

Governing Board of the Menzies School of Health Research. While the Menzies Research Institute was initially linked to Sydney University, Val was an important figure supporting the formation and running of the new Charles Darwin University. She became chairman of both Community Radio 8 TopFM and the Australian South East Asian Rehabilitation Foundation, which sends medical teams to Timor and Flores. In 2002 she chaired the Task Force on Illicit Drugs in the Northern Territory, commissioned by the Northern Territory Legislative Assembly.

Over her working life, Val has given great service to microbiology and to the Australian Society for Microbiology. She was Treasurer of the ASM National Council 1978–1984, then Membership Secretary 1984–1986. She edited six editions of *Recent Advances in Micro-*

biology, then became Chairman of the Northern Territory branch of the ASM. She also convened the Trust to raise \$2m for scholarships for young microbiologists. Portions of her interviews are in the Golden Jubilee issue of *Microbiology Australia*.

Dr Valerie Asche was an extraordinary woman, participating deeply in many aspects of life, especially in the Northern Territory. Scientist, community leader, craftswoman and friend to many. Her awards include: Distinguished Service Award Australian Society of Microbiology 1991; Dame of the Order of St John of Jerusalem 1993; Women's Achievement Award for Outstanding Contribution to Northern Territory 1998; Distinguished Service Award of Australia 2000; Senior Australian of Year 2000; and Member of the Order of Australia 2001.

An updated view on bacterial glycogen structure

Liang Wang and Michael J Wise

The authors advise that in Figure 1 of their published article (*Microbiology Australia*, Volume 40, Issue 4, pages 195–199, doi:10.1071/MA19056) they linked GlgE directly with branched (glucosyl units)_n. In fact, GlgE should work together with GlgB to synthesise glycogen. In addition, although Rv3032 was initially postulated to have a possible role in glycogen metabolism, recent study has shown no detectable evidence to support this postulation. The authors apologise for this error and state that this does not change the scientific conclusions of the article in any way. The correct Figure 1 is shown below.

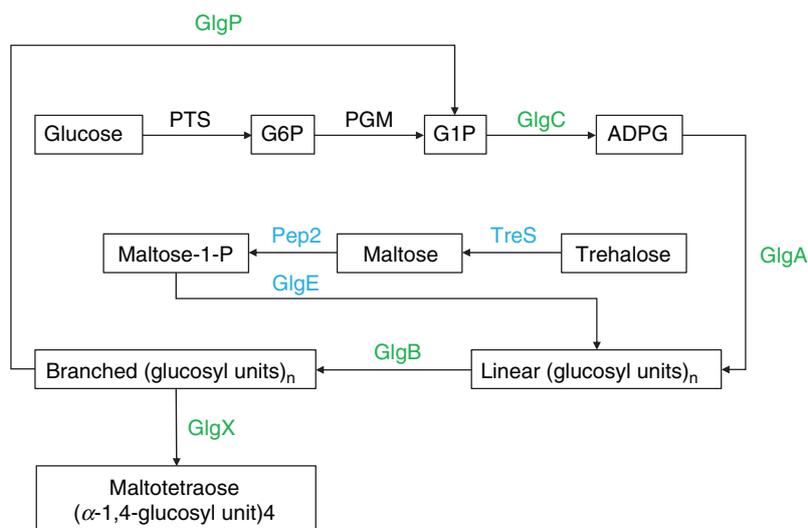


Figure 1. Schematic illustration of classical and non-classical glycogen metabolism pathways⁵. PTS, phosphotransferase system; PGM, phosphoglucomutase; G6P, glucose-6-phosphate; G1P, glucose-1-phosphate; ADPG, ADP-glucose.

An updated view on bacterial glycogen structure



Liang Wang

Department of Bioinformatics, School of Medical Informatics and Engineering, Xuzhou Medical University, Xuzhou, Jiangsu 221000, China
 Jiangsu Key Laboratory of New Drug Research and Clinical Pharmacy, School of Pharmacy, Xuzhou Medical University, Xuzhou, Jiangsu 221000, China
 Key Laboratory of Carbohydrate Chemistry and Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi, Jiangsu 214122, China
 Email: leonwang@xzhmu.edu.cn



Michael J Wise

The Marshall Centre for Infectious Diseases Research and Training
 University of Western Australia
 Perth, WA 6009, Australia
 Computer Science and Software Engineering, Faculty of Engineering and Mathematical Sciences
 University of Western Australia
 Perth, WA 6009, Australia

Glycogen is a homogenous and multi-disperse polysaccharide that is present in many clinically significant bacteria, such as *Escherichia coli*, *Vibrio cholera* and *Mycobacterium tuberculosis*. Its structure and metabolism have been linked with environmental viability, intracellular growth, pathogenicity and transmission capacity. However, due to the harsh extraction conditions and also the inconsistent methods for structure characterisation, understanding of bacterial glycogen structure and its association with bacterial metabolism and physiology has been hindered. Here we gave a concise overview of bacterial glycogen structure with a focus on its recently discovered higher level organisation, α particle. Standardised procedures for glycogen extraction and structure detection are also highlighted.

Glycogen metabolism

Glycogen is a widespread homogeneous polysaccharide and plays important roles in bacterial energy reserves and carbon supply with little effect on cellular osmolarity¹. Experimental studies have linked glycogen to bacterial environmental survival, intracellular growth, pathogenicity, transmission, etc., although controversial observations exist². Since glycogen plays a central role in bacterial carbohydrate metabolism, its synthesis and degradation are highly regulated. A systematic analysis of 1202 bacterial species proteomes confirmed that 402 of them harbour a complete set of enzymes in the classical glycogen metabolism pathway³. This pathway involves five enzymes operating concurrently to promote glycogen synthesis: glucose-1-phosphate adenylyltransferase (GlgC), glycogen synthase (GlgA), glycogen branching enzyme (GlgB); and degradation: glycogen phosphorylase (GlgP), and glycogen debranching enzyme (GlgX)¹. A second non-classical and widespread glycogen synthesis pathway was unexpectedly found in *Mycobacterium*

tuberculosis, which involves trehalose synthase (TreS), maltokinase (Mak1 or Pep2) and maltosyltransferase (GlgE)⁴. This pathway established a solid connection among glucose, trehalose, maltose and glycogen metabolism. Another branched α -glucan pathway involves two paralogues, glycogen synthase (Rv3032) and glycogen branching enzyme (Rv3031), which generates methyl-glucose lipopolysaccharide and is associated with fatty acid metabolism (Figure 1)^{4,5}. However, its connection with cytosolic glycogen metabolism is still under investigation. A comprehensive genome-wide screening of genes affecting glycogen accumulation, based on 3985 single-gene knockout mutants of nonessential genes in *E. coli* K-12 (Keio Collection), confirmed that 35 genes were related with glycogen-excess phenotypes while 30 genes were related with glycogen-deficient phenotypes⁶. Of the 65 gene products, their functions are mainly involved in direct glycogen synthesis and/or degradation, energy production, amino acid provision and cell envelope integrity⁶. Another systematic study using *E. coli* gene expression library ASKA observed that upregulation of 86 genes could influence glycogen accumulation⁷. The genes fell into the functional categories of general stress and stringent responses, aggregative and social behaviour, and intracellular communication, etc.⁷. Thus, glycogen metabolism is a highly interconnected process with a wide variety of cellular interactions. Among those sophisticated interactions, glycogen breakdown plays an important role in the interactions between the host and pathogenic bacteria, though the mechanisms and roles of glycogen during infection are still not fully elucidated¹.

Glycogen structure

Glycogen is characterised by a hyperbranched structure with α -1,4-glycosidic linkages at the linear chains and α -1,6-glycosidic linkages at the branching points¹. As an efficient energy storage

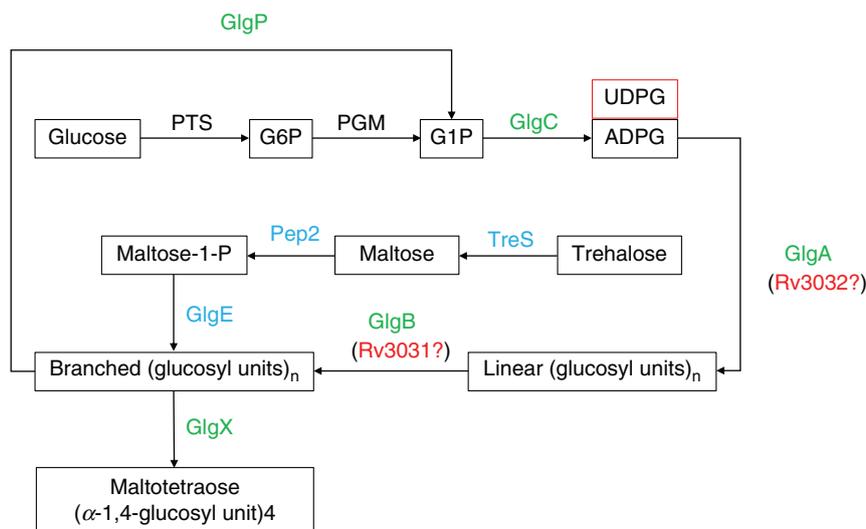


Figure 1. Schematic illustration of classical and non-classical glycogen metabolism pathways⁵. Rv3032 can use both ADPG and UDPG as substrates. PTS, phosphotransferase system; PGM, phosphoglucosylmutase; G6P, glucose-6-phosphate; G1P, glucose-1-phosphate; ADPG, ADP-glucose; UDPG, UDP-glucose.

molecule, a good structure-function relationship should be achieved for optimally providing energy and carbon source⁸. Although multiple models have been proposed to describe glycogen structure, such as the classical Whelan model, fractal structure model, and the newly proposed Monte-Carlo simulation, there is no settled model that accurately describes the polydisperse structures of glycogen, including particle size and chain length distributions, etc.^{3,9}. Currently, three structural levels have been defined in glycogen found in the liver of higher organisms, which are (1) linear α -D-glucosyl chains through α -1,4-glycosidic bonds, (2) highly branched β particles of around 20 nm in diameter, and (3) β -particle-aggregated rosette-shaped α particles up to 300 nm in diameter¹⁰. However, the molecular basis for the aggregation of β particles into α particles is still under investigation. Glycogen particles are also associated with a group of proteins (known as the glycogen proteome) that regulate its biological functions¹¹. Proteomic analysis of the rat, mouse and human liver inferred that a self-glycosylating homodimer protein, glycogenin, is the binding agent on the surface of β particles to form α particles¹². Intriguingly, no coding genes for glycogenin or its homologs have ever been identified in bacterial genomes; it is glycogen synthase that is involved in glucan initiation and elongation in bacteria¹³.

Until recently, only β particles were reported in bacteria, containing up to 55 000 glucosyl units with molecular mass of around 10^7 – 10^8 Daltons and varying in diameter from 20 to 50 nm¹. The average length of linear chains, g_c , in β particles varies between 6.6 to 23.5 glucose units in a species-dependent manner³. Correlation analysis between g_c and bacterial environmental viability showed that bacteria accumulating glycogen with short g_c have greater survival times in the external environment³. It was argued that short

g_c glycogen could lead to the formation of more compact β particles with higher branching degrees^{3,14}. These properties facilitate the slow utilisation of glycogen by hindering its degradation¹⁴, enhancing bacterial survivability³. Although maintaining glycogen structure requires a coordinated action of enzymatic activities and different structural proteins, GlgB plays a dominant role in chain length distribution patterns and corresponding branching degrees¹⁵. Recent analysis showed that domain organisation of GlgB is conserved, which consists of an N-terminus, a central (β/α)-barrel region and a C-terminus¹⁵. Based on N-terminal length variation, two groups of glycogen branching enzymes (GBEs) were initially identified: Group 1 with duplicated CBM48 domains (N1 and N2 domains) and Group 2 with a single CBM48 domain (N2 domains)¹⁶. A longer GBE N-terminus was further found to contribute to the transfer of shorter side chains, leading to shorter g_c glycogen¹⁷. A large comparison of 9387 bacterial GlgBs revealed that there might exist a third group with a longer N-terminus (N0, N1, and N2 domains) than that in Group 1 GlgBs (Figure 2)¹⁵. Thus, N-terminal diversity could be one of the determinant factors for the polydispersity of glycogen structures leading to the heterogeneity of degradation rates. However, experimental evidence is needed to validate the hypothesis.

Glycogen extraction methods

A variety of extraction methods have been used for bacterial glycogen isolation, such as the harsh boiling method with 30% potassium hydroxide solution (KOH-HW)¹⁸ and the comparatively mild trichloroacetic acid extraction methods with cold or hot water (TCA-CW or TCA-HW)¹⁹. Less commonly used reagents such as sodium dodecyl sulfate (SDS), thymol or glycine for bacterial

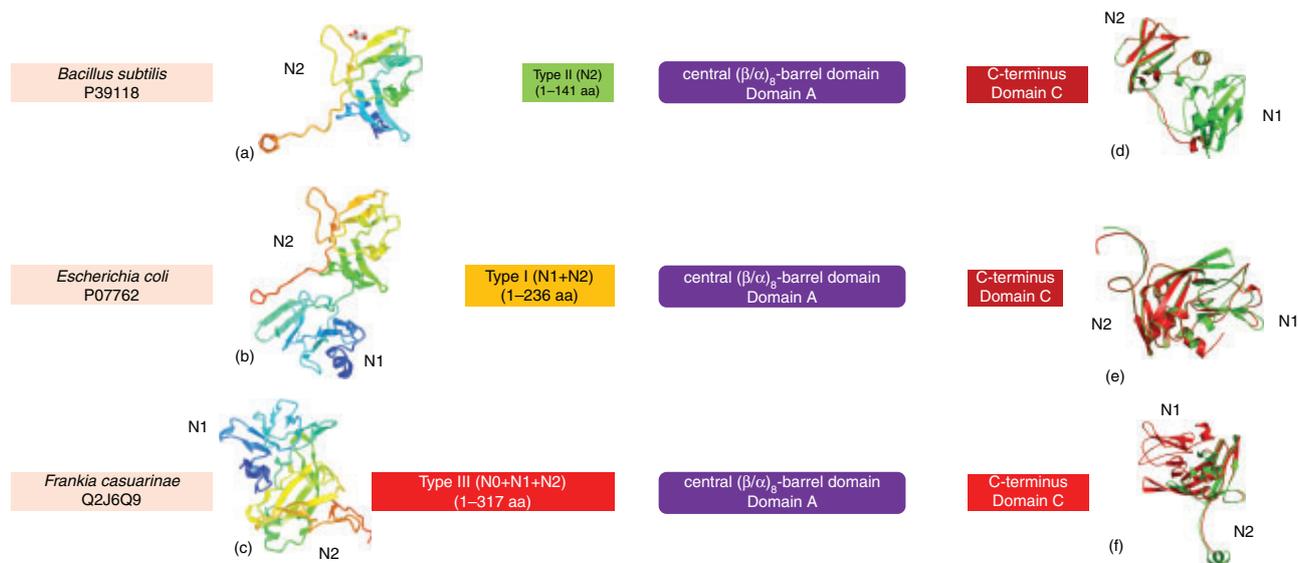


Figure 2. Three types of representative glycogen branching enzymes from *Escherichia coli* (Type 1 GlgB), *Bacillus subtilis* (Type 2 GlgB) and *Frankia casuarinae* (Type 3 GlgB)¹⁵. The N-terminal 3D structures of GlgBs (a–c) and corresponding pair-wise superimpositions – (d) *Escherichia coli* (green) and *Bacillus subtilis* (red), (e) *Escherichia coli* (green) and *Frankia casuarinae* (red), (f) *Bacillus subtilis* (green) and *Frankia casuarinae* (red) – are presented accordingly. Reprinted and modified with permission from Wang *et al.*¹⁵.

glycogen extraction have also been used in sporadic studies^{20–22}. Harsh extraction procedures with alkali, acid, and/or heat can degrade glycogen primary and tertiary structures²³, which could compromise experimental results and conclusions. On the other hand, the use of diverse extraction methods makes it difficult to compare glycogen structures due to the lack of uniform procedures. This is particularly the case with the fragile glycogen-associated proteins if one aims to investigate the functions of glycogen-associated proteomes. In higher organisms, sucrose density gradient ultracentrifugation with cold water (SDGU-CW) has been confirmed as less- or non-degradative method and is regularly used for glycogen extraction with the purpose of α particle study or glycogen proteomic analysis²³. SDGU-CW was previously used for glycogen extraction in *Selenomonas ruminantium* and *Fibrobacter succinogenes*^{20,24}. Interestingly, glycogen morphology via transmission electron microscopy (TEM) in both studies showed the possible existence of rosette-shaped α particles in bacteria, although this phenomenon did not garner much attention^{20,24}. Our lab recently explored this issue by comparing different extraction methods and confirmed the existence of bacterial glycogen α particles extracted via the SDGU-CW method in *E. coli* (Figure 3)⁹. Thus, we suggested that the mild SDGU-CW extraction method should be adopted as a standard method in bacterial glycogen study so as to reveal genuine comparative structural features. In addition, the discovery of glycogen α particles in both prokaryotes and eukaryotes indicates that any organism needing to store and then release glucose might have similar α and β particle structures: a type of convergent evolution, regardless of the presence of glycogenin⁹. Since α particles have been linked with release rate of glucose during degradation stage, further exploration of the formation

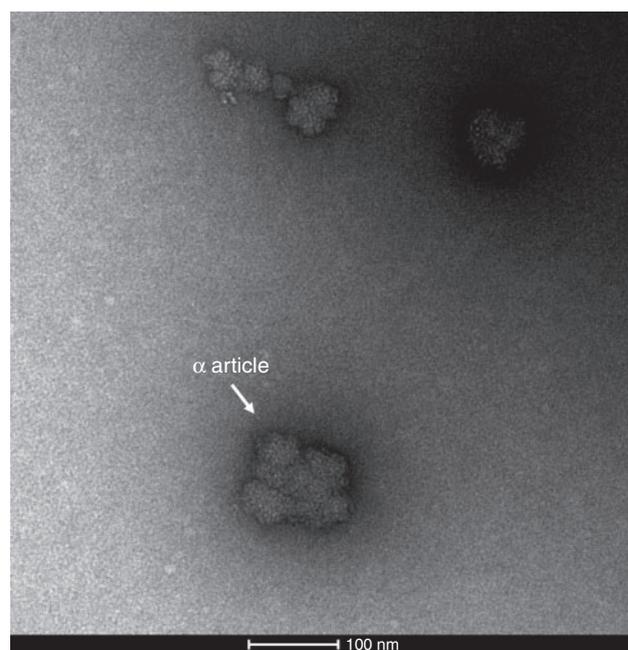


Figure 3. TEM image of a typical bacterial glycogen α particle extracted via the SDGU-CW method.

mechanisms of glycogen α particles in bacteria would be essential for better understanding its functions in bacterial physiology and pathogenicity. Relevant questions to ask include whether under certain circumstances more α particles will be synthesised while β particles will be more preferred in other situations based on the regulation of the activities and contents of α -particle forming enzyme(s).

Glycogen structural characterisation

Glycogen structure can be measured from different aspects, such as morphology, branching degree (percentage of α -1, 6-glycosidic

linkages), branch length distribution, and particle size distribution, etc. Glycogen morphology, such as α and β particles, can be most easily characterised using TEM⁹. Distributions of glycogen sizes can also be generated via histograms based on TEM images. However, with the latter method it is rather difficult to achieve quantitative results and it cannot give genuine particle sizes. Periodate oxidation is a commonly used method in the structural study of non-ionic polysaccharides in carbohydrate chemistry. The methods are done using sodium metaperiodate as the oxidising agent, and the presence of α -1, 6-glycosidic linkages can be confirmed by the free hydroxyl groups resulting from the consumption of periodate ions during the periodate oxidation reaction²⁵. From this, glycogen branching degree can be calculated. In addition, methylation²⁶, reducing end assay¹⁹, and nuclear magnetic resonance (NMR)²⁷ can also be used for measuring glycogen branching degree. In terms of chain lengths, iodine staining was originally used with the aid of spectrophotometry. It was shown that polysaccharide chains consisting of 8 to 12 glucose units stain a reddish colour at the wavelength of 520 nm, while at a length of 30 to 35 units, the stain appears blue, with a peak at 600 nm, similar to amylose²⁸. Although multiple studies have used this method to infer glycogen branching features, the method has mainly been applied to the analysis of amylose and amylopectin due to the weak interaction of glycogen with iodine²⁹. On the other hand, fluorophore-assisted capillary electrophoresis (FACE) is an advanced method for accurately quantifying the chain-length distributions of isoamylase-treated glycogen particles¹⁹. The average chain length of glycogen can be calculated based on percentages of oligosaccharides with specific degrees of polymerisation generated via FACE³. Until recently, FACE has been the only method that can give an accurate description of glycogen primary structure.

Size exclusion chromatography (SEC), together with a differential refractive index (DRI) detector, separates glycogen solely on hydrodynamic volumes V_b or the corresponding radius R_b ³⁰. With a multiple-angle laser light scattering (MALLS) detector, weight-average molecular weight (M_w) can be measured and molecular density of glycogen particles calculated as $\rho(R_b) = M_w(R_b) \times 4/3\pi R_b^3$. Thus, this method gives a quantitative overview of glycogen particle size distribution and should be preferred for glycogen analysis. The method can also be used for detecting fragility and stability of glycogen α particles. That is, fragile α particles are easily degraded into β particles after treatment with hydrogen bond disruptors like dimethyl sulfoxide (DMSO), which is reflected in the shift of glycogen particles toward smaller sizes on SEC graphs¹⁰. Our recent study also shows that both fragile and stable α particles exist in *E. coli* with unclear functions (Figure 4)⁹.

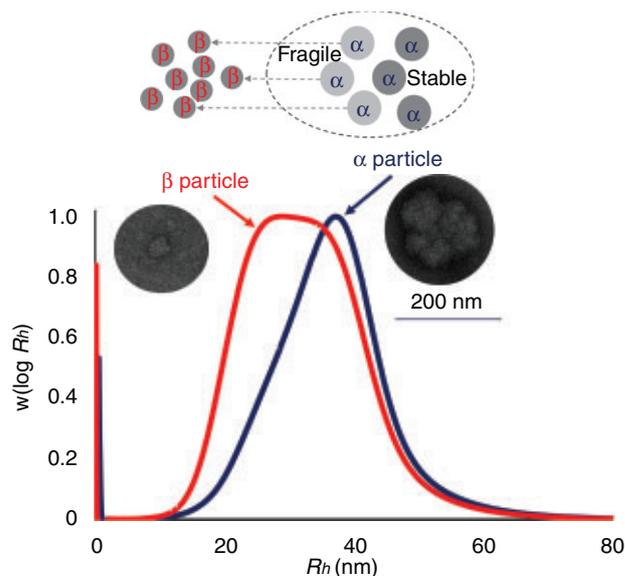


Figure 4. Schematic illustration of glycogen stability and fragility, together with SEC weight distributions, $w(\log R_h)$ (arbitrary units), in terms of particle sizes for *E. coli* BL21(DE3) glycogen extracted through the SDGU-CW method. Blue line: sample treated with water. Red line: sample treated with DMSO. Reprinted and modified with permission from Wang *et al.*⁹.

Summary and future perspectives

Glycogen is a central energy reserve in bacteria. Understanding the interactions between glycogen structure and metabolism has apparent clinical significance due to its associations with viability and virulence of bacterial pathogens. Lack of standardised extraction procedures and detection methods hinders the comprehensive understanding of glycogen functions across bacterial species. The pros and cons of various glycogen extraction procedures and detection methods were compared in recent studies and the SDGU-CW method is recommended for glycogen isolation due to its comparatively mild extraction conditions. On the other hand, SEC is suggested for measuring size, weight, and density of glycogen particles. In addition, TEM is recommended for studying glycogen morphology, while FACE is most suitable for dissecting glycogen primary structure. Most importantly, the discovery of fragile and stable glycogen α particles in bacteria creates a new avenue in the bacterial glycogen research field, from where it would be interesting for us to investigate how glucose concentration in the culture influences bacterial glycogen α particles formation, how glycogen α particles change in different stages of bacterial life, and what proteins are responsible for the formation and fragility of α particles in bacteria, etc. These studies might lead us to a better understanding of how glycogen contributes to bacterial environmental survival, intracellular growth, pathogenicity and transmission.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

This work was supported by the Excellent Researcher's Startup Foundation at Xuzhou Medical University (No. D2016007), Natural Science Foundation of Jiangsu Province (No. BK20180997), the Innovative and Entrepreneurial Talent Scheme in Jiangsu Province (2017), National Natural Science Foundation of China (No. 31900022), and the Open Project from the Key Laboratory of Carbohydrate Chemistry and Biotechnology, Ministry of Education at Jiangnan University (No. KLCCB-KF201902).

References

- Park, K.H. (2015) Roles of enzymes in glycogen metabolism and degradation in *Escherichia coli*. *J. Appl. Glycosci.* **62**, 37–45. doi:10.5458/jag.jag.JAG-2015_005
- Wilson, W.A. *et al.* (2010) Regulation of glycogen metabolism in yeast and bacteria. *FEMS Microbiol. Rev.* **34**, 952–985. doi:10.1111/j.1574-6976.2010.00220.x
- Wang, L. and Wise, M.J. (2011) Glycogen with short average chain length enhances bacterial durability. *Naturwissenschaften* **98**, 719–729. doi:10.1007/s00114-011-0832-x
- Chandra, G. *et al.* (2011) Unexpected and widespread connections between bacterial glycogen and trehalose metabolism. *Microbiology* **157**, 1565–1572. doi:10.1099/mic.0.044263-0
- Wang, L. *et al.* (2019) Bioinformatics analysis of metabolism pathways of archaeal energy reserves. *Sci. Rep.* **9**, 1034. doi:10.1038/s41598-018-37768-0
- Eydallin, G. *et al.* (2007) Genome-wide screening of genes affecting glycogen metabolism in *Escherichia coli* K-12. *FEBS Lett.* **581**, 2947–2953. doi:10.1016/j.febslet.2007.05.044
- Eydallin, G. *et al.* (2010) Genome-wide screening of genes whose enhanced expression affects glycogen accumulation in *Escherichia coli*. *DNA Res.* **17**, 61–71. doi:10.1093/dnares/dsp028
- Meléndez, R. *et al.* (1998) Physical constraints in the synthesis of glycogen that influence its structural homogeneity: a two-dimensional approach. *Biophys. J.* **75**, 106–114. doi:10.1016/S0006-3495(98)77498-3
- Wang, L. *et al.* (2019) Molecular structure of glycogen in *Escherichia coli*. *Biomacromolecules* **20**, 2821–2829. doi:10.1021/acs.biomac.9b00586
- Hu, Z. *et al.* (2018) Diurnal changes of glycogen molecular structure in healthy and diabetic mice. *Carbohydr. Polym.* **185**, 145–152. doi:10.1016/j.carbpol.2018.01.003
- Stapleton, D. *et al.* (2010) Analysis of hepatic glycogen-associated proteins. *Proteomics* **10**, 2320–2329. doi:10.1002/pmic.200900628
- Tan, X. *et al.* (2018) Proteomic investigation of the binding agent between liver glycogen β particles. *ACS Omega* **3**, 3640–3645. doi:10.1021/acsomega.8b00119
- Ugalde, J.E. *et al.* (2003) De novo synthesis of bacterial glycogen: *Agrobacterium tumefaciens* glycogen synthase is involved in glucan initiation and elongation. *Proc. Natl. Acad. Sci. USA* **100**, 10659–10663. doi:10.1073/pnas.1534787100
- Martinez-Garcia, M. *et al.* (2017) The glycogen of *Galdieria sulphuraria* as alternative to starch for the production of slowly digestible and resistant glucose polymers. *Carbohydr. Polym.* **169**, 75–82. doi:10.1016/j.carbpol.2017.04.004
- Wang, L. *et al.* (2019) Structure and evolution of glycogen branching enzyme N-termini from bacteria. *Front. Microbiol.* **9**, 3354. doi:10.3389/fmicb.2018.03354
- Leggio, L.L. *et al.* (2002) A structural model for the N-terminal N1 module of *E. coli* glycogen branching enzyme. *Biologia* **57**, 10.
- Devillers, C.H. *et al.* (2003) Characterization of the branching patterns of glycogen branching enzyme truncated on the N-terminus. *Arch. Biochem. Biophys.* **418**, 34–38. doi:10.1016/S0003-9861(03)00341-2
- Lou, J. *et al.* (1997) Glycogen formation by the ruminal bacterium *Prevotella ruminicola*. *Appl. Environ. Microbiol.* **63**, 1483–1488.
- Wang, L. *et al.* (2015) Influence of *in situ* progressive N-terminal is still controversial truncation of glycogen branching enzyme in *Escherichia coli* DH5 α on glycogen structure, accumulation, and bacterial viability. *BMC Microbiol.* **15**, 96. doi:10.1186/s12866-015-0421-9
- Kamio, Y. *et al.* (1981) Structure of glycogen produced by *Selenomonas ruminantium*. *Agric. Biol. Chem.* **45**, 209–216. doi:10.1271/bbb1961.45.209
- Sambou, T. *et al.* (2008) Capsular glucan and intracellular glycogen of *Mycobacterium tuberculosis*: biosynthesis and impact on the persistence in mice. *Mol. Microbiol.* **70**, 762–774. doi:10.1111/j.1365-2958.2008.06445.x
- Whyte, J.N.C. and Strasdine, G.A. (1972) An intracellular α -D-glucan from *Clostridium botulinum*, type E. *Carbohydr. Res.* **25**, 435–441. doi:10.1016/S0008-6215(00)81655-9
- Tan, X. *et al.* (2016) A new non-degradative method to purify glycogen. *Carbohydr. Polym.* **147**, 165–170. doi:10.1016/j.carbpol.2016.04.009
- Gong, J. and Forsberg, C.W. (1993) Separation of outer and cytoplasmic membranes of *Fibrobacter succinogenes* and membrane and glycogen granule locations of glycanases and cellobiase. *J. Bacteriol.* **175**, 6810–6821. doi:10.1128/jb.175.21.6810-6821.1993
- Northcote, D.H. (1953) The molecular structure and shape of yeast glycogen. *Biochem. J.* **53**, 348–352. doi:10.1042/bj0530348
- Manners, D.J. (1989) Some aspects of the structure of starch and glycogen. *J. Jpn. Soc. Starch Sci.* **36**, 311–323. doi:10.5458/jag1972.36.311
- Sillerud, L.O. and Shulman, R.G. (1983) Structure and metabolism of mammalian liver glycogen monitored by carbon-13 nuclear magnetic resonance. *Biochemistry* **22**, 1087–1094. doi:10.1021/bi00274a015
- Swanson, M.A. (1948) Studies on the structure of polysaccharides: IV. Relation of the iodine color to the structure. *J. Biol. Chem.* **172**, 825–837.
- Craig, S.A.S. *et al.* (1988) The iodine-staining properties and fine structure of some mammalian and invertebrate glycogens. *Carbohydr. Res.* **179**, 327–340. doi:10.1016/0008-6215(88)84129-6

Biographies

Associate Professor Liang Wang graduated with a PhD degree from the University of Western Australia in 2014, under the supervision of Associate Professor Michael J Wise and Associate Professor Charlene M Kahler. He worked as a postdoctoral researcher at Concordia University (Montreal, Canada) and then a research associate at Curtin University (Perth, Australia). He is currently the director of the Department of Bioinformatics at Xuzhou Medical University, China. He is also an adjunct researcher at Jiangsu Key Laboratory of New Drug Research and Clinical Pharmacy. Dr Wang was the recipient of the 2019 Australia-China *Helicobacter* Research Fellowship (ACHRF) funded by the Australia-China Council, Department of Foreign Affairs and Trade, Australia.

Associate Professor Michael J Wise completed a double degree in Engineering and Arts and a PhD in electrical engineering at the University of New South Wales. He then worked for the University Technology, Sydney for two years before lecturing in Computer Science at the University of Sydney. There he created computer software for use in plagiarism detection, but then realised that his programs had an alternative application for sequence alignment, which prompted an interest in bioinformatics. Professor Wise was subsequently employed as a Senior Research Fellow at Pembroke College in Cambridge. In 2004 he moved to The University of Western Australia and discovered the world of microbes.