

394 observed that *luxS* is involved in the activated methyl cycle and thus may alter the

395 metabolism and biofilm formation of bacteria [87].

396

397 The synthesis of autoinducer-II occurs in three enzyme enzymatic steps. The

398 substrate molecule is *S*-adenosylmethionine, a molecule found as a cofactor for many

399 DNA- and RNA-linked processes including protein synthesis. The presence of

400 methyltransferases results in S-adenosylmethionine donating methyl groups to a 401 variety of substrates in the methyl cycle to form the toxic intermediate S-402 adenosylhomocysteine. The nucleosidase enzyme Pfs (5'methylthioadenosine/S-403 adenosylhomocysteine nucleosidase) mediates the hydrolysis of S-404 adenosylhomocysteine to S-ribosylhomocysteine via the loss of adenine. At this stage 405 the transcription of *luxS* with the formation of LuxS leads to the catalysis of S-406 ribosylhomocysteine cleavage to 4,5-dihydroxy 2,3-pentanedione and homocysteine 407 [88]. The production of 4,5-dihydroxy 2,3-pentanedione to autoinducing peptide-II is 408 relatively uncharacterized in the literature with Xavier *et al* stating that 5-dihydroxy 409 2,3-pentanedione cyclizes to form pro-autoinducer-II, and subsequently boron is 410 added to form autoinducer-II in Gram-negative Vibrio harveyi. A similar mechanism 411 may exist in Gram-positives also (Figure 7) [85].

412

413 Conclusions

414 Biofilms are particularly prevalent in marine ecosystems where they constitute more 415 than 99.9% of bacteria present with these results correlating to the majority of 416 ecosystems [89]. This suggests a selective evolutionary advantage for biofilm 417 forming microorganisms over planktonic forms [25]. Infections of medical devices 418 are a significant problem due to their high impact on patient morbidity, mortality and 419 monetary expenditure. Most device related infections are due to contamination of the 420 device from environmental pathogens, such as staphylococcal skin flora, both before 421 and during implantation [90]. Biomolecular processes form a viable target by which 422 treatment strategies may be developed to prevent bacterial adherence and transfer 423 from planktonic to more resistant biofilm forms. In Gram-positive bacteria potential 424 treatment strategies include influencing the *agr* and *luxS* quorum sensing systems.

425 Inhibiting the *agr* quorum sensing signal has been show to increase attachment and 426 biofilm production in both *Staphylococcus epidermidis* and *Staphylococcus aureus* 427 [72][91]. This contrasts to what is seen with quorum sensing systems in Gram 428 negative microorganisms such as *Pseudomonas aeruginosa* [92], further evidence that 429 increased study is required in this area to positively affect clinical outcomes. For 430 staphylococcal biofilms, future work will be required to focus on the specific role and 431 action of teichoic acids, present at high density throughout the biofilm matrix, cell 432 surface adhesins and MSCRAMMs as promising drug targets for vaccine development. For example Stranger-Jones et al, showed a vaccine containing the 433 434 MSCRAMMs IsdA, IsdB, SdrD, and SdrE were identified as protective in a murine 435 model of Staphylococcus aureus abscess formation [93]. Inhibition of sortase A has 436 been hypothesised as a possible target for the prevention of surface protein anchoring 437 to the peptidoglycan cell wall and adhesin exposure with several distinct sortase 438 inhibitor classes identified whose aims are to irreversibly modify the thiol active site 439 of sortase [94][95].

440

441 **Future Perspectives**

442 The need to prevent bacterial adherence and eradicate existing established biofilms is 443 an increasing challenge for an innovative scientific community whose antimicrobial 444 arsenal is updating at a diminishing rate. Over the coming years the study of bacterial 445 biomolecular processes may hold the key to producing effective future antimicrobial 446 strategies that are targeted specifically to eradicate pathogenic bacteria thus allowing 447 mutualistic commensal bacteria to thrive in the host environment. Such an approach 448 would resolve infection, meet treatment goals and reduce potential systemic side effects, all without the threat of increased antimicrobial resistance. In order to 449

450	achieve these goals bacterial genotypes must be systematically linked to both
451	resistance and biomolecular pathways thereby allowing optimum processes to be
452	targeted. In order to be of greater success clinically and to reduce the potential for
453	resistance to develop, such biomolecular strategies will likely be required to be
454	utilised concurrently with novel biocidal approaches such as the use of antimicrobial
455	peptides [96] or ionic liquids [97].
456	
457	Executive Summary
458	Introduction
459	The ability of bacteria such as Staphylococcus epidermidis and Staphylococcus aureus
460	to produce exopolysaccharide biofilms allows for increased survival, maturation and
461	symbiotic relationships to be established at a solid surface environment such as that
462	present on a medical device.
463	
464	Processes involved in Gram-positive biofilm formation: Staphylococcus
465	epidermidis and Staphylococcus aureus
466	The biomolecular processes involved in formation of staphylococcal biofilms can be
467	divided into 5 key areas:
468	1) Adhesion in staphylococci: Cell surface hydrophobicity and cell surface
469	adhesins
470	• The primary or nonspecific adhesion of staphylococci is due mainly to the
471	cell and cell surface hydrophobicity.
472	• Capsular polysaccharide-adhesin is responsible for the process of surface
473	attachment. Slime associated antigen is linked to accumulation and
474	biofilm maturation at the surface.

475		• They are both synthesized from the proteins encoded by the <i>ica</i> operon.
476		• The secondary attachment of <i>Staphylococcus epidermidis</i> is improved by
477		the presence of the cell adhesin autolysin E, which binds to plasma
478		proteins such as vitronectin present in the conditioning layer formed on
479		implanted biomaterials.
480		• There are several surface bound proteins in <i>Staphylococcus epidermidis</i>
481		that are responsible for binding specifically to collagen, vitronectin,
482		fibronectin and fibrinogen and other proteins present in the extracellular
483		matrix.
484	2)	The role of teichoic acids
485		• Cell wall teichoic acids are the highest source of polyanionic charge on
486		the staphylococcal bacterial cell envelope.
487		• Increased cationic charge is provided by incorporation of D-alanine
488		into teichoic acids. This is a determinant in the successful attachment
489		of staphylococci to biomaterials.
490		• The production of teichoic acids is controlled by the <i>dlt</i> gene operon
491		
492	3)	Accumulation and the <i>ica</i> operon in staphylococci
493	•	The <i>ica</i> gene operon codes for the proteins and enzymes responsible for
494		polysaccharide intercellular adhesin production.
495	•	The <i>ica</i> gene cluster can be differentiated into the <i>ica</i> A, <i>ica</i> D, <i>ica</i> B and <i>ica</i> C
496		loci each responsible for relevant pathogenic and virulent factors involved in
497		polysaccharide intercellular adhesin synthesis.
498	•	The role of the <i>ica</i> gene operon in regulating biofilm formation, adhesion and
499		virulence has been proven by the introduction of the <i>icaRADBC</i> sequence into

500		strains of <i>Staphylococcus epidermidis</i> that were previously <i>icaADBC</i> negative
501		and biofilm negative.
502	•	Regulation of <i>icaR</i> transcription in <i>Staphylococcus epidermidis</i> is controlled
503		by the alternative sigma factor σ^{B} which itself is positively regulated by the
504		protein RsbU via activation of environmental stresses.
505	•	SarA is an essential element in the synthesis of polysaccharide intercellular
506		adhesin and biofilm development in Staphylococcus aureus through the
507		<i>icaADBC</i> operon it is influenced by environmental signals such as ethanol, salt
508		stress and iron limitation.
509	•	The staphylococcal accessory regulator protein A has been shown to be
510		positively regulated by σ^{B} .
511		
512	4)	The accumulation associated proteins in staphylococci
513	•	The <i>ica</i> operon itself is not a necessity for biofilm formation.
514		• Accumulation associated protein has been shown to be involved in the
515		accumulation of Staphylococcus epidermidis independently of polysaccharide
516		intercellular adhesin.
517		• Accumulation associated protein is a cell wall receptor for
518		polysaccharide intercellular adhesin.
519		• Proteolytic processing of accumulation associated protein leads to the
520		removal of the N-terminal domain by proteases resulting in the exposure of N-
521		acetylglucosamine binding domains.
522		• Protease production is controlled via quorum sensing pathways such as
523		the agr and sarA in staphylococci.
524		

525	5)	Quorum sensing in staphylococci:
526	Two quor	um sensing systems exist in staphylococci:
527	I.	The accessory gene regulator system (agr)
528	•	The accessory gene regulator system (agr) consists of two units RNA-II
529		and RNA-III. Transcription is dependent on the activation of their
530		respective P2 and P3 agr promoters.
531	•	RNA-II consists of four genes agrB, agrD, agrC and agrA.
532	•	An autoinductive pathway results in RNA-II and RNA-III (the effector
533		molecule of the agr system) transcription via the activation of the
534		promoters P2 and P3 by activated AgrA aided by SarA.
535	•	Staphylococcal accessory regulator gene has been implicated in the agr
536		quorum sensing system of staphylococci but mediates biofilm formation
537		via an <i>agr</i> independent pathway.
538	•	The importance of agr to the biofilm process is greatest at the detachment
539		phase of growth.
540	•	Detachment in staphylococci occurs due to the production of short
541		amphipathic peptides known as phenol-soluble modulins, e.g. δ -toxin,
542		encoded by regulatory RNA-III molecule and mediated by the agr
543		regulatory system.
544	II. Quoru	m sensing in staphylococci: the <i>luxS</i> system
545	•	<i>luxS</i> has been linked to preventing the production of polysaccharide
546	int	ercellular adhesin in staphylococci via downregulation of <i>icaADBC</i>
547	•	The <i>luxS</i> quorum sensing system is present in both Gram-positive and
548	Gr	am-negative bacteria and results in the formation of autoinducing peptide-II

549 • The synthesis of autoinducer-II occurs in three enzyme enzymatic
550 steps.

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- 867 Presenting emerging evidence for the existence of *ica*-independent biofilm
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