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Hypothesis

A different path: Revealing the function of staphylococcal proteins in biofilm formation

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ABSTRACT

***Staphylococcus aureus* and *Staphylococcus epidermidis* cause dangerous and difficult to treat medical device-related infections through their ability to form biofilms. Extracellular poly-*N*-acetylglucosamine (PNAG) facilitates biofilm formation and is a vaccination target, yet details of its biosynthesis by the *icaADBC* gene products is limited. IcaC is the proposed transporter for PNAG export, however a comparison of the Ica proteins to homologous exo-polysaccharide synthases suggests that the common IcaAD protein components both synthesise and transport the PNAG. The limited distribution of *icaC* to the Staphylococcaceae and its membership of a family of membrane-bound acyltransferases, leads us to suggest that IcaC is responsible for the known *O*-succinylation of PNAG that occurs in staphylococci, identifying a potentially new therapeutic target specific for these bacteria.**

1. Introduction

Staphylococcus aureus and *Staphylococcus epidermidis* infections associated with the insertion of medical devices can arise due to their ability to form biofilms on surfaces [1]. A biofilm is a multicellular, three-dimensional bacterial community anchored to a surface; bacteria within a biofilm are surrounded by an exopolymeric matrix, reducing bacterial exposure to antibiotics and the host immune system [2]. As a result, infections involving biofilms are very difficult to eradicate often leading to prolonged antibiotic therapy and/or surgical removal of the device and the surrounding infected tissue, placing a significant financial burden on health services [3]. The ability to form a biofilm is recognised as a major virulence factor of *S. epidermidis* in particular [4], and dissecting the molecular mechanisms underlying staphylococcal biofilm formation will aid the development of novel therapeutics. The first-discovered and most studied mechanism of intercellular adhesion in staphylococcal biofilms is mediated by the polysaccharide intercellular adhesin (PIA), which is a β-1,6-linked poly-*N*-acetylglucosamine (PNAG) molecule that is partially *N*-deacetylated [5–7]. More recently, both PNAG and *N*-deacetylated (dPNAG) have been studied as potential vaccine targets in therapeutic approaches against a range of human microbial pathogens [8,9].

In both *S. aureus* and *S. epidermidis*, biosynthesis of PNAG, and biofilm formation, requires the IcaADBC proteins, encoded in the *icaADBC* operon (Fig. 1); the sequences of the four proteins being highly conserved (⩾79% similarity) between the two staphylococci [10,11]. We have compared the *ica* encoded PNAG biosynthesis system to analogous systems in Gram-negative bacteria and also to Ica systems in other Gram-positive bacteria. Our analysis of published data suggests that PNAG is likely to be synthesised *and* translocated across the inner membrane by IcaA/D and that the integral membrane protein IcaC, rather than acting as the PNAG transporter (as proposed since its discovery in 1996) [11], is likely to be an enzyme that adds *O*-linked succinyl groups to PNAG residues. Thus we present a revised model for the function of the Ica proteins in PNAG synthesis in the Staphylococci and in Gram positive bacteria more generally.

2. Current models of PNAG synthesis in the Staphylococci

A role for the *ica* gene products in the synthesis of *S. epidermidis* PNAG was discovered in 1996 by Götz and colleagues [11]. Inactivation of genes in a three gene operon, *icaABC*, led to loss of detectable PNAG and cell aggregation. Later a fourth small gene, *icaD*, was identified between *icaA* and *icaB* [12], giving the final gene order of *icaADBC* (Fig. 1). The small gene *icaD* is unusual in that it

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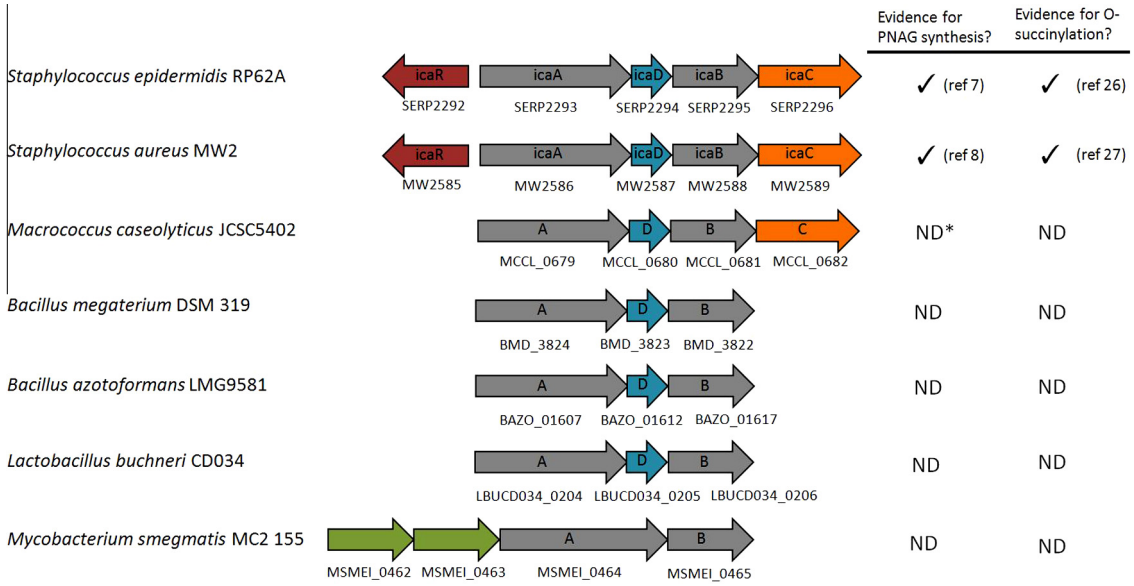

Fig. 1. Arrangement of the *ica* operon in *S. epidermidis*, *S. aureus* and other Gram-positive bacteria. The genes are centred on *icaB* and coloured based on similarity to the *ica* genes in *S. epidermidis*. Systematic gene names are indicated for each species and evidence for the presence of PNAG and *O*-succinylation of PNAG is indicated. No IcaR homologues were found outside staphylococcal species. *ND = Not Determined.

overlaps with both the 3′-end of *icaA* by 37 nucleotides and the 5′-end of *icaB* by four nucleotides [12]. Finally, a divergently transcribed transcriptional regulator *icaR* was identified in this region, which encodes a TetR family protein and is involved in environmental regulation of *icaADBC* operon expression [13]. The presence of the *ica* operon correlates with increased antibiotic resistance and biofilm formation in clinical isolates of *S. epidermidis*, where 45% of nosocomial infection isolates were found to be *ica* positive [14]. Several studies have also demonstrated that synthesis of PNAG is critical for staphylococcal biofilm formation in animal infection models [15,16], highlighting the key role for PNAG in persistence.

Since the discovery of the *ica* genes, a variety of models for Ica function have been proposed (reviewed in [17]) (Fig. 2A). Based on sequence homology, IcaA was identified as a probable *N*-acetylglucosamine transferase, with a likely function in the synthesis of the β-1,6-linked *N*-acetylglucosamine polymer [11]. IcaA is predicted to contain four transmembrane (TM) helices [11] with the glycosyltransferase 2 domain (GT-2, CAZY classification, EC 2.4.1) located between TM1 and TM2 in a cytoplasmic location. IcaD is a much smaller integral membrane protein, comprising two predicted TM helice, that is required for full activity of IcaA [12] but its precise role remains unclear. IcaB has been demonstrated experimentally to be a specific PNAG *N*-deacetylase, and a proportion of the PNAG (around 15–20% in *S. epidermidis*) is known to be *N*-deacetylated [18,19]. IcaC was originally (in 1996) described as an integral membrane protein with six TM helices and was suggested to play a role in PNAG export [11]; later work suggested that it must be coexpressed with IcaA/IcaD and was needed to produce ‘mature’ PNAG that could be recognised

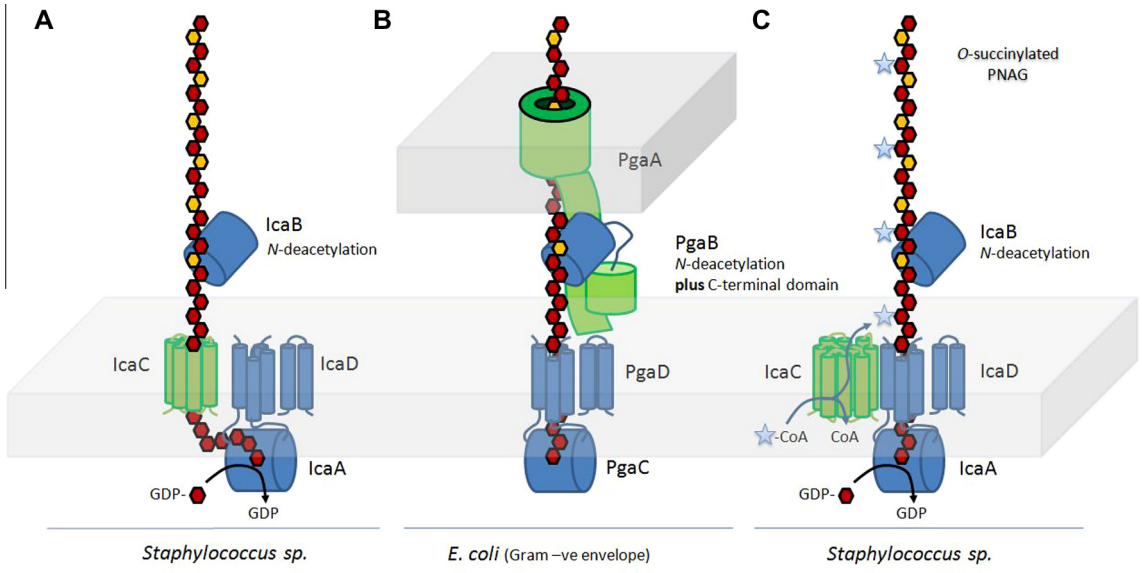

Fig. 2. Schematic comparison of the models for Ica and Pga gene functions in the biosynthesis of PNAG. (A) The current model of Ica gene function in PNAG biosynthesis in Gram-positive bacteria. (B) The Pga system from the Gram-negative bacterium *E. coli*. (C) Our refined model that has a different route for PNAG export through IcaA/D and the added *O*-succinylation of the PNAG which we propose is catalysed by IcaC. In each system shared components are coloured blue and unique components are coloured green. The indicated degree of deacetylation and *O*-succinylation are not stoichiometrically accurate and are for illustrative purposes only.

by a polyclonal antibody raised against PNAG isolated from wild-type cells [12]. Given its importance in infection, there is considerable interest in biofilm formation by *S. epidermidis* and *S. aureus* and thus there have been many reviews on the subject (for example [17]). However, in the absence of an alternative proposal, these reviews have reinforced the idea that the IcaAD complex only synthesises PNAG and its translocation across the bacterial cell membrane is performed by IcaC (represented in Fig. 2A).

3. Comparison of the Ica system with the Pga system of Gram-negative bacteria

As discussed above, the intercellular adhesin involved in *S. epidermidis* biofilm formation was identified as a linear array of β-1,6-linked *N*-acetylglucosamine residues [5]. A wide range of bacteria produce extracellular polysaccharides (EPS) and a recent excellent review drew together a diverse set of literature concerning Gram-negative bacteria which use the 'synthase'-type coupled synthesis and secretion pathways, as opposed to Wzx/Wxy or ABC transporter-type mechanisms [20]. One of the best studied of these systems is encoded by the *pga* operon of *E. coli*, which produces a similar PNAG molecule and which also mediates biofilm formation in this organism [21]. In the PgaABCD system the naming of the proteins is slightly different to the Ica system, but they share a number of key proteins (Fig. 2B). PgaC is the equivalent to IcaA, with a cytoplasmic GT-2 domain that is 43% identical at the amino acid level. Likewise PgaD is a small membrane protein required for full activity of PgaC [22], as observed for IcaD enhancing IcaA function. However, although IcaD and PgaD are both predicted to contain two TM helices, they share no detectable sequence homology. Together PgaC and PgaD are thought to be responsible for both the synthesis of the PNAG chain and its export across the inner membrane [22] (Fig. 2B). The PgaB protein is similar to IcaB in being the *N*-deacetylase for PNAG and the remaining protein, PgaA, is a unique outer membrane protein which is required for the PNAG to cross the extra barrier of the Gram-negative envelope. The only component of the Ica system that has no functional orthologue in the Pga system is IcaC. Given that the 'synthase'-like polysaccharide synthesis systems appear to function as stand-alone synthesis and export machinery, this suggests that the PgaABCD system and specifically the PgaCD proteins are necessary and sufficient for PNAG assembly and secretion through the cytoplasmic membrane. Hence, from this simple comparison to Gram-negative systems where the role of IcaAD is analogous to PgaCD, a role for IcaC as a direct route of PNAG export through the cytoplasmic membrane in *S. epidermidis* would require a significant alteration in the function of a typical 'synthase'-type polysaccharide biosynthesis system.

4. The *icaC* gene is not present in all *ica* operons in Gram-positive bacteria

To assess further the likely functions of the different *ica* genes in Gram-positive bacteria we examined the published genome sequences of Gram-positive organisms for orthologous *ica* gene clusters that included both IcaA and IcaB homologues (Fig. 1). We identified identical cluster organisations in other staphylococci, but some variations in *ica* clusters from a broader range of Gram-positive bacteria (Fig. 1). We found a range of bacteria outside of the staphylococci that also had clear *ica* clusters including *Bacillus megaterium* DSM 319 and *Bacillus azotoformans* LMG9581, but these clearly lack a linked *icaC* homologue. The only additional organisms that had an *icaC* gene present were *Macrococcus caseolyticus* JCSC5402 (Fig. 1) and *Tetragenococcus halophilus* (Fig. S1), suggesting a rather limited distribution of *icaC* genes even within Gram positive *ica* gene clusters. *M. caseolyticus* is the closest genus to the staphylococci and it is interesting that while many other *S. aureus* virulence factors are missing from this genome [23], it has retained what appears to be a fully functional PNAG biosynthesis system. The conservation of a full *ica* operon in this organism suggests that the addition of an *icaC* gene predates the diversification of the staphylococci.

5. *O*-succinylation of PNAG and a role for IcaC

As IcaC is not a conserved component of synthase-type polysaccharide biosynthesis systems and is not even widely found in *ica* operons in other Gram-positive bacteria, it appears to have a function specific for selected Staphylococcal species. Analysis of the IcaC sequence identifies it as being a member of a large family of acyltransferases (Pfam Acyl_transf_3 family (PF01757) and InterPro family IPR002656) (Fig. S2). Reanalysis of the sequence of IcaC using TMHMM confirms that it is a likely 10 TM helix protein characteristic of this family and not a six TM helix protein as sometimes reported previously [11,17]. Why might *S. epidermidis* require this large integral membrane protein if it is not involved directly in export of the PNAG through the cytoplasmic membrane?

Proteins within the Acyl_transf_3 family (PF01757) have a variety of biological functions but one of the best studied members is the *S. aureus* OatA protein. OatA catalyses the *O*-acetylation of *N*-acetylmuramic acid in peptidoglycan [24]. OatA and its relatives are thought to function by using acetyl-CoA as a donor molecule, which is then bound and cleaved to CoA and acetate on the cytoplasmic side of the membrane, with the acetate being moved across the membrane through the protein and attached to the synthesised polysaccharide on the outer surface of the cytoplasmic membrane. The closest Gram-negative match to IcaC identified using PSI-BLAST is YiaH (WecH) of *E. coli*. This protein similarly functions to transfer acetyl groups across the cytoplasmic membrane for the *O*-acetylation of the enterobacterial common antigen (ECA), a polymer of amino sugars [25].

There is significant evidence in the literature that PNAG is *O*-succinylated in both *S. epidermidis* [26] and *S. aureus* [27], but how this modification is catalysed has not been identified. It is important to note that PNAG (PIA) had been thought to be *N*-succinylated [28], but this has been subsequently demonstrated to be an experimental artefact [27], while evidence for *O*-succinylation is clear for both bacteria [26,27]. In *S. epidermidis* Mack and colleagues identified two forms of the PNAG structure, an abundant form that is around 80% of total polysaccharide, which is 'a linear glucosaminoglycan' [5], with around 15–20% of the GlcNAc residues being deacetylated in a random pattern (the function of the IcaB protein). However, the second form they found (polysaccharide II) was anionic and contained succinyl groups present on about 6% of the GlcNAc residues. In *S. aureus* the PNAG structure is similar and *O*-succinylation is thought to occur more frequently, in around 10% of the GlcNAc groups [27]. The modification is likely to be at the *O3* position, and a smaller amount was seen in the *O4* position, which might be due to spontaneous migration of the succinyl group around the ring after addition at *O3* [27]. Given the clear presence of this modification and its association with *S. epidermidis* and *S. aureus*, both of which contain an *icaC* gene in their *ica* clusters, we propose that IcaC is the missing *O*-succinyltransferase that adds succinyl groups to the PNAG polysaccharide as it emerges from the outer leaflet of the cytoplasmic membrane (Fig. 2C).

6. Concluding remarks

Given the critical health issues surrounding staphylococci and their ability to form biofilms, and the progress toward PNAG-based vaccines, it is important to fully characterise the PNAG synthase

system and *ica* proteins. Using genome sequence analysis and comparison to orthologous systems, we suggest a new model for the PNAG synthase system in *S. epidermidis* and *S. aureus*. The analyses presented here show that well-characterised Gram-negative bacteria such as *E. coli* can synthesise and export PNAG exopolysaccharide across their inner membrane without an IcaC homologue and that the minimal components required for an *ica* operon in Gram-positives are IcaAD and IcaB. As a result we conclude that IcaC cannot be a core functional component of PNAG biosynthesis systems and hence is unlikely to be the route of PNAG export (translocation is likely performed by IcaAD alone). Furthermore, we propose that, as IcaC is a member of the Acyl_transf_3 family (PF01757) and the PNAG in the staphylococci has a known modification with an unknown mechanism of addition, it is possible that IcaC functions as an *O*-succinyltransferase to modify the structure of the PNAG.

Enzymatic modification of the key virulence factor PNAG is likely to influence biofilm accumulation and aid evasion of the host immune system. If the positions of the *O*-succinyl modifications were randomly distributed on the carbohydrate chain then it would make it harder for the host to produce antibodies capable of recognising the bacterium [29]. A number of studies have identified a role for IcaC in formation of a mature biofilm, which in light of our new analysis would suggest that *O*-succinylation is important in vivo [11,12] and thus might represent a new therapeutic target.

7. Competing interests statement

The authors declare no competing financial interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.04.002.

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