Appl Microbiol Biotechnol (2014) 98:6947–6956
DOI 10.1007/s00253-014-5853-x

MINI-REVIEW

Insight into hyaluronic acid molecular weight control

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Received: 27 February 2014 / Revised: 22 May 2014 / Accepted: 24 May 2014 / Published online: 24 June 2014

Abstract Hyaluronic acid (HA) is a ubiquitous polysaccharide found in humans, animals, bacteria, algae and molluscs. Simple yet sophisticated, HA demonstrates unique and valuable rheological properties. In solution, HA behaves as a stiffened random coil and the resultant behaviour, even at low concentrations, is far from Newtonian or 'ideal'. These rheological properties are heavily influenced by molecular weight (MW), so it is not surprising that many of the biological functions of HA are dependent on molecular size. The current billion dollar market for HA continues to grow rapidly, both in gross production and the number of applications for its use. Increasing demand, in conjunction with a reticence to use animal-derived HA, has revitalised the market for HA produced by bacterial fermentation. Although the genes and pathways involved in bacterial production of HA are well characterised, the mechanisms that underlie HA MW control are less well understood. By performing a thorough analysis of the proposed mechanisms of MW control in bacterial fermentation, this mini-review tries to elucidate the challenges and future directions for bacterial HA biosynthesis.

Keywords Hyaluronic acid · Molecular weight control · Polysaccharides · *Streptococcus zooepidemicus*

Introduction

Hyaluronic acid (HA) is a polysaccharide of repeating units of glucuronic acid (GlcUA) and *N*-acetyl glucosamine (GlcNAc) joined by alternating β-1,3- and β-1,4-glycosidic bonds. This highly charged molecule is among the largest polysaccharides in nature. In solution, HA behaves as a stiffened random coil and, when the surrounding water volume is considered, HA is able to expand 1,000 times (Stern et al. 2006). The rheology of HA solutions is a function of both its molecular weight (MW) and concentration (Balazs 1974), and in turn, these properties determine the biological function and cellular distribution of HA within the body (Fraser et al. 1997). For example, high MW HA can be found within the umbilical cord (Weissmann and Meyer 1954), eyes (Meyer and Palmer 1934) and joints (Ogston and Stanier 1950) and is also responsible for hydration and lubrication of tissue surfaces (Juhlin 1997). Conversely, fragments of HA are sensed by the body as a sign of tissue injury and are mitogenic, immunostimulatory and highly angiogen (Ke et al. 2013).

For commercial applications, HA is sourced from rooster comb (MW≈5–6 MDa) or bacterial fermentation (MW≈0.5–4.5 MDa). Pathogenic bacteria such as groups A and C streptococci and the Gram-negative avian pathogen *Pasteurella multocida* produce a HA exopolysaccharide capsule (Fig. 1). These HA capsules are known virulence factors that protect the bacterial cell from complement-mediated killing and phagocytosis (Harper et al. 2006; Stollerman and Dale 2008). As the HA from these capsules is structurally identical to mammalian HA, native strains of the animal pathogen *Streptococcus equi* subsp. *zooepidemicus* (*S. zooepidemicus*) have been used to produce HA of up to 2 MDa (Chong et al. 2005). With the aid of genetic engineering, HA produced from *S. zooepidemicus* strains can reach 3.5 MDa (Chen et al. 2009, 2014).

HA has also been produced from recombinant strains of *Bacillus subtilis* (Widner et al. 2005), *Escherichia coli* (Yu and Stephanopoulos 2008), *Lactococcus lactis* (Chien and Lee 2007) and *Agrobacterium* sp. (Mao and Chen 2007). These hosts express the streptococcal hyaluronan synthase gene and can produce HA of up to 1 MDa in size. Although these recombinant hosts address concerns regarding the use of

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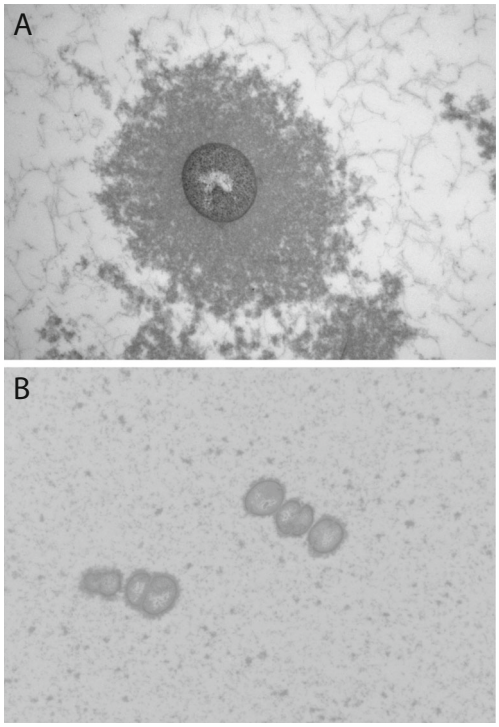

Fig. 1 **a** Electron micrograph section of *Streptococcus equi* subsp. *zooepidemicus* cells cultured in sheep blood agar plates and **b** cells treated with hyaluronidase. Cells were prepared for electron microscopy using the method of Fassel et al. (1998). Images were obtained at the Centre of Microscopy and Microanalysis of the University of Queensland with the help of Rick Webb and Andreas Weber

animal pathogens for production, HA of this size is still insufficient for many applications. In vitro enzyme approaches have also been developed using the hyaluronan synthase from *P. multocida* hyaluronan synthase (pmHAS) (DeAngelis 2008; DeAngelis et al. 2003; Jing and DeAngelis 2004). This system produces HA of defined size with low polydispersity by controlling the reaction stoichiometry (i.e. molar ratio of precursors and acceptor molecules). Although this approach has the added advantage of allowing for the incorporation of radioactive or fluorescent tags, it does not generate high MW polymers.

Most recently, recombinant *B. subtilis* strains that express the HA synthase gene have been used to produce HA polymers of up to 4.5 MDa (Jia et al. 2013). This MW is comparable to rooster comb HA without the risk of using animal products. Despite these advances, the biochemical mechanism(s) behind HA MW control remains elusive and much work remains to be done to understand this complex process. This mini-review describes factors known to influence the MW of HA MW including the composition of nutrient media, the physical and chemical variables of fermentation conditions, the concentration of the two precursor sugar nucleotides and the HA synthase.

Applications for HA

With its broad range of biological functions, HA is an important component of an increasing number of products and has an estimated market of around one billion US dollar per year (Videbaek 2011). Traditional medical applications for HA such as ophthalmic surgery and viscosupplementation generally require high MW HA, as do several new applications in fertilisation and tissue engineering (Choudhary et al. 2007). As HA is a natural component of the vitreous body of the eye, HA-based viscoelastic solutions are widely used in ophthalmology for cataract surgeries, intraocular lens implantation, corneal transplantation, glaucoma filtration and retinal attachment surgery (Goa and Benfield 1994). In the USA alone, more than 5 million units of ophthalmic HA viscoelastic devices (OVDs) are used each year.

To overcome the challenge of obtaining very high MW HA, companies such as Genzyme (Cambridge, UK) and Q-Med (Uppsala, Sweden) have turned to cross-linking to obtain HA with similar properties to high MW HA (Balazs and Leshchiner 1984; Balazs et al. 1987), and new cross-linking approaches continue to be developed (Collins and Birkinshaw 2013; Luo et al. 2000). Although cross-linking and other chemical modifications (reviewed in Schanté et al. 2011) increase the half-life and penetration of HA, it can also alter biocompatibility, rheological properties and biological function and impact on regulatory requirements. As cross-linking changes the side groups of the original unbranched polymer in an unpredictable way, cross-linking per se interferes with natural chain interactions and β-sheet formation of linear HA. Hence, cross-linked HA is used where half-life, more than rheology and biology, dictates product quality. As such, cross-linked HA is not suitable for OVDs or recently introduced fertilisation products (Huszar et al. 2006).

One example where half-life is critical is the use of viscosupplementation for the treatment of osteoarthritis. In patients affected by osteoarthritis, the concentration and MW of the HA present in the synovial fluid decreases, reducing the ability of HA to lubricate and distribute load-bearing stress within the joint. Injection of moderate to high MW HA into the intra-articular space can alleviate pain and delay progression. Treatments that use moderate MW HA (0.5–2 MDa) require five weekly injections for efficacy, whereas natural (~3.5 MDa) or cross-linked (~3.5–6 MDa) high MW HA requires only three injections, and one injection is sufficient for efficacy when using the Q-Med cross-linked gel.

Smaller molecules of HA are also involved in important biological functions, though they lack the rheological properties found in very high MW HA. These small HA molecules are involved in complex cellular signalling cascades and have demonstrated roles in signalling, fertilisation and embryogenesis (Vabres 2010) and in immune responses that trigger tissue repair (Santillan et al. 2008) including the induction of pro-

inflammatory cytokines (Noble 2002) and the development of new blood vessels (Rooney et al. 1995). Low MW HA is also associated with cancer (Lokeshwar et al. 2005; Speranza et al. 2005) and may induce proteolytic cleavage of CD44 on the surface of cancer cells, promoting cancer cell migration (Sugahara et al. 2003).

Measuring MW of HA

The rheological properties of HA are integral to its commercial value; however, as rheology is dictated by MW and concentration, determining the average molecular mass (the abundant weighted mean of the molecular masses in the sample) is generally an accurate predictor of the behaviour of HA in solution.

MW has been extensively studied using various approaches including size-exclusion chromatography (SEC), multiangle light scattering (MALS), SEC-MALS (Bothner et al. 1988; Mendichi et al. 2003) and capillary viscometry and asymmetric field flow fractionation-MALS (AFF-MALS) (Ali et al. 2012; Moon 2010; Moon et al. 2008). Despite some satisfactory results, the cylindrical nature of HA, its solvent interference and its large polydispersity have made column-based techniques unreliable. Size-exclusion chromatography does not resolve high MW HA, and the reported distribution is generally artificial. Shear degradation, concentration effects, poor column resolution and, in general, poor reproducibility are among some of the major problems encountered. Sizing of molecules based on MALS with a MW greater than 1 MDa relies on non-linear extrapolation of scattering data to a zero angle. The extrapolation relies heavily on the measurement of the low angle; however, such measurements are notoriously noisy in water-based systems. As such, many studies of HA by SEC-MALS differ on the reported value of the radius of gyration (Rg).

The poor resolution is reflected in the polydispersity numbers reported in the literature. For low MW HA, numbers in the 1.6–2 values are common corresponding to close to random termination. On the contrary, for high MW HA, it is common to see numbers in the 1.02–1.10 region, suggesting that cells have the ability to count out of 20–60,000 residues with very high accuracy. In reality, samples are just not resolved by any of those methods, so the MW determined for each time interval is for a mixture of chain lengths. In MALS-based sizing, MW is characterised by an extremely flat MW line as a function of elution time. If the MW determined by MALS was accurate, this would not be so much of a problem in terms of establishing average MW. However, the lowest angles on the MALS, which are the most critical for accurately determining the zero angle value, have to be dropped due to high noise in aqueous solution. Studies have shown that when you drop an angle, the fitted MW changes dramatically; in reality, there is no clean decision on the trade-off between using noisy data and using the lower angles needed to accurately extrapolate. As such, the claim of absolute MW determination for high MW HA has to be considered spurious. Today, the intrinsic viscosity measurement using a Canon or *Ubbelohde* viscometer remains to be the most reliable technique to evaluate HA MW.

Synthesis of HA

HA is made following the polymerisation of the precursor sugars UDP-GlcUA and UDP-GlcNAc by the HAS. In bacteria, UDP-GlcUA is produced from glucose-6-phosphate via glucose-1-phosphate and UDP-glucose intermediates while UDP-GlcNAc is produced from fructose-6-phosphate via glucosamine-6-phosphate, glucosamine-1-phosphate and *N*-acetyl glucosamine-1-phosphate intermediates (Fig. 2). Many of these intermediate products are required for the production of essential cell wall components including peptidoglycan and teichoic acid.

The synthesis of HA requires a single enzyme, HAS, which is responsible for the polymerisation of both sugar precursors as demonstrated by in vitro and in vivo studies (DeAngelis et al. 1998, 1993; Hubbard et al. 2012; Itano et al. 1999). HAS enzymes belong to the GT2 family of glycosyltransferases, which include other β-glycosyltransferases such as cellulose and chitin synthases (Chong et al. 2005). Two classes of hyaluronan synthase have been identified and can be delineated by the number of GT2 modules present in the enzyme and the nature of the polymerisation reaction (processive/non-processive) (reviewed in Weigel and DeAngelis 2007). Class I enzymes are found in mammals (Itano and Kimata 1996; Watanabe and Yamaguchi 1996), groups A and C streptococci (Dougherty and van de Rijn 1994; Kumari and Weigel 1997), *Xenopus* (frog) (Meyer and Kreil 1996) and *Chlorella* virus PCBV-1 (DeAngelis et al. 1997). pmHAS, from the Gram-negative avian pathogen *P. multocida*, is the only class II HAS identified to date (DeAngelis et al. 1998).

Genes involved in the bacterial production of HA are often expressed in an operon and may include gene homologues of enzymes required for the production of HA precursors (Blank et al. 2008; Chung et al. 1998). In *Streptococcus* sp., this operon consists of two, three or five genes (*hasAB*, *hasABC*, *hasABCDE*). These operons always encode the HAS encoded by *hasA* and a UDP-glucose dehydrogenase (*hasB*) but may also encode a UDP-glucose pyrophosphorylase (*hasC*), a second pyrophosphorylase (*hasD*/*glmU*) and an extra copy of phosphoglucoisomerase (*hasE*/*pgi*) (Blank et al. 2008). In *P. multocida*, the genetic arrangement of the HA biosynthesis locus is closely related to that of the capsule loci from *E. coli* and *Haemophilus influenzae* (Chung et al. 1998). This locus is divided into three functional regions encoding enzymes for

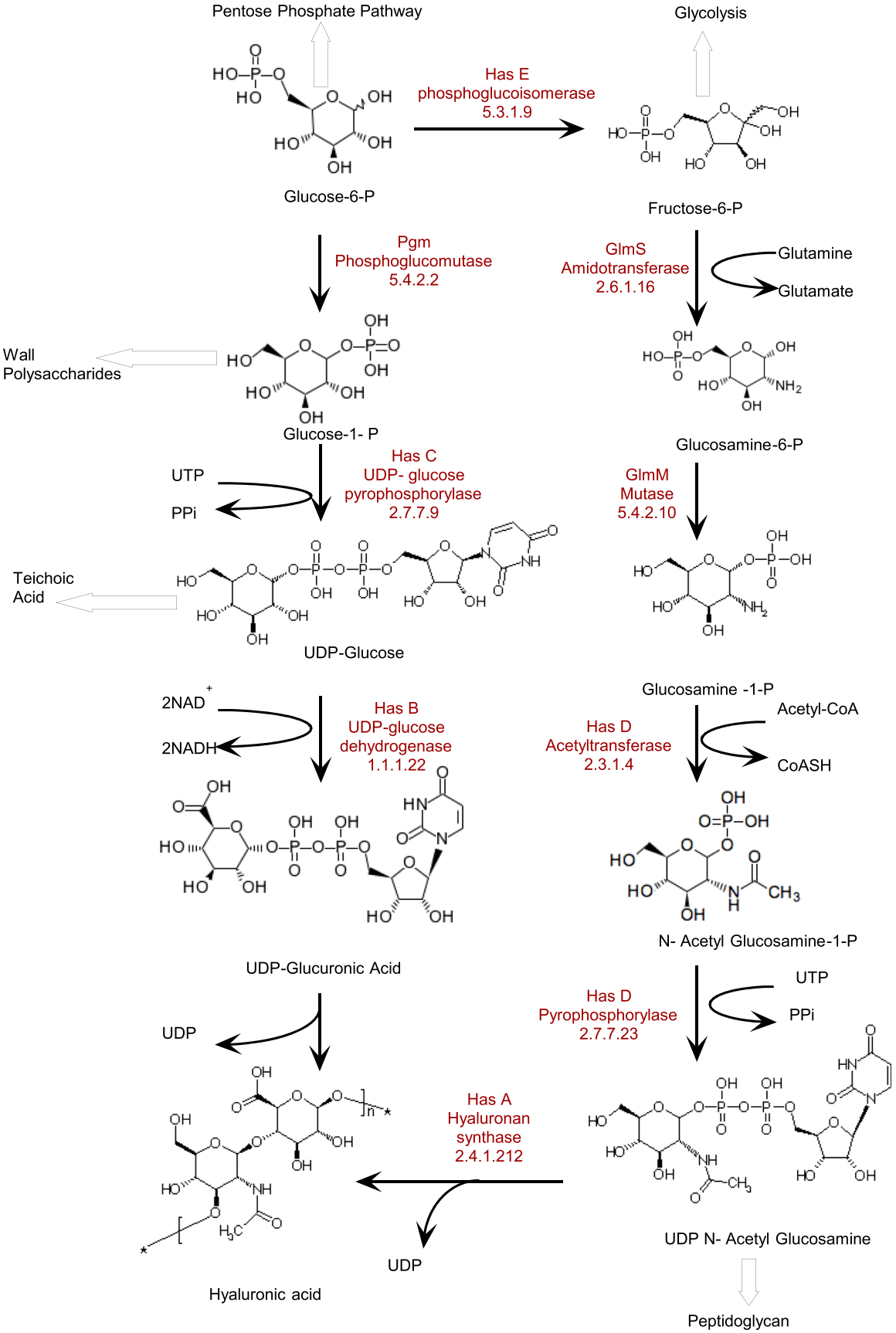

Fig. 2 Biosynthetic pathway of HA production in streptococci

capsule export (region 1), capsule biosynthesis (region 2) and phospholipid modification of the polymer (region 3). Although the genetic arrangement is different, the *P. multocida* biosynthesis region also encodes five genes (*hyaABCDE*) (Chung et al. 1998). As yet, only pmHAS has been functionally identified as the product of *hyaD* (DeAngelis et al. 1998); however, sequence homology has been used to ascribe *hyaA* and *hyaC* as a putative glycosyltransferase and a UDP-glucose dehydrogenase, respectively (Chung et al. 1998).

As the production of HA only requires the activity of the HAS enzyme, the duplication of other biosynthetic genes into a new operonic structure allows for the independent regulation of capsule expression. In streptococci, operons exhibit a significant strain-to-strain variation in expression levels (Anzai et al. 1999). Known regulators of capsule expression include the complex global virulence-associated regulators Mga (McIver and Scott 1997) and CsrR/S in streptococci (Levin and Wessels 1998) and Fis in *P. multocida* (Steen et al. 2010). In addition, these operons are also influenced by *cis*-acting promoter sequences (Albertí et al 1998) which have been modified to increase *hasA* expression and HA yield (Tlusta et al. 2013).

Fermentation conditions influence HA MW

In streptococcal systems, the availability of oxygen is critical to HA yield and MW. Studies have shown that small increases in dissolved oxygen (DO) can increase HA MW. As DO was increased from 0 to 10 and 50 %, MW increased from 1.2 to 1.75 and 2.19 MDa, respectively (Duan et al. 2009). Under these conditions, the addition of as little as 5 % DO was sufficient to increase HA yield (Huang et al. 2006) while 10 % DO was sufficient to increase *hasA* expression by sevenfold and double HAS activity (Duan et al. 2009) without affecting cell growth. The increases to HA yield have been attributed to an increase in mass transfer and metabolic efficiency as indicated by an increased in the sugar uptake rate (Blank et al. 2005; Chong and Nielsen 2003a). Further increases in DO or high levels of agitation were associated with a decrease in MW which was attributed to HA degradation from reactive oxygen species. The amount of reactive oxygen species increased in cultures with DO ≥50 % (Duan et al. 2009); however, their role in HA degradation can be mitigated by the addition of oxygen scavengers to the culture media (Cazzola et al. 2003). Oxygen also induced expression of *glmU* which encodes the enzyme for the last step in the pathway to produce UDP-GlcNAc (Wu et al. 2009).

In addition to the increased expression of *hasA* and *glmU*, the switch from anaerobic to aerobic fermentation changes streptococci metabolism, increasing theoretical ATP production and NADH oxidase activity (Chong and Nielsen 2003a). As such, it was proposed that HA synthesis was limited by the availability of energy resources. To investigate, Chong and Nielsen (2003a) used maltose fermentation and the overexpression of the NADH oxidase gene (*nox*) (Chong and Nielsen 2003b) to further increase the availability of ATP and NADH. As expected, both conditions resulted in a shift to almost homo-acetic metabolism, increasing ATP yield and NADH activity; however, it made a little difference to the MW and yield of HA. Culture condition such as temperature, carbon source, pH and oxygen availability have all been found to influence MW and yield (Armstrong et al. 1997; Armstrong and Johns 1997; Huang et al. 2006; Kim et al. 1996, 2006; Park et al. 1996). Altogether, these fermentation studies led to the formation of three proposed mechanisms of MW control in bacterial fermentation: (1) the 'torque' mechanism postulates that MW is limited by factors that cause the polymer to disassociate from the HAS enzyme, (2) HA MW is determined by the availability and ratio of UDP-sugar precursors, and (3) HA MW is an intrinsic feature of HAS, including its sugar binding affinity, glycosyltransferase activity and polymerisation rate.

Torque mechanism of MW control

Observations, showing that external environmental conditions influence HA MW, led to the hypothesis that the more time the HA chain is held by the HAS enzyme, the larger the polymer; conversely, if the chains are readily released by the enzyme, the resulting HA is of smaller size (Weigel and DeAngelis 2007). During batch fermentation of *S. zooepidemicus*, the accumulation of HA in the culture increases the viscosity of the broth, reducing mass transfer and limiting HA production to 6–10 g/L. To counter the increase in viscosity, cultures are agitated, to increase the mass transfer and HA yield; however, this approach reduces HA MW. This results suggested that larger HA molecules could be damaged by shear stress caused by the increased impeller speed; however, Duan et al. (2008) attribute the decrease in MW to the premature release of the HA chain from the HAS enzyme. In support of this theory, studies have shown that increasing the ionic strength in vitro results in longer HA (Gibbs et al. 1968), indicating that hydrophobic forces may also influence HA MW.

Within the cell, membrane stability (longevity) of HAS may also contribute to HA MW. The stability of class I HAS enzymes in the membrane relies on specific cardiolipin molecules which can be altered to affect HAS activity (Tlapak-Simmons et al. 1999a; Tlapak-Simmons et al. 1998). Although there is no data on the effect of cardiolipin on HA MW, polar residues within membrane domains 2 and 4 (Lys48 and Glu327, respectively) have been shown to be involved in the synthesize of very large HA (Kumari et al. 2006).

Availability of UDP-sugar determines HA MW

In 1997, Armstrong and Johns observed an inverse correlation between the growth rate and MW which led to the hypothesis that precursor concentration could influence MW. Overwhelming evidence that the availability of activated sugar precursors influence HA MW has come from studies that examined sugar phosphates and UDP-sugar concentration (Marcellin et al. 2009) in genetically modified *S. zooepidemicus* (Chen et al. 2009, 2014). Under these conditions, over-expression of *hasA* in the native streptococcal host was insufficient to increase HA MW (Chen et al. 2009), though this could be because *hasA* expression is already elevated in aerobic cultures (Duan et al. 2009). Using numerous over-expression strains (*hasA*, *hasB*, *hasC*, *hasD*(*glmU*), *hasE*(*pgi*), *hasED*, *pgm*, *glmS*, *glmM*, *pgi*/*glmS*/*glmU*, *pgi*/*glmS*/*glmM*/*glmU*), these studies demonstrated a strong correlation between the levels of UDP-GlcNAc and MW (Fig. 3), allowing for an increase in MW from 1.8±0.1 MDa in the WT strain to 3.4±0.1 MDa in strains over-expressing both *pgi* and *glmU* (Chen et al. 2009).

Interestingly, engineered strains carrying only the vector control also produced higher MW that of the wild-type strain (Chen et al. 2009). This plasmid effect was found to increase the expression of *glmU* (the last step in UDP-GlcNAc biosynthesis) and decrease the expression of *murA* (the first step in peptidoglycan biosynthesis) (Marcellin et al. 2010), providing additional evidence that MW is controlled by the availability of UDP precursors. Similarly, strains cultured in the presence of oxygen, which also demonstrate increased HA yield and MW, also demonstrate an increased expression of *glmU* (Wu et al. 2009). These findings have also been applied to HA production in heterologous hosts. Regardless of the source of the HAS enzyme, heterologous hosts that express genes involved in the production of UDP-GlcNAc produce higher MW HA (Badle et al. 2014; Jia et al. 2013; Sheng et al. 2009).

As UDP-GlcNAc levels are critical to MW in engineered strains, feeding strategies to increase UDP-GlcNAc availability have been investigated (Chen et al. 2014). Feeding glucosamine was found to increase UDP-GlcNAc availability significantly, but it was at the expense of UDP-GlcUA which dropped below the limit of detection, and the end result was a reduction in HA MW. Switching to a mixed feeding strategy that used glucose and GlcNAc increased the levels of both UDP-precursor sugars; however, MW remained unchanged. Subsequent transcriptomic analysis identified an increase in expression of *nagB* and a slight decrease in *glmS* expression which would direct much of the GlcNAc feedstock away from UDP-GlcNAc and toward glycolysis (Chen et al. 2014). Importantly, expression of genes required for the production of UDP-GlcNAc was not altered. As over-expression of genes in the UDP-GlcNAc pathway leads to high MW HA, this result indicates the presence of an underlying regulation influencing MW. Although UDP-GlcNAc concentration is critical, it appears that the mechanism of HA MW control is more complex than precursor concentration alone.

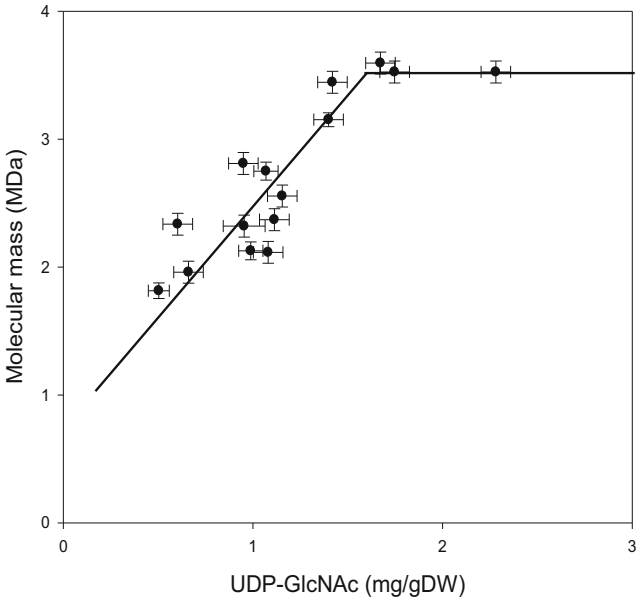

Fig. 3 Relationship between HA molecular weight and UDP-GlcNAc concentration. Analysis of UDP-sugar concentration and mean MW of HA produced in a range of genetically modified strains of *S. zooepidemicus*. As UDP-GlcNAc concentration exceeds 1.5 g/g DCW and MW approaches 3.5 MDa, this correlation ceases, presumably due to UDP-GlcNAc saturation of the system. Adapted from Chen et al. (2009, 2014)

HA MW is determined by innate features of the HAS enzyme

The multifunctional nature of HAS, with its role in sugar binding, transferase activity and polymerisation rate (Fig. 4), also makes this enzyme a likely candidate for MW control (Hubbard et al. 2012; Thomas and Brown 2010; Weigel et al. 1997). As previously indicated, a correlation between UDP-GlcNAc concentration and HA MW has been observed in *S. zooepidemicus* and in recombinant strains (Badle et al. 2014; Chen et al. 2009). This is consistent with the available affinity data of class I HAS enzymes in which the K_m value for UDP-GlcNAc (150–1,000 μM) is significantly higher than that for UDP-GlcUA (30–75 μM) (Itano and Kimata 2002; Tlapak-Simmons et al. 1999b). Biologically, the requirement for higher concentrations of UDP-GlcNAc for HAS function probably ensures that this valuable precursor remains available for essential functions. Equally, these values may explain

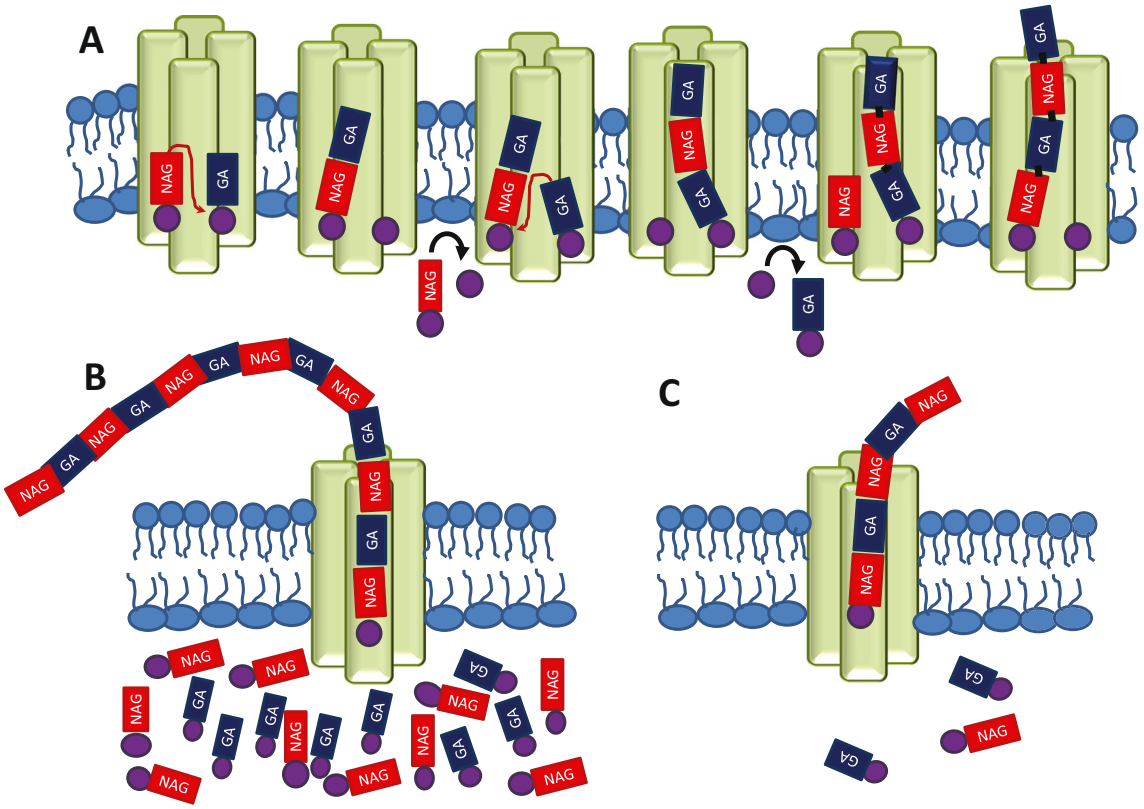

Fig. 4 Model for HA synthesis and translocation as proposed by Tlapak-Simmons et al. (2004) and revised by Hubbard et al. (2012). **a** HAS is a membrane-bound multifunctional enzyme conferring all of the activities required to continuously attach the alternating saccharide units from the cytoplasmic pool and to extend the growing hyaluronan chain through the cell wall to form the capsule. The HAS enzyme alternates between GlcUA- and GlcNAc-specific binding pockets and energizes the translocation of the growing polysaccharide chain through a transmembrane (TM) pore. The MW of the polymer is dependent on the availability and balance of UDP-GlcNAc or UDP-GlcUA precursors as results in the production of either high (**b**) or low (**c**) molecular weight HA

why increasing UDP-GlcNAc via over-expression of the pathway increases HA MW (Chen et al. 2009, 2014).

The two transferase activities could also influence HA MW. The in vitro analysis of membranes predicts that it take 8–16 min to produce a 2-MDa chain (Weigel 2004). If this V_{max} is representative, it may explain why slowing growth allows for larger HA; however, it is also possible that V_{max} is much faster in vivo than what is reported in vitro. Analysis of a unique HAS capable of producing ultra-high MW HA of up to 12 MDa (Tian et al. 2013) identified two asparagine-to-serine substitutions within the putative GlcNAc active site (Nagahashi et al. 1995; Watanabe and Yamaguchi 1996) which may contribute to HA MW. It stands to reason that modification of the UDP-GlcNAc binding and/or glycosyltransferase sites in HAS could increase HA MW; however, functional studies identifying the streptococcal HAS sugar binding and/or glycosyltransferase sites have not been performed. Conversely, analysis of pmHAS has confirmed the presence of two distinct and independent glycosyltransferase sites containing DGS motifs (Jing and DeAngelis 2000), though enzyme engineering to modify these active sites in pmHAS has not been performed either.

Future perspectives for high MW, low polydisperse HA production

Extensive studies of HA production have identified numerous factors that influence HA MW; however, the mechanism underpinning MW control remains elusive. Fortunately, even in the absence of a defined mechanism, much of this knowledge has been used to engineer new strains with improved production characteristics. Most recently, *B. subtilis* strains expressing pmHAS have been shown to produce very high MW HA of a controlled size with low polydispersity (Jia et al. 2013). These strains address expression and precursor concentrations via the use of two inducible artificial operons: the first encodes the pmHAS gene (*hyaD*) and the second encodes extra copies of the *B. subtilis* genes for the biosynthesis of UDP-GlcUA or UDP-GlcNAc precursors. With this strategy, high MW HA (yield=6.8 g/L; MW=4.5 MDa) was produced for the first time in a recombinant host. Perhaps, further characterisation of HAS UDP-sugar binding and transferase activity may provide avenues for enzyme engineering allowing for additional advances in HA production and insights into MW control of this important biological polymer.

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