Extracellular Polymeric Substances of "Candidatus Accumulibacter"

Composition, application and turnover

Tomas Martinez, S.

DOI
10.4233/uuid:88ce92a5-153e-47e4-bb21-999237161ab7

Publication date
2023

Document Version
Final published version

Citation (APA)
Tomas Martinez, S. (2023). *Extracellular Polymeric Substances of "Candidatus Accumulibacter": Composition, application and turnover*. [Dissertation (TU Delft), Delft University of Technology]. https://doi.org/10.4233/uuid:88ce92a5-153e-47e4-bb21-999237161ab7

Extracellular Polymeric Substances of "*Candidatus* Accumulibacter"

Composition, application and turnover

Sergio Tomás Martínez

Propositions

accompanying the dissertation

Extracellular Polymeric Substances of "*Candidatus* Accumulibacter"

Composition, application and turnover

by

Sergio Tomás Martínez

1. The widespread presence of nonulosonic acids among non-pathogenic prokaryotes, including "*Ca.* Accumulibacter", questions the role of nonulosonic acids. (Chapter 2)
2. The similarities of some components of the extracellular polymeric substances (EPS) with mucin-derived products, opens up the potential of microbial EPS as source of these products. (Chapter 3)
3. The observation that the turnover of EPS is similar to the turnover of intracellular proteins challenges the believe of a targeted consumption of EPS as nutrient source. (Chapter 5)
4. There is no ideal microbial quantification technique. One should choose accordingly depending on the goal of the project. (This thesis)
5. The similarities between multicellular tissues and microbial biofilms can serve as inspiration for the study of EPS.
6. Overinterpretation of original results in review papers leads to a misinformation cascade effect.
7. The fact that the EPS acronym is used for extracellular polymeric substances and for exopolysaccharides can underestimate the complexity of the extracellular matrix.
8. EPS components are like Pokémon, whenever we start to know them all, new ones are discovered.
9. Diversity in the workplace is necessary to avoid the feeling of "onlyness" of minorities, even if that requires the establishment of quotas.
10. Growing up gay creates a fear of "disappointing" the people around you, which, in many cases, leads to the search of personal success as compensation.

These propositions are regarded as opposable and defendable, and have been approved as such by the promotor prof. dr. ir. M. C. M. van Loosdrecht, copromotor prof. dr. ir. D. G. Weissbrodt and copromotor dr. Y. Lin.

Extracellular Polymeric Substances of "*Candidatus* Accumulibacter"

Composition, application and turnover

Extracellular Polymeric Substances of "*Candidatus* Accumulibacter"

Composition, application and turnover

Dissertation

for the purpose of obtaining the degree of doctor
at Delft University of Technology,
by the authority of the Rector Magnificus, prof. dr. ir. T.H.J.J. van der Hagen,
chair of the Board of Doctorates
to be defended publicly on
Friday 20 January 2023 at 10:00 o'clock

by

Sergio Tomás Martínez

Master of Science in Life Science and Technology,
Delft University of Technology, the Netherlands,
born in Villena, Spain

This dissertation has been approved by the

Promotor: prof. dr. ir. M.C.M. van Loosdrecht
Copromotor: prof. dr. ir. D.G. Weissbrodt
Copromotor: dr. Y. Lin

Composition of the doctoral committee:

Rector Magnificus	chairperson
prof. dr. ir. M.C.M. van Loosdrecht	Delft University of Technology, promotor
prof. dr. ir. D.G. Weissbrodt	Norwegian University of Science and Technology, Norway former Delft University of Technology, copromotor
dr. Y. Lin	Delft University of Technology, copromotor

Independent members:

prof. dr. G.H. Koenderink	Delft University of Technology
prof. dr. ir. H.J. Busscher	University of Groningen
prof. dr. M.A.M. Reis	Universidade Nova de Lisboa, Portugal
dr. T. Wennekes	Utrecht University
prof. dr. U. Hanefeld	Delft University of Technology, reserve

The research presented in this thesis was performed at the Environmental Biotechnology Section, Department of Biotechnology, Faculty of Applied Sciences, Delft University of Technology, The Netherlands. This work is part of the research project "Nature inspired biopolymer nanocomposites towards a cyclic economy" (Nanocycle) funded by the programme Closed cycles – Transition to a circular economy (grant no. ALWGK.2016.025) of the Earth and Life Sciences Division of the Dutch Research Council (NWO).

Printed by Proefschriftspecialist

Cover illustration and design : Sergio Tomás Martínez and Quentin Aubry

ISBN: 978-94-6384-401-7

An electronic version of this dissertation is available at http://repository.tudelft.nl/

A mi familia

Contents

Summary

The majority of bacteria grow in the form of microbial aggregates known as biofilms. In these biofilms, microorganisms are embedded in a mixture of extracellular polymeric substances (EPS) produced by the microorganisms themselves. EPS is a complex mixture of biopolymers of different nature, such as polysaccharides, proteins, nucleic acids or lipids, among others. In spite of the significant progress over the last decades, EPS is still a black box waiting to be opened, in terms of specific composition, function, structure and production.

Biofilms have great importance in many environmental engineering processes, as for example, aerobic granular sludge (AGS). AGS is a novel biological wastewater treatment where microorganisms are stimulated to form compact granules. Among the complex microbial community in AGS, polyphosphate accumulating organisms (PAOs) are of great importance, due to their role in phosphate removal and granule stabilization. Because of their dominance in AGS and their rapid anaerobic carbon sequestration, they are assumed to be the main EPS producer in AGS. Therefore, PAOs (specifically the well-studied "*Candidatus* Accumulibacter phosphatis") can be used as model microorganism for the study of EPS of AGS.

The goal of this thesis is to study the EPS of "*Ca.* Accumulibacter" in terms of specific composition, application and synthesis/consumption. A better characterization of the EPS of "*Ca.* Accumulibacter" will lead to a comprehensive understanding of this microorganism and further optimization of the granular sludge processes, and their application.

In **chapter 2** the presence of nonulosonic acids (NulOs) in the EPS of "*Ca.* Accumulibacter" was confirmed. Fluorescence lectin-binding analysis in granules enriched with "*Ca.* Accumulibacter" revealed a wide distribution of NulOs across the granules, mainly detected at the surface of the bacterial cells. Mass spectrometry showed a variety of NulOs in the EPS. Phylogenetic analysis of the NulOs biosynthetic pathway of "*Ca.* Accumulibacter" confirmed its potential to produce different types of NulOs. Additionally, proteomic analysis showed the ability of "*Ca.* Accumulibacter" to reutilize and reincorporate NulOs into its EPS.

Chapter 3 aimed to locate NulOs within the EPS of "*Ca.* Accumulibacter". After EPS extraction, and subsequent separation by size exclusion chromatography, different molecular weight (MW) fractions were obtained. This separation resulted in two high MW fractions dominated by polysaccharides with a NulO content up to four times higher than the extracted EPS, suggesting that NulOs in "*Ca.* Accumulibacter" are likely located in high MW polysaccharides. Additionally, it was seen that the extracted EPS and the NulO-rich fractions can bind and neutralize histones. This suggest that they can serve as source for sepsis treatment drugs.

In **chapter 4**, we examined the catabolism of sialic acids in the microbial community dominated by "*Ca.* Accumulibacter" using protein alignment tools. The results showed the ability of *Clostridium* to release sialic acids from the glycan chains by the action of a sialidase. *Clostridium* and *Chryseobacterium* can take up free sialic acids and utilize them as nutrient. These results display similarities with the catabolism of sialic acids by the gut microbiota.

Chapter 5 focused on the turnover of EPS of "*Ca.* Accumulibacter" in terms of polysaccharides and proteins. A stable enrichment of "*Ca.* Accumulibacter" was fed with ^{13}C-labelled acetate and the label incorporation in proteins and polysaccharides was analyzed over time. The results showed an average turnover rate of sugars and proteins higher than the expected value based on solid removal rate. Additionally, no significant difference was observed between intracellular and secreted proteins. These findings indicated that the EPS from the "*Ca.* Accumulibacter" enriched community is not selectively degraded by flanking populations under stable process conditions. Instead, we observed a general decay of biomass.

The main findings from this thesis are summarized in **chapter 6**, which also provides an outlook for future work following the results from this research to further expand the known area in EPS research.

SAMENVATTING

De meeste bacteriën groeien in de vorm van microbiële aggregaten die bekend staan als biofilms. In deze biofilms zijn micro-organismen ingebed in een mengsel van extracellulaire polymerische substanties (EPS) die door de micro-organismen zelf worden geproduceerd. EPS is een complex mengsel van biopolymeren van verschillende aard, zoals onder andere polysachariden, eiwitten, nucleïnezuren of lipiden. Ondanks de aanzienlijke vooruitgang in de afgelopen decennia, is EPS nog steeds een zwarte doos die wacht om open te gaan, wat betreft de specifieke samenstelling, functie, structuur en productie.

Biofilms zijn van groot belang in veel milieutechnologische processen, zoals aeroob korrelslib (AGS). AGS is een nieuwe biologische afvalwaterzuiverings technologie waarbij micro-organismen worden gestimuleerd om compacte korrels te vormen. Binnen de complexe microbiële gemeenschap in AGS zijn polyfosfaat accumulerende organismen (PAO's) van groot belang, vanwege hun rol bij fosfaatverwijdering en korrelstabilisatie. Vanwege hun dominantie in AGS en hun snelle anaërobe koolstofvastlegging, worden zij aangenomen als de belangrijkste EPS-producent in AGS. Daarom kunnen PAO's (met name de goed bestudeerde "*Candidatus* Accumulibacter phosphatis") worden gebruikt als modelmicro-organismen voor het onderzoek van EPS in AGS.

Het doel van dit proefschrift is het bestuderen van de EPS van "*Ca.* Accumulibacter" wat betreft despecifieke samenstelling, toepassing en synthese/verbruik. Een betere karakterisatie van de EPS van "*Ca.* Accumulibacter" zal leiden tot een uitgebreid begrip van dit micro-organisme en verdere optimalisatie van de korrelslibprocessen en de toepassing hiervan.

In **hoofdstuk 2** wordt de aanwezigheid van nonulosonic zuren (NulOs) in de EPS van "*Ca.* Accumulibacter" werd bevestigd. Fluorescentie-lectine-bindingsanalyse in korrels dominant met "*Ca.* Accumulibacter" onthulde een brede verdeling van NulOs over de korrels, voornamelijk gedetecteerd aan het oppervlak van de bacteriële cellen. Massaspectrometrie onthulde een verscheidenheid aan NulOs in de EPS. Fylogenetische analyse van de NulOs-biosynthetische route van "*Ca.* Accumulibacter" bevestigde zijn potentie om verschillende soorten NulOs te produceren. Bovendien toonde proteomische analyse de mogelijkheid van "*Ca.*

Accumulibacter" om NulOs opnieuw te gebruiken en opnieuw op te nemen in zijn EPS.

Hoofdstuk 3 doelde om NulOs te lokaliseren binnen de EPS van "*Ca.* Accumulibacter". Na EPS extractie en daaropvolgende scheiding door chromatografie op basis van de grootte werden fracties met verschillend molecuulgewicht (MW) verkregen. Deze scheiding resulteerde in twee fracties met een hoog MW die gedomineerd werden door polysachariden met een NulO-gehalte dat tot vier keer hoger is dan het geëxtraheerde EPS, wat suggereert dat de NulOs in "*Ca.* Accumulibacter" zich waarschijnlijk in polysachariden met een hoog MW bevinden. Bovendien werd geobserveerd dat de geëxtraheerde EPS en de NulO-rijke fracties histonen kunnen binden en neutraliseren. Dit suggereert dat ze kunnen dienen als bron voor geneesmiddelen voor de behandeling van sepsis.

In **hoofdstuk 4** onderzochten we het katabolisme van siaalzuren in de microbiële gemeenschap die gedomineerd wordt door "*Ca.* Accumulibacter" met behulp van hulpmiddelen voor het vergelijken van eiwit-sequenties. De resultaten toonden het vermogen van *Clostridium* om siaalzuren uit de glycaanketens vrij te maken door middel van een sialidase. *Clostridium* en *Chryseobacterium* kunnen vrije siaalzuren opnemen en als voedingsstof gebruiken. Deze resultaten vertonen overeenkomsten met het katabolisme van siaalzuren door de darmmicrobiota.

Hoofdstuk 5 richtte zich op de omzet van EPS van "*Ca.* Accumulibacter" in termen van polysachariden en eiwitten. Een stabiele verrijking van "*Ca.* Accumulibacter" werd gevoed met ^{13}C-gelabeld acetaat en de opname van het label in eiwitten en polysachariden werd over tijd geanalyseerd. De resultaten lieten een gemiddelde omloopsnelheid van suikers en eiwitten zien die hoger was dan de verwachte waarde op basis van de retentietijd van vaste stoffen. Bovendien werd er geen significant verschil waargenomen tussen intracellulaire en uitgescheiden eiwitten. Deze bevindingen gaven aan dat de EPS van de microbiële gemeenschap dominant met "*Ca.* Accumulibacter" niet selectief wordt afgebroken door flankerende populaties onder stabiele procesomstandigheden. In plaats daarvan zagen we een algemeen verval van biomassa.

De belangrijkste bevindingen van dit proefschrift zijn samengevat in **hoofdstuk 6**, dat ook een vooruitzicht biedt voor toekomstig werk opvolgend op de resultaten van dit onderzoek om het bekende gebied in EPS-onderzoek verder uit te breiden.

Resumen

La mayoría de las bacterias crecen en forma de agregados microbianos conocidos como biofilms o biopelículas. En estas biofilms, los microorganismos están integrados en una mezcla de sustancias poliméricas extracelulares (SPEs) producidas por los propios microorganismos. Las SPEs son una mezcla compleja de biopolímeros de distinta naturaleza, como polisacáridos, proteínas, ácidos nucleicos o lípidos, entre otros. A pesar de los importantes avances durante las últimas décadas, las SPEs son todavía una "caja negra" esperando ser abierta, en cuanto a composición específica, función, estructura y producción.

Las biofilms tienen una gran importancia en muchos procesos de ingeniería medioambiental, como por ejemplo, la biomasa granular aerobia (AGS). El AGS es un novedoso tratamiento de aguas residuales en el que los microorganismos son estimulados a formar gránulos compactos. Entre la compleja comunidad microbiana en el AGS, los organismos acumuladores de polifosfato (PAOs) tienen gran importancia, debido a su rol en la eliminación de fosfato y en la estabilización de los gránulos. Por su predominio en el AGS y su rápido secuestro del carbón anaeróbico, se les considera los principales productores de SPEs en el AGS. Por lo tanto, los PAOs (concretamente el bien estudiado "*Candidatus* Accumulibacter phosphatis") se pueden usar como microorganismos modelos para el estudio de las SPEs del AGS.

El objetivo de esta tesis es estudiar las SPEs de "*Ca.* Accumulibacter" en cuanto a composición específica, aplicación y síntesis/consumo. Una mejor caracterización de las SPEs de "*Ca.* Accumulibacter" llevará a un profundo entendimiento de este microorganismo y a una mayor optimización de los procesos de biomasa granular y su aplicación.

En el **capítulo 2** se confirmó la presencia de ácidos nonulosónicos (NulOs) en las SPEs de "*Ca.* Accumulibacter". El análisis de la unión de lectinas fluorescentes en gránulos enriquecidos con "*Ca.* Accumulibacter" reveló una amplia distribución de NulOs a través de los gránulos, detectados principal mente en la superficie de las células bacterianas. La espectrometría de masas mostró una variedad de NulOs en las SPEs. El análisis filogenético de la ruta biosintética de NulOs de "*Ca.* Äccumulibacter" confirmó su potencial para producir diferentes tipos de NulOs.

Además, el análisis proteómico mostró la capacidad de "*Ca.* Accumulibacter" para reutilizar y reincorporar NulOs en sus SPEs.

El objetivo del **capítulo 3** fue localizar los NulOs en las SPEs de "*Ca.* Accumulibacter". Tras la extracción de las SPEs y su subsecuente separación mediante cromatografía de exclusión por tamaño se obtuvieron fracciones de distinto peso molecular (MW). Esta separación resultó en dos fracciones de alto MW dominadas por polisacáridos y con un contenido de NulOs hasta cuatro veces mayor que las SPEs extraídas, lo que sugiere que los NulOs en "*Ca.* Accumulibacter" probablemente se encuentran en polisacáridos de alto MW. Además, se vio que las SPEs extraídas y las fracciones con más NulOs pueden unirse a histonas y neutralizarlas. Esto sugiere que pueden servir como fuente de fármacos para el tratamiento de sepsis.

En el **capítulo 4**, examinamos el catabolismo de los ácidos siálicos en la comunidad microbiana dominada por "*Ca.* Accumulibacter" usando herramientas de alineación de proteínas. Los resultados mostraron la capacidad de *Clostridium* para liberar ácidos siálicos de las cadenas de glicanos mediante la acción de una sialidasa. *Clostridium* y *Chryseobacterium* pueden tomar los ácidos siálicos libres y utilizarlos como nutrientes. Estos resultados muestran similitudes con el catabolismo de los ácidos siálicos en la microbiota intestinal.

El **capítulo 5** se centró en el recambio (o "turnover") de las SPEs de "*Ca.* Accumulibacter" en cuanto a polisacáridos y proteínas. Un enriquecimiento estable de "*Ca.* Accumulibacter" se alimentó con acetato marcado con ^{13}C y se analizó la incorporación de ^{13}C en proteínas y polisacáridos a lo largo del tiempo. Los resultados mostraron un "turnover" de proteínas y polisacáridos mayor que el esperado por la eliminación de sólidos. Además, no se observó diferencias significantes entre las proteínas intracelulares y secretadas. Estos hallazgos indicaron que las SPEs de la comunidad enriquecida con "*Ca.* Accumulibacter" no son degradadas selectivamente por las poblaciones flanqueantes. En cambio, observamos un deterioro general de la biomasa.

Los resultados principales de esta tesis están resumidos en el **capítulo 6**, el cual también aporta una perspectiva para el futuro trabajo que siga los resultados de esta investigación para así expandir más el área de conocimiento en la investigación de las SPEs.

1

General Introduction

1.1. Extracellular polymeric substances and biofilms

Although one could imagine microorganisms living as individual particles floating around in natural aquatic systems, the majority of bacteria grow in the form of microbial aggregates known as biofilms, either attached on a surface or in the form of floccular or granular bioaggregates. In these biofilms, microorganisms are embedded in a mixture of extracellular polymeric substances (EPS) produced by the microorganisms themselves (Flemming and Wingender 2010). Biofilms have been studied for decades since they were described as "bacterial slime" in the 40s (Heukelekian and Heller 1940). However, little is known still about the complex composition and function of this polymeric matrix, and EPS is often referred as "the dark matter of biofilms" (Flemming et al. 2007). EPS differ between different types of biofilms as it depends on the microorganisms present and on external conditions such as temperature, shear stress or nutrient type or availability. However, some general consensus has been reached with respect to EPS functions and composition.

Initially, the extracellular matrix was believed to be composed purely by polysaccharides and EPS denoted "exopolysaccharides". However, this term was renamed to "extracellular polymeric substances" after discovering the real complexity of the matrix. EPS do not only encompass polysaccharides, but also other biopolymers such as (glyco-)proteins, nucleic acids, lipids and humic substances (Flemming and Wingender 2010; Seviour et al. 2019). Moreover, bacterial surface structures (*e.g.*, flagella, S-layer proteins, lipopolysaccharides), contribute to the stabilization of the extracellular matrix (Zogaj et al. 2001). EPS do not only contain structural elements, but it can also contain enzymes or DNA that modify the properties of the polymers. Besides the difference in EPS composition, it is assumed that the roles of EPS are conserved across biofilms. These functions include adhesion and aggregation, water retention and sorption of organic compounds and inorganic ions or protective barrier among others (Flemming et al. 2007).

The study of structural and functional properties of EPS sometimes require their solubilization and extraction from the matrix, which can result in a great challenge. Despite that EPS can be extracted by different chemical or physical methods, there is not a standard extraction method that fits to all. This

complicates the quantification and comparison between different biofilm sample. Extraction procedures are normally designed to maximize the extraction yield avoiding cell lysis (Gehr and Henry 1983). Although they might be effective to some types of biofilms, these extraction protocols often result in the characterization of EPS which are irrelevant for the structure of the biofilm. In order to identify and characterize functional EPS of the biofilm, harsh methods that damage the cells but ensure the solubilization and recovery of a broader range of key EPS components are needed (Felz et al. 2016). This approach requires the confirmation that the identified polymers are extracellular, which could be done by microscopic imaging techniques (*e.g.*, different lectins and fluorescent probes for carbohydrates) (Neu and Lawrence 2014) or sequence-based tools for proteins (*e.g.*, signal peptide prediction algorithms based on conserved amino acid sequences) (Erickson et al. 2010).

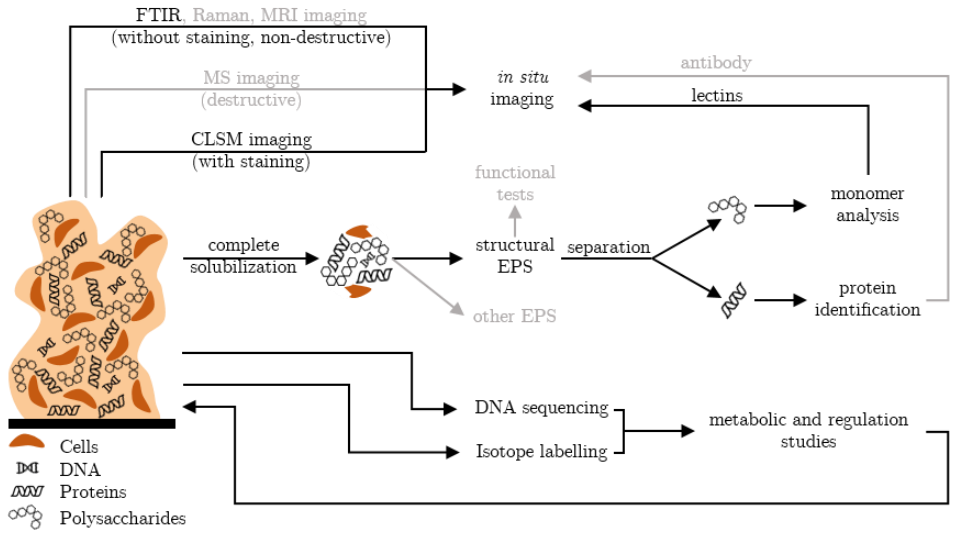

Figure 1.1. Proposed multidisciplinary roadmap for resolving the identities and functions of extracellular polymeric substances in biofilms, involving complementary chemical, biochemical and 'omic' analysis of biofilms and isolated constituents. Adapted from Seviour et al. (2019). The approaches that were used in this thesis appear highlighted in black.

In spite of the significant progress over decades, EPS is still a black box waiting to be open, in terms of composition, function, structure and production. In order to improve the understanding of this fascinating matrix, integrated and multidisciplinary approaches need to be applied, either *in situ* in biofilms or to the extracted EPS, as summarized in Figure 1.1. Improved EPS extraction

methods and chemical characterization can reveal the molecular structure of EPS. Imaging and biophysical methods can contribute to verify the identity and distribution of the different components. Genetic and metabolic labelling methods can derive into a better understanding on the formation of EPS (Seviour et al. 2019).

1.2. Aerobic granular sludge and "*Candidatus* Accumulibacter"

Biofilms have great importance in many environmental engineering processes. In some cases, such as membrane filtration or water distribution systems, biofilms cause significant problems due to biofouling or biocorrosion (Flemming and Schaule 1988; Ma et al. 2020). On the other hand, biofilm formation is beneficial and needed for other applications such as wastewater treatment or biological air purification (Qureshi 2009).Traditionally, biological wastewater treatment plants (WWTP) have employed activated sludge (in the form of flocs) to remove contaminants from wastewater. Novel biological treatments have emerged in the last decades, such as the aerobic granular sludge (AGS) process (de Bruin et al. 2004). Here, microorganisms are stimulated to form compact granules, which reduces energy and space requirements due to the increased settleability (Pronk et al. 2015). This technology is being commercialized and applied worldwide under the name of Nereda® (Royal HaskoningDHV 2022a)

Organic carbon, phosphorus and nitrogen are removed by the microorganisms present in AGS (De Kreuk et al. 2005). The microbial community in AGS is diverse, which brings significant difficulty in finding out EPS "producers" for further identification of specific genetic information of metabolic routes involved in EPS production. Among the various microorganisms, polyphosphate accumulating organisms (PAOs) are an important functional group in AGS because of their role in phosphate removal and granule stabilization (de Kreuk and van Loosdrecht 2004; Weissbrodt et al. 2013). PAOs rapidly take up volatile fatty acids anaerobically and store them in the form of intracellular polyhydroxyalkanoates (PHAs). When electron acceptors are available, they utilize these PHAs as carbon and energy source to grow and to take up phosphorus, which they store as intracellular polyphosphate (Smolders et al. 1995). Due to their dominance in AGS and the rapid anaerobic carbon

sequestration, they are assumed to be the main EPS producer in AGS (de Kreuk and van Loosdrecht 2004). Therefore, PAOs can be used as model microorganisms to study the EPS of AGS.

Different types of PAOs have been described in WWTPs performing enhanced biological phosphorus removal (EBPR), such as *Tetrasphaera*, "*Candidatus* Accumulibacter", *Dechloromonas* or *Tessaracoccus* (Nielsen et al. 2019). Among these bacteria, "*Ca.* Accumulibacter phosphatis" has been identified as the dominant PAO responsible for EBPR (Seviour et al. 2003). Currently, "*Ca.* Accumulibacter" has not been isolated as pure culture yet, however, it has been successfully cultivated in mixed cultures in laboratory for decades (Smolders et al. 1994), reaching very high levels of enrichment (Guedes da Silva et al. 2020). "*Ca.* Accumulibacter" is a model PAO that has been well studied by genomic, proteomic, metabolic and modelling studies (Oehmen et al. 2010; Barr et al. 2016; Oyserman et al. 2016; Guedes da Silva et al. 2019; Rubio-Rincón et al. 2019; Guedes da Silva et al. 2020). However, most of the studies neglected the EPS formation. A better characterization of the EPS of "*Ca.* Accumulibacter" will lead to a comprehensive understanding of this microorganism and further optimization of the granular sludge processes.

1.3. EPS-BASED PRODUCTS

During the wastewater treatment process, a large amount of excess sludge is generated. In most cases, this sludge is considered as a waste and needs to be disposed. The cost of handling the waste sludge represents up to 50 % of the total wastewater treatment cost (Kroiss 2004). However, this excess sludge can be a potential resource where useful materials can be recovered. The recovery of biomaterials from waste sludge will contribute to a circular economy, increasing the sustainability and economics of the wastewater treatment process (Lin et al. 2015). EPS represent a large fraction of the sludge dry weight. Previous research have demonstrated that a significant amount of EPS can be recovered from AGS (*i.e.*, 25 % of extraction yield) (Felz et al. 2016). EPS extraction from AGS is currently being developed to an industrial level. This has led to the opening of two extraction plants in the Netherlands, located in Zutphen and in Epe. The resulting extracted polymers from the excess granules of the Nereda® are being commercialized under the name Kaumera. (Royal HaskoningDHV 2022b).

EPS recovered from AGS can be used as raw material for several applications. Lin et al. (2015) showed the water repellent properties of the recovered polymers, which can be used as coating material, forming water-resistant films. Kim et al. (2020) explored the flame retardant properties of fabric coated with extracted EPS, which met the regulation standards. Extracted EPS from AGS can also be used as a sustainable biosorbent for dye removal from water (Ladnorg et al. 2019). This biomaterial also can be applied in agriculture as coating material for the slow release of fertilizers or as biostimulant (van Leeuwen et al. 2018; ChainCraft 2022). Further applications are being explored, such as curing agent for concrete or to form bionanocomposite materials (Royal HaskoningDHV 2022b).

Even though the commercial stage of these recovered polymers has been reached, fundamental knowledge about their biosynthesis, stability and detailed molecular composition, which are essential for expanding their application, is still lacking. Especially, insights into the biosynthesis and turnover of EPS can ensure a stable production of the biomaterial. A more in-depth identification of the specific molecular composition can open doors to new applications and markets for EPS-based products.

It is indicated by previous research that a few important carbohydrate polymers, which resemble those produced by vertebrates, can be found in the EPS of granular sludge. Felz et al. (2020) have described that the extracellular matrix of AGS contains hyaluronic acid-like and sulfated glycosaminoglycans (GAGs)-like polymers . Hyaluronic acid and sulfated GAGs have been used in therapeutic applications, such as, anticoagulant and antithrombotic drugs (Volpi 2006). Xue et al. (2019) proved that the sulfated polysaccharides purified from saline activated sludge have anti-angiogenesis, anticoagulant and antioxidant properties. The presence of these carbohydrate polymers in the EPS of AGS suggests the potential of EPS as a raw material for medical products, increasing the value of EPS-derived materials and highlighting the potential of products recovered from wastewater residuals to promote circular economy and health.

In addition to these exciting findings about carbohydrate polymers, the existence of sialic acids, a type of carbohydrate monomer which belongs to nonulosonic acid family, was reported in the EPS of seawater adapted AGS (de Graaff et al. 2019).

1.4. Nonulosonic acids

Nonulosonic acids (NulOs) are a family of α-keto-acid carbohydrates with a nine-carbon backbone, with a wide variety of chemical forms. The most studied representatives are derivatives of neuraminic (Neu) and ketodeoxynonulosonic (Kdn) acids (also known as sialic acids) (Chen and Varki 2010). Sialic acids are typically found as terminal residues on the glycan chains of vertebrate extracellular glycoconjugates, making them the "bridging" or recognition molecules between cells, as well as between cells and extracellular matrices. Apart from sialic acids, other NulOs have been found only in microbes, such as the isomers pseudaminic (Pse) and legionaminic (Leg) acids, which are often referred as "bacterial sialic acids" (Knirel et al. 2003).

Investigations of NulOs have been predominantly focused in animal cells and pathogenic or commensal bacteria due to their importance in animal physiology and pathogenesis. In animals, sialic acids are crucial to physiological processes, such as recognition between cells and neuronal transmission, or diseases such as cancer and autoimmune diseases (Traving and Schauer 1998). Pathogenic bacteria can either de novo synthesize NulOs or scavenge them from the host's glycoconjugates to mimic the host's glycosylation pattern, contributing to a delay of the immune response (Carlin et al. 2009). Some pathogenic and commensal bacteria are able to use host's sialic acids as nutrients (Vimr 2013).

Although NulOs have been overlooked in non-pathogenic bacteria, they have recently been described as ubiquitous in the microbial world, including environmentally relevant bacteria (Lewis et al. 2009; Kleikamp et al. 2020), making their study and understanding highly relevant. Different types of NulOs have been described in the EPS of biofilms forming in cooling towers (Pinel et al. 2020), anammox granular sludge (Boleij et al. 2020) and seawater adapted AGS dominated with "*Ca.* Accumulibacter" (de Graaff et al. 2019). Although the role of NulOs in environmental non-pathogenic bacteria is not known, they might play roles in biofilm formation, bacterial motility or stabilization of biomolecules (Goon et al. 2003; Jurcisek et al. 2005; Hanisch et al. 2013).

Sialic acids and derivatives show interesting medical applications, such as chemical inhibitors for cancer treatment, inactivation of cytotoxicity, biopharmaceutical stabilizers or drug deliverers (Bauer and Osborn 2015; Zlatina

et al. 2017). The presence of NulOs in the EPS of AGS suggest a new possible application of the recovered polymers. Sialic acids have been traditionally synthesized chemically or extracted from natural sources. Engineered bacteria have also been explored for their production. However, the complex structure of bacterial sialic acids makes their production difficult (Flack et al. 2020). Moreover, the dependency of organic solvents for their chemical synthesis is a concern for the sustainability of the production. The presence of NulOs in AGS points towards a potential new sustainable source.

To summarize, significant effort has been made to unravel the composition and characterization of EPS, "the dark matter of biofilms", which leads to a great development of both fundamental understanding and exciting application opportunities. However, as new EPS components continue popping up, it is realized that the EPS in biofilms are far more complicated than we currently appreciated. To open up the black box of EPS and manipulate their production, knowledge covering chemical composition, genetic, proteomic and metabolic aspects has to be generated. To this end, instead of studying EPS as a complicated mixture, a well-controlled model system (*e.g.*, an enriched microbial community) and well-defined EPS components (*e.g.*, carbohydrates with known metabolic pathways or total proteins and carbohydrates) has to be employed.

1.5. Scope and outline of the thesis

This thesis focuses on the study of EPS of granules enriched with "*Ca.* Accumulibacter". For this work, a lab-scale reactor was employed to obtain a highly enriched "*Ca.* Accumulibacter" culture performing EBPR activity. As it has been pointed through this introduction, there are only a few in depth detailed studies of the EPS of microbial communities. Therefore, the study of EPS can continue in many different directions. This thesis contributes to further development on the field of EPS and focuses on three specific main aspects (Figure 1.2), namely the:

1. *Detailed specific composition of EPS from "Ca.* Accumulibacter" *granules* by evaluating the presence, diversity and localization of NulOs in the EPS (**chapters 2** and **3**).

2. *Application of NulOs-rich polymers extracted from "Ca.* Accumulibacter" *granules* by evaluating the potential medical use of the isolated fractions of EPS (**chapter 4**).
3. *Metabolism and stability of EPS in "Ca.* Accumulibacter" *enrichment cultures* by studying the catabolism of sialic acids (**chapter 4**) and the overall EPS turnover (**chapter 5**).

The focus of **chapter 2** was to confirm the presence and explore the diversity of NulOs in "*Ca.* Accumulibacter". To achieve this, granules highly enriched with "*Ca.* Accumulibacter" were analyzed with a combination of techniques, such as fluorescence lectin-binding analysis, enzymatic release and mass spectrometry. Additionally, genomic and proteomic information were studied to evaluate the NulOs biosynthetic pathway in "*Ca.* Accumulibacter".

In **chapter 3**, EPS from "*Ca.* Accumulibacter" were extracted and fractionated by size exclusion chromatography to obtain NulOs-rich polymers, with the aim of determining the location of these NulOs. Additionally, the potential application of the NulOs-rich polymers against sepsis was evaluated.

Chapter 4 examined the catabolism of sialic acids in environmental bacterial aggregates. The pathways involved in the degradation of sialic acids in pathogenic and commensal bacteria were used as reference. Proteins alignment tools were employed to evaluate these pathways within the EBPR microbial community.

In **chapter 5**, the turnover dynamics of the main components of the EPS of PAOs (polysaccharides and proteins) were researched. A stable enrichment was fed with uniformly ^{13}C-labelled acetate and the incorporation patterns in extracellular proteins and polysaccharides were analyzed and compared to the ones of intracellular proteins.

Finally, the concluding **chapter 6** summarizes the main findings of this thesis and provides an outlook for future work following the results from this research.

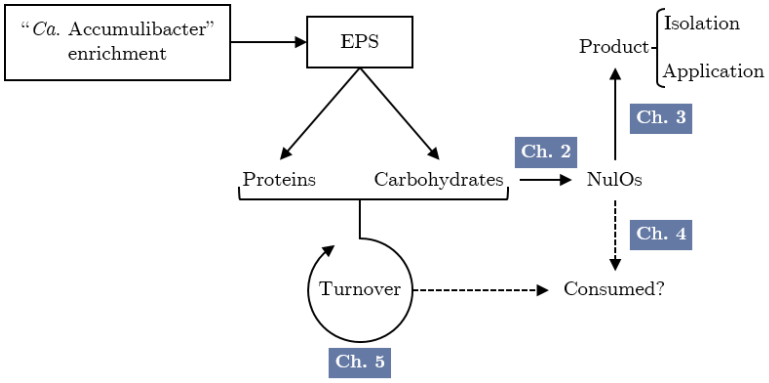

Figure 1.2. Schematic representation of the different chapters of this thesis studying the EPS of "*Ca.* Accumulibacter".

References

Barr JJ, Dutilh BE, Skennerton CT, Fukushima T, Hastie ML, Gorman JJ, Tyson GW, Bond PL (2016) Metagenomic and metaproteomic analyses of *Accumulibacter phosphatis*-enriched floccular and granular biofilm. Environ Microbiol 18:273–287 . https://doi.org/10.1111/1462-2920.13019

Bauer J, Osborn HMI (2015) Sialic acids in biological and therapeutic processes: opportunities and challenges. http://dx.doi.org/104155/fmc15135 7:2285–2299 . https://doi.org/10.4155/FMC.15.135

Boleij M, Kleikamp H, Pabst M, Neu TR, van Loosdrecht MCM, Lin Y (2020) Decorating the Anammox House: Sialic Acids and Sulfated Glycosaminoglycans in the Extracellular Polymeric Substances of Anammox Granular Sludge. Environ Sci Technol 54:5218–5226 . https://doi.org/10.1021/ACS.EST.9B07207

Carlin AF, Uchiyama S, Chang YC, Lewis AL, Nizet V, Varki A (2009) Molecular mimicry of host sialylated glycans allows a bacterial pathogen to engage neutrophil Siglec-9 and dampen the innate immune response. Blood 113:3333–3336 . https://doi.org/10.1182/blood-2008-11-187302

ChainCraft (2022) Agrochemicals – ChainCraft. https://www.chaincraft.nl/agrochemicals/. Accessed 9 Aug 2022

Chen X, Varki A (2010) Advances in the biology and chemistry of sialic acids. ACS Chem. Biol. 5:163–176

de Bruin LMM, de Kreuk MK, van der Roest HFR, Uijterlinde C, van Loosdrecht MCM (2004) Aerobic granular sludge technology: an alternative to activated sludge? Water Sci Technol 49:1–7 . https://doi.org/10.2166/WST.2004.0790

de Graaff DR, Felz S, Neu TR, Pronk M, van Loosdrecht MCM, Lin Y (2019) Sialic acids in the extracellular polymeric substances of seawater-adapted aerobic granular sludge. Water Res 155:343–351 . https://doi.org/10.1016/J.WATRES.2019.02.040

De Kreuk MK, Heijnen JJ, Van Loosdrecht MCM (2005) Simultaneous COD, nitrogen, and phosphate removal by aerobic granular sludge. Biotechnol Bioeng 90:761–769 . https://doi.org/10.1002/BIT.20470

de Kreuk MK, van Loosdrecht MCM (2004) Selection of slow growing organisms as a means for improving aerobic granular sludge stability. Water Sci Technol 49:9–17

Erickson BK, Mueller RS, Verberkmoes NC, Shah M, Singer SW, Thelen MP, Banfield JF, Hettich RL (2010) Computational prediction and experimental validation of signal peptide cleavages in the extracellular proteome of a natural microbial community. J Proteome Res 9:2148–2159 . https://doi.org/10.1021/PR900877A/SUPPL_FILE/PR900877A_SI_001.PDF

Felz S, Al-Zuhairy S, Aarstad OA, van Loosdrecht MCM, Lin YM (2016) Extraction of Structural Extracellular Polymeric Substances from Aerobic Granular Sludge. J Vis Exp e54534 . https://doi.org/10.3791/54534

Felz S, Neu TR, van Loosdrecht MCM, Lin Y (2020) Aerobic granular sludge contains Hyaluronic acid-like and sulfated glycosaminoglycans-like polymers. Water Res 169:115291 . https://doi.org/10.1016/J.WATRES.2019.115291

Flack EKP, Chidwick HS, Best M, Thomas GH, Fascione MA (2020) Synthetic Approaches for Accessing Pseudaminic Acid (Pse) Bacterial Glycans. ChemBioChem 21:1397–1407 . https://doi.org/10.1002/CBIC.202000019

Flemming H-C, Neu TR, Wozniak DJ (2007) The EPS matrix: the "house of biofilm

cells";. J Bacteriol 189:7945–7 . https://doi.org/10.1128/JB.00858-07

Flemming H-C, Wingender J (2010) The biofilm matrix. Nat Rev Microbiol 8:623–633 . https://doi.org/10.1038/nrmicro2415

Flemming HC, Schaule G (1988) Biofouling on membranes - A microbiological approach. Desalination 70:95–119 . https://doi.org/10.1016/0011-9164(88)85047-1

Gehr R, Henry JG (1983) Removal of extracellular material techniques and pitfalls. Water Res 17:1743–1748 . https://doi.org/10.1016/0043-1354(83)90195-1

Goon S, Kelly JF, Logan SM, Ewing CP, Guerry P (2003) Pseudaminic acid, the major modification on *Campylobacter flagellin*, is synthesized via the Cj1293 gene. Mol Microbiol 50:659–671 . https://doi.org/10.1046/J.1365-2958.2003.03725.X

Guedes da Silva L, Olavarria Gamez K, Castro Gomes J, Akkermans K, Welles L, Abbas B, van Loosdrecht MCM, Wahl SA (2020) Revealing the Metabolic Flexibility of "*Candidatus* Accumulibacter phosphatis" through Redox Cofactor Analysis and Metabolic Network Modeling. Appl Environ Microbiol 86: . https://doi.org/10.1128/AEM.00808-20

Guedes da Silva L, Tomás-Martínez S, van Loosdrecht MCM, Wahl SA (2019) The environment selects: Modeling energy allocation in microbial communities under dynamic environments. bioRxiv 689174 . https://doi.org/10.1101/689174

Hanisch F, Weidemann W, Großmann M, Joshi PR, Holzhausen HJ, Stoltenburg G, Weis J, Zierz S, Horstkorte R (2013) Sialylation and muscle performance: Sialic acid is a marker of muscle ageing. PLoS One 8: . https://doi.org/10.1371/journal.pone.0080520

Heukelekian H, Heller A (1940) Relation between Food Concentration and Surface for Bacterial Growth. J Bacteriol 40:547 . https://doi.org/10.1128/JB.40.4.547-558.1940

Jurcisek J, Greiner L, Watanabe H, Zaleski A, Apicella MA, Bakaletz LO (2005) Role of Sialic Acid and Complex Carbohydrate Biosynthesis in Biofilm Formation by Nontypeable *Haemophilus influenzae* in the Chinchilla Middle Ear. Infect Immun 73:3210 . https://doi.org/10.1128/IAI.73.6.3210-3218.2005

Kim NK, Mao N, Lin R, Bhattacharyya D, van Loosdrecht MCM, Lin Y (2020) Flame retardant property of flax fabrics coated by extracellular polymeric substances recovered from both activated sludge and aerobic granular sludge. Water Res 170:115344 . https://doi.org/10.1016/J.WATRES.2019.115344

Kleikamp HBC, Lin YM, McMillan DGG, Geelhoed JS, Naus-Wiezer SNH, van Baarlen P, Saha C, Louwen R, Sorokin DY, van Loosdrecht MCM, Pabst M (2020) Tackling the chemical diversity of microbial nonulosonic acids – a universal large-scale survey approach. Chem Sci. https://doi.org/10.1039/C9SC06406K

Knirel YA, Shashkov AS, Tsvetkov YE, Jansson PE, Zähringer U (2003) 5,7-Diamino-3,5,7,9-tetradeoxynon-2-ulosonic acids in bacterial glycopolymers: Chemistry and biochemistry. Adv Carbohydr Chem Biochem 58:371–417 . https://doi.org/10.1016/S0065-2318(03)58007-6

Kroiss H (2004) What is the potential for utilizing the resources in sludge? Water Sci Technol 49:1–10 . https://doi.org/10.2166/WST.2004.0595

Ladnorg S, Junior NL, Dall'Agnol P, Domingos DG, Magnus BS, Wichern M, Gehring T, Da Costa RHR (2019) Alginate-like exopolysaccharide extracted from aerobic granular sludge as biosorbent for methylene blue: Thermodynamic, kinetic and isotherm studies. J Environ Chem Eng 7:103081 . https://doi.org/10.1016/J.JECE.2019.103081

Lewis AL, Desa N, Hansen EE, Knirel YA, Gordon JI, Gagneux P, Nizet V, Varki A (2009) Innovations in host and microbial sialic acid biosynthesis revealed by phylogenomic prediction of nonulosonic acid structure

Lin YM, Nierop KGJ, Girbal-Neuhauser E, Adriaanse M, van Loosdrecht MCM (2015) Sustainable polysaccharide-based biomaterial recovered from waste aerobic granular sludge as a surface coating material. Sustain Mater Technol 4:24–29 . https://doi.org/10.1016/J.SUSMAT.2015.06.002

Ma Y, Zhang Y, Zhang R, Guan F, Hou B, Duan J (2020) Microbiologically influenced corrosion of marine steels within the interaction between steel and biofilms: a brief view. Appl Microbiol Biotechnol 104:515–525 . https://doi.org/10.1007/S00253-019-10184-8/FIGURES/1

Neu TR, Lawrence JR (2014) Advanced techniques for *in situ* analysis of the biofilm matrix (Structure, Composition, Dynamics) by means of laser scanning microscopy. Methods Mol Biol 1147:43–64 . https://doi.org/10.1007/978-1-4939-0467-9_4/TABLES/4

Nielsen PH, McIlroy SJ, Albertsen M, Nierychlo M (2019) Re-evaluating the microbiology of the enhanced biological phosphorus removal process. Curr Opin Biotechnol 57:111–118 . https://doi.org/10.1016/J.COPBIO.2019.03.008

Oehmen A, Lopez-Vazquez CM, Carvalho G, Reis MAM, van Loosdrecht MCM (2010) Modelling the population dynamics and metabolic diversity of organisms relevant in anaerobic/anoxic/aerobic enhanced biological phosphorus removal processes. Water Res 44:4473–4486 . https://doi.org/10.1016/j.watres.2010.06.017

Oyserman BO, Noguera DR, del Rio TG, Tringe SG, McMahon KD (2016) Metatranscriptomic insights on gene expression and regulatory controls in *Candidatus* Accumulibacter phosphatis. ISME J 10:810–22 . https://doi.org/10.1038/ismej.2015.155

Pinel ISM, Kleikamp HBC, Pabst M, Vrouwenvelder JS, van Loosdrecht MCM, Lin Y (2020) Sialic Acids: An Important Family of Carbohydrates Overlooked in Environmental Biofilms. Appl Sci 2020, Vol 10, Page 7694 10:7694 . https://doi.org/10.3390/APP10217694

Pronk M, de Kreuk MK, de Bruin B, Kamminga P, Kleerebezem R, van Loosdrecht MCM (2015) Full scale performance of the aerobic granular sludge process for sewage treatment. Water Res 84:207–217 . https://doi.org/10.1016/J.WATRES.2015.07.011

Qureshi N (2009) Beneficial biofilms: wastewater and other industrial applications. Biofilms Food Beverage Ind 474–498 . https://doi.org/10.1533/9781845697167.4.474

Royal HaskoningDHV (2022a) Nereda® Technology. https://global.royalhaskoningdhv.com/nereda. Accessed 9 Aug 2022

Royal HaskoningDHV (2022b) Kaumera. https://kaumera.com. Accessed 9 Aug 2022

Rubio-Rincón FJ, Weissbrodt DG, Lopez-Vazquez CM, Welles L, Abbas B, Albertsen M, Nielsen PH, van Loosdrecht MCM, Brdjanovic D (2019) "*Candidatus* Accumulibacter delftensis": A clade IC novel polyphosphate-accumulating organism without denitrifying activity on nitrate. Water Res 161:136–151 . https://doi.org/10.1016/J.WATRES.2019.03.053

Seviour RJ, Mino T, Onuki M (2003) The microbiology of biological phosphorus removal in activated sludge systems. FEMS Microbiol. Rev. 27:99–127

Seviour T, Derlon N, Dueholm MS, Flemming H-C, Girbal-Neuhauser E, Horn H, Kjelleberg S, van Loosdrecht MCM, Lotti T, Malpei MF, Nerenberg R, Neu TR, Paul

E, Yu H, Lin Y (2019) Extracellular polymeric substances of biofilms: Suffering from an identity crisis. Water Res 151:1–7 . https://doi.org/10.1016/J.WATRES.2018.11.020

Smolders GJF, van der Meij J, van Loosdrecht MCM, Heijnen JJ (1995) A structured metabolic model for anaerobic and aerobic stoichiometry and kinetics of the biological phosphorus removal process. Biotechnol Bioeng 47:277–287 . https://doi.org/10.1002/BIT.260470302

Smolders GJF, van der Meij J, van Loosdrecht MCM, Heijnen JJ (1994) Model of the anaerobic metabolism of the biological phosphorus removal process: Stoichiometry and pH influence. Biotechnol Bioeng 43:461–470 . https://doi.org/10.1002/BIT.260430605

Traving C, Schauer R (1998) Structure, function and metabolism of sialic acids. Cell. Mol. Life Sci. 54:1330–1349

van Leeuwen K, de Vries E, Koop S, Roest K (2018) The Energy & Raw Materials Factory: Role and Potential Contribution to the Circular Economy of the Netherlands. Environ Manage 61:786–795 . https://doi.org/10.1007/S00267-018-0995-8/FIGURES/2

Vimr ER (2013) Unified theory of bacterial sialometabolism: how and why bacteria metabolize host sialic acids. ISRN Microbiol 2013:1–26 . https://doi.org/10.1155/2013/816713

Volpi N (2006) Therapeutic Applications of Glycosaminoglycans. Curr Med Chem 13:1799–1810 . https://doi.org/10.2174/092986706777452470

Weissbrodt DG, Neu TR, Kuhlicke U, Rappaz Y, Holliger C (2013) Assessment of bacterial and structural dynamics in aerobic granular biofilms. Front Microbiol 4: . https://doi.org/10.3389/fmicb.2013.00175

Xue W, Zeng Q, Lin S, Zan F, Hao T, Lin Y, van Loosdrecht MCM, Chen G (2019) Recovery of high-value and scarce resources from biological wastewater treatment: Sulfated polysaccharides. Water Res 163:114889 . https://doi.org/10.1016/J.WATRES.2019.114889

Zlatina K, Lütteke T, Galuska SP (2017) Individual Impact of Distinct Polysialic Acid Chain Lengths on the Cytotoxicity of Histone H1, H2A, H2B, H3 and H4. Polymers (Basel) 9: . https://doi.org/10.3390/POLYM9120720

Zogaj X, Nimtz M, Rohde M, Bokranz W, Römling U (2001) The multicellular morphotypes of *Salmonella typhimurium* and *Escherichia coli* produce cellulose as the second component of the extracellular matrix. Mol Microbiol 39:1452–1463 . https://doi.org/10.1046/J.1365-2958.2001.02337.X

2

PRODUCTION OF NONULOSONIC ACIDS IN THE EXTRACELLULAR POLYMERIC SUBSTANCES OF "*CANDIDATUS* ACCUMULIBACTER PHOSPHATIS"

Abstract

Nonulosonic acids (NulOs) are a family of acidic carbohydrates with a nine-carbon backbone, which include different related structures, such as sialic acids. They have mainly been studied for their relevance in animal cells and pathogenic bacteria. Recently, sialic acids have been discovered as important compound in the extracellular matrix of virtually all microbial life and in "*Candidatus* Accumulibacter phosphatis", a well-studied polyphosphate-accumulating organism, in particular. Here, bioaggregates highly enriched with these bacteria (approx. 95 % based on proteomic data) were used to study the production of NulOs in an enrichment of this microorganism. Fluorescence lectin-binding analysis, enzymatic quantification, and mass spectrometry were used to analyze the different NulOs present, showing a wide distribution and variety of these carbohydrates, such as sialic acids and bacterial NulOs, in the bioaggregates. Phylogenetic analysis confirmed the potential of "*Ca.* Accumulibacter" to produce different types of NulOs. Proteomic analysis showed the ability of "*Ca.* Accumulibacter" to reutilize and reincorporate these carbohydrates. This investigation points out the importance of diverse NulOs in non-pathogenic bacteria, which are normally overlooked. Sialic acids and other NulOs should be further investigated for their role in the ecology of "*Ca.* Accumulibacter" in particular, and biofilms in general.

Highlights

- "*Ca.* Accumulibacter" has the potential to produce a range of nonulosonic acids.
- Mass spectrometry and lectin binding can reveal the presence and location of nonulosonic acids.
- Role of nonulosonic acid in non-pathogenic bacteria needs to be studied in detail.

Published as:
Tomás-Martínez S, Kleikamp HBC, Neu TR, Pabst M, Weissbrodt DG, van Loosdrecht MCM, Lin Y (2021) Production of nonulosonic acids in the extracellular polymeric substances of "*Candidatus* Accumulibacter phosphatis". Appl Microbiol Biotechnol

2.1. Introduction

Wastewater transports polluting nutrients, such as organic matter, phosphorus (P), or nitrogen (N). When P and/or N are in excess, discharging this wastewater into surface waters leads to eutrophication. Thus, these pollutants must be eliminated from wastewater streams (Mainstone and Parr 2002). Enhanced biological phosphorus removal (EBPR) has become a widely applied treatment to eliminate inorganic phosphorus and organic matter from wastewater. This technology exploits the metabolic capacity of polyphosphate-accumulating organisms (PAOs) to take up inorganic phosphorus and to store it in the form of intracellular polyphosphate. "*Candidatus* Accumulibacter phosphatis", a well-studied model PAO, has been identified as a dominant species responsible for EBPR (Seviour et al. 2003). This microorganism has not been isolated yet. It grows in the form of compact microcolonies and bioaggregates (flocs, granules or biofilms) held together by extracellular polymeric substances (EPS) (Weissbrodt et al. 2013; Barr et al. 2016).

EPS is a complex mixture of biopolymers of different nature, such as polysaccharides, proteins, nucleic acids or lipids, among others. These biopolymers are synthesized or released by microorganisms across their life cycle, forming matrices that provide mechanical stability and act as scaffold for the microorganisms in biofilms (Flemming and Wingender 2010). Although research in the past years led to analytical advances for the extraction and characterization of EPS (Felz et al. 2016; Boleij et al. 2018; Felz et al. 2019; Boleij et al. 2019), the EPS matrix still represents the "dark matter" of biofilms that need to be studied in more detail (Neu and Lawrence 2016; Neu and Lawrence 2017; Seviour et al. 2019). The pragmatic study of individual components (*i.e.*, proteins and carbohydrates like monosaccharides and polysaccharides) can give new insights in the understanding of EPS as a whole. Recently, sialic acids have been detected and described in the EPS of both EBPR and salt-adapted aerobic granular sludge with the presence of "*Ca.* Accumulibacter" using fluorescence lectin-binding analysis (FLBA) coupled to confocal laser scanning microscopy (CSLM) (Weissbrodt et al. 2013; de Graaff et al. 2019).

Sialic acids are a subset of a family of α-keto acids with a nine-carbon backbone, called nonulosonic acids (NulOs). These carbohydrates are unusual among the various monosaccharide building blocks of extracellular glycoconjugates, which

normally have five or six carbons. Sialic acids are typically found as terminal residues on the glycan chains of vertebrate extracellular glycoconjugates, making them the "bridging" or recognition molecules between cells, as well as between cells and extra-cellular matrix (Chen and Varki 2010). The distinct features of sialic acids contribute to higher structural complexity and the potential for more unique and varied biological functions, in comparison to other monosaccharides (Deng et al. 2013).

Looking into the specific chemical structure, sialic acids are derivatives of neuraminic (Neu) and ketodeoxynonulosonic (Kdn) acids. The most studied one is *N*-acetylneuraminic acid (Neu5Ac) (Varki et al. 2017). Apart from these acids, other NulOs have been found only in microbes, such as the isomers pseudaminic (Pse) and legionaminic (Leg) acids, which are structurally similar to sialic acids (Figure 2.1A) (Knirel et al. 2003). These NulOs have recently been described as ubiquitous in the microbial world (Lewis et al. 2009; Kleikamp et al. 2020a), making their study and understanding highly relevant.

Despite the different chemical structure, the NulOs share similarities in their metabolic pathway (Figure 2.1C). The common steps in each NulO biosynthetic pathway (NAB) are catalyzed by homologous enzymes: the condensation of a 6-carbon intermediate with phosphoenol pyruvate (PEP) produces a 9-C α-keto acid (catalyzed by the enzyme NAB-2); its activation results from the addition of CMP by the enzyme NAB-1 (Lewis et al. 2009). The different NulOs can be further modified by additional substitutions on the hydroxyl groups such as *O*-acetyl, *O*-methyl, *O*-sulfate, *O*-hydroxybutiryl, *O*-formyl or *O*-lactyl groups (Figure 2.1B) (Angata and Varki 2002). A phylogenetic analysis of the NAB-2 enzyme, the most conserved one in the pathway, can be used to predict the NulO types synthesized by an organism (Lewis et al. 2009). In the case of the sialic acids NeuAc and Kdn, the same biosynthetic machinery leads to their synthesis, using *N*-acetylmannosamine or mannose as starting substrate respectively (Varki et al. 2017).

Investigations of NulOs have been predominantly focused in animal cells and pathogenic bacteria. Among the large diversified NulOs, Neu5Ac is often assumed as the most dominant one present in biological samples. It plays important roles in recognition processes or stabilization of biomolecules (Hanisch et al. 2013). In animals, it is crucial to physiological processes, such as recognition between cells

and neuronal transmission, or diseases such as cancer and autoimmune diseases (Traving and Schauer 1998). In pathogenic bacteria, NulOs contribute in delaying the host's immune response by mimicking the host's glycosylation pattern (Carlin et al. 2009).

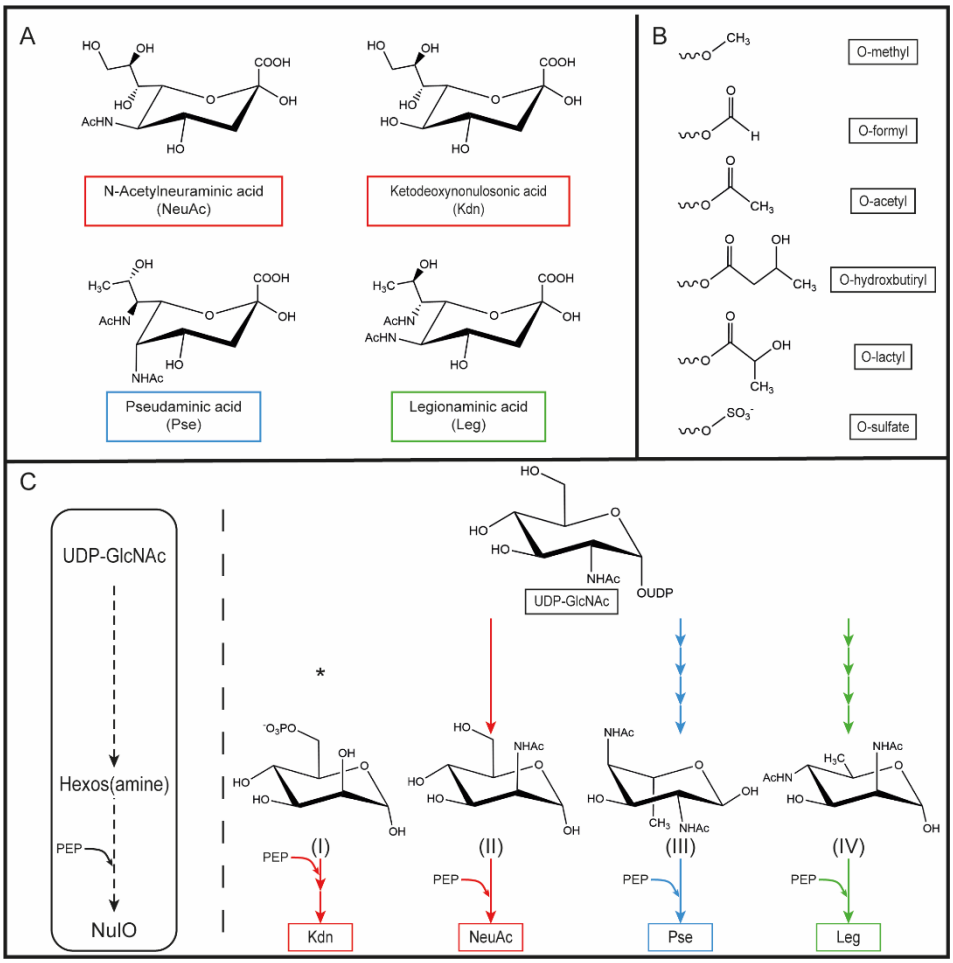

Figure 2.1. Common metabolic pathway for the biosynthesis of different NulOs. A) Chemical structure of different NulOs. B) Possible modifications of the hydroxyl groups NulOs. C) Core (left) and specific (right) biosynthetic pathways for the different NulOs. The biosynthetic pathways of the different NulOs branch from UDP-GlcNAc, with the exception of Kdn. Each arrow represents one enzymatic step. NeuAc and Kdn share the enzymes involved in the synthesis. (I) mannose-6-phosphate; (II) *N*-acetylmannosamine; (III) 2,4-diacetamido-2,4,6-trideoxy-L-altropyranose; (IV) 2,4-diacetamido-2,4,6-trideoxy-d-mannopyranose. Adapted from Lewis et al. (2009).

Regardless of the intensive studies of NulOs in animal tissue and on the surface-related structure of pathogenic bacteria cells, the presence, production and function of NulOs in non-pathogenic bacteria have not been widely realized and

studied. Only very recently, a genome level study (Lewis et al. 2009) and a NulOs universal survey by high resolution mass spectrometry (Kleikamp et al. 2020a) revealed the unexpectedly wide distribution of nonulosonic acid biosynthesis (NAB) pathway genes and wide-spread occurrence of NulOs in non-pathogenic bacteria. These discoveries indicate that NulOs must be an important component in the EPS of bacterial aggregates in natural and engineered ecosystems, which has been completely overlooked at present. It also indicates that the current model of evolution and utilization of sialic acids in prokaryotes which is driven by host–pathogen interactions may not reflect the complete picture and need to be questioned (Lewis et al. 2009; Kleikamp et al. 2020a).

It is known that "*Ca.* Accumulibacter" is not only the most abundant and well-studied PAO in EBPR systems, but also contributes to phosphate sequestration and phosphate cycling in estuarine systems (Watson et al. 2019). It is unknown how many types of NulOs can be produced and what the potential pathways are. Studying the diversity, production and utilization of NulOs with an enriched culture of "*Ca.* Accumulibacter", will add new information to the ecology of this important microorganism, *i.e.*, if diverse NulOs production is one of the strategies to win the competition over other microorganisms in the system. Furthermore, it will provide valuable insights into the synthesis and turnover of NulOs (or sialic acids) by non-pathogenic environmental bacteria. The study of the role of NulOs outside the pathogen-host interaction will extend the current understanding of ecology and evolution of these carbohydrates.

The objective of this research is to confirm the presence, predict the production and explore the diversity of NulOs in "*Ca.* Accumulibacter". To achieve this, "*Ca.* Accumulibacter" was enriched using a lab-scale sequencing batch reactor with EBPR performance (Guedes da Silva 2020). NulOs produced by this biomass were analyzed by a combination of techniques, such as fluorescence lectin-binding analysis (FLBA), enzymatic release, and mass spectrometry. Genomic and proteomic investigations were conducted to evaluate the diversity of pathways involved NulOs formation and utilization by "*Ca.* Accumulibacter".

2.2. Materials & Methods

"*Ca.* Accumulibacter" enriched biomass and seawater-adapted aerobic granules

An in-house enrichment culture of "*Ca.* Accumulibacter" was used (Guedes da Silva 2020). The enrichment was maintained in a 1.5 L sequencing batch reactor (SBR), with slight modifications from the SBR-2 described in Guedes da Silva et al. (2018). The COD-based acetate:propionate ratio in the feed was 65:35 gCOD/gCOD. FISH showed the dominance of PAO in the system (approx. 95 % of biovolume), and 16S rRNA gene amplicon sequencing confirmed "*Ca.* Accumulibacter" as the dominant PAO. Proteomic investigations by (Kleikamp et al. 2020b) further confirmed this dominance (approx. 95 %). Seawater-adapted aerobic granules from (de Graaff et al. 2019) were also used in the study. FISH showed the dominance of PAO in these granules as well.

Nonulosonic acids analyses

Fluorescence lectin-binding analysis (FLBA)

Lectin staining of the biomass was done according to earlier works (Weissbrodt et al. 2013; Boleij et al. 2018; de Graaff et al. 2019). Bioaggregates enriched with "*Ca.* Accumulibacter" were stained and mounted in coverwell chambers with a 0.5 mm spacer in order to avoid squeezing of the samples. Glycoconjugates of the biomass were examined by means of barcoding with green fluorescent lectins (Neu and Kuhlicke 2017). Thus, all commercially available lectins labelled with a green fluorophore (FITC or Alexa488) were applied as probes individually to different aggregates. A total of 77 lectins were used to screen glycoconjugates (Bennke et al. 2013). The binding sites of the sialic acid-specific lectins that gave the strongest signal are listed in Table 2.1. After incubation with the lectin solution, the sample was washed with tap water for three times in order to remove unbound lectins. For 3D imaging a TCS SP5X confocal laser scanning microscope (Leica, Wetzlar, Germany) was employed. The system comprised an upright microscope and a super continuum light source (white laser). The hardware setup was controlled by the software LAS AF 2.4.1. Confocal datasets were recorded by using 25x NA 0.95 and 63x NA 1.2 water immersion lenses. Excitation was at 490 nm and emission signals were detected simultaneously with two photomultipliers from 480 to 500 nm (reflection) and 505-580 nm (fluorescence). Image data sets were deconvolved with Huygens version 18.04 using blind deconvolution (SVI,

Hilversum, The Netherlands) and projected with Imaris version 9.2 (Bitplane, Belfast, UK). Images were printed from Photoshop CS6 (Adobe).

Table 2.1. Sialic acid-specific lectin used in this analysis.

Lectin name	Abbreviation	Ligand motif	Ref.*
Cancer antennarius lectin	CCA	9-*O*-Ac-NeuAc; 4-*O*-Ac-NeuAc	1
Maackia amurensis lectin	MAA	Neu5Ac(α2-3)Gal(β1-4)GlcNac/Glc	2
Sambucus nigra lectin	SNA	Neu5Ac(α2-6)Gal/GalNAc	3
Wheat germ agglutinin	WGA	Internal GlcNAc; Neu5Ac	4

*References:
1. Ravindranath et al. (1985)
2. Knibbs et al. (1991)
3. Shibuya et al. (1987)
4. Gallagher et al. (1985)

Nonulosonic acid diversity and enzymatic quantification

The diversity of NulOs in bioaggregates from the "*Ca.* Accumulibacter" enrichment and in seawater-adapted aerobic granules (de Graaff et al. 2019) was analyzed by high resolution mass spectrometry according to Kleikamp et al. (2020a), with the addition of manually verification of lower abundant species. The Sialic Acid Quantitation Kit (Sigma-Aldrich, Burlington, MA) was used to estimate the content of sialic acids (Neu5Ac as model one) in the enriched "*Ca.* Accumulibacter" biomass. The whole cell assay described in the kit was conducted. Fresh samples were washed with 20 mM Tris-HCl buffer (pH 7.5) and resuspended in 80 µL of demineralized water. Then 20 µL of sialidase buffer and 1 µL of α(2-3,6,8,9)-neuraminidase were added. This enzyme releases α-2,3-, α-2,6-, α-2,8-, and α-2,9-linked *N*-acetylneuraminic acid from complex carbohydrates. Samples were incubated overnight at 37°C. After incubation, supernatants were collected, volumes were adjusted to 980 µL with Tris-HCl buffer, 20 µL of 0.01 M β-NADH solution was added and the absorbance at 340 nm was measured. Afterwards, 1 µL of *N*-acetylneuraminic acid aldolase and 1 µL of lactic dehydrogenase were added to each sample, and they were incubated at 37 °C for 1 h. Absorbance at 340 nm was measured again after incubation. Sialic acid concentration was calculated using a calibration line of Neu5Ac provided in the kit.

Genomic analysis of pathways for biosynthesis of different nonulosonic acids

BLAST (Basic Local Alignment Search Tool) analysis of key enzymes

In order to predict the potential production of different NulOs by "*Ca.* Accumulibacter", different near-complete draft metagenome-assembled genomes (MAGs) of "*Ca.* Accumulibacter" (Rubio-Rincón et al. 2019) were studied. These MAGs were used to get the amino acid sequences of nonulosonic acid synthases (NAB-2), *i.e.*, the most conserved enzymes of the biosynthetic pathway, which condensates a 6-carbon intermediate with pyruvate to produce a 9-carbon α-keto acid (Figure 2.1C). A protein sequence alignment versus a protein database (BLASTp), or a protein sequence alignment versus a translated nucleotide sequence database (TBLASTn; when only nucleotide sequences were available) from the NCBI website (blast.ncbi.nlm.nih.gov/Blast.cgi) was performed using the sequence of the known *N*-acetylneuraminic acid synthase from *Campylobacter jejuni* (accession number: CAL35431.1) as query. E-values lower than 5e-15 were set as positive result and chosen for the phylogenetic analysis (Petit et al. 2018).

Phylogenetic analysis of NAB-2 sequences

In order to predict the specific NulO synthesized by the different NAB-2 enzymes from the MAGs of "*Ca.* Accumulibacter", the phylogenetic method developed by Lewis et al. (2009) was performed. For this analysis, NAB-2 amino acid sequences with known specificity from different bacteria, archaea and animals were employed. Less conserved enzymes from "*Ca.* Accumulibacter" (Table 2.2 m-s) were removed from the analysis in order to improve the final alignment. The removed sequences might represent unknown specificities not included in the analysis (Lewis et al. 2009).

Shotgun proteomics analysis

Shotgun proteomic analysis of the "*Ca.* Accumulibacter" enrichment was performed as described in the study of Kleikamp et al. (2020b). Briefly, biomass material was disrupted using beads beating in B-PER reagent (Thermo Fisher Scientific, Waltham, MA)/TEAB buffer (50 mM TEAB, 1 % (w/w) NaDOC, adjusted to pH 8.0) buffer. The cell debris was further pelleted and the proteins were precipitated using ice cold acetone. The protein pellet was redissolved in 200 mM ammonium bicarbonate, reduced using DTT and alkylated using iodoacetamide and digested using sequencing grade Trypsin (Promega, Madison,

WI). An aliquot of approx. 100 ng protein digest was further analyzed by a one dimensional shotgun proteomics approach (Köcher et al., 2012) using an ESAY nano LC 1200 coupled to a QE plus Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA). The flow rate was maintained at 300 nL/min over a linear gradient from 5 % to 30 % solvent B over 85 minutes, and finally to 75 % B over 25 minutes. Solvent A was H_2O containing 0.1 % formic acid, and solvent B consisted of 80 % acetonitrile in H_2O and 0.1 % formic acid. The Orbitrap was operated in data depended acquisition mode acquiring peptide signals form 400-1200 m/z at 70K resolution, where the top 10 signals fragmented using a NCE of 30. Raw data were analyzed using PEAKS Studio 8.5 (Bioinformatics Solutions Inc., Waterloo, Canada) allowing 20 ppm parent ion and 0.02 Da fragment mass error tolerance. Search conditions further included considering 3 missed cleavages, carbamidomethylation as fixed and methionine oxidation and N/Q deamidation as variable modifications. Data were matched against a global "*Ca.* Accumulibacter phosphatis" database (Uniprot, Date, Tax ID 327159). Peptide search included the GPM crap contaminant database and a decoy fusion for determining false discovery rates. Peptide spectrum matches were filtered against 1 % false discovery rate (FDR) and protein identifications with 2 or more unique peptides were considered as significant.

2.3. Results

The enrichment culture of "*Ca.* Accumulibacter" was derived from the system described by Guedes da Silva (2020). Data describing the performance of the enrichment are given by the authors. As shown by fluorescence *in situ* hybridization (FISH) and proteomic data (Kleikamp et al. 2020b), the bioaggregates used in this research were highly enriched with "*Ca.* Accumulibacter" (approx. 95 %).

Nonulosonic acid analyses

Fluorescence lectin-binding analysis (FLBA)

Lectins are proteins that bind to specific carbohydrate groups. Fluorescence-labelled lectins can be used as probes for the *in situ* analysis of glycoconjugates in the EPS of bioaggregates (Neu and Kuhlicke 2017). Intact biomass samples collected from the "*Ca.* Accumulibacter" enrichment culture were screened with 77 lectins (data not shown). Some sialic acid-specific lectins gave a positive result,

such as CCA, WGA, MAA and SNA (the binding site of these lectins can be found in Table 2.1). Especially, MAA and SNA gave the strongest signal (Figure 2.2A and B). The signals from both lectins were widely distributed across the aggregate, mainly detected at the surface of the bacterial cells (a more detailed view can be seen in Figure 2.3). SNA recognizes α-2,6-linked sialic acid; while MAA recognize α-2,3-linked sialic acid (Soares et al. 2000) (Table 2.1). The strong signals from both SNA and MAA lectins indicated that the sialic acids on the cell surface of "*Ca.* Accumulibacter" present both types of linkages. In contrast with SNA and MAA, the distribution of the signal of WGA showed the presence of lectin-specific glycoconjugates in other parts of the biomass (Figure 2.2C). This may be due to the wider specificity of WGA (*e.g.*, towards GlcNAc). In addition, although CCA gave a low signal (Figure 2.2D), it indicated the presence of sialic acids with different modifications, as it is specific for the staining of 9-*O* or 4-*O*-acetyl NeuAc.

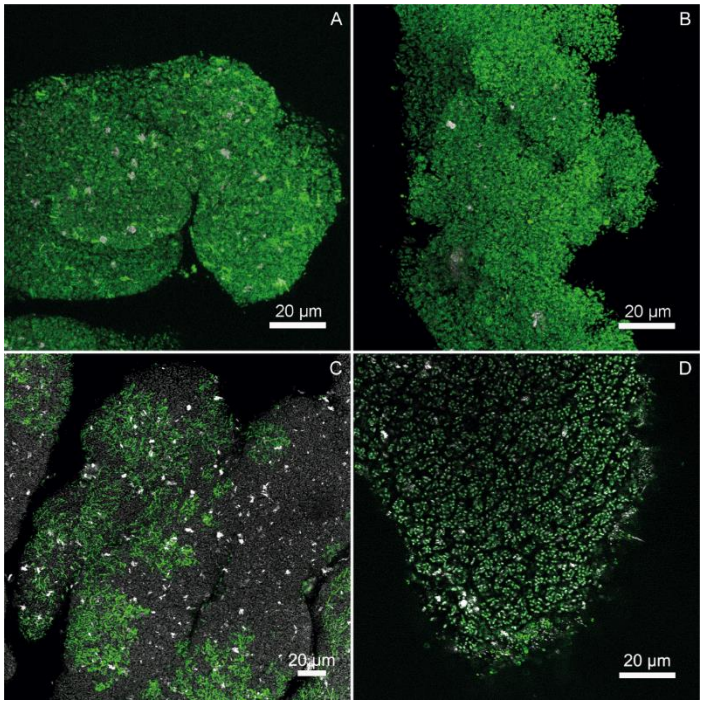

Figure 2.2. Confocal laser scanning microscopy (CLSM) after fluorescence lectin-binding analysis (FLBA). Images show bioaggregates enriched in "*Ca.* Accumulibacter". The glycoconjugates visualized in A, B, C, D show four different sialic acid-specific lectins (A - MAA, B - SNA, C - WGA, D - CCA). The reflection signals either mark reflective particles associated with the granules (A, B) or outline the shape of the granule due to cell internal reflections (C, D). Color allocation: green – glycoconjugates, white – reflection signal.

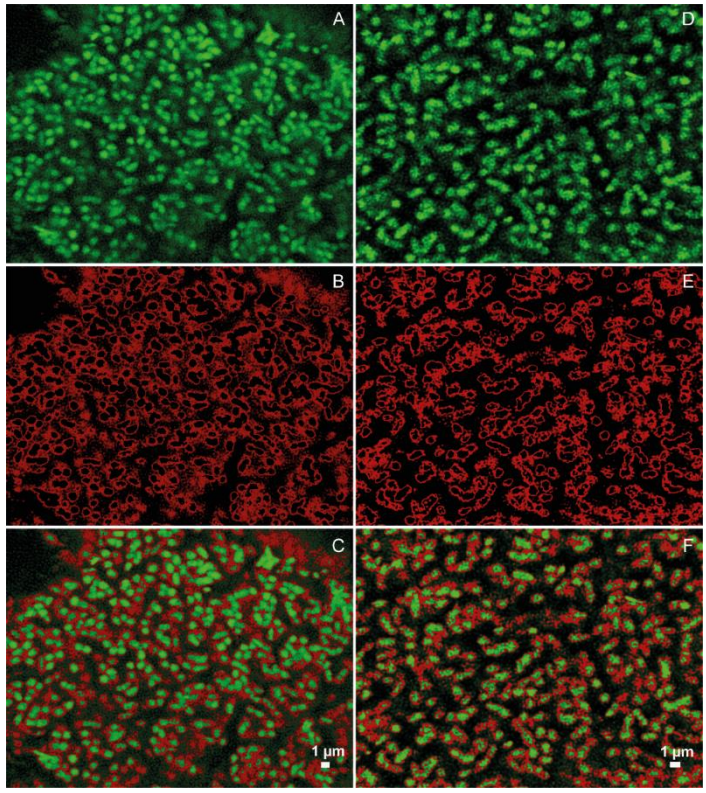

Figure 2.3. Zoomed in section of a granule showing more details of glycoconjugate distribution after lectin staining. The settings for projections were defined in a way that strong and weak signal can be differentiated in different colors. A) Strong signal of lectin MAA at the surface of the bacterial cells (shown in green). Please take notice of the blob-like appearance of the glycoconjugates at the bacterial cell surface. B) Weak signal of the lectin MAA in the space in between bacterial cells (shown in red). C) Overlay of A and B. In Figure D), E) and F) the same is shown for the lectin SNA.

Nonulosonic acid diversity by mass spectrometry analysis

Lectin staining showed a wide distribution of different sialic acids in the "*Ca.* Accumulibacter" enriched bioaggregates. However for this, not only the type of NulO but also the same motifs (linkage type and subterminal monosaccharide) can result in binding. In order to fully explore the diversity of NulOs, manually verification was added in mass spectrometry analysis, which allowed the detection of relatively lower abundant species. Similar as what was reported in Kleikamp et al. (2020a): bacterial NulOs (Pse/Leg) were found dominant both in the enrichment and seawater-adapted aerobic granular sludge, NeuAc was only detected in the latter biomass. Unlike Kleikamp et al. (2020a), small amount of Kdn was detected in the enrichment. Since both samples were enriched with "*Ca.*

Accumulibacter", it seems that bacterial NulOs (Pse/Leg), NeuAc and Kdn might be the potential NulOs that this microorganism can produce.

Sialic acid enzymatic release

Bacterial NulOs and sialic acids are widely distributed in the enriched "*Ca.* Accumulibacter" biomass. In order to quantify the amount of sialic acids present in the enrichment, a commercial enzymatic assay was performed, using a broad spectrum sialidase (α-(2-3,6,8,9)-neuraminidase). The enzyme releases α-2,3-, α-2,6-, α-2,8-, and α-2,9-linked NeuAc. The liberated sialic acids are then detected and quantified after a reaction with an aldolase and dehydrogenase. Unfortunately, no sialic acids were released. This could be due to the specificity of the sialidase which might recognize only NeuAc, but not other variants of sialic acids (*e.g.*, Kdn) and bacterial NulOs. According to MS analysis, NeuAc was present in seawater-adapted aerobic granules, but not in the enriched "*Ca.* Accumulibacter" biomass, which explains why the amount of sialic acids was successfully quantified by the enzymatic assay in seawater-adapted granules as described in de Graaff et al. (2019), but was unsuccessful for "*Ca.* Accumulibacter" enrichment. In fact, sialidases have been reported to differ in their sensitivity, *e.g.*, it was found that Kdn is linked to almost all glycan structures in place of NeuAc, but it has lower sensitivity to sialidase which is specific for NeuAc (Lambre et al. 1982).

Phylogenetic analysis

sVarious metagenome-assembled genomes (MAGs) of "*Ca.* Accumulibacter" available in public repositories and surveyed in literature (Rubio-Rincón et al. 2019) were used to predict the potential diversity of NulOs that "*Ca.* Accumulibacter" can produce. The prediction was focused on the NulO synthase (NAB-2), the enzyme that condenses a 6-carbon intermediate with phosphoenolpyruvate to yield a 9-carbon α-keto acid. This is a common step in the biosynthetic pathway of the different NulOs and the most conserved enzyme in the metabolic route (Lewis et al. 2009). The enzyme NeuAc synthase from *Campylobacter jejuni* (accession number: CAL35431.1) was used to obtain NAB-2 amino acid sequences from the different genomes of "*Ca.* Accumulibacter" (Table 2.2). Different reported NAB-2 amino acid sequences were used as query, giving similar results (data not shown). All the potential NAB-2 enzymes listed present low e-value, ranging from 6e-53 to 5e-19. Although the NeuAc synthase from *C. jejuni* matched with most of the available MAGs of "*Ca.* Accumulibacter",

some of them did not show the presence of this enzyme, which can be both due to a poor assembly or annotation of the genome or to the genetic incapacity of some genotypes to produce NulOs.

These amino acid sequences were used to predict their potential specificity using the phylogenetic analysis method developed by Lewis et al. (2009). The sequences of "*Ca.* Accumulibacter" with the higher e-values (m-s in Table 2.2) were eliminated from the analysis as they appeared to be less-conserved and affected the multiple alignment and therefore, the phylogenetic analysis. These divergent sequences might indicate specificity for a different NulO than the ones used for the final analysis (Lewis et al. 2009). The rest of enzymes of "*Ca.* Accumulibacter", together with sequences from animal, bacteria and archaea, were used to generate a distance-based neighbor-joining tree (Figure 2.4).

The different NAB-2 sequences were grouped based on their predicted specificities. Four groups are generated corresponding to legionaminic acid (Leg), pseudaminic acid (Pse), neuraminic acid (Neu) and "animal-like" NulOs, which reflects a novel phylogenetic class for which no biochemical data currently exist (Lewis et al. 2009). Within these four groups, NAB-2 enzymes from "*Ca.* Accumulibacter" were located in the three groups: Pse, Leg and Neu. For the genomes that presented more than one copy of NAB-2, each of the copy was predicted to produce either the sialic acid Neu, or the one of the bacterial structures (Pse/Leg). Therefore, it is predicted that "*Ca.* Accumulibacter" has the potential to synthesize NulOs with one or two different core structures (Pse/Leg and Neu). Looking back at the mass spectrometry (MS) results, the enriched "*Ca.* Accumulibacter" in this research produced bacterial NulOs (as Leg and Pse are isomers, they cannot be differentiated by MS analysis) and Neu (including Kdn and derived forms of Neu (*e.g.*, NeuAc) as well since they share the same pathway with Neu), which is in consistence with the phylogenetic prediction.

Table 2.2. Selected NAB-2 enzymes (nonulosonic acid synthase) from "*Ca.* Accumulibacter" used for the phylogenetic analysis. Amino acid sequences were obtained by performing BLASTp using the NAB-2 enzyme from *C. jejuni* (accession number: CAL35431.1) as query. No accession numbers are provided for the enzymes where no protein sequences were available as the whole nucleotide sequence was used. Genomes were recovered from on-line public databases such as NIH GenBank (GCA accession numbers), NCBI RefSeq (GCF accession numbers), and JCI MGM (Ga accession numbers).

Enzyme reference	**Enzyme accession number**	**E-value**	**Genome name**	**Genome accession number**
a	2626517415	6.00e-53	"*Ca.* Accumulibacter sp. UW-2"	Ga0078784
b	2689798196	7.00e-53	"*Ca.* Accumulibacter phosphatis Type IA UW-3"	Ga0131788
c	2626510243	7.00e-53	"*Ca.* Accumulibacter sp. UW-?"	Ga0078783
d	OJW49354.1	2.00e-49	"*Ca.* Accumulibacter sp. 66-26"	GCA 001897745
e	KFB70901.1	3.00e-49	"*Ca.* Accumulibacter sp. BA-91"	GCF 000585035
f	OJW47994.1	2.00e-42	"*Ca.* Accumulibacter sp. 66-26"	GCA 001897745
g	KFB74173.1	5.00e-40	"*Ca.* Accumulibacter sp. BA-91"	GCF 000585035
h	KFB66819.1	6.00e-39	"*Ca.* Accumulibacter sp. SK-01"	GCA 000584955
i	KFB76891.1	1.00e-38	"*Ca.* Accumulibacter sp. SK-02"	GCA 000584975
j	WP_046535243.1	3.00e-38	"*Ca.* Accumulibacter phosphatis UW-1"	GCA 000024165
k	-	7.00e-38	"*Ca.* Accumulibacter sp. UBA 5574"	GCA 002425405
l	-	2.00e-37	"*Ca.* Accumulibacter sp. HKU-2"	GCF 000987395
m	HCZ15346.1	5.00e-22	"*Ca.* Accumulibacter sp. UBA11064"	GCA 003538495
n	HCN68329.1	2.00e-21	"*Ca.* Accumulibacter sp. UBA 11070"	GCA 003535635
o	EXI67255.1	7.00e-21	"*Ca.* Accumulibacter sp. SK-12"	GCA 000585015
p	-	3.00e-20	"*Ca.* Accumulibacter sp. UBA 2783"	GCA 002352265
q	EXI83869.1	6.00e-20	"*Ca.* Accumulibacter sp. BA-94"	GCA 000585095
r	EXI78685.1	7.00e-20	"*Ca.* Accumulibacter sp. BA-92"	GCA 000585055
s	RDE52394.1	5.00e-19	"*Ca.* Accumulibacter sp. UW-LDO-IC"	GCA 003332265

Figure 2.4. Distance-based neighbor-joining tree of the NAB-2 sequences (nonulosonic acid synthase). Sequences from bacteria, archaea and animals were used. Enzymes are grouped based on their predicted nonulosonic acid specificity (color shading). Letters (a-l) indicate the enzymes present in the different available genomes of "*Ca.* Accumulibacter" as shown in Table 2.2.

The production of NulOs by "*Ca.* Accumulibacter" was confirmed by lectin staining, the recent mass spectrometry survey (Kleikamp et al. 2020a) and the phylogenetic analysis. To understand also the metabolism of NulOs, the proteome of the "*Ca.* Accumulibacter" enriched biomass was studied using mass spectrometry. Out of the complete list of identified proteins (Table S2.1), it was found that "*Ca.* Accumulibacter" expressed neuraminic acid receptor, permease and tripartite ATP-independent periplasmic (TRAP) transporter proteins (Table 2.3). Those three proteins are used by some pathogenic bacteria as mechanism to decorate their surface molecules, such as capsule polysaccharides,

lipopolysaccharides or flagellum, with sialic acids scavenged from the host, *i.e.*, an extracellular sialic acid molecule is captured via a receptor (*i.e.*, neuraminic acid receptor) and transported through the plasma membrane into the cell via a transporter (*e.g.*, TRAP transporter); linking the sialic acid to a glycoconjugate and finally embedding the glycoconjugate within the plasma membrane (Honma et al. 2015). This suggests the presence of a NulOs-specific utilization/recycling system in the "*Ca.* Accumulibacter" enrichment, similar to the one in pathogenic bacteria. Other enzymes involved in the utilization of NulOs were not found, such as sialidases or enzymes involved in the catabolism of NulOs.

Table 2.3. Expressed proteins by "*Ca.* Accumulibacter" involved in the transport of NulOs. The complete list of identified proteins in the enriched biomass can be found in Table S2.1.

Protein	**Accession**	**# Unique Peptides**	**Coverage (%)**
Neu5Ac-binding protein	A0A011QNL6	11	58
Neu5Ac permease	A0A011P7S7	4	6
TRAP transporter solute receptor	A0A011QP03	3	73

2.4. Discussion

"*Ca.* Accumulibacter" and nonulosonic acid production

Nonulosonic acids (NulOs) are a family of acidic carbohydrates with a nine-carbon backbone. They include sialic acids and other bacterial monosaccharides such as pseudaminic (Pse) and legionaminic acids (Leg). NulOs have been observed at the surface of animal cells and pathogenic bacteria, but they have been generally overlooked in non-pathogenic microorganisms (Varki et al. 2017).

Recently, sialic acids were discovered in glycoproteins within the extracellular polymeric substances (EPS) of seawater-adapted aerobic granular sludge (de Graaff et al. 2019). "*Ca.* Accumulibacter" was suggested to be responsible for sialic acid production. However, to prove the link between the specific microorganism and sialic acid production, a study on a highly enriched culture was necessary. The granular biomass used in this study was proven to be highly enriched in "*Ca.* Accumulibacter" (approx. 95 %, by proteomic investigations (Kleikamp et al. 2020b) and by FISH staining (Guedes da Silva et al. 2018)). Sialic acid-specific lectin staining displayed that sialic acids with α-2,3- and α-2,6-linkage to the sub-terminal monosaccharide were distributed widely on the cell surface of "*Ca.* Accumulibacter". In fact, these sialic acids visualized by lectin staining consist of diverse NulOs: *i.e.*, Kdn (a common sialic acid) and Pse/Leg (bacterial NulOs)

with various modifications, as the actual structure cannot be determined by lectin staining (Song et al. 2011). The most conserved enzyme (nonulosonic acid synthase, NAB-2) of the NulOs biosynthetic pathway can be traced back from the available genomes of "*Ca.* Accumulibacter". The lack of this enzyme in some genomes might be attributed to a low quality or an incomplete state of those genomes but also to the genetic inability to produce NulOs of some genotypes of "*Ca.* Accumulibacter". Phylogenetic analysis based on different sequences of the NAB-2 enzyme predicted the capacity of "*Ca.* Accumulibacter" to produce Pse, Leg and/or Neu (including Kdn). Therefore, this study provides evidence that "*Ca.* Accumulibacter" can synthesize sialic acids and other NulOs. Moreover, it shows the significant diversity of NulOs available in biological environments, in addition to the most common sialic acid Neu5Ac.

Importance of sialic acids and other nonulosonic acids in non-pathogenic bacteria

The ability of bacteria to synthesize sialic acids has been mainly studied in a number of pathogens, where sialic acids or NulOs serve as a way of abolishing the immune response of the host by molecular mimicry (Carlin et al. 2009). Three different types of NulOs are frequently reported as produced by pathogenic bacteria: NeuAc, Pse and Leg. Most NulOs producing pathogens synthesize one type of NulOs, *e.g.*, *Pasteurella multocida* can synthesize NeuAc, *Pseudomonas aeruginosa* can synthesize Pse, and *Clostridium botulinum* can synthesize Leg. Only few pathogens, such as *Campylobacter jejuni* and *Vibrio vulnificus* can synthesize multiple types of NulOs depending on the strain examined (Almagro-Moreno and Boyd 2010). The production of NulOs confers specific advantages to these bacteria in the host-pathogen interaction. Surprisingly, "*Ca.* Accumulibacter", a non-pathogenic bacterium that was cultivated in a bioreactor without any host-pathogen interaction, were found to produce different types of NulOs. Moreover, these compounds were also present in most bacteria and archaea recently tested by Kleikamp et al. (2020a). Thus, the synthesis of NulOs is not necessarily connected with the host-pathogen interaction.

Apart from the synthesis of NulOs, mammalian commensal and pathogenic bacteria that colonize sialic acid rich tissues, such as the respiratory or the gastrointestinal tract, use host-derived sialic acids as competitive advantage. These bacteria take up sialic acids released from the host by means of dedicated transporters, either incorporating them into their cell surface macromolecules or

metabolizing them as a source of carbon, nitrogen and energy source (Almagro-Moreno and Boyd 2010). These bacteria that uptake/utilize sialic acids are closely associated with the host and exposed to a sialic acids rich environment. However, it is extremely interesting to see that "*Ca.* Accumulibacter", cultivated without any NulOs in the media, still expressed Neu receptor, permease and TRAP transporter proteins, which are essential for the uptake of NulOs. Therefore, the common understanding that both the abilities to synthesize and utilize NulOs are limited within pathogenic and/or commensal bacteria is not correct. These abilities might be widely spread in bacteria.

Most of the studies of NulOs to date have been focused on Neu5Ac, since it is the most abundant one in mammals (especially in humans). The findings that there were multiple NulOs produced by "*Ca.* Accumulibacter" enrichment and microorganisms in seawater-adapted aerobic granules, together with other findings reported in literature, suggest the need to extend the consideration of NulOs beyond Neu5Ac alone when bacteria are involved.

Nonulosonic acids and the EBPR process

To avoid eutrophication due to phosphorus pollution, inorganic phosphorus is removed from wastewater by a process called EBPR. "*Ca.* Accumulibacter" has been identified as the dominant organisms responsible for EBPR (Zilles et al. 2002). This microorganism has been well studied in the past decades: different genomic, proteomic, metabolic and modelling studies are available (Oehmen et al. 2010; Barr et al. 2016; Oyserman et al. 2016; Guedes da Silva et al. 2018; Guedes da Silva et al. 2019; Rubio-Rincón et al. 2019). However, most of the studies overlook the extracellular matrix.

Sialic acids are known to participate in cell-cell interactions in mammals (Schnaar et al. 2014). Especially due to their electronegative nature, together with their bulky structure, they form a protective layer surrounding the cell. NulOs might play a similar role in "*Ca.* Accumulibacter" and provide advantages in competing with other microorganisms in the EBPR process. "*Ca.* Accumulibacter" is able to synthesize multiple NulOs with various modifications. These structures cannot be recognized by a single type of sialidase (as shown in the enzymatic analysis), therefore they can protect the cells from enzymatic degradation. On the other hand, when NulOs become available (*e.g.*, released from their own macromolecules), the expression of specific transporters by "*Ca.* Accumulibacter"

allows them to re-uptake these carbohydrates and re-utilize them, avoiding synthesizing them de novo. Through this recycling, less nutrients and cellular energy resources are required, perhaps this strategy provides "*Ca.* Accumulibacter" advantages in competition as well . Moreover, in vertebrates, sialic acids are typically found as terminal residues on the glycan chains of extracellular glycoconjugates, acting as "bridging" molecules between cells, and between cells and extracellular matrices (Chen and Varki 2010). Although NulOs have been overlooked in environmental bacteria, their known roles as recognition molecules in other organisms suggest that they may be involved in functions such as regulation of "*Ca.* Accumulibacter" bioaggregates formation in wastewater treatment process and natural estuarine systems or mediating recognition for bacteriophages. Therefore, sialic acids and other nonulosonic acids should be investigated in further detail to understand their role in the ecology of "*Ca.* Accumulibacter" and even in the EBPR process in particular, and biofilms in general.

Supplementary information

SI is available online at DOI: 10.1007/s00253-021-11249-3

Table S2.1. Complete list of unique proteins identified in the enrichment of "*Ca.* Accumulibacter".

References

Almagro-Moreno S, Boyd EF (2010) Bacterial catabolism of nonulosonic (sialic) acid and fitness in the gut. Gut Microbes 1:45–50 . https://doi.org/10.4161/gmic.1.1.10386

Angata T, Varki A (2002) Chemical diversity in the sialic acids and related α-keto acids: An evolutionary perspective. Chem Rev 102:439–469 . https://doi.org/10.1021/cr000407m

Barr JJ, Dutilh BE, Skennerton CT, Fukushima T, Hastie ML, Gorman JJ, Tyson GW, Bond PL (2016) Metagenomic and metaproteomic analyses of *Accumulibacter phosphatis*-enriched floccular and granular biofilm. Environ Microbiol 18:273–287 . https://doi.org/10.1111/1462-2920.13019

Bennke CM, Neu TR, Fuchs BM, Amann R (2013) Mapping glycoconjugate-mediated interactions of marine *Bacteroidetes* with diatoms. Syst Appl Microbiol 36:417–425 . https://doi.org/10.1016/j.syapm.2013.05.002

Boleij M, Pabst M, Neu TR, van Loosdrecht MCM, Lin Y (2018) Identification of Glycoproteins Isolated from Extracellular Polymeric Substances of Full-Scale Anammox Granular Sludge. Environ Sci Technol 52:13127–13135 . https://doi.org/10.1021/acs.est.8b03180

Boleij M, Seviour T, Wong LL, van Loosdrecht MCM, Lin Y (2019) Solubilization and characterization of extracellular proteins from anammox granular sludge. Water Res 164:114952 . https://doi.org/10.1016/J.WATRES.2019.114952

Carlin AF, Uchiyama S, Chang YC, Lewis AL, Nizet V, Varki A (2009) Molecular mimicry of host sialylated glycans allows a bacterial pathogen to engage neutrophil Siglec-9 and dampen the innate immune response. Blood 113:3333–3336 . https://doi.org/10.1182/blood-2008-11-187302

Chen X, Varki A (2010) Advances in the biology and chemistry of sialic acids. ACS Chem. Biol. 5:163–176

de Graaff DR, Felz S, Neu TR, Pronk M, van Loosdrecht MCM, Lin Y (2019) Sialic acids in the extracellular polymeric substances of seawater-adapted aerobic granular sludge. Water Res 155:343–351 . https://doi.org/10.1016/J.WATRES.2019.02.040

Deng L, Chen X, Varki A (2013) Exploration of sialic acid diversity and biology using sialoglycan microarrays. Biopolymers 99:650–665

Felz S, Al-Zuhairy S, Aarstad OA, van Loosdrecht MCM, Lin YM (2016) Extraction of Structural Extracellular Polymeric Substances from Aerobic Granular Sludge. J Vis Exp e54534 . https://doi.org/10.3791/54534

Felz S, Vermeulen P, van Loosdrecht MCM, Lin YM (2019) Chemical characterization methods for the analysis of structural extracellular polymeric substances (EPS). Water Res 157:201–208 . https://doi.org/10.1016/J.WATRES.2019.03.068

Flemming H-C, Wingender J (2010) The biofilm matrix. Nat Rev Microbiol 8:623–633 . https://doi.org/10.1038/nrmicro2415

Gallagher JT, Morris A, Dexter TM (1985) Identification of two binding sites for wheat-germ agglutinin on polylactosamine-type oligosaccharides. Biochem J 231:115–122 . https://doi.org/10.1042/bj2310115

Guedes da Silva L (2020) Life in changing environments: The intriguing cycles of Polyphosphate Accumulating Organisms. Dissertation. Delft University of Technology

Guedes da Silva L, Gamez KO, Gomes JC, Akkermans K, Welles L, Abbas B, van Loosdrecht MCM, Wahl SA (2018) Revealing metabolic flexibility of Candidatus Accumulibacter phosphatis

through redox cofactor analysis and metabolic network modeling. bioRxiv 458331 . https://doi.org/10.1101/458331

Guedes da Silva L, Tomás-Martínez S, van Loosdrecht MCM, Wahl SA (2019) The environment selects: Modeling energy allocation in microbial communities under dynamic environments. bioRxiv 689174 . https://doi.org/10.1101/689174

Hanisch F, Weidemann W, Großmann M, Joshi PR, Holzhausen HJ, Stoltenburg G, Weis J, Zierz S, Horstkorte R (2013) Sialylation and muscle performance: Sialic acid is a marker of muscle ageing. PLoS One 8: . https://doi.org/10.1371/journal.pone.0080520

Honma K, Ruscitto A, Frey AM, Stafford GP, Sharma A (2015) Sialic acid transporter NanT participates in *Tannerella forsythia* biofilm formation and survival on epithelial cells. Microb Pathog 94:12–20 . https://doi.org/10.1016/j.micpath.2015.08.012

Kleikamp HBC, Lin YM, McMillan DGG, Geelhoed JS, Naus-Wiezer SNH, van Baarlen P, Saha C, Louwen R, Sorokin DY, van Loosdrecht MCM, Pabst M (2020a) Tackling the chemical diversity of microbial nonulosonic acids – a universal large-scale survey approach. Chem Sci. https://doi.org/10.1039/C9SC06406K

Kleikamp HBC, Pronk M, Tugui C, Guedes da Silva L, Abbas B, Mei Lin Y, van Loosdrecht MCM, Pabst M (2020b) Quantitative profiling of microbial communities by 2 de novo metaproteomics. bioRxiv 2020:2020.08.16.252924 . https://doi.org/10.1101/2020.08.16.252924

Knibbs RN, Goldstein IJ, Ratcliffe RM, Shibuya N (1991) Characterization of the carbohydrate binding specificity of the leukoagglutinating lectin from *Maackia amurensis*. Comparison with other sialic acid-specific lectins. J Biol Chem 266:83–88

Knirel YA, Shashkov AS, Tsvetkov YE, Jansson PE, Zähringer U (2003) 5,7-Diamino-3,5,7,9-tetradeoxynon-2-ulosonic acids in bacterial glycopolymers: Chemistry and biochemistry. Adv Carbohydr Chem Biochem 58:371–417 . https://doi.org/10.1016/S0065-2318(03)58007-6

Lambre CR, Kazatchkine MD, Maillet F, Thibon M (1982) Guinea pig erythrocytes, after their contact with influenza virus, acquire the ability to activate the human alternative complement pathway through virus-induced desialation of the cells. J Immunol 128:629–634

Lewis AL, Desa N, Hansen EE, Knirel YA, Gordon JI, Gagneux P, Nizet V, Varki A (2009) Innovations in host and microbial sialic acid biosynthesis revealed by phylogenomic prediction of nonulosonic acid structure. Proc Natl Acad Sci U S A 106:13552–13557 . https://doi.org/10.1073/pnas.0902431106

Mainstone CP, Parr W (2002) Phosphorus in rivers - Ecology and management. Sci Total Environ 282–283:25–47 . https://doi.org/10.1016/S0048-9697(01)00937-8

Neu T, Kuhlicke U (2017) Fluorescence Lectin Bar-Coding of Glycoconjugates in the Extracellular Matrix of Biofilm and Bioaggregate Forming Microorganisms. Microorganisms 5:5 . https://doi.org/10.3390/microorganisms5010005

Neu TR, Lawrence JR (2016) Laser Microscopy for the Study of Biofilms: Issues and Options. In: Romani AM, Guasch H, Balaguer MD (eds) Aquatic Biofilms: Ecology, Water Quality and Wastewater Treatment. Caister Academic Press, Norfolk, pp 29–46

Neu TR, Lawrence JR (2017) The extracellular matrix - an intractable part of biofilm systems. In: Flemming H-C, Neu TR, Wingender J (eds) The perfect slime - Microbial extracellular polymeric substances (EPS). IWA Publishing, London, pp 25–60

Oehmen A, Lopez-Vazquez CM, Carvalho G, Reis MAM, van Loosdrecht MCM (2010) Modelling the population dynamics and

metabolic diversity of organisms relevant in anaerobic/anoxic/aerobic enhanced biological phosphorus removal processes. Water Res 44:4473–4486 . https://doi.org/10.1016/j.watres.2010.06.017

Oyserman BO, Noguera DR, del Rio TG, Tringe SG, McMahon KD (2016) Metatranscriptomic insights on gene expression and regulatory controls in *Candidatus* Accumulibacter phosphatis. ISME J 10:810–22 . https://doi.org/10.1038/ismej.2015.155

Petit D, Teppa E, Cenci U, Ball S, Harduin-Lepers A (2018) Reconstruction of the sialylation pathway in the ancestor of eukaryotes. Sci REPORTS | 8:2946 . https://doi.org/10.1038/s41598-018-20920-1

Ravindranath MH, Higa HH, Cooper EL, Paulson JC (1985) Purification and characterization of an O-acetylsialic acid-specific lectin from a marine crab *Cancer antennarius*. J Biol Chem 260:8850–6

Rubio-Rincón FJ, Weissbrodt DG, Lopez-Vazquez CM, Welles L, Abbas B, Albertsen M, Nielsen PH, van Loosdrecht MCM, Brdjanovic D (2019) "*Candidatus* Accumulibacter delftensis": A clade IC novel polyphosphate-accumulating organism without denitrifying activity on nitrate. Water Res 161:136–151 . https://doi.org/10.1016/J.WATRES.2019.03.053

Schnaar RL, Gerardy-Schahn R, Hildebrandt H (2014) Sialic acids in the brain: Gangliosides and polysialic acid in nervous system development, stability, disease, and regeneration. Physiol Rev 94:461–518 . https://doi.org/10.1152/physrev.00033.2013

Seviour RJ, Mino T, Onuki M (2003) The microbiology of biological phosphorus removal in activated sludge systems. FEMS Microbiol. Rev. 27:99–127

Seviour T, Derlon N, Dueholm MS, Flemming H-C, Girbal-Neuhauser E, Horn H, Kjelleberg S, van Loosdrecht MCM, Lotti T, Malpei MF, Nerenberg R, Neu TR, Paul E, Yu H, Lin Y (2019) Extracellular polymeric substances of biofilms: Suffering from an identity crisis. Water Res 151:1–7 . https://doi.org/10.1016/J.WATRES.2018.11.020

Shibuya N, Goldstein IJ, Broekaert WF, Nsimba-Lubaki M, Peeters B, Peumans WJ (1987) The elderberry (*Sambucus nigra* L.) bark lectin recognizes the Neu5Ac(alpha 2-6)Gal/GalNAc sequence. J Biol Chem 262:1596–1601

Soares RMA, Rosangela RM, Alviano DS, Angluster J, Alviano CS, Travassos LR (2000) Identification of sialic acids on the cell surface of *Candida albicans*. Biochim Biophys Acta - Gen Subj 1474:262–268 . https://doi.org/10.1016/S0304-4165(00)00003-9

Song X, Yu H, Chen X, Lasanajak Y, Tappert MM, Air GM, Tiwari VK, Cao H, Chokhawala HA, Zheng H, Cummings RD, Smith DF (2011) A sialylated glycan microarray reveals novel interactions of modified sialic acids with proteins and viruses. J Biol Chem 286:31610–31622 . https://doi.org/10.1074/jbc.M111.274217

Traving C, Schauer R (1998) Structure, function and metabolism of sialic acids. Cell. Mol. Life Sci. 54:1330–1349

Varki A, Schnaar RL, Schauer R (2017) Sialic Acids and Other Nonulosonic Acids. In: Varki A (ed) Essentials of Glycobiology, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor (NY), pp 179–195

Watson SJ, Needoba JA, Peterson TD (2019) Widespread detection of *Candidatus* Accumulibacter phosphatis, a polyphosphate-accumulating organism, in sediments of the Columbia River estuary. Environ Microbiol 21:1369–1382 . https://doi.org/10.1111/1462-2920.14576

Weissbrodt DG, Neu TR, Kuhlicke U, Rappaz Y, Holliger C (2013) Assessment of bacterial and structural dynamics in aerobic granular biofilms. Front Microbiol 4: . https://doi.org/10.3389/fmicb.2013.00175

Zilles JL, Peccia J, Kim MW, Hung CH, Noguera DR (2002) Involvement of *Rhodocyclus*-related organisms in phosphorus removal in full-scale wastewater treatment plants. Appl Environ Microbiol 68:2763–2769 . https://doi.org/10.1128/AEM.68.6.2763-2769.2002

3

Enrichment and application of bacterial sialic acids containing polymers from the extracellular polymeric substances of "*Candidatus* Accumulibacter"

Abstract

Pseudaminic and legionaminic acids are a subgroup of nonulosonic acids (NulOs) unique to bacterial species. There is a lack of advances in the study of these NulOs due to their complex synthesis and production. Recently, it was seen that "*Candidatus* Accumulibacter" can produce Pse or Leg analogues as part of its extracellular polymeric substances (EPS). In order to employ a "*Ca.* Accumulibacter" enrichment as production platform for bacterial sialic acids, it is necessary to determine which fractions of the EPS of "*Ca.* Accumulibacter" contain NulOs and how to enrich and/or isolate them. We extracted the EPS from granules enriched with "*Ca.* Accumulibcater" and used size-exclusion chromatography to separate them into different molecular weight fractions. This separation resulted in two high molecular weight (> 5,500 kDa) fractions dominated by polysaccharides, with a NulO content up to 4 times higher than the extracted EPS. This suggests that NulOs in "*Ca.* Accumulibacter" are likely located in high molecular weight polysaccharides. Additionally, it was seen that the extracted EPS and the NulO-rich fractions can bind and neutralize histones. This opens the possibility of EPS and NulO-rich fractions as potential source for sepsis treatment drugs.

Highlights

- NulOs in "*Ca.* Accumulibacter" are likely located in high molecular weight polysaccharides.
- Size-exclusion chromatography allows to obtain high molecular weight polysaccharide-rich fractions enriched with NulOs.
- EPS and the NulOs-rich fractions are a potential source for sepsis treatment drugs.

Published as:

Tomás-Martínez S, Chen LM, Pabst M, Weissbrodt DG, van Loosdrecht MCM, Lin Y (2022) Enrichment and application of extracellular nonulosonic acids containing polymers of *Accumulibacter*. Appl Microbiol Biotechnol

3.1. Introduction

Nonulosonic acids (NulOs) are a family of α-keto-acid carbohydrates with a nine-carbon backbone, with a wide variety of chemical forms. The different NulOs are normally found as terminal residues of extracellular glycoconjugates, acting as recognition molecules. The most studied representatives are derivatives of neuraminic (Neu) and ketodeoxynonulosonic (Kdn) acids (also known as sialic acids), specially N-acetyl-neuraminic acid (Neu5Ac), due to their importance in human physiology (Chen and Varki 2010). However, there is a subgroup of NulOs that are unique to bacterial species. Examples of these are the derivatives of pseudaminic (Pse) or legionaminic (Leg) acids, which are often referred as "bacterial sialic acids" (Knirel et al. 2003). Bacteria have been reported to use these NulOs to decorate their surface polymers, such as capsular polysaccharides, lipopolysaccharides, flagella or S-layer glycocoproteins. Bacteria can also polymerize NulOs in these structures, forming polysialic acid (polySia) chains, with different degrees of polymerization (Haines-Menges et al. 2015). Bacterial sialic acids have been mainly studied for their role in pathogenesis. In pathogenic bacteria, these molecules serve as virulence factor and as mechanism of evading the host's immune response by molecular mimicking, due to the structural similarities with human sialic acids (Varki et al. 2017). Bacterial sialic acids have also been suggested to play important roles in bacterial motility and biofilm formation (Goon et al. 2003; Jurcisek et al. 2005). However, further research is needed to fully understand the exact role of Pse and Leg and their derivatives.

An important reason for the lack of advances in this field is the lack of chemical access to NulOs in general, and Pse and Leg and their derivatives in particular. Neu5Ac has been traditionally synthesized chemically or extracted from natural sources. Engineered bacteria (*i.e.*, *Escherichia coli*) have also been explored for its production. However, the complex structure of Pse and Leg makes their synthesis, production and commercialization difficult (Flack et al. 2020). The biosynthetic pathway of these carbohydrates is complex and requires several steps (Tomek et al. 2017). This makes enzymatic methods for the production of these compounds and derivatives too complex and their production would be very costly (Chidwick et al. 2021). There have nonetheless been advances in the chemical synthesis of Pse and Leg, the production yields are still low for a proper commercial production (Carter and Kiefel 2018). Moreover, the dependency of

organic solvents for their chemical synthesis is a concern for the sustainability of the production. Microbial biosynthesis of Pse and Leg has been mainly studied in pathogenic bacteria, which complicates the use of these organisms as production method. Therefore, new sustainable and efficient ways of production need to be explored.

A genome level study revealed that the biosynthetic pathway for different NulOs is widespread among archaea and bacteria (Lewis et al. 2009). However, NulOs have been overlooked in non-pathogenic bacteria. Very recently, a mass spectrometry based survey revealed an unexpectedly wide distribution of NulOs among non-pathogenic environmental bacteria (Kleikamp et al. 2020). Pinel et al. (2020) described the presence of Kdn and bacterial sialic acids in biofilms forming in cooling towers. In wastewater environments, Boleij et al. (2020) detected NeuAc, Kdn and bacterial sialic acids in the extracellular polymeric substances (EPS) of anammox granular sludge. Sialic acids were also identified in aerobic granular sludge dominated with "*Candidatus* Accumulibacter" (de Graaff et al. 2019). Further research with a highly enriched culture of "*Ca.* Accumulibacter" revealed its potential to produce different types of NulOs as part of its EPS, primarily the bacterial sialic acids Pse and/or Leg, which have the same molecular weight (Tomás-Martínez et al. 2021). Although the role of these carbohydrates in non-pathogenic environmental bacteria is still unknown, these findings point towards a new potential sustainable source of bacterial sialic acids.

"*Ca.* Accumulibacter" is the most abundant and well-studied polyphosphate accumulating organism (PAO) in wastewater treatment plants with biological phosphorus removal. Even though this microorganism has never been isolated, it has been successfully cultivated in laboratory bioreactors for decades (Smolders et al. 1994), reaching levels of enrichment of more than 95 % (Guedes da Silva et al. 2020). This successful enrichment has been achieved by employing ecological selection principles. "*Ca.* Accumulibacter" grows in the form of compact bioaggregates (granules) held together by the EPS (Weissbrodt et al. 2013; Barr et al. 2016). If bacterial sialic acids can be produced by a natural enrichment of "*Ca.* Accumulibacter" in a mixed-culture bioreactor, the aforementioned problem of involving pathogenic bacteria in the production process and the high cost of employing pure cultures will be avoided. This will be beneficial for the large scale industrial production of NulOs.

In order to employ a "*Ca.* Accumulibacter" enrichment to produce bacterial sialic acids, it is necessary to determine which fractions of the EPS of "*Ca.* Accumulibacter" contain NulOs and how to enrich and/or isolate them. In addition, NulOs can be polymerized into polysialic acid chains, conferring a high negative charge density. The polyanionic characteristics of these polymers allows their application in binding and neutralizing positively charged compounds, such as against histone-mediated cytotoxicity. Positively charged histones act as antimicrobial peptides to combat against pathogens. However, they are also toxic for host cells and excessive extracellular histones are associated with the development of sepsis or other diseases (Xu et al. 2009). Negatively charged polysialic acid inactivate the cytotoxic characteristics of histones (Galuska et al. 2017; Zlatina et al. 2017).

The aim of the present research was to determine in which EPS component of "*Ca.* Accumulibacter" NulOs are by fractionation and to evaluate the potential application of the NulOs-rich fractions against sepsis. EPS were extracted and characterized from granules from a lab enrichment of "*Ca.* Accumulibacter". Extracted EPS was separated into different molecular weight fractions using size-exclusion chromatography. The anionic characteristic of the fractions was evaluated and the NulOs content was measured. Finally, the potential application against sepsis was evaluated performing a histone-binding assay.

3.2. Materials & Methods

Reactor operation and characterization

The PAO enrichment was obtained in a 2 L (1.5 L working volume) sequencing batch reactor (SBR), following conditions similar to the one described by Guedes da Silva et al. (2020) with some adaptations. The reactor was inoculated using activated sludge from a municipal wastewater treatment plant (Harnaschpolder, The Netherlands). Each SBR cycle lasted 6 hours, consisting of 20 minutes of settling, 15 minutes of effluent removal, 5 minutes of N_2 sparging, 5 minutes of feeding, 135 minutes of anaerobic phase and 180 minutes of aerobic phase. The hydraulic retention time (HRT) was 12 hours (removal of 750 mL of broth per cycle). The average solids retention time (SRT) was controlled to 8 days by the removal of effluent at the end of the mixed aerobic phase. The pH was controlled

at 7.0 ± 0.1 by dosing 0.2 M HCl or 0.2 M NaOH. The temperature was maintained at 20 ± 1 °C.

The reactor was fed with two separate media: a concentrated COD medium (400 mg COD/L) of 68:32 gCOD/gCOD acetate:propionate (5.53 g/L NaAc·3H2O, 1.20 g/L NaPr, 0.04 g/L yeast extract) and a concentrated mineral medium (1.53 g/L NH4Cl, 1.59 g/L $MgSO_4 \cdot 7H_2O$, 0.40 g/L $CaCl_2 \cdot 2H_2O$, 0.48 KCl, 0.04 g/L N-allylthiourea (ATU), 2.22 g/L $NaH_2PO_4 \cdot H_2O$, 6 mL/L of trace element solution prepared following Smolders et al. (1994). In each cycle, 75 mL of each medium were added to the reactor, together with 600 mL of demineralized water. The final feed contained 400 mg COD/L of acetate.

Monitoring of the SBR

Electrical conductivity in the bulk liquid was used to follow phosphate release and uptake patterns and to verify the steady performance of the reactor (Weissbrodt et al. 2014). Extracellular concentrations of phosphate and ammonium were measured with a Gallery Discrete Analyzer (Thermo Fisher Scientific, Waltham, MA). Acetate was measured by high performance liquid chromatography (HPLC) with an Aminex HPX-87H column (Bio-Rad, Hercules, CA), coupled to RI and UV detectors (Waters, Milford, MA), using 0.01 M phosphoric acid as eluent supplied at a flowrate of 0.6 mL/min.

Microbial community analysis

The microbial community was characterized by 16S rRNA gene amplicon sequencing. DNA was extracted from the granules using the DNeasy UltraClean Microbial kit (Qiagen, Venlo, The Netherlands), using the manufacturer's protocol. The extracted DNA was quantified using a Qubit 4 (Thermo Fisher Scientific, Waltham, MA). Samples were sent to Novogene Ltd. (Hong Kong, China) for amplicon sequencing of the V3-4 hypervariable region of the 16S rRNA gene (position 341-806) on a MiSeq desktop sequencing platform (Illumina, San Diego, CA) operated under paired-end mode. The raw sequencing reads were processed by Novogene Ltd. (Hong Kong, China) and quality filtered using the QIIME software (Caporaso et al. 2010). Chimeric sequences were removed using UCHIME (Edgar et al. 2011) and sequences with ≥97 % identity were assigned to the same operational taxonomic units (OTUs) using UPARSE (Edgar 2013). Each OTU was taxonomically annotated using the Mothur software against the SSU rRNA database of the SILVA Database (Quast et al. 2013).

EPS extraction

Biomass samples collected at the end of the aerobic phase were freeze-dried prior to EPS extraction. EPS were extracted in alkaline conditions at high temperature, using a method adapted from Felz et al. (2016). Freeze-dried biomass were stirred in of 0.1 M NaOH (1 % w/v of volatile solids) at 80 ºC for 30 min. Extraction mixtures were centrifuged at 4000xg at 4 ºC for 20 min. Supernatants were collected and dialyzed overnight in dialysis tubing with a molecular cut-off of 3.5 kDa, frozen at -80 ºC and freeze-dried. The freeze-dried extracted EPS samples were stored for further analysis.

Size-exclusion chromatography fractionation

Freeze-dried EPS was solubilized in NaOH 0.01 M to a concentration of 10 mg/mL. Size-exclusion chromatography (SEC) was performed using a Hiload 16/600 Superose 6 prepacked column (Cytiva Lifesciences, Marlborough, MA) fitted on a system containing GX-271 dispenser/dilutor, a 307 pump and a 112 UV (280 nm) detector (Gilson, Middleton, WI). Fractions of molecular weights (MW) were determined after calibration with a HMW gel filtration calibration kit (44-669 kDa) (Cytiva Lifesciences, Marlborough, MA) and Blue Dextran (2,000 kDa). Molecular weights higher than the standards were calculated by linear extrapolation of the calibration line. A total of 15 mL of dissolved EPS was injected in the column with a flow rate of 1 mL/min. The running buffer consisted of 0.15 M NaCl and 0.05 M glycine-NaOH at pH 10. Seven different fractions were collected with MW ranges as shown in Table 3.1. The different fractions were dialyzed overnight in dialysis tubing with a molecular cut-off of 3.5 kDa, frozen at -80 ºC and freeze-dried. The freeze-dried fractions were stored for further analysis.

EPS and fractions characterization

Protein and carbohydrate content

Protein content was estimated using the bicinchoninic acid (BCA) assay (Smith et al. 1985) with bovine serum albumin (BSA) as standard. Carbohydrate content was determined using the phenol-sulfuric acid assay (Dubois et al. 1956) with glucose as standard. Both methods were used as described by Felz et al. (2019).

Fourier-transformed infra-red (FT-IR) spectroscopy

The FT-IR spectra of the different fractions was recorded on a FT-IR spectrometer (Perkin Elmer, Shelton, CT) at room temperature, with a

wavenumber range from 550 to 4000 cm^{-1}. Resolution of 1 cm^{-1} and accumulation of 8 scans were applied to each sample.

Nonulosonic acid analysis

NulOs were analyzed by high resolution mass spectrometry according to Kleikamp et al. (2020). Freeze-dried biomass were hydrolyzed in of diluted (2 M) acetic acid during 2 hours at 80 °C. After centrifugation, samples were dried using a Speedbac concentrator and labelled using DMB (1,2-diamino-4,5- methylene dioxybenzene dihydrochloride) during 2.5 hours at 50 °C. Labelled NulOs were analyzed by reverse phase chromatography Orbitrap mass spectrometry (QE plus quadrupole Orbitrap, Thermo Fisher Scientific, Waltham, MA). NulOs were identified according to their mass. To estimate the relative amount of NulOs in the samples, the peak area of a standard of Kdn was used as reference.

SDS-PAGE analysis and staining with Alcian Blue

SDS-PAGE was performed using NuPage® Novex 4-12 % Bis-Tris gels (Invitrogen, Waltham, MA) as described by Boleij et al. (2018). After dissolving in NaOH 0.1 M, each fraction was prepared in NuPAGE LDS-buffer and DTT (dithiothreitol) was added to a final concentration of 10 mM. Samples were incubated at 70 °C for 10 min for protein denaturation. A volume of 10 µL of sample was loaded per well. The Spectra Multicolor Broad Range Protein Ladder (Thermo Fisher Scientific, Waltham, MA) was used as MW marker. Gel electrophoresis was performed at 200 V for 35 min. After electrophoresis, the gel was stained with Alcian Blue at pH 2.5 for the visualization of carboxylate groups (R-COO-). The gel was extensively washed in solution I (25 % (v/v) ethanol and 10 % (v/v) acetic acid) for 2.5 hours, refreshing the solution 4 times. After washing, the gel was stained in 0.125 % (v/v) Alcian Blue in solution I for 30 min and washed in solution I overnight.

Histone binding and agarose gel electrophoresis

Interaction of EPS and the obtained fractions with histones was tested using a method adapted from Zlatina et al. (2017). A mass of 5 µg of histones (H1, H2A, or H2B) was incubated with different amounts of EPS (in a ratio of 1:1, 1:2 or 1:3 histone:EPS), fractions (in a ratio of 1:2, 1:3 or 1:4 histone:fraction) or free Neu5Ac (in a ratio 1:3 histone:Neu5Ac) in 50 mM Tris for 1 hour at 30 °C and 300 rpm. Subsequently, 1 µL glycerol was added to each sample and samples were loaded on a 0.8 % agarose gel in 500 mM Tris/HCl, 90 mM boric acid, pH 8.5.

The electrophoresis was performed at 80 V for 90 min with a running buffer (90 mM Tris/HCl, 90 mM boric acid, pH 8.5). The agarose gel was stained with Coomassie Blue for 1 hour and washed in demineralized water overnight.

3.3. Results

EPS extraction

For this study, the EPS of a lab-scale enrichment of "*Ca.* Accumulibacter" performing phosphate removal were extracted. The reactor performance and microbial community composition was similar as in earlier reports (Oehmen et al. 2005; Guedes da Silva et al. 2020), showing high PAO activity and enrichment of "*Ca.* Accumulibacter" (Figure S3.1). The EPS extraction yield was 58.3±14.7 % w/w of volatile solids. The protein and carbohydrate content of the extracted EPS accounted for 60.7±6.8 and 19.0±4.3 % w/w of volatile solids of EPS, respectively. Due to the limitation of the total carbohydrate assay, NulOs are not detected by this method (de Graaff et al. 2019). Thus, the amount of NulOs was not included in the carbohydrate content of EPS.

EPS fractionation and characterization

The extracted pool of EPS was solubilized and fractionated in different molecular weight (MW) ranges using size-exclusion chromatography (SEC). Extracted EPS was separated in seven different fractions with apparent molecular weights ranging from 3 to more than 15,000 kDa. Notably, part of the EPS could not be solubilized and was not injected for the fractionation (non-soluble fraction). Table 3.1 shows the contribution of each fraction to the overall EPS. Most of the fractions (F1-F5) contributed similarly, with weight percentages ranging from 7.7 to 12.1 %, with the exception of the smaller fractions. F6 (12-100 kDa) showed the highest contribution, corresponding to 34.7 % of the total extracted EPS. On the other hand, F7 represented the lowest amount and was excluded from the subsequent analyses due to insufficient sample.

It is worth pointing out that, the fractionation range of the Hiload 16/600 Superose 6 column used in this research is between 5 kDa to 5,000 kDa, and the elution limitation is 40,000 kDa. Although the molecular weight of fraction F1 and F2 are out of the fractionation range of the column, as it is still within the elution limitation, they were collected and analyzed. Their molecular weight range was calculated by extrapolating the calibration curve. Moreover, one should be

aware that all the molecular weight specification of the column corresponds to "globular proteins". As the EPS may not be globular proteins, the molecular weight measured by SEC can only be considered as a relative value.

Table 3.1. Weight distribution of the fractions obtained from SEC of the extracted EPS after dialysis and lyophilization. The non-soluble fraction represents the remaining solids after solubilization of the extracted EPS prior to the fractionation.

Fraction Name	**MW range (kDa)**	**Weight percentage of EPS (%)**
F1	>15,000	7.7
F2	5,500-15,000	8.4
F3	738-5,500	9.8
F4	100-738	12.1
F5	12-100	8.9
F6	5-12	34.7
F7	3-5	5.7
Non-soluble fraction		12.7

For each of the fractions, the contents of total proteins and carbohydrates were estimated using colorimetric methods and BSA and glucose as standards, respectively. Figure 3.1 shows the carbohydrate to protein ratio for the extracted EPS and each of the fractions obtained from SEC. Although extracted EPS was dominated by proteins, the high MW fractions (F1 and F2) were dominated by carbohydrates (PS/PN ratio > 1). The decrease of the MW in the fractions was accompanied with an increase of protein content relative to the carbohydrate content. The smallest MW fractions (F4-F6) were dominated by proteins (PS/PN ratio < 1). Interestingly, F5 and F6 were mainly composed of proteins and the carbohydrate fraction was negligible (PS/PN ratio of 0.01). Thus, SEC allowed the separation of the extracted EPS in high MW carbohydrates dominated fractions.

In order to get a better evaluation of the differences between the obtained fractions, FT-IR spectroscopy was used to analyze their composition. Figure 3.2 shows the individual FT-IR spectrum of each of the fractions. These results confirmed the decrease of carbohydrate content and increase of protein content as the MW decreases. F1 shows a high peak at ~1030 cm^{-1}, corresponding to the C-O stretching of carbohydrates. This peak decreases in F2 and becomes negligible in the rest of the fractions. The opposite tendency occurs with the peaks at ~1530 and ~1640 cm^{-1}, corresponding to the N-O stretching and N-H bending of proteins,

respectively. This peak becomes dominant in the fractions with the lowest PS/PN ratio (F4 and F5). Additionally, F1 shows a peak at ~1730 cm^{-1}, which is associated to the α-keto aldonic acid structure of NulOs (de Graaff et al. 2019). This peak appears subtly in the spectrum of F2 and it is absent in the rest of the fractions. These results suggest the presence of NulOs in the high MW fractions (F1 and F2).

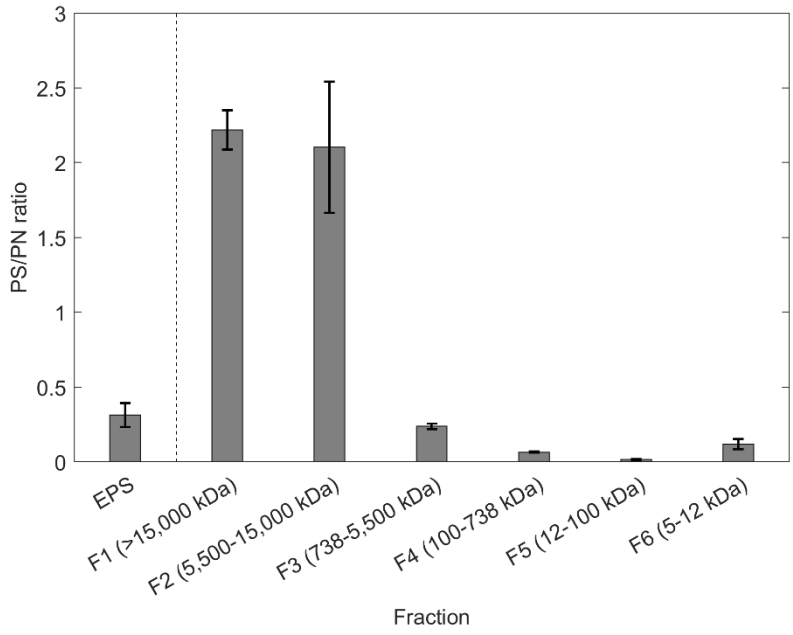

Figure 3.1. Carbohydrate to protein ratio (PS/PN) of the different MW fractions obtained by SEC. Carbohydrate and protein content are expressed as glucose and BSA equivalents.

To confirm the presence and type of NulOs in the EPS and the fractions obtained by SEC, the different samples were analyzed using mass spectrometry. It revealed the presence of double acetylated Pse or Leg ($PseAc_2$ or $LegAc_2$), which cannot be distinguished as they have the same molecular mass. This NulO was detected in the extracted EPS and in the high MW fractions (F1, F2 and F3). The rest of the fractions showed negligible amount of NulO. In order to estimate the amount of NulO in each sample, the area of $PseAc_2$ or $LegAc_2$ was compared to a reference amount of standard Kdn. Although this cannot be used as absolute quantification, it can give a relative estimate of the NulO content of each sample. The estimated content of $PseAc_2$ or $LegAc_2$ of each sample is given in Figure 3.3. The fractions F1 and F2 showed a higher content of $PseAc_2$ or $LegAc_2$ than the original EPS

(4 and 3 times higher, respectively). The fractionation with SEC allowed to obtain fractions highly enriched with $PseAc_2$ or $LegAc_2$,. Those fractions are also carbohydrate-rich and with a MW >5,500 kDa.

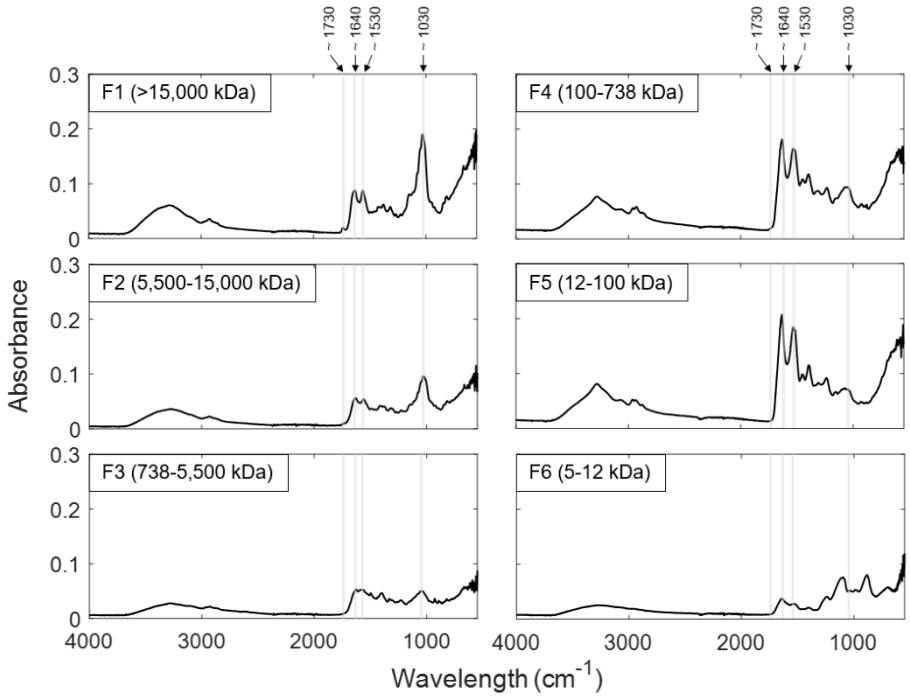

Figure 3.2. FT-IR spectra of the different MW fractions obtained by SEC. Gray areas highlight peaks corresponding to carbohydrates (~1030 cm^{-1}), proteins (~1530 and ~1640 cm^{-1}) and NulOs (~1730 cm^{-1}).

The carboxylic group of NulOs can confer a negative charge to the polymer, which binds with Alcian Blue. In order to confirm and visualize the strongly acidic carboxylic groups in the extracted EPS and the separated fractions, samples were loaded in a SDS-PAGE gel. After the separation, the gel was stained using Alcian Blue at pH 2.5, which is specific for acidic glyconjugates (Figure 3.4). The extracted EPS and fractions F1 and F2 were heavily stained at the position corresponding to high molecular weight, implying the presence of acidic glycoconjugates. In comparison, F3 was slightly stained and F4-F6 were not stained at all. This confirmed the presence of strong acidic groups in the high MW fractions, which is in line with the high amount of $PseAc_2$ or $LegAc_2$ in these fractions.

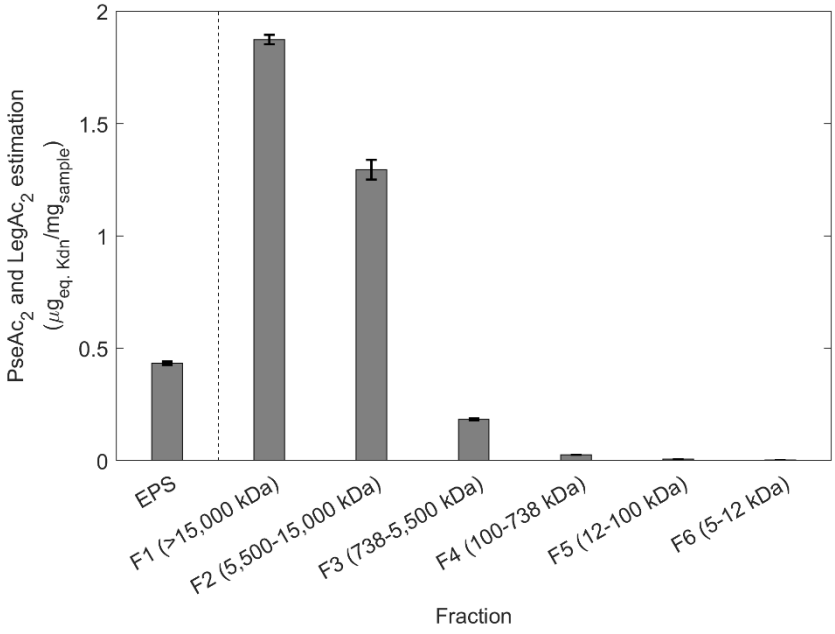

Figure 3.3. Relative quantification of NulOs in the extracted EPS and in the different MW fractions obtained by SEC. The detected NulO is $PseAc_2$ or $LegAc_2$, which could not be distinguished as the have the same molecular weight. The amount of NulOs was estimated based on the relative area of a spiked standard of Kdn.

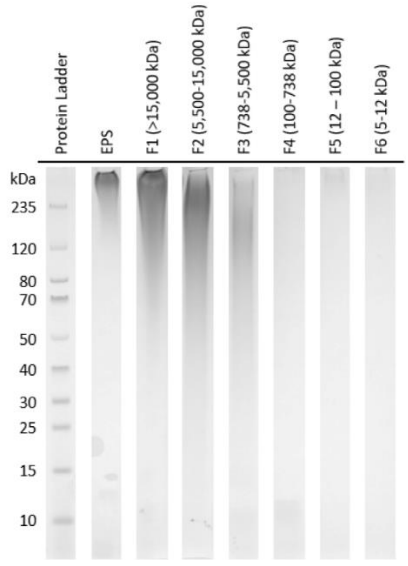

Figure 3.4. SDS-PAGE gel of the extracted EPS and the different MW fractions obtained by SEC. The gel was stained with Alcian Blue at pH 2.5 for acidic carbohydrates.

Histone binding assay

Negatively charged polymers, such as polysialic acids or heparin, can be used as treatment of sepsis due to their capacity to bind histones. To test the potential of extracted EPS and the NulO-rich fractions (F1 and F2) for application in the treatment of sepsis, a histone binding assay was performed. Histones were incubated with the different samples and the migration characteristics was evaluated (Figure 3.5). Histones (*e.g.*, H1, H2A and H2B) are positively charged and they migrate towards the cathode (negative pole). When they are incubated with negatively charged polymers, if the interaction result to the neutralization of the charge of histones, their migration towards the cathode will be reduced.

Firstly, the histone-binding capacity of EPS was tested with three different histones (H1, H2A and H2B) by dosing different amounts of EPS (Figure 3.5A). In the case of histone H1, the migration was slightly only reduced and only when a dosage ratio of 1:3 histone:EPS was used. When a lower dosage of EPS was used, no migration reduction was observed. In the case of histones H2A and H2B, a dosage ratio of 1:1 histone:EPS was already effective, as the migration was only half of the histone control. An increase of dosage ratio to 1:2 and 1:3, significantly decreased the migration characteristics of the histones.

As H2A and H2B are the most abundant histones causing sepsis (Zlatina et al. 2017), they were further tested with the NulO-rich fractions (F1 and F2) (Figure 3.5B). Both fractions reduced the migration of the histone H2B but only F2 reduced the migration of histone H2A. This indicated that they bind with the histones and neutralized their charge. Once the dosage ratio was increased from 1:2 to 1:4 histone:EPS, the neutralization effect increased as well. Generally, the neutralization effect of both NulO-rich fractions is stronger with H2B than with H2A. F2 had a higher reduction of the migration of both histones, when compared to F1, even though the NulO content was lower in F2. It was also noticed that the binding capacity of the extracted EPS was higher than the NulO-rich fractions, a dosage ratio of 1:3 histone:EPS already inhibited the migration of both histones. The exact interaction between EPS (including the fractions) and histones is unclear: probably the polymer conformation or the polymerization degree of NulOs play a role in addition to the charge, or there are other binding sites besides the NulO, for example sulfated glycosaminoglycans (Zlatina et al. 2017, Wang et al. 2020b).

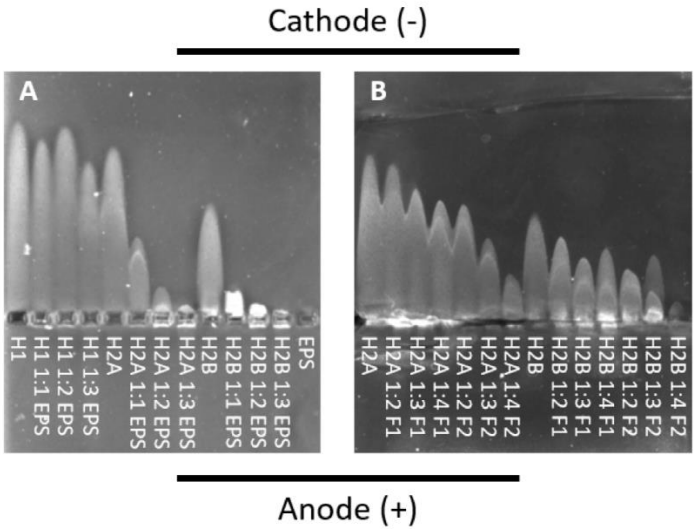

Figure 3.5. Histone binding assay. Histones were incubated with different amounts (as indicated by the mass ratio) of extracted EPS (A) or NulO-rich fractions (F1 (>15,000 kDa) and F2 (5,500-15,000 kDa)) (B). After electrophoresis, gels were stained with Coomassie Blue.

3.4. Discussion

An enrichment of "*Ca.* Accumulibacter" can be a potential production platform of Pse or Leg derivatives

The lack of chemical access to Pse and Leg and their derivatives hinders the study of these type of NulOs. Although their production has been attempted through engineered bacteria or chemical synthesis (Carter and Kiefel 2018; Flack et al. 2020), it is still very costly and too complex. A sustainable alternative can be the use of mixed cultures, which requires non-sterile conditions and reduces the cost substantially (Kleerebezem and van Loosdrecht 2007). This mixed culture biotechnology has been employed for the production of different chemicals, such as, polyhydroxyalkanoates, organic acids or medium-chain fatty acids, among others (Serafim et al. 2008; Dionisi and Silva 2016; Stamatopoulou et al. 2020).

Recently, it was demonstrated that "*Ca.* Accumulibacter" has the potential to produce different types of NulOs in its EPS (Tomás-Martínez et al. 2021). In the current work, we showed the production of $PseAc_2$ or $LegAc_2$ by "*Ca.* Accumulibacter" under our reactor conditions. This bacteria can be cultivated in lab reactors, reaching enrichment levels of up to 95 % of the total community

(Guedes da Silva et al. 2020). This type of reactors allows a good control of the conditions, ensuring the long term reproducibility of the production. Moreover, culture conditions can be easily adapted for specific requirements. For example, it could be determined under which conditions the NulOs production is optimized, and by manipulating the operational conditions, to reach the optimized production.

After NulOs production by "*Ca.* Accumulibacter", a purification strategy needs to be implemented. Here, we managed to increase the NulO content 4 times by a simple purification. Firstly, EPS was extracted by alkaline conditions. The resulting extracted EPS was then fractionated based on molecular weight using size-exclusion chromatography (SEC). This resulted in a NulO enrichment in the fractions corresponding to the highest molecular weights (>5,500 kDa). Further purification has to be explored to obtain $PseAc_2$ or $LegAc_2$. Mild acetic acid hydrolysis has been used previously to release and purify Pse from polysaccharides (Lee et al. 2018). After the release, additional separation steps would be needed to obtain the final product.

NulOs in "*Ca.* Accumulibacter" are likely located in high MW carbohydrates

The separation of the extracted EPS into different MW fractions revealed that $PseAc_2$ and/or $LegAc_2$ are present in high MW polymers (F1 and F2). These fractions, as oppose to the rest, are rich in carbohydrates. Typically, polysaccharides have a much higher MW than proteins. Gómez-Ordóñez et al. (2012) described polysaccharides from seaweed with MW higher than 2,400 kDa. Liu et al. (2016) showed the presence of polymers with MW higher than 1,000 kDa in the EPS of aerobic granules. However, most of the studies in bacterial EPS report a MW to a maximum of 670 kDa (Garnier et al. 2005; Simon et al. 2009). It is noted that, the fractionation range and elution limitation of the SEC column in those studies were much lower than the current research. Probably due to the separation limitation of the column, EPS with higher molecular weight was overlooked. On the other hand, glycosylation of proteins can significantly increase their apparent molecular weight in SEC separation. Human mucus is a complex polymeric mixture with protein biomolecules ranging from 6 kDa to 100 MDa. Specifically, mucins have a typical MW of 200 kDa to 100 MDa (Radicioni et al. 2016). Mucins are highly glycosylated proteins linked with sialic acids and represent 20-30 % by weight of the mucus. As the PS/PN ratio in fractions F1

and F2 is higher than 1, there is a possibility that these two fractions are highly glycosylated proteins comparable to mucins, with similar MW range (5.5 MDa – 40 MDa), linked with bacterial sialic acids.

Pathogenic bacteria have been described to decorate some of their surface polymers with NulOs, such as capsular polysaccharides, lipopolysaccharides, flagella or S-layer glycocoproteins (Haines-Menges et al. 2015). The MW of these polymers has been reported to range from tens to hundreds kDa. The capsular polysaccharide of *Streptococcus pneumonia* ranged from 606 to 1,145 kDa (Bednar and Hennessey 1993). For some strains of *E. coli*, the described MW was lower, ranging from 51.3 to 130.6 kDa (Restaino et al. 2019). Their results showed that lipopolysaccharides have a higher MW than capsular polysaccharide, judging from their elution time in SEC.

Although it was not determined exactly which type of polymer contains $PseAc_2$ or $LegAc_2$ in "*Ca.* Accumulibacter", definitely the NulO-containing polymer is highly glycosylated with a high MW. This could be a glycoprotein similar to mucins, or lipopolysaccharides with a high carbohydrate content. Further purification and analysis could reveal the exact location of these NulOs.

NulOs-rich EPS and fractions as potential source for sepsis treatment drugs

Negatively charged polysaccharides such as heparin or polysialic acids have a cytoprotective effect by neutralization of extracellular histones (Ulm et al. 2013; Wang et al. 2020b). We demonstrated the potential use of the extracted EPS from "*Ca.* Accumulibacter" for this application. The extracted EPS can bind histones H2A and H2B and neutralize them, as indicated by the decrease of migration distance in Figure 3.5. However, the complex composition of EPS will hinder their direct application in the medical field. Separation and purification techniques are needed to obtain compounds that can act as final sepsis treatment drugs.

In this study, the use of SEC for the separation of the extracted EPS allowed to obtain fractions rich in NulOs and dominated by polysaccharides (F1 and F2). For medical application, polysaccharides are preferred over proteins, as proteins show stability and immunogenicity problems (Wang et al. 2020a). Fractions F1 and F2 were tested for their capacity to neutralize histones. A slightly lower capacity than the extracted EPS was observed, which can be compensated by a

higher dosage to achieve a similar effect as the extracted EPS. Although F1 had a higher NulOs content than F2, F2 had a higher histone neutralization effect. Additionally, the higher the dosage, the stronger the effect was. According to Zlatina et al. (2017), polysialic acids rather than single sialic acid monomer manifest the neutralization capacity to histones. Moreover, this capacity of polysialic acids depends on the degree of polymerization. This might explain the higher effect of F2, where $PseAc_2$ or $LegAc_2$ might have a higher degree of polymerization than in F1.

It was noticed that the extracted EPS displayed a stronger neutralizing effect than the NulOs-rich fractions. These differences could be caused by the presence of binding sites other than NulOs in the EPS. For instance, sulfated polysaccharides have been described in the EPS of aerobic and anaerobic granular sludge (Felz et al. 2020; de Bruin et al. 2022), which could potentially contribute to the histone binding capacity of EPS. Further research is needed to examine all the histone binding sites in the EPS and the fractions, in order to fully understand the neutralization mechanisms.

3.5. Conclusion

In this study we showed that enrichments of "*Ca.* Accumulibacter" can be a potential sustainable alternative for the production of bacterial NulOs (*e.g.*, $PseAc_2$ or $LegAc_2$). Size-exclusion chromatography equipped with high molecular weight separation column can be used as initial purification step to obtain NulOs-rich fractions. This separation obtained high molecular weight fractions ($>$ 5,500 kDa) dominated by polysaccharides, where the NulO content was increased up to 4 times, compared with the extracted EPS. Additionally, the capacity of EPS and these fractions to bind histones suggest that they can serve as source for sepsis treatment drugs, although further purification needs to be evaluated.

Supplementary information

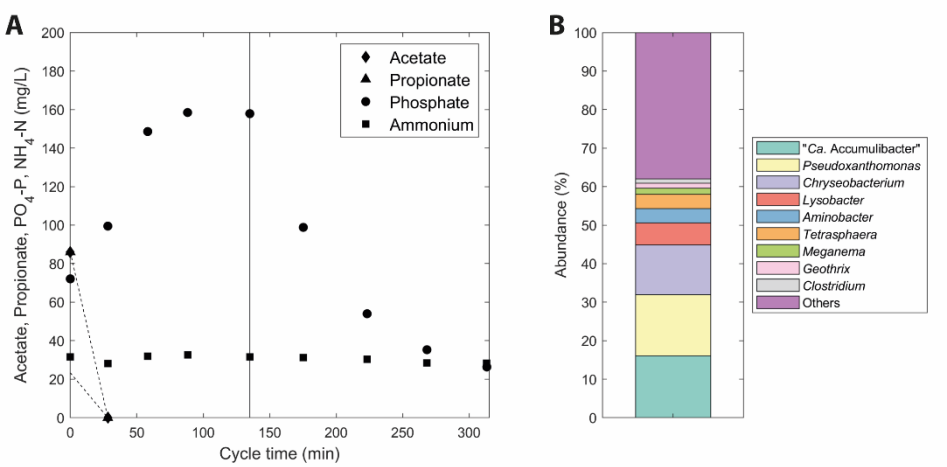

Figure S3.1. A: Concentration of acetate, propionate, phosphate and ammonium during a single SBR cycle after feeding. Black line represents the transition from anaerobic to aerobic phase. B: Relative genus-level microbial community distribution based on 16S rRNA gene amplicon sequencing. All OTUs contributing < 1 % are grouped as "Others".

References

Barr JJ, Dutilh BE, Skennerton CT, Fukushima T, Hastie ML, Gorman JJ, Tyson GW, Bond PL (2016) Metagenomic and metaproteomic analyses of Accumulibacter phosphatis-enriched floccular and granular biofilm. Environ Microbiol 18:273–287 . https://doi.org/10.1111/1462-2920.13019

Bednar B, Hennessey JP (1993) Molecular size analysis of capsular polysaccharide preparations from Streptococcus pneumoniae. Carbohydr Res 243:115–130 . https://doi.org/10.1016/0008-6215(93)84085-K

Boleij M, Kleikamp H, Pabst M, Neu TR, van Loosdrecht MCM, Lin Y (2020) Decorating the Anammox House: Sialic Acids and Sulfated Glycosaminoglycans in the Extracellular Polymeric Substances of Anammox Granular Sludge. Environ Sci Technol 54:5218–5226 . https://doi.org/10.1021/ACS.EST.9B07207

Boleij M, Pabst M, Neu TR, van Loosdrecht MCM, Lin Y (2018) Identification of Glycoproteins Isolated from Extracellular Polymeric Substances of Full-Scale Anammox Granular Sludge. Environ Sci Technol 52:13127–13135 . https://doi.org/10.1021/acs.est.8b03180

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pẽa AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R (2010) QIIME allows analysis of high-throughput community sequencing data. Nat Methods 2010 75 7:335–336 . https://doi.org/10.1038/nmeth.f.303

Carter JR, Kiefel MJ (2018) A new approach to the synthesis of legionaminic acid analogues. RSC Adv 8:35768–35775 . https://doi.org/10.1039/C8RA07771A

Chen X, Varki A (2010) Advances in the biology and chemistry of sialic acids. ACS Chem. Biol. 5:163–176

Chidwick HS, Flack EKP, Keenan T, Walton J, Thomas GH, Fascione MA (2021) Reconstitution and optimisation of the biosynthesis of bacterial sugar pseudaminic acid (Pse5Ac7Ac) enables preparative enzymatic synthesis of CMP-Pse5Ac7Ac. Sci Reports 2021 111 11:1–12 . https://doi.org/10.1038/s41598-021-83707-x

de Bruin S, Vasquez-Cardenas D, Sarbu SM, Meysman FJR, Sousa DZ, van Loosdrecht MCM, Lin Y (2022) Sulfated glycosaminoglycan-like polymers are present in an acidophilic biofilm from a sulfidic cave. Sci Total Environ 829:154472 . https://doi.org/10.1016/J.SCITOTENV.2022.154472

de Graaff DR, Felz S, Neu TR, Pronk M, van Loosdrecht MCM, Lin Y (2019) Sialic acids in the extracellular polymeric substances of seawater-adapted aerobic granular sludge. Water Res 155:343–351 . https://doi.org/10.1016/J.WATRES.2019.02.040

Dionisi D, Silva IMO (2016) Production of ethanol, organic acids and hydrogen: an opportunity for mixed culture biotechnology? Rev Environ Sci Bio/Technology 2016 152 15:213–242 . https://doi.org/10.1007/S11157-016-9393-Y

Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric Method for Determination of Sugars and Related Substances. Anal Chem 28:350–356 . https://doi.org/10.1021/AC60111A017/ASSET/AC60111A017.FP.PNG_V03

Edgar RC (2013) UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat Methods 2013 1010 10:996–998 . https://doi.org/10.1038/nmeth.2604

Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R (2011) UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27:2194–2200 . https://doi.org/10.1093/BIOINFORMATICS/BTR381

Felz S, Al-Zuhairy S, Aarstad OA, van Loosdrecht MCM, Lin YM (2016) Extraction of Structural Extracellular Polymeric Substances from Aerobic Granular Sludge. J Vis Exp e54534 . https://doi.org/10.3791/54534

Felz S, Neu TR, van Loosdrecht MCM, Lin Y (2020) Aerobic granular sludge contains Hyaluronic acid-like and sulfated glycosaminoglycans-like polymers. Water Res 169:115291 . https://doi.org/10.1016/J.WATRES.2019.115291

Felz S, Vermeulen P, van Loosdrecht MCM, Lin YM (2019) Chemical characterization methods for the analysis of structural extracellular polymeric substances (EPS). Water Res 157:201–208 . https://doi.org/10.1016/J.WATRES.2019.03.068

Flack EKP, Chidwick HS, Best M, Thomas GH, Fascione MA (2020) Synthetic Approaches for Accessing Pseudaminic Acid (Pse) Bacterial Glycans. ChemBioChem 21:1397–1407 . https://doi.org/10.1002/CBIC.202000019

Galuska SP, Galuska CE, Tharmalingam T, Zlatina K, Prem G, Husejnov FCO, Rudd PM, Vann WF, Reid C, Vionnet J, Gallagher ME, Carrington FA, Hassett SL, Carrington SD (2017) In vitro generation of polysialylated cervical mucins by bacterial polysialyltransferases to counteract cytotoxicity of extracellular histones. FEBS J 284:1688–1699 . https://doi.org/10.1111/FEBS.14073

Garnier C, Görner T, Lartiges BS, Abdelouhab S, De Donato P (2005) Characterization of activated sludge exopolymers from various origins: A combined size-exclusion chromatography and infrared microscopy study. Water Res 39:3044–3054 . https://doi.org/10.1016/J.WATRES.2005.05.007

Gómez-Ordóñez E, Jiménez-Escrig A, Rupérez P (2012) Molecular weight distribution of polysaccharides from edible seaweeds by high-performance size-exclusion chromatography (HPSEC). Talanta 93:153–159 . https://doi.org/10.1016/J.TALANTA.2012.01.067

Goon S, Kelly JF, Logan SM, Ewing CP, Guerry P (2003) Pseudaminic acid, the major modification on Campylobacter flagellin, is synthesized via the Cj1293 gene. Mol Microbiol 50:659–671 . https://doi.org/10.1046/J.1365-2958.2003.03725.X

Guedes da Silva L, Olavarria Gamez K, Castro Gomes J, Akkermans K, Welles L, Abbas B, van Loosdrecht MCM, Wahl SA (2020) Revealing the Metabolic Flexibility of "Candidatus Accumulibacter phosphatis" through Redox Cofactor Analysis and Metabolic Network Modeling. Appl Environ Microbiol 86: . https://doi.org/10.1128/AEM.00808-20

Haines-Menges BL, Whitaker WB, Lubin JB, Boyd EF (2015) Host Sialic Acids: A Delicacy for the Pathogen with Discerning Taste. In: Metabolism and Bacterial Pathogenesis. American Society of Microbiology, pp 321–342

Jurcisek J, Greiner L, Watanabe H, Zaleski A, Apicella MA, Bakaletz LO (2005) Role of Sialic Acid and Complex Carbohydrate Biosynthesis in Biofilm Formation by Nontypeable Haemophilus influenzae in the Chinchilla Middle Ear. Infect Immun 73:3210 . https://doi.org/10.1128/IAI.73.6.3210-3218.2005

Kleerebezem R, van Loosdrecht MC (2007) Mixed culture biotechnology for bioenergy production. Curr Opin Biotechnol 18:207–212 . https://doi.org/10.1016/J.COPBIO.2007.05.001

Kleikamp HBC, Lin YM, McMillan DGG, Geelhoed JS, Naus-Wiezer SNH, van Baarlen P, Saha C, Louwen R, Sorokin DY, van Loosdrecht MCM, Pabst M (2020) Tackling the chemical diversity of microbial nonulosonic acids – a universal large-scale survey approach. Chem Sci. https://doi.org/10.1039/C9SC06406K

Knirel YA, Shashkov AS, Tsvetkov YE, Jansson PE, Zähringer U (2003) 5,7-Diamino-3,5,7,9-tetradeoxynon-2-ulosonic acids in bacterial glycopolymers: Chemistry and biochemistry. Adv Carbohydr Chem Biochem 58:371–417 . https://doi.org/10.1016/S0065-2318(03)58007-6

Lee IM, Yang FL, Chen TL, Liao KS, Ren CT, Lin NT, Chang YP, Wu CY, Wu SH (2018) Pseudaminic Acid on Exopolysaccharide of Acinetobacter baumannii Plays a Critical Role in Phage-Assisted Preparation of Glycoconjugate Vaccine with High Antigenicity. J Am Chem Soc 140:8639–8643 . https://doi.org/10.1021/JACS.8B04078/ASSET/IMAGES/LARGE/JA-2018-04078A_0003.JPEG

Lewis AL, Desa N, Hansen EE, Knirel YA, Gordon JI, Gagneux P, Nizet V, Varki A (2009) Innovations in host and microbial sialic acid biosynthesis revealed by phylogenomic prediction of nonulosonic acid structure. Proc Natl Acad Sci U S A 106:13552–13557 . https://doi.org/10.1073/pnas.0902431106

Liu X, Sun S, Ma B, Zhang C, Wan C, Lee DJ (2016) Understanding of aerobic granulation enhanced by starvation in the perspective of quorum sensing. Appl Microbiol Biotechnol 100:3747–3755 . https://doi.org/10.1007/S00253-015-7246-1/TABLES/2

Oehmen A, Yuan Z, Blackall LL, Keller J (2005) Comparison of acetate and propionate uptake by polyphosphate accumulating organisms and glycogen accumulating organisms. Biotechnol Bioeng 91:162–168 . https://doi.org/10.1002/BIT.20500

Pinel ISM, Kleikamp HBC, Pabst M, Vrouwenvelder JS, van Loosdrecht MCM, Lin Y (2020) Sialic Acids: An Important Family of Carbohydrates Overlooked in Environmental Biofilms. Appl Sci 2020, Vol 10, Page 7694 10:7694 . https://doi.org/10.3390/APP10217694

Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO (2013) The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res 41:D590 . https://doi.org/10.1093/NAR/GKS1219

Radicioni G, Cao R, Carpenter J, Ford AA, Wang TT, Li Y, Kesimer M (2016) The innate immune properties of airway mucosal surfaces are regulated by dynamic interactions between mucins and interacting proteins: the mucin interactome. Mucosal Immunol 2016 96 9:1442–1454 . https://doi.org/10.1038/mi.2016.27

Restaino OF, D'ambrosio S, Cassese E, Ferraiuolo SB, Alfano A, Ventriglia R, Marrazzo A, Schiraldi C, Cimini D (2019) Molecular weight determination of heparosan- and chondroitin-like capsular polysaccharides: figuring out differences between wild -type and engineered Escherichia coli strains. Appl Microbiol Biotechnol 103:6771–6782 . https://doi.org/10.1007/S00253-019-09969-8/TABLES/2

Serafim LS, Lemos PC, Albuquerque MGE, Reis MAM (2008) Strategies for PHA production by mixed cultures and renewable waste materials. Appl Microbiol Biotechnol 81:615–628 . https://doi.org/10.1007/S00253-008-1757-Y/TABLES/2

Simon S, Païro B, Villain M, D'Abzac P, Hullebusch E Van, Lens P, Guibaud G (2009) Evaluation of size exclusion chromatography (SEC) for the characterization of extracellular polymeric substances (EPS) in anaerobic granular sludges. Bioresour Technol 100:6258–6268 .

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC (1985) Measurement of protein using bicinchoninic acid. Anal Biochem 150:76–85 . https://doi.org/10.1016/0003-2697(85)90442-7

Smolders GJF, van der Meij J, van Loosdrecht MCM, Heijnen JJ (1994) Model of the anaerobic metabolism of the biological phosphorus removal process: Stoichiometry and pH influence. Biotechnol Bioeng 43:461–470 . https://doi.org/10.1002/BIT.260430605

Stamatopoulou P, Malkowski J, Conrado L, Brown K, Scarborough M (2020) Fermentation of Organic Residues to Beneficial Chemicals: A Review of Medium-Chain Fatty Acid Production. Process 2020, Vol 8, Page 1571 8:1571 . https://doi.org/10.3390/PR8121571

Tomás-Martínez S, Kleikamp HBC, Neu TR, Pabst M, Weissbrodt DG, van Loosdrecht MCM, Lin Y (2021) Production of nonulosonic acids in the extracellular polymeric substances of "Candidatus Accumulibacter phosphatis." Appl Microbiol Biotechnol 2021 1058 105:3327–3338 . https://doi.org/10.1007/S00253-021-11249-3

Tomek MB, Janesch B, Maresch D, Windwarder M, Altmann F, Messner P, Schäffer C (2017) A pseudaminic acid or a legionaminic acid derivative transferase is strain-specifically implicated in the general protein O-glycosylation system of the periodontal pathogen Tannerella forsythia. Glycobiology 27:555–567 . https://doi.org/10.1093/GLYCOB/CWX019

Ulm C, Saffarzadeh M, Mahavadi P, Müller S, Prem G, Saboor F, Simon P, Middendorff R, Geyer H, Henneke I, Bayer N, Rinné S, Lütteke T, Böttcher-Friebertshäuser E, Gerardy-Schahn R, Schwarzer D, Mühlenhoff M, Preissner KT, Günther A, Geyer R, Galuska SP (2013) Soluble polysialylated NCAM: A novel player of the innate immune system in the lung. Cell Mol Life Sci 70:3695–3708 . https://doi.org/10.1007/S00018-013-1342-0/FIGURES/8

Varki A, Schnaar RL, Schauer R (2017) Sialic Acids and Other Nonulosonic Acids. In: Varki A (ed) Essentials of Glycobiology, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor (NY), pp 179–195

Wang K, Liu M, Mo R (2020a) Polysaccharide-Based Biomaterials for Protein Delivery. Med Drug Discov 7:100031 . https://doi.org/10.1016/J.MEDIDD.2020.100031

Wang Z, Wang L, Cao C, Jin H, Zhang Y, Liu Y, Gao Y, Liang X, Li G, Shou S (2020b) Heparin Attenuates Histone-Mediated Cytotoxicity in Septic Acute Kidney Injury. Front Med 7:755 . https://doi.org/10.3389/FMED.2020.586652/BIBTEX

Weissbrodt DG, Maillard J, Brovelli A, Chabrelie A, May J, Holliger C (2014) Multilevel correlations in the biological phosphorus removal process: From bacterial enrichment to conductivity-based metabolic batch tests and polyphosphatase assays. Biotechnol Bioeng 111:2421–2435 . https://doi.org/10.1002/BIT.25320

Weissbrodt DG, Neu TR, Kuhlicke U, Rappaz Y, Holliger C (2013) Assessment of bacterial and structural dynamics in aerobic granular biofilms. Front Microbiol 4: . https://doi.org/10.3389/fmicb.2013.00175

Xu J, Zhang X, Pelayo R, Monestier M, Ammollo CT, Semeraro F, Taylor FB, Esmon NL, Lupu F, Esmon CT (2009) Extracellular histones are major mediators of death in sepsis. Nat Med 15:1318–1321 . https://doi.org/10.1038/NM.2053

Zlatina K, Lütteke T, Galuska SP (2017) Individual Impact of Distinct Polysialic Acid Chain Lengths on the Cytotoxicity of Histone H1, H2A, H2B, H3 and H4. Polymers (Basel) 9: . https://doi.org/10.3390/POLYM9120720

4

CATABOLISM OF SIALIC ACIDS IN AN ENVIRONMENTAL MICROBIAL COMMUNITY

Abstract

Sialic acids are a family of nine-carbon negatively charged carbohydrates. In animals, they are abundant on mucosa surfaces as terminal carbohydrates of mucin glycoproteins. Some commensal and pathogenic bacteria are able to release, take up, and catabolize sialic acids. Recently, sialic acids have been discovered to be widespread among most microorganisms. Although the catabolism of sialic acids has been intensively investigated in the field of host-microbe interactions, very limited information is available on microbial degradation of sialic acids produced by environmental microorganisms. In this study, the catabolic pathways of sialic acids within an microbial community dominated by "*Candidatus* Accumulibacter" was evaluated. Protein alignment tools were used to detect the presence of the different proteins involved in the utilization of sialic acids in the flanking populations detected by 16S rRNA gene amplicon sequencing. The results showed the ability of *Clostridium* to release sialic acids from the glycan chains by the action of a sialidase. *Clostridium* and *Chryseobacterium* can take up free sialic acids and utilize them as nutrient. Interestingly, these results display similarities with the catabolism of sialic acids by the gut microbiota. This study points at the importance of sialic acids in environmental communities in the absence of eukaryotic hosts.

Highlights

- Catabolism of sialic acids in the EBPR system resembles the one of the gut microbiota.
- *Clostridium* and *Chryseobacterium* can take up free sialic acids and utilize them as nutrients.
- *Clostridium* can release sialic acids from glycan chains by the action of a sialidase, while *Chryseobacterium* lacks sialidases.

Published as:

Tomás-Martínez S, Chen LM, Neu TR, Weissbrodt DG, van Loosdrecht MCM, Lin Y (2022) Catabolism of sialic acids in an environmental microbial community. FEMS Microbiol Ecol

4.1. Introduction

Sialic acids are part of a family of nine-carbon, negatively charged, carbohydrates called nonulosonic acids. These compounds are normally found as terminal residues on the glycan chain of the extracellular glycoconjugates of vertebrates, with *N*-acetylneuraminic acid (Neu5Ac) as the most studied representative (Chen and Varki 2010). In animals, sialic acids are extremely important for recognition, interaction processes, and immune responses (Traving and Schauer 1998). In order to delay the immune response of the host, pathogenic bacteria utilize sialic acids for mimicking the host glycosylation pattern. They can either *de novo* synthesize these molecules or scavenge them after releasing them from the host glycoconjugates. (Carlin et al. 2009). Additionally, some pathogenic bacteria are able to use host sialic acids as carbon, nitrogen, and energy source, which is crucial during bacterial colonization (Vimr 2013).

In animals, a major reservoir of sialic acids is found on mucosal surfaces, where sialic acids appear as the terminal carbohydrate of mucin glycoproteins. These mucins form a protective mucosal layer that acts as barrier for pathogens (McGuckin et al. 2011). In intestinal mucins, oligosaccharides make up to 80 % of the mass (Capon et al. 1992), and over 65 % of glycans contain residues of sialic acids as terminal carbohydrates (Robbe et al. 2004). These oligosaccharides can be used as nutrient sources by gastrointestinal commensal and pathogenic bacteria. The consumption of these complex oligosaccharides requires the synergic action of different bacteria. The presence of sialic acids as terminal carbohydrate, makes them the first target of this degradation (Haines-Menges et al. 2015).

The utilization of sialic acids as nutrients starts with their release from the host glycoconjugates under the action of sialidases (NanH), in the form of, *e.g.,* Neu5Ac. Free sialic acids are then taken up by bacteria by the action of specific transporters, which can belong to four different families (*i.e.,* ATP-binding cassette (ABC), tripartite ATP-independent periplasmic (TRAP), major facilitator superfamily (MFS), and sodium solute symporter (SSS)). Gram-negative bacteria first transport sialic acids across the outer membrane by a general porin (Thomas 2016). In the intracellular space, pyruvate is cleaved from Neu5Ac generating *N*-acetylmannosamine (ManNAc) by the action of *N*-acetylneuraminic acid lyase (NanA). ManNAc is then phosphorylated and converted to *N*-acetylglucosamine-6-phosphate (GlcNAc-6-P) by the action of *N*-

acetylmannosamine kinase (NanK) and *N*-acetylmannosamine-6-phosphate epimerase (NanE). GlcNAc-6-P is further deacetylated and deaminated by the action of *N*-acetylglucosamine-6-phopshate deacetylase (NagA) and glucosamine-6-phosphate deaminase (NagB), producing fructose-6-phosphate (Fru-6-P) which can be catabolized in the central carbon metabolism (Vimr 2013; Haines-Menges et al. 2015). Figure 4.1 shows the canonical catabolic Neu5Ac pathway. In addition, other pathways have been described as well. For example, *Bacteroides fragilis* utilizes a novel *N*-acetylmannosamine-6 epimerase converting ManNAc into GlcNAc, which is further phosphorylated to GlcNAc-6P, and the rest of the pathway is the same as the canonical catabolic Neu5Ac pathway (Brigham et al. 2009). On the other hand, *Rumminococcus gnavus* releases sialic acids in the form of 2,7-anhydro-Neu5Ac by the action of a *trans*-sialidase. This chemical form is transported into the cell and converted back to Neu5Ac, which is processed as described in Figure 4.1 (Bell et al. 2020).

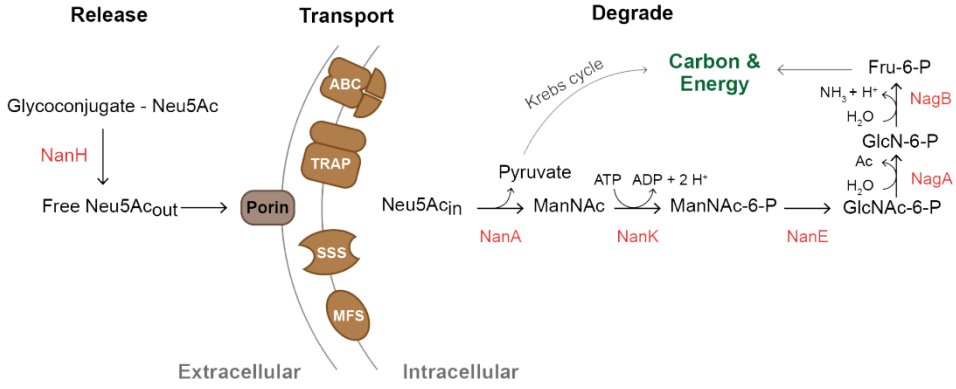

Figure 4.1. Schematic representation of the catabolic pathway of sialic acids (*i.e.,* Neu5Ac) in bacteria. NanH: sialidase; MFS: major facilitator superfamily; TRAP: tripartite ATP-independent periplasmic; ABC: ATP-binding cassette; SSS: sodium solute symporter; NanA: *N*-acetylneuraminic acid lyase; NanK: *N*-acetylmannosamine acid kinase; NanE: *N*-acetylmannosamine-6-P epimerase; NagA: *N*-acetylglucosamine-6-P deacetylase; NagB: glucosamine-6-P deaminase.

Various interactions have been described between gastrointestinal bacteria and sialic acids present in the host glycans. Some bacteria, such as the pathogen *Vibrio cholerae* can secrete extracellular sialidases that release sialic acids from the host glycans, which are then transported into the cells and used as nutrients (Sharma et al. 2011). On the other hand, other microorganisms can excrete sialidases to release sialic acids, but are not able to consume them as nutrients (*i.e., Bacteroides thetaiotaomicron*) (Brigham et al. 2009). This action exposes the

underlying sugars, making them available for consumption. As a result, the released sialic acids can be used by other bacteria, such as some commensal strains of *Escherichia coli*. These strains of *E. coli* lack sialidases to liberate sialic acids from the host glycans but have the enzymes necessary for the uptake and catabolism of free sialic acids (Huang et al. 2015).

Sialic acids have been mainly studied in animal cells and pathogenic bacteria, due to the assumption that presence of sialic acids is confined to pathogenic and commensal species. Only very recently, a mass spectrometry based survey has revealed an unexpectedly wide distribution of sialic acids among non-pathogenic environmental bacteria (Kleikamp et al. 2020). This suggests that sialic acids might be an important component of the extracellular polymeric substances (EPS) of environmental bacteria, where they have been largely overlooked. In addition, although catabolism of sialic acids has been intensively investigated under host-microbe circumstances, little information is available on the microbial degradation of sialic acids produced by other environmental microorganisms. Therefore, understanding the catabolism of sialic acids among environmental bacteria is important to reveal the role of sialic acids within the microbial world other than pathogens and commensals.

"*Candidatus* Accumulibacter phosphatis", a well-studied polyphosphate-accumulating organism (PAO) dominant in wastewater treatment systems designed for enhanced biological phosphorus removal (EBPR), has been recently described to produce different types of sialic acids as part as their EPS (Tomás-Martínez et al. 2021). In these engineered ecosystems alternating anaerobic feast and aerobic famine regimes, the carbon sources are rapidly scavenged by "*Ca.* Accumulibacter" under anaerobic conditions making them unavailable for other organisms in the community. Under substrate limitation, it is assumed that, the extracellular polymers, such as polysaccharides and glycoproteins, produced by "*Ca.* Accumulibacter" might be utilized as substrate by other organisms, especially during the aerobic phase of the EBPR process. As sialic acids mostly appear as the terminal carbohydrate of the glycan chain in glycoproteins present in the EPS (de Graaff et al. 2019), they are likely the first target for cleavage and degradation. In this study, the pathways involved in the catabolism of sialic acids within the EBPR microbial community were evaluated by using protein alignment tools. This gave insight on the catabolism of sialic acids in environmental bacterial aggregates, where no interaction with a vertebrate host is present.

4.2. Materials & Methods

EBPR community characterization

Biomass enriched with "*Ca.* Accumulibacter" from a laboratory scale sequencing batch reactor (SBR) performing EBPR was used for this study (Tomás-Martínez et al. 2021). In order to characterize the microbial community and identify flanking populations present in the biomass, 16S rRNA gene amplicon sequencing was applied. DNA was extracted from the granules using the DNeasy UltraClean Microbial kit (Qiagen, Venlo, The Netherlands), using the manufacturer's protocol. The extracted DNA was quantified using a Qubit 4 (Thermo Fisher Scientific, Waltham, MA). Samples were sent to Novogene Ltd. (Hong Kong, China) for amplicon sequencing of the V3-4 hypervariable region of the 16S rRNA gene (position 341-806) on a MiSeq desktop sequencing platform (Illumina, San Diego, CA) operated under paired-end mode. The raw sequencing reads were processed by Novogene Ldt. (Hong Kong, China) and quality filtered using the QIIME software (Caporaso et al. 2010). Chimeric sequences were removed using UCHIME (Edgar et al. 2011) and sequences with ≥97 % identity were assigned to the same operational taxonomic units (OTUs) using UPARSE (Edgar 2013). Each OTU was taxonomically annotated using the Mothur software against the SSU rRNA database of the SILVA Database (Quast et al. 2013).

BLASTp analysis of enzymes involved in the catabolism of sialic acids

Amino acid sequences of enzymes involved in the catabolic pathway of sialic acids (Figure 4.1) from different well-described sialic acid consumers were retrieved from the online database NCBI RefSeq (O'Leary et al. 2016). The accession number of the sequences and the reference microorganisms are listed in Table S4.1. These sequences were used to predict the degradation capability of the different bacterial populations of the EBPR community. A protein sequence alignment versus a protein database (BLASTp) from the NCBI website (blast.ncbi.nlm.nih.gov/Blast.cgi) with default parameters was performed for each of the most abundant members detected in the EBPR community by 16S rRNA gene amplicon sequencing (Figure 4.2). The presence of the enzymes involved in the catabolism of sialic acids in the flanking populations was confirmed with an e-value lower than 5e-25 (Petit et al. 2018).

Fluorescence lectin binding analysis

Lectin staining of bioaggregates of the EBPR biomass was conducted according to earlier works (Weissbrodt et al. 2013; Boleij et al. 2018; de Graaff et al. 2019; Tomás-Martínez et al. 2021). Bioaggregates were stained and mounted in CoverWell imaging chambers with various spacers in order to avoid squeezing of the samples. The basis for the final staining protocol was fluorescent lectin barcoding (*i.e.,* screening with all commercially available lectins). In a second step, the examination was detailed with a small screening using lectins specific to sialic acids such as CCA, HMA, LFA, LPA, MAA, SNA, and WGA. Finally, the lectin approach was combined with a number of other fluorochromes specific for nucleic acids or proteins. For the image data set shown (Figure 4.3), protein staining with Sypro Orange was applied according to the suppliers instruction (Thermo Fischer Scientific, Waltham, MA). After incubating for 1 h, samples were washed three times with tap water and stained with the sialic acid-specific lectin HMA-Alexa633 (Turonova et al. 2016). After incubation with the lectin solution, the sample was washed again with tap water for three times in order to remove unbound lectins. Samples were examined with a TCS SP5X confocal laser scanning microscope (CLSM; Leica, Weltzlar, Germany) equipped with an upright microscope and a super continuum light source (white laser). The microscope hardware was controlled by the software LAS AF 2.4.1. Confocal datasets were recorded by using 25x NA 0.95 and 63x NA 1.2 water immersion lenses. Excitation was performed at 475 nm and 630 nm, and fluorescence emission signals were detected simultaneously with two photomultipliers from 520 to 600 nm (Sypro Orange) and 645 to 720 nm (HMA-Alexa633). Image data sets were deconvolved with Huygens version 21.04 using blind deconvolution (SVI, Hilversum, The Netherlands) and projected with Imaris version 9.8 (Oxford Instruments, Abingdon, UK).

4.3. Results & Discussion

Predominance of "*Ca.* Accumulibacter" and flanking populations in the EBPR biomass

Although "*Ca.* Accumulibacter" has been widely studied for its importance in the EBPR process (Mino et al. 1998; Barr et al. 2016; Rubio-Rincón et al. 2019; Guedes da Silva et al. 2020; Tomás-Martínez et al. 2021), this organism has not been isolated as pure culture up to date. In this study, one of our previously

described enrichment culture of "*Ca.* Accumulibacter" was used (Guedes da Silva 2020; Tomás-Martínez et al. 2021). Although the relative abundance of "*Ca.* Accumulibacter" is underrepresented by 16S rRNA gene sequencing, this method was used to determine the flanking populations present in the bioreactor. Methods such as fluorescence *in situ* hybridization (FISH) or metaproteomics allow a better representation of the contribution of the different microorganisms to the population mass (Stokholm-Bjerregaard et al. 2017; Kleiner et al. 2017; Kleikamp et al. 2021). "*Ca.* Accumulibacter" was shown to account for 95 % of the mass of the enriched microbial community (Kleikamp et al. 2021). Genomics based techniques are more suitable to identify the low abundance flanking population. The genomics based microbial community composition of the enriched EBPR biomass can be observed in Figure 4.2. "*Ca.* Accumulibacter" appeared as dominant organism (65 % of 16S rRNA gene amplicon sequencing read counts). "*Ca.* Accumulibacter" was mainly accompanied by the genera *Pseudoxanthomonas*, *Chryseobacterium*, *Cloacibacterium*, and *Clostridium*, which all showed an abundance of more than 1 % of the total 16S rRNA gene counts. Since these genera are not capable to sequester acetate under anaerobic conditions it is inferred that they grow on products produced by the dominant population of "*Ca.* Accumulibacter".

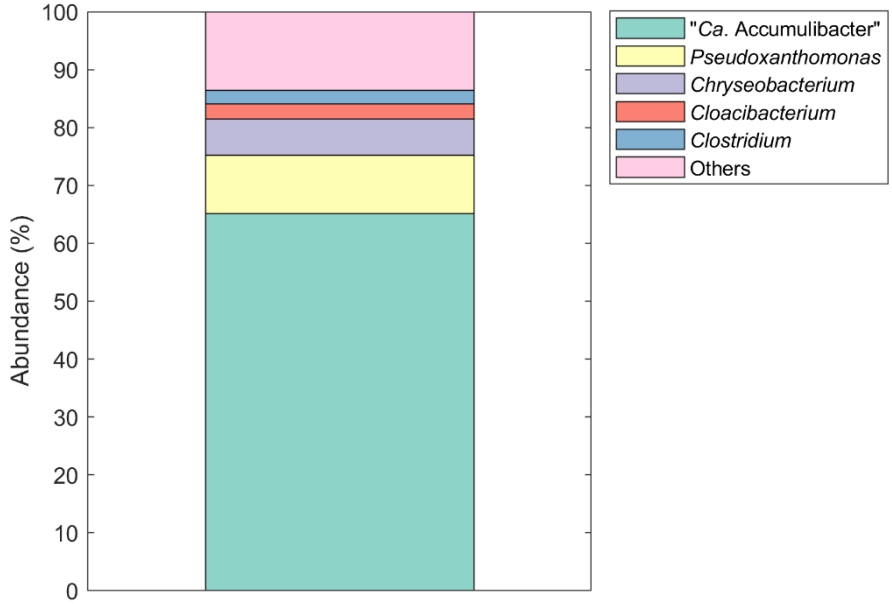

Figure 4.2. Relative genus-level microbial community distribution based on 16S rRNA gene copy numbers. All OTUs contributing < 1 % are grouped as "Others".

Abundancy of sialic acids on the surface of EBPR bioaggregates

EPS consist of different biopolymers, such as polysaccharides or (glyco)proteins (Flemming and Wingender 2010). Recently, it was shown that "*Ca.* Accumulibacter" can produce different types of sialic acids (*i.e.,* neuraminic, pseudaminic, and legionaminic acids) as part of their extracellular matrix (Tomás-Martínez et al. 2021). In the current research, aggregates were first stained with Sypro Orange (specific for proteins), washed and counterstained with the fluorescent labelled lectin HMA (specific for sialic acids) to visualize the distribution of extracellular proteins and glycoconjugates containing sialic acids (Figure 4.3). The protein specific stain indicates a foam-like and tubular frequently covering bacterial cells, showing that proteins (and/or glycoproteins that do not contain sialic acids) are widely distributed throughout the EPS matrix as a fibrous network. The sialic acid specific signal appears directly on the individual bacterial cell surfaces or around microcolonies as a more extended signal linking groups of bacteria, which are embedded in the fibrous network. Those heavily sialylated "covers" could be part of cell surface polymers, such as capsular polysaccharides, lipopolysaccharides, flagella or S-layer glycoproteins as reported in literature (Haines-Menges et al. 2015). The corresponding volume view of the same area as Figure 4.3 is shown in Figure S4.1. Furthermore, for clarity, Figure S4.2 shows a single slice of the data stack after deconvolution revealing a dotted protein signal around the bacterial cell surface (Saarimaa et al. 2006; Peltola et al. 2008).

The extracellular polymers could potentially be used as carbon, nitrogen, and energy source by the organisms present in the community when no other carbon source is available (Weissbrodt et al. 2013). In this case, both the proteinaceous fibrous network and the sialylated glycoconjugates could be used. The complexity of those polymers might require the synergetic action of different microorganisms to fully hydrolyze and metabolize them; this is common in other microbial communities such as the gut microbiota (Flint et al. 2012). Sialic acids appear as terminal carbohydrate residue in glycoconjugates in the EPS and have been described to protect the underlying carbohydrates and proteins from hydrolysis (Nishiyama et al. 2000; de Graaff et al. 2019; Boleij et al. 2020). Their protective role and terminal position at the surface makes sialic acid the first target for the utilization of glycoproteins as substrate.

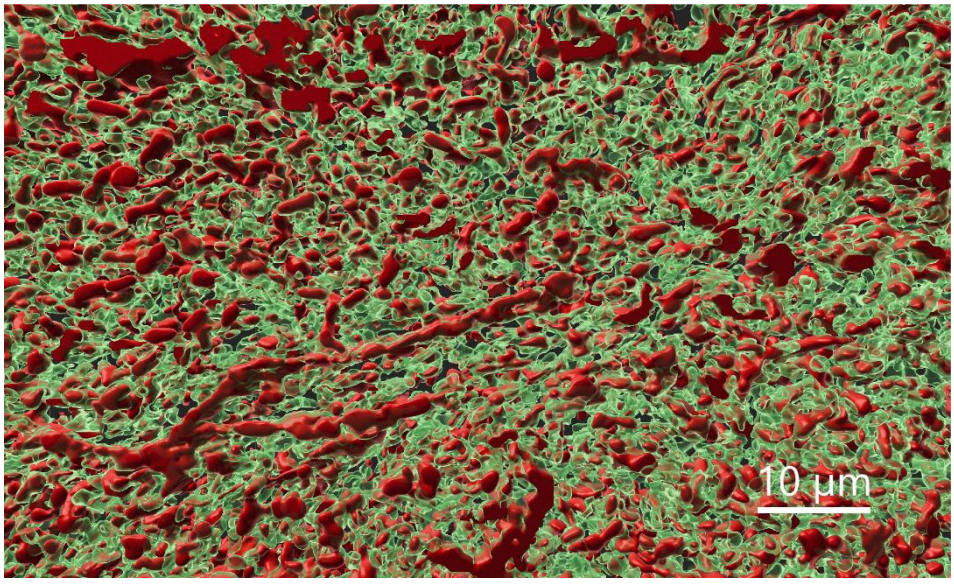

Figure 4.3. Confocal laser scanning microscopy of a bioaggregate enriched with "*Ca.* Accumulibacter" presented as a 3-dimensional isosurface view. The green channel is projected semi-transparent in order to show the foam like protein matrix. The red channel shows the glycoconjugates indicating individual bacterial cell surfaces and microcolonies as well as groups of bacteria linked via a glycoconjugate matrix. Color allocation: green – Sypro Orange protein stain, red – HMA-Alexa633 lectin. A volume view of the same area is shown in Figure S4.1.

Enzymes involved in sialic acids utilization in the genomes of predominant populations

To check the potential of different organisms in the EBPR system to utilize sialic acids as nutrient source, the amino acid reference sequences of the enzymes involved in the release (NanH), transport (MFS, TRAP, ABC, SSS), and degradation (NanA, NanK, NanE, NagA, NagB) of sialic acids (Figure 4.1) were blasted against the sequences of the different genera present in the EBPR community (Figure 4.2). The presence or absence of the gene encoding for each protein for each of the organisms are summarized in Table 4.1. More details can be found in Table S4.2.

These results reflect different possible interactions between the microorganisms present in the EBPR biomass and the sialic acids. Organisms affiliating with the genera *Pseudoxanthomonas* and *Cloacibacterium* do not harbour the necessary enzymatic machinery for the catabolism of this type of carbohydrates. Although *Pseudoxanthomonas* showed a positive match for different transporters, this can

be a false positive due to the absence of most of the catabolic proteins and the similarity with other transporters from the same families (Thomas 2016).

Table 4.1. Presence (blue) or absence (white) of genes coding for the main proteins involved in the utilization of sialic acids in the genomes of the five most abundant populations present in the examined EBPR biomass. Detailed information can be found in Table S4.2. NanH: sialidase; NanA: *N*-acetylneuraminic acid lyase; NanK: *N*-acetylmannosamine acid kinase; NanE: *N*-acetylmannosamine-6-P epimerase; NagA: *N*-acetylglucosamine-6-P deacetylase; NagB: glucosamine-6-P deaminase; MFS: major facilitator superfamily; TRAP: tripartite ATP-independent periplasmic; ABC: ATP-binding cassette; SSS: sodium solute symporter.

	Organims	*Ca.* Accumulibacter	*Pseudoxanthomonas*	*Chryseobacterium*	*Cloacibacterium*	*Clostridium*
	TaxID	327159	83618	59732	501783	1485
Catabolic proteins	**NanH**					
	NanA					
	NanK					
	NanE					
	NagA					
	NagB					
Transporters	**MFS**					
	TRAP					
	ABC					
	SS					

In comparison, microorganisms from the genus *Clostridium* can encode all the proteins involved in the degradation of sialic acids. This starts with the cleavage from the glycoconjugates by the action of a sialidase (NanH). After sialic acids are liberated, they can be taken up into the intracellular space with the help of a transporter. Once sialic acids are in the cytoplasm, they can be degraded by the action of the different enzymes and enter the general carbon catabolism. Bacteria belonging to the genus *Chryseobacterium* lack the genes encoding for sialidases, but possess the transporters and the rest of enzymes for the use of sialic acids as nutrients. This means that after the cleavage by *Clostridium* sialidases,

Clostridium and *Chryseobacterium* might compete for the uptake of free sialic acids.

Additionally, "*Ca.* Accumulibacter" only presented the genes encoding for the transporters, which could act as a reuptake mechanism to compete with the organisms consuming sialic acids, as proposed previously (Tomás-Martínez et al. 2021). In this way, "*Ca.* Accumulibacter" can re-utilize this specific type of carbohydrate, saving cellular resources by not synthesizing them *de novo*. A proposed illustration of the interaction between the bacteria in the EBPR system and the sialic acids is shown in Figure 4.4.

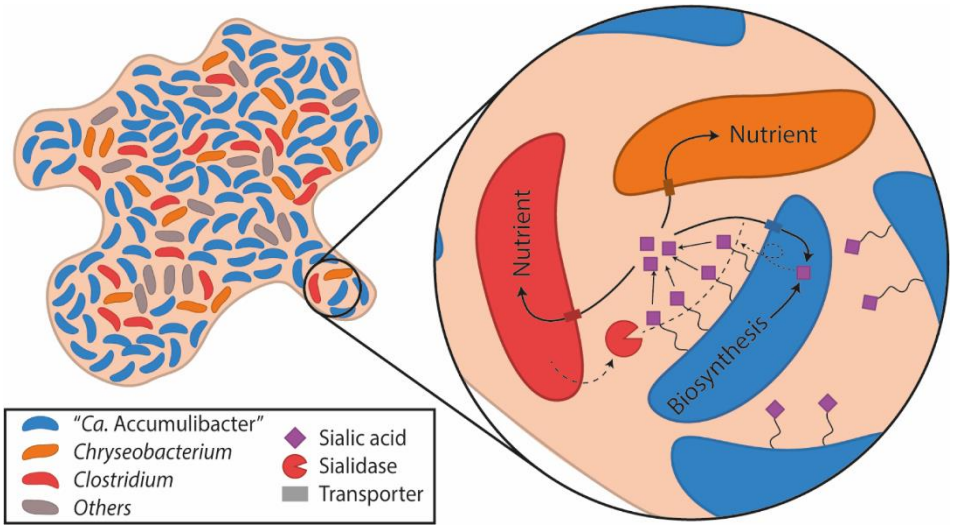

Figure 4.4. Illustration of the interaction between bacteria and sialic acids in bioaggregates of the EBPR biomass, proposed from the protein elucidations out of the genomes retrieved for the predominant populations. Sialic acids are synthesized by "*Ca.* Accumulibacter" and placed in their extracellular molecules. Once in the extracellular matrix, sialic acids are exposed to sialidases produced by *Clostridium*, releasing them as free sialic acids. *Clostridium* and *Chryseobacterium* compete for these free sialic acids and take them up for utilization as carbon, nitrogen, and energy sources. Additionally, "*Ca.* Accumulibacter" can re-uptake the free sialic acids and re-utilize them as building blocks.

Similarities in the catabolism of sialic acids between EBPR biomass and gut microbiota

Interestingly, the possible catabolism of sialic acids in EBPR bioaggregates resembles what has been described for gut microbiota. In the fermentative environment of guts, there is a fierce competition for scarce resources among densely populated gut inhabitants. One way many bacterial species compete is

specialisation on alternative carbon sources instead of the highly utilized glucose (Hooper et al. 2002). One of the nutrient sources are host mucins, which are the main structural component of the mucus layer covering the epithelium surface, while sialic acids are present as terminal carbohydrates in mucin glycoproteins (McGuckin et al. 2011).

Mucosal sialic acids serve as nutrients for some organisms like the commensal bacterium *Akkermansia muciniphila* or the pathogen *Vibrio cholerae*. These bacteria can excrete sialidases that cleave and release sialic acids present in mucins. Sialic acids can be transported into the bacteria, where they can be fully catabolized (van Passel et al. 2011; Sharma et al. 2011). *Clostridium* present in the EBPR community might occupy a similar niche in the EBPR community as *A. muciniphila* or *V. cholerae* in the gut microbiome.

Some commensal strains of *Escherichia coli* lack sialidases genes, but are still able to take up and utilize sialic acids as nutrients. The growth of these *E. coli* strains relies on the release of sialic acids by the sialidase activity of other microorganisms (Huang et al. 2015). This behavior correspond to the strategy of *Chryseobacterium* in the EBPR system.

In addition, other organisms (*e.g., Bacteroides thetaiotaomicron*) that can secrete sialidases but cannot take up free sialic acids have been described in the gut microbiota (Brigham et al. 2009). However, there is no comparable behavior observed by the genomic search in the current research.

Tracking the catabolism of sialic acids in environmental microbiology

Although this study focused on one specific type of sialic acids (*i.e., N*-acetylneuraminic acid, Neu5Ac), other sialic acid structures have been described in "*Ca.* Accumulibacter", such as pseudaminic or legionaminic acids (Tomás-Martínez et al. 2021). Currently, most of the studies reported in literature have focused on the catabolism of Neu5Ac or other related structures and no biochemical data is available for the degradation of other bacterial sialic acids (*i.e.,* pseudaminic or legionaminic acids). Because of their chemical differences, different types of sialidases are needed to cleave the sialic acids structures present in the matrices (Juge et al. 2016). It could be speculated that "*Ca.* Accumulibacter" (and most environmental bacteria) take advantage of the need for specific catabolisms for each sialic acid as protection mechanism: different

enzymes would be needed to degrade their variety of sialic acids. However, no matter what type of sialic acids it is, in microbial communities, due to its crucial terminal position at the glycan chain present in the EPS, it is likely that the consumption of any type of sialic acids will follow a similar pattern as Neu5Ac. Further investigations should be engaged to validate the catabolic conversion of the various sialic acids present in EBPR and other microbial communities.

The catabolism of sialic acids might be connected with the degradation of the relevant glycan chain as well. Once sialic acids are released, the underlying carbohydrates become available as substrate for the organisms in the community. Interestingly, it is reported that members of the here-detected genus *Chryseobacterium* can grow on complex polymeric compounds by degrading them into simpler substrates, which can be further used by other microorganisms (McBride 2014). Apparently, the degradation of these complex polymers requires the synergic action of different organisms, similar to cross-feeding phenomena in the gut microbiota (Flint et al. 2012). Such synergistic action can possibly apply to EPS macromolecules as well.

Up-to-date studies on the sialic acid metabolism (sialometabolism) have mainly focused on commensal or pathogenic bacteria in contact with animal cells (Almagro-Moreno and Boyd 2009). Based on our investigations, we suggest that the complex microbial interactions related to sialic acid metabolism in the gut microbiota can occur in an environmental community without the presence of a host. The catabolism of the host sialic acids plays an important role in microbial colonization. The same situation can arise in a microbial competitive environment, where sialic acids can be heavily expressed by the dominant species in the EPS for their protective roles. As a survival strategy, other bacteria may develop mechanisms to utilize the sialic acids in many different ways. This implies that the study of sialometabolism needs to take microbe-microbe interaction into account in environmental microbiomes besides the current main focus of host-microbe interactions. Using this study as a starting point, future research on this emerging field with sialic acids as important monosaccharides in EPS and source of nutrients, electrons and energy for microbial growth will help elucidate the complex food networks in microbial communities.

Supplementary information

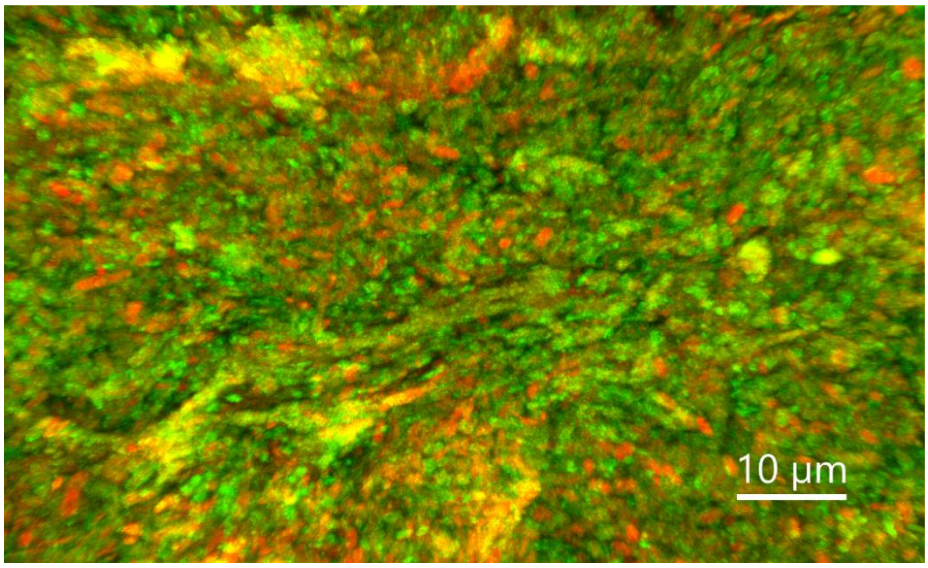

Figure S4.1. Confocal laser scanning microscopy of a bioaggregate enriched with "*Ca.* Accumulibacter" presented as a 3-dimensional volume view. The image shows the same location and magnification as in Figure 4.3 of the manuscript. Due to the volumetric view, areas with both signals in close neighborhood appear as overlay in yellow. Color allocation: green – Sypro Orange protein stain, red – HMA-Alexa633 lectin.

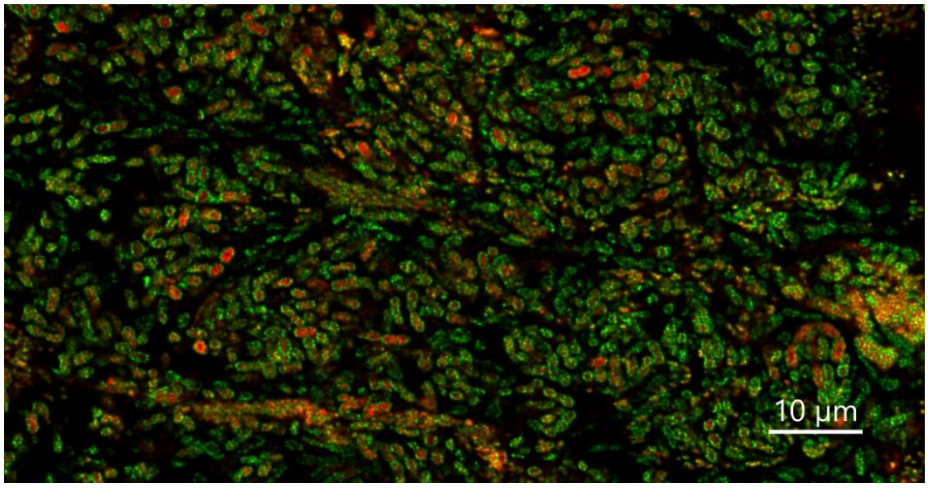

Figure S4.2. Confocal laser scanning microscopy of a bioaggregate enriched with "*Ca.* Accumulibacter". The image shows a single optical slice at the same magnification as in the data stack shown in Figure 4.3 of the manuscript. The image originates from the mid-section and is presented after deconvolution. Please take notice of the dotted protein signal at the bacterial cell surface, which becomes visible after deconvolving the dataset. Color allocation: green – Sypro Orange protein stain, red – HMA-Alexa633 lectin.

Table S4.1. Selected enzymes involved in the catabolism of sialic acids from well-described sialic acid consumers used as queries in the analysis.

Protein	**Description**	**Organism of Origin**	**Accession number**
NanH	Neuraminidase	*Bacteroides fragilis*	SUV39464
NanH	Neuraminidase	*Parabacteroides merdae*	SUV33336
NanH	Exo-alpha-sialidase	*Chloroflexi bacterium*	RPI96845
NanH	Exo-alpha-sialidase	*Flavobacteria bacterium*	WP_008866912
NanH	Exo-alpha-sialidase	*Streptomyces achromogenes*	WP_030611728
NanA	*N*-acetylneuraminate lyase	*Vibrio cholerae*	AWA78177
NanA	*N*-acetylneuraminate lyase	*Staphylococcus aureus*	BAB41528
NanK	*N*-acetylmannosamine kinase	*Vibrio cholerae*	AWA78172
NanK	*N*-acetylmannosamine kinase	*Escherichia coli*	OAF92132
NanK	*N*-acetylmannosamine kinase	*Staphylococcus aureus*	AKJ48184
NanE	*N*-acetylmannosamine-6-P epimerase	*Vibrio cholerae*	KNH51097
NanE	*N*-acetylmannosamine-6-P epimerase	*Escherichia coli*	ESD69545
NanE	*N*-acetylmannosamine-6-P epimerase	*Streptococcus pneumoniae*	ELU59687
NanE	*N*-acetylmannosamine-6-P epimerase	*Staphylococcus aureus*	KXA32796
NagA	*N*-acetylglucosamine-6-phosphate deacetylase	*Vibrio*	WP_031847727
NagA	*N*-acetylglucosamine-6-phosphate deacetylase	*Staphylococcus aureus*	BBA23352
NagB	Glucosamine-6-phosphate deaminase	*Vibrio cholerae*	TYC39340
NagB	Glucosamine-6-phosphate deaminase	*Staphylococcus aureus*	BBA23212
NagB	Glucosamine-6-phosphate deaminase	*Escherichia coli*	ESE05957
Transport (MFS)	MFS transporter, sialate:H+ symporter (SHS)	*Escherichia coli*	EFK25661
Transport (ABC)	ABC transporter substrate-binding protein	*Haemophilus*	WP_010945473
Transport (SSS)	acetylneuraminate ABC transporter	*Salmonella enterica*	NP_460100.1
Transport (TRAP)	Sialic acid-binding periplasmic protein SiaP	*Haemophilus influenzae*	P44542.1
Transport (ABC)	sugar ABC transporter, permease protein	*Streptococcus pneumoniae*	ABJ54339.1

Table S4.2. Positive match of genes coding for the main proteins involved in the utilization of sialic acids in the genomes of the five most abundant populations present in the examined EBPR biomass.

Target Organism	Protein	Result Accession	Result organism	E-value	Query Accession
"*Ca.* Accumulibacter"	Transport (TRAP)	EXI89098.1	Ca. Accumulibacter sp. BA-93	1.64E-48	P44542.1
Pseudoxanthomonas	NanA	PZO61833.1	Pseudoxanthomonas suwonensis	1.64E-26	AWA78177
	NagA	WP_183640104.1	unclassified Pseudoxanthomonas	7.09E-54	WP_031847727
	NagA	WP_183640104.1	unclassified Pseudoxanthomonas	1.34E-63	BBA23352
	Transport (MFS)	WP_122228458.1	Pseudoxanthomonas spadix	3.09E-31	EFK25661
	Transport (ABC)	WP_125166337.1	Pseudoxanthomonas sp. SGD-5-1	3.40E-26	WP_010945473
	Transport (SSS)	WP_192311846.1	Pseudoxanthomonas sp. PXM02	2.00E-75	NP_460100.1
	Transport (TRAP)	WP_097121491.1	Pseudoxanthomonas wuyuanensis	2.00E-25	P44542.1
	Transport (ABC)	WP_162309918.1	Pseudoxanthomonas broegbernensis	2.00E-55	ABJ54339.1
Chryseobacterium	NanA	WP_159760295.1	Chryseobacterium sp. 18061	4.60E-26	AWA78177
	NanA	WP_159760295.1	Chryseobacterium sp. 18061	3.64E-70	BAB41528
	NanK	WP_159760289.1	Chryseobacterium sp. 18061	9.66E-67	AWA78172
	NanK	WP_159760289.1	Chryseobacterium sp. 18061	5.33E-139	OAF92132
	NanE	WP_159760291.1	Chryseobacterium sp. 18061	1.67E-79	KNH51097
	NanE	WP_159760291.1	Chryseobacterium sp. 18061	6.72E-29	ESD69545
	NanE	WP_159760291.1	Chryseobacterium sp. 18061	1.59E-38	ELU59687
	NanE	WP_159760291.1	Chryseobacterium sp. 18061	3.62E-28	KXA32796
	NagA	WP_201175330.1	Chryseobacterium sp. 18061	7.22E-70	WP_031847727
	NagA	WP_201175330.1	Chryseobacterium sp. 18061	5.06E-64	BBA23352
	NagB	WP_097164779.1	Chryseobacterium sp. 18061	8.34E-167	TYC39340
	NagB	WP_027379244.1	Chryseobacterium daeguense	1.63E-45	BBA23212
	NagB	WP_097164779.1	Chryseobacterium sp. 18061	0	ESE05957
	Transport (MFS)	WP_159760293.1	Chryseobacterium sp. 18061	0	EFK25661
	Transport (ABC)	WP_159753995.1	Chryseobacterium sp. 18061	3.48E-37	WP_010945473
	Transport (SSS)	RZJ89996.1	Chryseobacterium sp.	1.00E-73	NP_460100.1

	Transport (TRAP)	WP_201166205.1	Chryseobacterium sp. 18061	7.00E-47	P44542.1
	Transport (ABC)	WP_159755754.1	Chryseobacterium sp. 18061	8.00E-46	ABJ54339.1
Cloacibacterium	NagB	WP_188616846.1	Cloacibacterium rupense	2.31E-51	TYC39340
	NagB	WP_188616846.1	Cloacibacterium rupense	2.62E-50	BBA23212
	NagB	WP_188616846.1	Cloacibacterium rupense	5.79E-50	ESE05957
Clostridium	NanH	WP_131371760.1	Clostridium perfringens	2.05E-32	SUV39464
	NanH	WP_152889052.1	Clostridium tarantellae	4.99E-38	SUV33336
	NanH	WP_152888621.1	Clostridium tarantellae	7.05E-62	RPI96845
	NanH	WP_152890918.1	Clostridium tarantellae	2.99E-47	WP_008866912
	NanH	WP_152890918.1	Clostridium tarantellae	1.16E-28	WP_030611728
	NanA	WP_118619555.1	Clostridium sp.	1.07E-40	AWA78177
	NanA	WP_035289317.1	Clostridium sp. KNHs214	3.36E-132	BAB41528
	NanK	MBI6872137.1	Clostridium aciditolerans	2.18E-37	AWA78172
	NanK	MBI6031139.1	Clostridium perfringens	1.58E-41	OAF92132
	NanK	WP_072892235.1	Clostridium fallax	1.71E-40	AKJ48184
	NanE	WP_187005086.1	Clostridium sp.	4.77E-42	KNH51097
	NanE	WP_050607702.1	Clostridium niameyense	9.21E-94	ESD69545
	NanE	WP_055263529.1	Clostridium disporicum	3.96E-95	ELU59687
	NanE	SCI02745.1	uncultured Clostridium sp.	1.09E-87	KXA32796
	NagA	WP_039257308.1	Clostridium botulinum	6.27E-111	WP_031847727
	NagA	WP_128749700.1	Clostridium sp. JN-9	1.07E-99	BBA23352
	NagB	WP_008681234.1	Clostridium sp.	1.02E-68	TYC39340
	NagB	WP_078681760.1	Clostridium sp.	1.22E-69	BBA23212
	NagB	CCY67659.1	Clostridium sp. CAG:678	4.73E-64	ESE05957
	Transport (ABC)	WP_169007896.1	Clostridium jeddahense	1.59E-49	WP_010945473
	Transport (SSS)	WP_039259152.1	Clostridium botulinum	9.00E-104	NP_460100.1
	Transport (TRAP)	MBS5509900.1	Clostridium sp.	3.00E-50	P44542.1
	Transport (ABC)	TGY40235.1	Clostridium sartagoforme	3.00E-165	ABJ54339.1

References

Almagro-Moreno S, Boyd EF (2009) Insights into the evolution of sialic acid catabolism among bacteria. BMC Evol Biol 9:1–16 . https://doi.org/10.1186/1471-2148-9-118/FIGURES/6

Barr JJ, Dutilh BE, Skennerton CT, Fukushima T, Hastie ML, Gorman JJ, Tyson GW, Bond PL (2016) Metagenomic and metaproteomic analyses of *Accumulibacter phosphatis*-enriched floccular and granular biofilm. Environ Microbiol 18:273–287 . https://doi.org/10.1111/1462-2920.13019

Bell A, Severi E, Lee M, Monaco S, Latousakis D, Angulo J, Thomas GH, Naismith JH, Juge N (2020) Uncovering a novel molecular mechanism for scavenging sialic acids in bacteria. J Biol Chem 295:13724–13736 . https://doi.org/10.1074/JBC.RA120.014454

Boleij M, Kleikamp H, Pabst M, Neu TR, van Loosdrecht MCM, Lin Y (2020) Decorating the Anammox House: Sialic Acids and Sulfated Glycosaminoglycans in the Extracellular Polymeric Substances of Anammox Granular Sludge. Environ Sci Technol 54:5218–5226 . https://doi.org/10.1021/ACS.EST.9B07207

Boleij M, Pabst M, Neu TR, van Loosdrecht MCM, Lin Y (2018) Identification of Glycoproteins Isolated from Extracellular Polymeric Substances of Full-Scale Anammox Granular Sludge. Environ Sci Technol 52:13127–13135 . https://doi.org/10.1021/acs.est.8b03180

Brigham C, Caughlan R, Gallegos R, Dallas MB, Godoy VG, Malamy MH (2009) Sialic Acid (*N*-Acetyl Neuraminic Acid) Utilization by *Bacteroides fragilis* Requires a Novel *N*-Acetyl Mannosamine Epimerase. J Bacteriol 191:3629 . https://doi.org/10.1128/JB.00811-08

Capon C, Laboisse CL, Wieruszeski JM, Maoret JJ, Augeron C, Fournet B (1992) Oligosaccharide structures of mucins secreted by the human colonic cancer cell line CL.16E. J Biol Chem 267:19248–19257 . https://doi.org/10.1016/S0021-9258(18)41768-1

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pẽa AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R (2010) QIIME allows analysis of high-throughput community sequencing data. Nat Methods 2010 75 7:335–336 . https://doi.org/10.1038/nmeth.f.303

Carlin AF, Uchiyama S, Chang YC, Lewis AL, Nizet V, Varki A (2009) Molecular mimicry of host sialylated glycans allows a bacterial pathogen to engage neutrophil Siglec-9 and dampen the innate immune response. Blood 113:3333–3336 . https://doi.org/10.1182/blood-2008-11-187302

Chen X, Varki A (2010) Advances in the biology and chemistry of sialic acids. ACS Chem. Biol. 5:163–176

de Graaff DR, Felz S, Neu TR, Pronk M, van Loosdrecht MCM, Lin Y (2019) Sialic acids in the extracellular polymeric substances of seawater-adapted aerobic granular sludge. Water Res 155:343–351 . https://doi.org/10.1016/J.WATRES.2019.02.040

Edgar RC (2013) UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat Methods 2013 1010 10:996–998 . https://doi.org/10.1038/nmeth.2604

Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R (2011) UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27:2194–2200 . https://doi.org/10.1093/BIOINFORMATICS/BTR381

Flemming H-C, Wingender J (2010) The biofilm matrix. Nat Rev Microbiol 8:623–633 . https://doi.org/10.1038/nrmicro2415

Flint HJ, Scott KP, Duncan SH, Louis P, Forano E (2012) Microbial degradation of complex carbohydrates in the gut. Gut Microbes 3:289

Guedes da Silva L (2020) Life in changing environments: The intriguing cycles of Polyphosphate Accumulating Organisms. Dissertation. Delft University of Technology

Guedes da Silva L, Olavarria Gamez K, Castro Gomes J, Akkermans K, Welles L, Abbas B, van Loosdrecht MCM, Wahl SA (2020) Revealing the Metabolic Flexibility of "*Candidatus* Accumulibacter phosphatis" through Redox Cofactor Analysis and Metabolic Network Modeling. Appl Environ Microbiol 86: . https://doi.org/10.1128/AEM.00808-20

Haines-Menges BL, Whitaker WB, Lubin JB, Boyd EF (2015) Host Sialic Acids: A Delicacy for the Pathogen with Discerning Taste. In: Metabolism and Bacterial Pathogenesis. American Society of Microbiology, pp 321–342

Hooper L V., Midwedt T, Gordon JI (2002) How host-microbial interactions shape the nutrient environment of the mammalian intestine. Annu Rev Nutr 22:283–307 . https://doi.org/10.1146/ANNUREV.NUTR.22.011602.092259

Huang YL, Chassard C, Hausmann M, Von Itzstein M, Hennet T (2015) Sialic acid catabolism drives intestinal inflammation and microbial dysbiosis in mice. Nat Commun 2015 61 6:1–11 . https://doi.org/10.1038/ncomms9141

Juge N, Tailford L, Owen CD (2016) Sialidases from gut bacteria: a mini-review. Biochem Soc Trans 44:166 . https://doi.org/10.1042/BST20150226

Kleikamp HBC, Lin YM, McMillan DGG, Geelhoed JS, Naus-Wiezer SNH, van Baarlen P, Saha C, Louwen R, Sorokin DY, van Loosdrecht MCM, Pabst M (2020) Tackling the chemical diversity of microbial nonulosonic acids – a universal large-scale survey approach. Chem Sci. https://doi.org/10.1039/C9SC06406K

Kleikamp HBC, Pronk M, Tugui C, Guedes da Silva L, Abbas B, Lin YM, van Loosdrecht MCM, Pabst M (2021) Database-independent *de novo* metaproteomics of complex microbial communities. Cell Syst 12:375-383.e5 . https://doi.org/10.1016/J.CELS.2021.04.003

Kleiner M, Thorson E, Sharp CE, Dong X, Liu D, Li C, Strous M (2017) Assessing species biomass contributions in microbial communities via metaproteomics. Nat Commun 2017 81 8:1–14 . https://doi.org/10.1038/s41467-017-01544-x

McBride MJ (2014) The family flavobacteriaceae. In: The Prokaryotes: Other Major Lineages of Bacteria and The Archaea. Springer-Verlag Berlin Heidelberg, pp 643–676

McGuckin MA, Lindén SK, Sutton P, Florin TH (2011) Mucin dynamics and enteric pathogens. Nat Rev Microbiol 9:265–278 . https://doi.org/10.1038/NRMICRO2538

Mino T, Van Loosdrecht MCM, Heijnen JJ (1998) Microbiology and biochemistry of the enhanced biological phosphate removal process. Water Res 32:3193–3207 . https://doi.org/10.1016/S0043-1354(98)00129-8

Nishiyama T, Kimura N, Jitsuhara Y, Uchida M, Ochi F, Yamaguchi H (2000) *N*-Glycans Protect Proteins from Protease Digestion through Their Binding Affinities for Aromatic Amino Acid Residues. J Biochem 127:427–433 . https://doi.org/10.1093/oxfordjournals.jbchem.a022624

O'Leary NA, Wright MW, Brister JR, Ciufo S, Haddad D, McVeigh R, Rajput B, Robbertse B, Smith-White B, Ako-Adjei D, Astashyn A, Badretdin A, Bao Y, Blinkova O, Brover V, Chetvernin V, Choi J, Cox E, Ermolaeva O, Farrell CM, Goldfarb T, Gupta T, Haft D, Hatcher E, Hlavina W,

Joardar VS, Kodali VK, Li W, Maglott D, Masterson P, McGarvey KM, Murphy MR, O'Neill K, Pujar S, Rangwala SH, Rausch D, Riddick LD, Schoch C, Shkeda A, Storz SS, Sun H, Thibaud-Nissen F, Tolstoy I, Tully RE, Vatsan AR, Wallin C, Webb D, Wu W, Landrum MJ, Kimchi A, Tatusova T, DiCuccio M, Kitts P, Murphy TD, Pruitt KD (2016) Reference sequence (RefSeq) database at NCBI: current status, taxonomic expansion, and functional annotation. Nucleic Acids Res 44:D733–D745 . https://doi.org/10.1093/NAR/GKV1189

Peltola M, Neu TR, Raulio M, Kolari M, Salkinoja-Salonen MS (2008) Architecture of *Deinococcus geothermalis* biofilms on glass and steel: a lectin study. Environ Microbiol 10:1752–1759 . https://doi.org/10.1111/J.1462-2920.2008.01596.X

Petit D, Teppa E, Cenci U, Ball S, Harduin-Lepers A (2018) Reconstruction of the sialylation pathway in the ancestor of eukaryotes. Sci REPORTS | 8:2946 . https://doi.org/10.1038/s41598-018-20920-1

Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO (2013) The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res 41:D590 . https://doi.org/10.1093/NAR/GKS1219

Robbe C, Capon C, Coddeville B, Michalski JC (2004) Structural diversity and specific distribution of *O*-glycans in normal human mucins along the intestinal tract. Biochem J 384:307 . https://doi.org/10.1042/BJ20040605

Rubio-Rincón FJ, Weissbrodt DG, Lopez-Vazquez CM, Welles L, Abbas B, Albertsen M, Nielsen PH, van Loosdrecht MCM, Brdjanovic D (2019) "*Candidatus* Accumulibacter delftensis": A clade IC novel polyphosphate-accumulating organism without denitrifying activity on nitrate. Water Res 161:136–151 . https://doi.org/10.1016/J.WATRES.2019.03.053

Saarimaa C, Peltola M, Raulio M, Neu TR, Salkinoja-Salonen MS, Neubauer P (2006) Characterization of adhesion threads of *Deinococcus geothermalis* as type IV pili. J Bacteriol 188:7016–7021 . https://doi.org/10.1128/JB.00608-06

Sharma SK, Moe TS, Srivastava R, Chandra D, Srivastava BS (2011) Functional characterization of VC1929 of *Vibrio cholerae* El Tor: Role in mannose-sensitive haemagglutination, virulence and utilization of sialic acid. Microbiology 157:3180–3186 . https://doi.org/10.1099/MIC.0.050245-0/CITE/REFWORKS

Stokholm-Bjerregaard M, McIlroy SJ, Nierychlo M, Karst SM, Albertsen M, Nielsen PH (2017) A Critical Assessment of the Microorganisms Proposed to be Important to Enhanced Biological Phosphorus Removal in Full-Scale Wastewater Treatment Systems. Front Microbiol 0:718 . https://doi.org/10.3389/FMICB.2017.00718

Thomas GH (2016) Sialic acid acquisition in bacteria–one substrate, many transporters. Biochem Soc Trans 44:760–765 . https://doi.org/10.1042/BST20160056

Tomás-Martínez S, Kleikamp HBC, Neu TR, Pabst M, Weissbrodt DG, van Loosdrecht MCM, Lin Y (2021) Production of nonulosonic acids in the extracellular polymeric substances of "*Candidatus* Accumulibacter phosphatis." Appl Microbiol Biotechnol 2021 1058 105:3327–3338 . https://doi.org/10.1007/S00253-021-11249-3

Traving C, Schauer R (1998) Structure, function and metabolism of sialic acids. Cell. Mol. Life Sci. 54:1330–1349

Turonova H, Neu TR, Ulbrich P, Pazlarova J, Tresse O (2016) The biofilm matrix of *Campylobacter jejuni* determined by fluorescence lectin-binding analysis. https://doi.org/101080/0892701420161169402 32:597–608 . https://doi.org/10.1080/08927014.2016.1169402

van Passel MWJ, Kant R, Zoetendal EG, Plugge CM, Derrien M, Malfatti SA, Chain PSG, Woyke T, Palva A, de Vos WM, Smidt H (2011) The Genome of *Akkermansia muciniphila*, a Dedicated Intestinal Mucin Degrader, and Its Use in Exploring Intestinal Metagenomes. PLoS One 6: . https://doi.org/10.1371/JOURNAL.PONE.0016876

Vimr ER (2013) Unified theory of bacterial sialometabolism: how and why bacteria metabolize host sialic acids. ISRN Microbiol 2013:1–26 . https://doi.org/10.1155/2013/816713

Weissbrodt DG, Neu TR, Kuhlicke U, Rappaz Y, Holliger C (2013) Assessment of bacterial and structural dynamics in aerobic granular biofilms. Front Microbiol 4: . https://doi.org/10.3389/fmicb.2013.00175

5

Turnover of the extracellular polymeric matrix of granules performing biological phosphate removal

Abstract

Polyphosphate accumulating organisms (PAOs) are responsible for enhanced biological phosphate removal (EBPR) from wastewater, where they grow embedded in a matrix of extracellular polymeric substances (EPS). EPS comprise a mixture of biopolymers like polysaccharides or (glyco)proteins. Despite previous studies, little is known about the dynamics of EPS in mixed cultures, and their production by PAOs and potential consumption by flanking microbes. EPS are biodegradable and have been suggested to be a substrate for other organisms in the community. Studying EPS turnover can help elucidate their biosynthesis and biodegradation cycles. We analyzed the turnover of proteins and polysaccharides in the EPS of an enrichment culture of PAOs relative to the turnover of internal proteins. An anaerobic-aerobic sequencing batch reactor (SBR) simulating EBPR conditions was operated to enrich for PAOs. After achieving a stable culture, carbon source was switched to uniformly ^{13}C-labelled acetate. Samples were collected at the end of each aerobic phase. EPS were extracted by alkaline treatment. ^{13}C enrichment in proteins and sugars (after hydrolysis of polysaccharides) in the extracted EPS were measured by mass spectrometry. The average turnover rate of sugars and proteins (0.167 and 0.192 d^{-1} respectively) was higher than the expected value based on the solid removal rate (0.132 d^{-1}), and no significant difference was observed between intracellular and extracellular proteins. This indicates that EPS from the PAO enriched community is not selectively degraded by flanking populations under stable EBPR process conditions. Instead, we observed general decay of biomass, which corresponds to a value of 0.048 d^{-1}.

Highlights

- Proteins showed a higher turnover rate than carbohydrates.
- Turnover of EPS was similar to the turnover of intracellular proteins.
- EPS is not preferentially consumed by flanking populations.

Published as:

Tomás-Martínez S, Zwolsman EJ, Merlier F, Pabst M, Lin Y, van Loosdrecht MCM, Weissbrodt DG (2023) Turnover of the extracellular polymeric matrix of granules performing biological phosphate removal. Appl Microbiol Biotechnol

5.1. Introduction

Enhanced biological phosphate removal (EBPR) is a widely utilized treatment to eliminate inorganic phosphate and organic matter from wastewater. Polyphosphate-accumulating organisms (PAOs) have the capacity to anaerobically take up volatile fatty acids (*e.g.*, acetate) and to store them intracellularly as polyhydroxyalkanoate (PHA). When an electron donor is available, PAOs consume the stored PHA and take up inorganic phosphate, storing it as intracellular polyphosphate (Smolders et al. 1995). PAOs are a very important group of organisms in wastewater treatment plants performing EBPR, including the aerobic granular sludge process. "*Candidatus* Accumulibacter phosphatis" has been described as the dominant species responsible for phosphate removal, although other PAOs have been identified, such as *Tetrasphaera* or *Dechloromonas* (Marques et al. 2017; Petriglieri et al. 2021). In wastewater treatment plants, these microorganisms grow as part of the microbial community in the form of bioaggregates (flocs, granules or biofilms) embedded in a matrix of extracellular polymeric substances (EPS) (Weissbrodt et al. 2013; Barr et al. 2016).

EPS are metabolically synthesized and released by the microorganisms forming scaffolds that provide mechanical stability in biofilms. EPS consist of a mixture of biopolymers, such as polysaccharides, proteins, nucleic acids or lipids, among others (Flemming and Wingender 2010). The EPS matrix provides certain benefits to the microbial community such as water retention, nutrient sorption or protection against viruses and predators (Seviour et al. 2019). Therefore, it is important to keep the extracellular structure stable.

EPS is regularly indicated as a potential nutrient source for the microorganisms present in the community (Flemming and Wingender 2010). A few studies have shown the biodegradability of EPS, *e.g.*, Zhang and Bishop (2003) have demonstrated that extracted EPS can be consumed by their own producers when supplied as substrate. The same results have been obtained by Wang, Liu and Tay (2007) using extracted EPS from aerobic granules. Pannard et al. (2016) have shown the degradation of EPS under nitrogen and carbon limitation. Some microorganisms present in the EBPR systems, *e.g.*, *Sphingobacterium* or *Chryseobacterium*, have been described to consume exopolysaccharides and other complex polymeric compounds (Matsuyama et al. 2008; McBride 2014).

Therefore, granules could be seen as an ecosystem containing EPS producers and consumers (Weissbrodt et al. 2013). Despite that the biodegradability of EPS has been proven, the consumption has been only shown under substrate limited conditions and using extracted EPS (*ex situ*). The *in situ* consumption of EPS under stable culture conditions has not been shown, although often implicit or explicit assumed to occur based on the above described type of experiments. Therefore, the consumption of EPS by flanking populations (*i.e.*, low abundant bacteria that do not grow on the primary substrate) needs to be evaluated to prove these assumptions. During normal growth conditions, the consumption of a certain compound or polymer needs to be balanced by the production of newly synthesized ones, to maintain the steady state. This renewal or replacement is referred as turnover, and the frequency at which it happens, as turnover rate (Doherty and Whitfield 2011).

In order to evaluate the turnover of biopolymers, newly synthesized polymers have to be differentiated from the already existing ones. Metabolic incorporation of a stable isotope (*e.g.*, ^{13}C or ^{15}N) is a widely used technique for the study of the dynamics of different polymers. Although most of the research focuses on the isotope incorporation in proteins (Jehmlich et al. 2016), some studies have also focused on the incorporation into carbohydrates or lipids (Liang et al. 2017; Schlame et al. 2020). In some pure cultures, the use of labelled building blocks (*e.g.*, a specific amino acid or monosaccharide) is preferred. However, this is not suitable for most organisms, especially for microbial communities, where these building blocks can be used for other purposes rather than anabolism. In these cases, the use of a labelled substrate is preferred (*e.g.*, ^{13}C-labelled carbon source) (Taubert et al. 2011). This metabolic labelling technique does not limit the study to a certain polymer (*e.g.*, the use of labelled amino acids can only be used for the study of proteins), but allows to study all microbial products as the stable isotope is incorporated through the central metabolism (Lawson et al. 2021). Label incorporation can be analyzed by mass spectrometric techniques obtaining parameters such as the fraction of stable isotope incorporation (relative isotope abundance, RIA) and labelling ratio (LR). The LR describes the ratio of the labelled compound to the non-labeled compound and can be used for the estimation of the turnover rate of biomolecules (Jehmlich et al. 2016).

Several studies have applied stable isotope labelling for the analysis of EPS metabolism (Moerdijk-Poortvliet et al. 2018; Maqbool et al. 2020; Arshad et al.

2021). Specifically, Maqbool et al. (2020) employed ^{13}C and ^{15}N labelled substrates to evaluate the turnover of the EPS of activated sludge. Although they have demonstrated that carbon content of the EPS is replenished faster than nitrogen, specific components such as proteins or polysaccharides were not analyzed. Additionally, Arshad et al. (2021) have shown no difference between the carbon and nitrogen assimilation to biomass or to EPS. Since the main components of the EPS contain carbon and nitrogen in their chemical structures, such as amino acids, amino-sugars or sialic acids, it is not possible to derive conclusions on the different specific components of the EPS.

The aim of the current research is to evaluate the turnover dynamics of the main components of the EPS of PAOs (*i.e.*, polysaccharides and proteins) in comparison with the intracellular polymer turnover. We evaluated the specific label (*i.e.*, ^{13}C) incorporation patterns in the proteins and polysaccharides. A sequencing batch reactor (SBR) simulating EBPR conditions was operated to enrich a PAO dominated microbial community. Once stable conditions were achieved, the enrichment was fed with uniformly ^{13}C-labelled acetate over one solid retention time (SRT). Samples were collected at the end of each SBR cycle and EPS were extracted and incorporation of ^{13}C in proteins and sugar monomers (after the hydrolysis of the original polysaccharides) from the extracted EPS was measured by mass spectrometry. Sequence prediction tools were used to differentiate between intracellular and extracellular proteins.

5.2. Materials & Methods

Sequencing batch reactor operation

The PAO enrichment was obtained in a 1.5 L (1 L working volume) sequencing batch reactor (SBR), following conditions similar to the one described by Guedes da Silva et al. (2020) with some adaptations. The reactor was inoculated using activated sludge from a municipal wastewater treatment plant (Harnaschpolder, The Netherlands). Each SBR cycle lasted 6 hours, consisting of 20 minutes of settling, 15 minutes of effluent removal, 5 minutes of N_2 sparging to maintain strict anaerobic conditions, 5 minutes of feeding, 135 minutes of anaerobic phase and 180 minutes of aerobic phase. The hydraulic retention time (HRT) was 12 hours (removal of 500 mL of broth per cycle). The average solids retention time (SRT) was controlled to 7.55 ± 0.15 days by the removal of effluent at the end

of the mixed aerobic phase. The pH was controlled at 7.4 ± 0.1 by dosing 0.2 M HCl or 0.2 M NaOH. The temperature was maintained at 20 ± 1 °C.

The reactor was fed with two separate media: a concentrated COD medium (8.51 g/L $NaAc{\cdot}3H_2O$, 0.04 g/L yeast extract) and a concentrated mineral medium (1.53 g/L NH_4Cl, 1.59 g/L $MgSO_4{\cdot}7H_2O$, 0.40 g/L $CaCl_2{\cdot}2H_2O$, 0.48 KCl, 0.04 g/L N-allylthiourea (ATU), 2.22 g/L $NaH_2PO_4{\cdot}H_2O$, 6 mL/L of trace element solution prepared following Smolders et al. (1994)). In each cycle 50 mL of each media was added to the reactor, together with 400 mL of demineralized water. The final feed contained 400 mg COD/L of acetate.

Monitoring of the SBR

Conductivity in the bulk liquid was used to follow phosphate release and uptake patterns and to verify the steady performance of the reactor. Extracellular concentrations of phosphate and ammonium were measured with a Thermo Fisher Gallery Discrete Analyzer (Thermo Fisher Scientific, Waltham, MA). Acetate was measured by high performance liquid chromatography (HPLC) with an Aminex HPX-87H column (Bio-Rad, Hercules, CA), coupled to an RI and UV detectors (Waters, Milford, MA), using 0.01 M phosphoric acid as eluent supplied at a flowrate of 0.6 mL/min. Biomass samples were taken at the end of the aerobic phase for microbial community characterization before and ater the ^{13}C labelling experiment.

Microbial community characterization

Fluorescence *in situ* hybridization (FISH)

Samples were handled, fixed and stained as described by Winkler et al. (2011). All bacteria were targeted using a mixture of EUB338, EUB338-II and EUB338-III probes (Amann et al. 1990; Daims et al. 1999). "*Ca.* Accumulibacter" was visualized using a mixture of PAO462, PAO651 and PAO846 probes (PAOmix) (Crocetti et al. 2000). *Dechloromonas* was targeted using the probe Dech69 and Dech209 (Dechmix). Hybridized samples were examined with Axio Imager 2 fluorescence microscope (Zeiss, Oberkochen, Germany).

16S rRNA gene amplicon sequencing

DNA was extracted from the granules using the DNeasy UltraClean Microbial kit (Qiagen, Venlo, The Netherlands), using the manufacturer's protocol. The extracted DNA was quantified using a Qubit 4 (Thermo Fisher Scientific,

Waltham, MA). Samples were sent to Novogene Ltd. (Hong Kong, China) for amplicon sequencing of the V3-4 hypervariable region of the 16S rRNA gene (position 341-806) on a MiSeq desktop sequencing platform (Illumina, San Diego, CA) operated under paired-end mode. The raw sequencing reads were processed by Novogene Ltd. (Hong Kong, China) and quality filtered using the QIIME software (Caporaso et al. 2010). Chimeric sequences were removed using UCHIME (Edgar et al. 2011) and sequences with ≥97 % identity were assigned to the same operational taxonomic units (OTUs) using UPARSE (Edgar 2013). Each OTU was taxonomically annotated using the Mothur software against the SSU rRNA database of the SILVA Database (Quast et al. 2013). Sequences obtained are deposited under the Bioproject accession number PRJNA874531 in the NCBI database.

Metagenome sequencing

The previous DNA samples were sent to Novogene Ltd. (Hong Kong, China) for shotgun metagenome sequencing. DNA sample was fragmented by sonication to a size of 350 bp and the fragmented DNA was used for libraries preparation using NEB Next Ultra DNA Library Prep Kit, following manufacturer's recommendations. Libraries were sequenced on an Illumina NovaSeq platform (Illumina, San Diego, CA) as 2 x 150 bp paired-end reads. The raw sequencing reads were processed by Novogene Ldt. (Hong Kong, China) for quality-trimming, adapter removal and contaminant-filtering. The trimmed reads were assembled into scaffolds using MEGAHIT (Li et al. 2015). Scaffolds (≥500 bp) were used for open reading frame (ORF) prediction by MetaGeneMark (Zhu et al. 2010). KEGG, EggNog and CAZy databases were used for functional annotation. Additionally, BLASTp from the NCBI website (blast.ncbi.nlm.nih.gov/Blast.cgi) for functional and taxonomic annotation. SignalP5.0 was employed for prediction of secreted proteins (referred as extracellular proteins) (Almagro Armenteros et al. 2019). Sequences obtained are deposited under the Bioproject accession number PRJNA874531 in the NCBI database.

Proteomic composition

Proteomic data annotated with taxonomies from an unlabeled sample (prior to the addition of ^{13}C-acetate) was used to estimate the composition of the microbial community. Taxonomic composition was estimated based on the combined peptide areas corresponding to each genus.

^{13}C labelling experiment

After a stable enrichment was achieved, the feeding media was replaced in order to perform the labelling experiment. Some changes in the media were made for the experiment: yeast extract was added to the mineral media and COD media was prepared using only uniformly labelled sodium U-^{13}C-acetate (99 atom % ^{13}C, Cortecnet, Les Ulis, France), to a concentration of 5.25 g/L. Online conductivity measurements were used to ensure the correct performance of the enrichment during the experiment. These conditions were maintained during 8 days (corresponding to 32 cycles). Biomass samples were manually collected at the end of each aerobic phase maintaining the SRT. Collected biomass samples were washed and stored at -80 ^{o}C.

EPS extraction and general analysis

Biomass samples were freeze-dried prior to the EPS extraction. EPS were extracted in alkaline conditions at high temperature, using a method adapted from Felz et al. (2016). Around 150 mg of freeze-dried biomass were stirred in 10 mL of 0.1 M NaOH (1.5 % w/v) at 80 ^{o}C for 30 min. Extraction mixtures were centrifuged at 4000xg at 4 ^{o}C for 20 min. Supernatants were collected and dialyzed overnight in dialysis tubing with a molecular cut-off of 3.5 kDa, frozen at -80 ^{o}C and freeze-dried. The freeze-dried extracted EPS samples were stored for further analysis.

The organic and ash fraction of the extracted EPS samples were determined by combusting them at 550 ^{o}C (APHA 1998). Protein content was estimated using the bicinchoninic acid (BCA) assay (Smith et al. 1985) with bovine serum albumin (BSA) as standard. Saccharides content was determined using the phenol-sulfuric acid assay (Dubois et al. 1956) with glucose as standard. Both methods were used as described by Felz et al. (2019). Protein and polysaccharide content are expressed as BCA and glucose equivalents respectively.

Analysis of ^{13}C incorporation into polysaccharides

Extracted EPS samples were hydrolyzed in 1 M HCl with a sample concentration of 10 g/L. Hydrolysis was performed at 105 ^{o}C during 8 hours with mixing every hour. After hydrolysis samples were neutralized with 1 M NaOH. Samples were centrifuged at 17000 x g for 5 min and the supernatant was filtered through a 0.22 µm PVDF filter. Quantification of the enrichment of sugars was performed by liquid chromatography-high resolution mass spectrometry (LC-HRMS) on an

HPLC Agilent 1290 with DAD connected to Agilent Q-TOF 6538. HPLC was carried out on an Agilent Poroshell 120 HILIC-Z (100 x 2.1 mm ID, 2.7 µm) column connected to an Agilent Infinity 1290 HPLC kept at 40 °C. The solvent system was A: 25 mM of ammonium formate in H2O adjusted at pH 11 with ammonium hydroxide and B: Acetonitrile. The gradient program began with 97 % B, then ramped to 89 % B at 12 min, for decreased to 80 % B in 8 min, and to 30 % in 5 minutes returned to the initial conditions and kept constant for 3 min. The flow-rate was 0.500 mL/min and injection volume was 5 µL. All compounds response were measured in ESI- and calibrated externally. The ESI Gas Temp was 200 °C, Vcap -3000V, Drying Gaz was set at 10 L/min and Nebuliser at 30 psig. Fragmentor was set at 100 V. HRMS spectrum was registered at 2 Hz in the mass range of 60 to 1100 m/z with internal calibration. MassHunter software was used for data processing. The isotopic ions abundances were extracted from the extracted ions chromatogram calculate from the monoisotopic ions $[M-H]^-$ or $[M-COO]^-$ peak. The average of both values was used for the analysis.

Analysis of ^{13}C incorporation into proteins

Briefly, biomass material was disrupted using beads beating in B-PER reagent (Thermo Fisher Scientific, Waltham, MA)/TEAB buffer (50 mM TEAB, 1 % (w/w) NaDOC, adjusted to pH 8.0) buffer. The cell debris was further pelleted and the proteins were precipitated using ice cold acetone. The protein pellet was redissolved in 200 mM ammonium bicarbonate, reduced using DTT and alkylated using iodoacetamide and digested using sequencing grade Trypsin (Promega, Madison, WI). Aliquots of *ca.* 100 ng protein digest were further analyzed by a one dimensional shotgun proteomics approach using an ESAY nano LC 1200 coupled to a QE plus Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) using methods described recently (Lawson et al. 2021; Kleikamp et al. 2021). Raw data were analyzed using PEAKS Studio (Bioinformatics Solutions Inc., Waterloo, Canada) for determining the community composition and MetaProSIP (OpenMS, University of Tubingen, Germany) (Sachsenberg et al. 2015) integrated into the KNIME 4.0.1 analytics platform as described recently (Lawson et al. 2021), was used to determine ^{13}C stable isotope incorporation into the proteome. The most abundant ^{13}C enriched isotope peak cluster (compared to the native peptide peak) was used to represent the labelling ratio of each time point. All peptide spectra were matched against a protein database generated from predicted open reading frames from the total metagenomic assembly.

Proteomic raw data are deposited under the project PXD036522 in the ProteomeXchange Consortium.

Calculation of turnover rates

The evolution of the labelling ratio (LR) of the different compounds during the cultivation time was evaluated in order to estimate the different turnover rates. The label incorporation in sugars followed an exponential cumulative dynamic, described by Eq. 5.1. Non-linear regression was used to calculate the different parameters: "a", which represents the asymptotic value of the curve, and "k" (d-1), which is the turnover rate.

$$LR = a - e^{(-k \cdot t)} \qquad \text{Eq. 5.1}$$

Proteins followed a sigmoidal dynamic, which can be described by the Gompertz function (Taubert et al. 2011). However, in order to compare the behavior of proteins to sugars, LR of proteins were also fitted using Eq. 5.1. Interestingly, the turnover rate calculated using Eq. 5.1 was similar to the one using the Gompertz function.

5.3. Results

PAO enrichment culture

In this study we analyzed the protein and polysaccharide turnover of a PAO enrichment, that was continuously cultivated in a sequencing batch reactor fed with acetate. A stable enrichment was confirmed by online pH and conductivity measurements as well as off-line measurements of acetate, phosphate and ammonium. The performance of a typical SBR cycle is shown in Figure 5.1. Acetate was completely consumed anaerobically within the first 32 minutes, meaning that for most part of the cycle the only potential external carbon sources are EPS or other side products. Acetate consumption was linked to a net phosphate release of 134.2 mg PO_4-P/L was achieved. This anaerobic release corresponds to 0.76 P-mol/C-mol of anaerobic phosphate release per carbon uptake. This is similar to previous highly enriched PAO communities (Smolders et al. 1994; Oehmen et al. 2005). During the aerobic phase, a phosphate removal of 207.9 mg PO_4-P/L was achieved, resulting in a final phosphate concentration of 2.2 mg PO_4-P/L. The decreasing ammonium concentration (7.2 mg NH_4-N/L)

was only associated to ammonium uptake for biomass growth, as nitrification was inhibited by the addition of ATU.

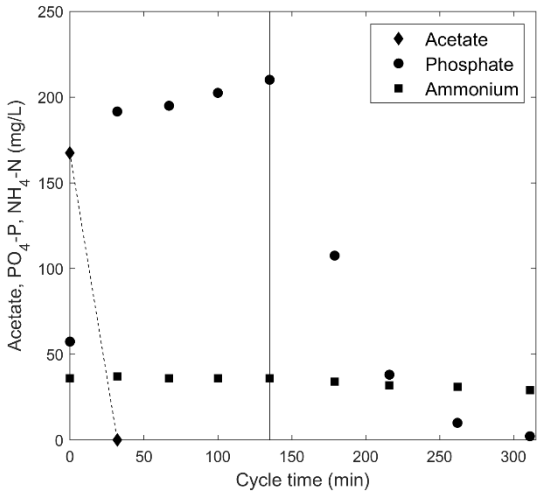

Figure 5.1. Concentration of acetate, phosphate and ammonium during a single SBR cycle after feeding. Black line represents the transition from anaerobic to aerobic phase.

After a stable enrichment was obtained, the bacterial community composition was analyzed by means of 16S rRNA gene amplicon sequencing, metaproteomic analysis (Figure 5.2A) and FISH (Figure 5.2B). Sequencing results showed a predominance of *Dechloromonas* (39.6 %) followed by the model PAO "*Ca.* Accumulibacter" (26.3 %). However, protein counts showed a higher predominance of "*Ca.* Accumulibacter" (60.7 %) compared to *Dechloromonas* (32.6 %). The microbial community compositions observed with these methods is further detailed in Figure S5.1. Although glycogen accumulating organisms (GAOs) are typically seen in EBPR systems, they were not present in this microbial community. FISH results showed a large predominance of "*Ca.* Accumulibacter" relative to *Dechloromonas*. FISH was not quantified exactly but the ratio of "*Ca.* Accumulibacter" to *Dechloromonas* was far higher than suggested by 16S rRNA amplicon sequencing, while other eubacteria had a very low fraction in the community (Figure 5.2B). Both populations of "*Ca.* Accumulibacter" and *Dechloromonas* have been described as PAO (Goel et al. 2012). After the ^{13}C labelling experiment, the bacterial community composition was analyzed and no changes were observed. Together with the reactor performance described in Figure

5.1, the molecular data collectively demonstrate that a high PAO enrichment was achieved.

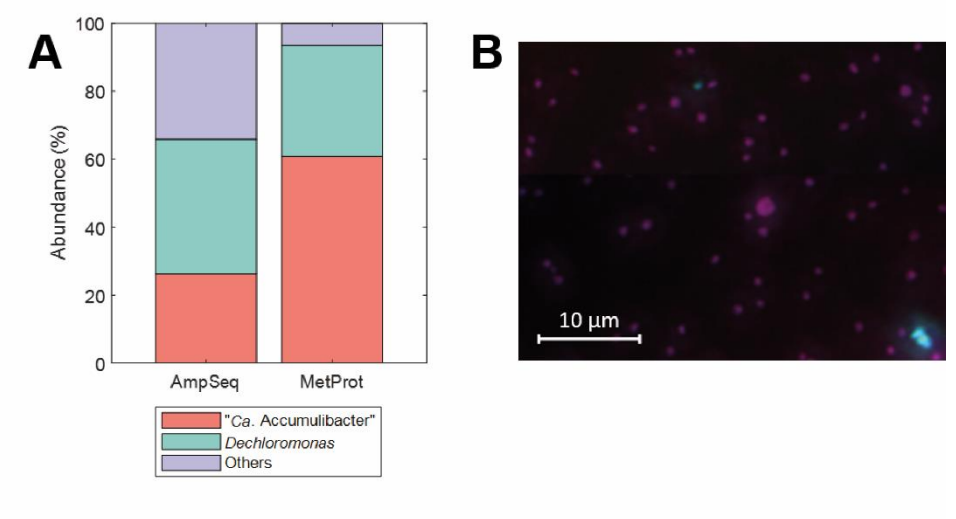

Figure 5.2. Microbial community analysis. A: Abundance of "*Ca.* Accumulibacter" and *Dechloromonas* based on 16S rRNA gene amplicon sequencing (AmpSeq) and metaproteomics (MetProt) based on identified peptides. B: Fluorescence *in situ* hybridization (FISH) image of the PAO enrichment, with PAOmix probes (targeting "*Ca.* Accumulibacter", in red), Dechmix probes (targeting *Dechloromonas*, in green) and EUBmix probes (targeting all bacteria, in blue). Magenta color represents the overlap of "*Ca.* Accumulibacter" (red) and eubacteria (blue); cyan color represents the overlap of Dechloromonas (green) and eubacteria (blue).

Analysis of ^{13}C-labelled extracellular polymeric substances

After the enrichment achieved steady state conditions, the carbon source was switched from ^{12}C-acetate to uniformly ^{13}C-labelled acetate during approximately one SRT (8 days, 32 SBR cycles). Samples were taken at the end of each SBR cycle (*i.e.*, at the end of the aerobic phase). EPS was extracted and analyzed. The protein and polysaccharide content of the extracted EPS was similar in all the samples and accounted for 55.9±3.7 and 6.2±0.7 % w/w of volatile solids of EPS, expressed as BCA and glucose equivalents respectively. Note that these generally used measurements can have a significant bias (Felz et al. 2019). Mass spectrometric methods were used to determine the incorporation of ^{13}C into polysaccharides and proteins. However, due to the harsh extraction conditions, other intracellular compounds may also be present in the extracted EPS as shown previously (Felz et al. 2016).

^{13}C INCORPORATION INTO SUGARS

In order to estimate the label incorporation in the extracellular polysaccharides, extracted EPS samples were hydrolyzed with HCl and the resulting sugars were analyzed by LC-HRMS. The labelling ratio of the sugars over the cultivation time is shown in Figure 5.3. Label incorporation in sugars follow the same behavior as the theoretical dynamics due to excess sludge removal (solid line in Figure 5.3). However, at the end of the experiment all analyzed sugars incorporated a higher amount of ^{13}C than calculated based on the SRT (65 % enrichment). Moreover, two main trends can be distinguished. Glucose shows a clearly faster incorporation of the label when compared to the rest of the sugars. However, due to the extraction method, we cannot exclude presence of intracellular glycogen in our samples. In PAOs, intracellular glycogen is consumed and synthesized in every cycle, resulting in a high turnover rate, and therefore faster incorporation of the label (Mino et al. 1998). Thus, due to the possible presence of intracellular glycogen, glucose was excluded from the analysis of extracellular sugars. The rest of the analyzed sugars present a common behavior, with lower dynamics, but still higher incorporation at the end of the experiment than expected on SRT dynamics only (solid line in Figure 5.3).

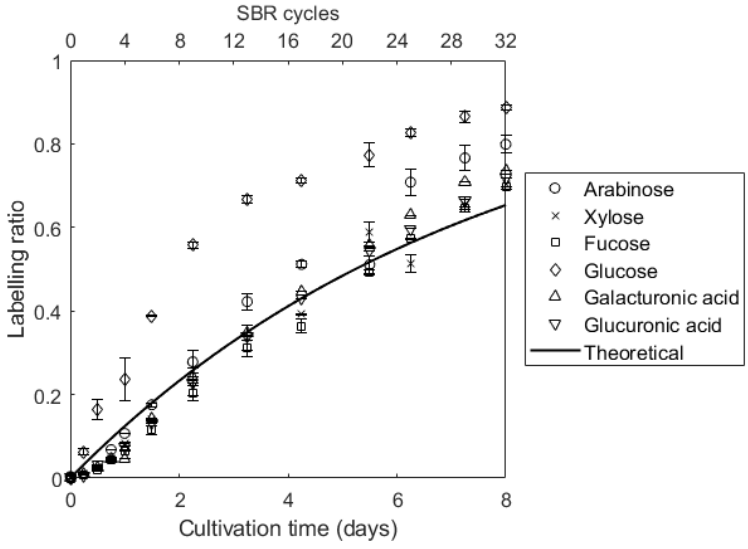

Figure 5.3. Labelling ratio for the several common monosaccharides over the different SBR cycles. Each point represents a sample at the end of a SBR cycle (top x-axis). The total duration of the experiment is shown in the bottom x-axis. The solid line represents the theoretical incorporation

according to Eq. 5.1, with values of a = 1 and k = 0.132 d^{-1} (inverse of SRT). Error bars represent the standard deviation.

A nonlinear regression analysis of the measured labelling ratio of the different sugars (Figure 5.3) was performed to estimate kinetic parameters as described by Eq. 5.1. This regression showed a high R^2 value for all of the sugars (>0.97). Glucose showed a much faster turnover rate than the rest of the analyzed sugars. However, all the analyzed sugars exhibited a higher turnover rate than expected from the SRT (Table 5.1). This difference with the SRT indicates the presence of some degradation, compensated by a higher synthesis, and therefore, increased label incorporation.

Table 5.1. Turnover rate estimation of individual sugars. Calculated parameters of the regression analysis of time-dependent label incorporation in sugars according to Eq. 5.1. As a reference, the theoretical incorporation (solid line in Figure 5.3) corresponds to values of a = 1 and k = 0.132 d^{-1} (inverse of SRT).

Sugar	**Asymptote, a**	**Turnover rate, k (d^{-1})**	**R^2**
Arabinose	0.96	0.190	0.97
Xylose	0.96	0.151	0.98
Fucose	0.95	0.146	0.98
Glucose	0.98	0.326	0.99
Galacturonic acid	0.94	0.173	0.98
Glucuronic acid	0.96	0.158	0.99

^{13}C incorporation into proteins

Extracted EPS samples were also analyzed by means of mass spectrometry based proteomics to monitor the ^{13}C incorporation into the proteins. For a total of 782 proteins incorporation of ^{13}C label could be detected. Detailed information about each individual protein can be further found in Table S1. The percentage of proteins showing labelling increased linearly over time. However, with increasing incorporation of ^{13}C into the proteins, fewer proteins were identified. This is a known challenge in protein stable isotope probing due to broadening of peak isotope envelopes and the loss of fully unlabeled (native) peptide mass peaks in the spectra (Kleiner et al. 2021).

The labelling ratio of the most abundant stable isotope enriched cluster for all detected proteins was averaged, which value is shown in Figure 5.4. Unlike the incorporation of label in sugars, label incorporation in proteins did not follow an exponential cumulative dynamic, but instead a sigmoidal incorporation. Label

incorporation showed an initial slow incorporation up to approximately day 3 of the experiment. After this delay, the protein labelling ratio quickly increased until it started to approach an asymptotic value at the end of the experiment. The final average labelling ratio was higher than the value expected based on wash-out of biomass to maintain a constant SRT. Some proteins showed a longer or shorter delay in label incorporation, however, no specific trend was detected (Table S5.1). In order to compare the behavior of proteins to sugars, protein data were also fitted using Eq. 5.1, and the derived parameters where in this case a = 0.92; k = 0.192 d^{-1}; R^2 = 0.88.

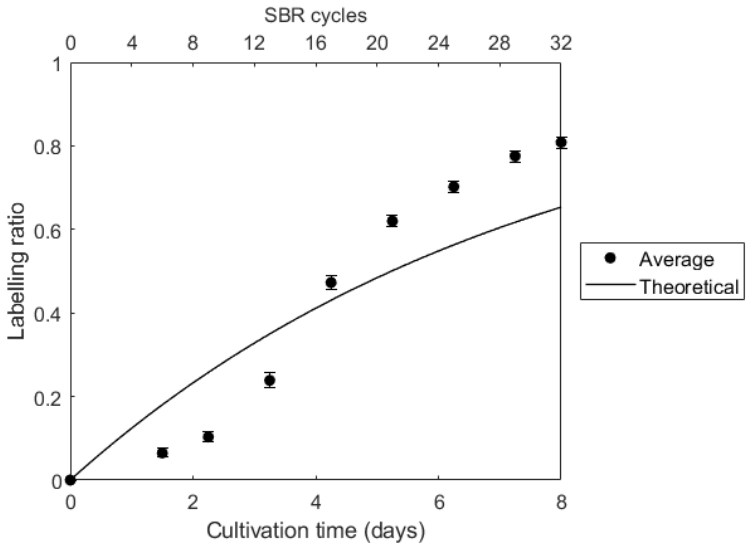

Figure 5.4. Labelling ratio for proteins over the different SBR cycles. Each point represents a sample at the end of a SBR cycle (top x-axis). The total duration of the experiment is shown in the bottom x-axis. The solid line represents the theoretical incorporation according to Eq. 5.1, with values of a = 1 and k = 0.132 d^{-1} (inverse of SRT). Error bars represent the 95 % confidence interval of the mean.

Due to the harsh extraction method, intracellular proteins will also be present in the extracted EPS (Felz et al. 2016). In order to evaluate the differences of label incorporation between intracellular and extracellular proteins, a secretion-signal prediction tool (Almagro Armenteros et al. 2019) was used to distinguish intracellular from extracellular proteins. This tool recognizes the conserved amino acid sequences in the proteins that determine their secretion (Almagro Armenteros et al. 2019). Extracellular proteins can result from other mechanisms such as outer membrane vesicles or cell lysis, which do not require signal sequences

and cannot be predicted (Bonnington and Kuehn 2014). Therefore, the analysis focused only on extracellular proteins with secretion-signal peptide. The analysis revealed that 131 of the detected proteins (representing *ca.* 20 % of total protein signal intensity) have a signal peptide for secretion. This information was used to compare the label incorporation in extracellular and intracellular proteins (Figure 5.5A). No difference was seen between both types of proteins as the average labelling ratio of both groups overlapped.

Additionally, proteins from the two PAO populations of "*Ca.* Accumulibacter" and *Dechloromonas* in the enrichment were analyzed separately to evaluate if they behaved differently (Figure 5.5B). Proteins from both organisms showed the same trend with an initial delay. Although the labelling ratio in the proteins from *Dechloromonas* was slightly higher, no significant differences were observed.

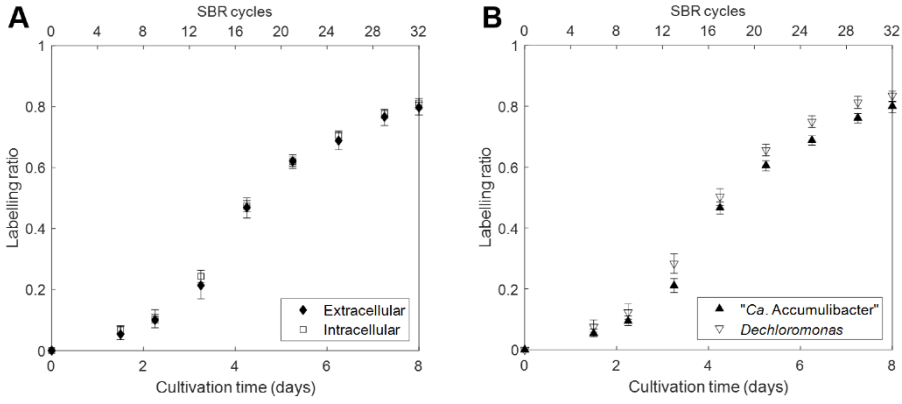

Figure 5.5. Labelling ratio observed for extracellular *vs.* intracellular (A) proteins or proteins from "*Ca.* Accumulibacter" *vs. Dechloromonas* (B) over the different SBR cycles. Each point represents a sample at the end of a SBR cycle (top x-axis). The total duration of the experiment is shown in the bottom x-axis. Error bars represent the 95 % confidence interval of the mean.

5.4. Discussion

"*Ca.* Accumulibacter" and *Dechloromonas* showed a comparable incorporation behavior in the PAO enrichment

A SBR system simulating EBPR conditions was used to enrich for PAOs. The reactor conditions were similar to previous in-house work where the enrichments were dominated by "*Ca.* Accumulibacter" (Welles et al. 2016; Rubio-Rincón et al. 2019). Periodic acetate and phosphate measurements and online conductivity and

pH monitoring showed a stable and successful PAO enrichment. Anaerobic phosphate release coupled to acetate uptake was in the range of previously reported values (Oehmen et al. 2005; Welles et al. 2015), and the same as for a previous highly enriched culture of "*Ca.* Accumulibacter" (Guedes da Silva et al. 2020).

The microbial community analysis revealed that both "*Ca.* Accumulibacter" and *Dechloromonas* dominated the PAO enrichment. *Dechloromonas* has also been described as a PAO abundant in wastewater treatment plants (Petriglieri et al. 2021). The different community analysis methods (16S rRNA gene amplicon sequencing, proteome, FISH) gave different ratios for the two main species in the community. Analysis by 16S rRNA gene amplicon sequencing has been described to strongly underestimate the abundance of "*Ca.* Accumulibacter" (Albertsen et al. 2015; Kleikamp et al. 2022), but it can be useful to determine flanking populations. Therefore, we gave stronger credibility to FISH and proteomic results, which showed that these two PAOs formed the majority of the microbial community. *Dechloromonas* represented a substantial and stable fraction of the population based on protein counts, the exact reason for its selection in this enrichment culture is as yet unknown. *Dechloromonas* have been suggested to be denitrifying PAOs and play important roles in nitrogen cycle (Lv et al. 2014). However, no NO_3^- was present in this cultivation and ATU was added to inhibit ammonium oxidation, which was only consumed for anabolic processes. Protein label incorporation patterns were highly comparable between *Dechloromonas* and "*Ca.* Accumulibacter". Both PAOs have been described to share physiological characteristics (Kong et al. 2007), and both show a similar expressed proteome profile.

No difference in turnover was observed between extracellular and intracellular components

PAOs grow embedded in a matrix of EPS. EPS are a mixture of different biopolymers such as proteins or polysaccharides. In pure cultures and fast-growing organisms, EPS is mainly composed by polysaccharides (Sadovskaya et al. 2005). For slower growing organisms the EPS is more often composed of (glycol-) proteins (Lin et al. 2018). Also for "*Ca.* Accumulibacter" (56 %), anammox bacteria (Boleij et al. 2018) and ammonium oxidizing bacteria (Yin et al. 2015) such glycoproteins have been described. EPS have been reported to be utilized by their producers or other neighboring microbes during nutrient limited conditions

(Zhang and Bishop 2003; Pannard et al. 2016; Tomás-Martínez et al. 2022). In EBPR systems like the one presented in this research, carbon is rapidly taken up and stored intracellularly by PAOs, therefore no carbon source is available to other organisms present in the community and EPS could potentially be used as carbon source.

We examined the incorporation of ^{13}C into proteins and polysaccharides, as constituents of EPS of PAOs. Due to the harsh extraction procedure, intracellular compounds can also be present in the extracted EPS (Felz et al. 2016). To differentiate intracellular and extracellular proteins, sequence analysis tools were used which revealed that *ca.* 20 % of the proteins corresponded to proteins containing a signal peptide for secretion. Although information about the contribution of intracellular components in the extracted EPS is limited, this value was higher than reported in the literature. For example, Zhang et al. (2015) detected 3 extracellular proteins out of 131 in activated sludge. The comparison of extracellular and intracellular proteins showed no difference of label incorporation as both profiles overlapped (Figure 5.5A). This indicates no difference between synthesis/degradation dynamics of proteins present in the extracellular or intracellular space, as ^{13}C label incorporation follows the same dynamics. A higher degradation of a certain protein is balanced with a higher synthesis and therefore, faster label incorporation. Since the extracellular and intracellular proteins behaved the same, there was no indication for a significant higher turnover of extracellular proteins due to being used as carbon source by other bacteria present in the community.

Label incorporation into sugars did not reveal any unique labelling characteristics, with the exception of glucose, which was finally excluded from the analysis due to the potential intracellular glycogen present in the extracted EPS. The other sugars have been described previously as part of the EPS of aerobic granular sludge enriched with "*Ca.* Accumulibacter" (Weissbrodt et al. 2013; Felz et al. 2019). Overall, the turnover rate of polysaccharides (as combination of sugars) was lower than the one of proteins. Intracellular proteins showed a higher turnover rate than polysaccharides, indicating that extracellular components (such as polysaccharides) did not show a higher label incorporation (and therefore degradation) in the PAO enrichment. Recently, Arshad and colleagues demonstrated that there was no difference in the incorporation of label (^{13}C or

^{15}N) into the EPS and biomass of activated sludge (Arshad et al. 2021), which goes in line with the results obtained in our study.

Overall these results indicate that there is no faster turnover of extracellular polymers relative to intracellular biopolymers. In the literature it is often suggested that in biofilms a flanking community is growing on the extracellular polymers (Xu et al. 2022). However, this might be partly based on a too focused interpretation of previous studies. For instance, Zhang and Bishop (2003) only showed that extracted EPS can be degraded by starving bacteria. This is often used to state that EPS might have a preferential degradation by flanking populations. Using this *in situ* labelling approach, we identified equal turnover rates of intracellular and extracellular biopolymers. This indicates a general decay of biomass rather than a preferential consumption of EPS by the flanking populations.

SRT DOES NOT SET THE ACTUAL BACTERIAL GROWTH RATE

Although extracellular and intracellular components did not show a difference in label incorporation, ^{13}C was incorporated faster in proteins and sugars than what was expected based on the SRT. Based on the SRT (7.55 days), the average growth rate imposed to the experimental biosystem over the whole SBR cycle was 0.132 d^{-1} ($\mu_{imposed}$), as inverse of SRT. Based on the average turnover rates (Table 5.1, excluding glucose) of the different polymers, an average actual growth rate of 0.180 d^{-1} (μ_{actual}) can be estimated, which would correspond to an SRT of 5.6 days. This difference between the imposed and the actual growth rate is the result of biomass decay. The actual growth rate is a combination of the imposed growth rate and the decay rate (k_{decay}):

$$\mu_{actual} = \mu_{imposed} + k_{decay} \qquad \text{Eq. 5.2}$$

Based on the experimental observations a decay rate of 0.048 d^{-1} was calculated. Some models include decay rates for their growth simulations. For example, the general activated sludge model (Gujer et al. 1999), a decay rate for PAOs of 0.2 d^{-1} is included, which is an overestimation based on our observations.

This decay might be caused by different processes, such as, cell lysis, protein degradation or by the action of viruses or predators (*e.g.*, other bacteria, protozoa). The observed decay corresponds to *ca.* 27 % of the actual growth rate of the system. This ratio of decay could explain the presence of *ca.* 7 % of flanking

populations according to proteomic data (Figure 5.2A). The label incorporation pattern in the different flanking populations could give more insights in this decay. However, due to the loss of resolution in the mass spectra, flanking populations could not be resolved in detail.

Incorporation of label in polysaccharides and proteins showed different dynamics

The incorporation of label in sugars and proteins showed a completely different behavior. For sugars the incorporation was according to the expected dynamics in line with most labelling studies (Cargile et al. 2004; Hong et al. 2012). On the other hand, incorporation in proteins appeared to show and initial delay comparable to a sigmoidal dynamics. A sigmoidal incorporation has previously been reported by (Taubert et al. 2011). However, in their case, the reason of this delay was a lag phase due to change of substrate. For environmental studies, however, often there are not enough data to determine whether the observed delay is also present in their experiments. On the other hand, in the study of (Martin et al. 2012) a short delay can be observed, but albeit at lower magnitude compared to our experiments. PAOs (and GAOs) present a unique metabolism involving the anaerobic-aerobic cycling of intracellular carbon storage polymers (*i.e.*, glycogen and PHA). These polymers are initially non-labelled. In the first cycles the ^{13}C would first build-up in these storage polymers and would then with a certain delay get incorporated in the microbial cell. It can however be calculated that this delay is only a few hours and not several days, Therefore the presence of these storage polymer pools cannot explain the observed delayed label incorporation in the proteins. Moreover if this was significant there would also be a delay in labelling of the sugars.

During the experiment, unlabelled yeast extract was present in low amounts in the medium. The main constituent of yeast extract are proteins, polypeptides and amino acids. The direct incorporation of these amino acids is likely not the cause of the observed sigmoidal incorporation curve for proteins. The yeast extract contributed only to *ca.* 1 % of the total COD of the feed, and its effect is marginal.

Another reason for this delay can be that synthesis of amino acids is more complex than sugar biosynthesis. It takes longer for bacteria to label their pool of amino acids. Moreover, the recycling of amino acids present in old proteins can also contribute to this delayed phenomenon. Bacteria can reutilize amino acids from

misfolded or non-functional proteins through degradation by the proteasome or other less complex proteases (Elharar et al. 2014). The newly formed proteins would incorporate old non-labelled amino acids and newly synthesized labelled amino acids.

Finally, the employed approach to study ^{13}C incorporation into proteins may not be sufficiently quantitative to resolve the low levels of incorporation in particular at early stages. The detection of the isotope labelled fraction is based on the detection of new mass spectrometric features that arise from the labelled peptide. Therefore, the detection requires a significant incorporation (estimated >10 % incorporation, or more in the case of complex samples), and lower levels therefore might remain undetected. Isotope patterns of metabolites (*e.g.*, sugars) are easier to resolve, more homogenous, and therefore easier to measure and quantify. Nevertheless, all possible causes may contribute to the observed incorporation differences between proteins and sugars at the early stages, and deconvoluting their individual contributions will remain subject for further studies.

Supplementary information

SI is available online at DOI: 10.1101/2022.08.11.503576

Table S5.1. Labelling ratio incorporation into the individual proteins over the different SBR cycles. Functional and taxonomic annotation and subcellular location prediction is shown for each protein.

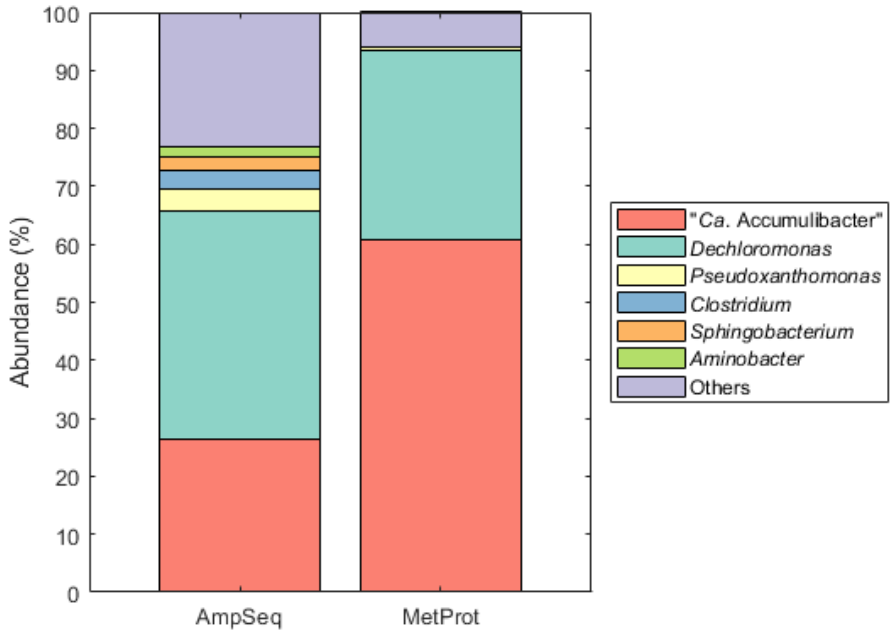

Figure S5.1. Detailed microbial community composition. Relative genus-level microbial community distribution based on 16S rRNA gene amplicon sequencing (AmpSeq) and metaproteomics (MetProt) based on identified peptides. For AmpSeq, all OTUs contributing < 1 % are grouped as "Others". For MetProt, all the groups cointributign < 0.5 % are represented by "Others".

References

Albertsen M, Karst SM, Ziegler AS, Kirkegaard RH, Nielsen PH (2015) Back to Basics – The Influence of DNA Extraction and Primer Choice on Phylogenetic Analysis of Activated Sludge Communities. PLoS One 10:e0132783 . https://doi.org/10.1371/JOURNAL.PONE.0132783

Almagro Armenteros JJ, Tsirigos KD, Sønderby CK, Petersen TN, Winther O, Brunak S, von Heijne G, Nielsen H (2019) SignalP 5.0 improves signal peptide predictions using deep neural networks. Nat Biotechnol 2019 374 37:420–423 . https://doi.org/10.1038/s41587-019-0036-z

Amann RI, Binder BJ, Olson RJ, Chisholm SW, Devereux R, Stahl DA (1990) Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl Environ Microbiol 56:1919 . https://doi.org/10.1128/AEM.56.6.1919-1925.1990

APHA (1998) Standard methods for the examination of water and wastewater, 20th ed. American Public Health Association, Washington D.C.

Arshad Z, Maqbool T, Shin KH, Kim SH, Hur J (2021) Using stable isotope probing and fluorescence spectroscopy to examine the roles of substrate and soluble microbial products in extracellular polymeric substance formation in activated sludge process. Sci Total Environ 788: . https://doi.org/10.1016/J.SCITOTENV.2021.147875

Barr JJ, Dutilh BE, Skennerton CT, Fukushima T, Hastie ML, Gorman JJ, Tyson GW, Bond PL (2016) Metagenomic and metaproteomic analyses of *Accumulibacter phosphatis*-enriched floccular and granular biofilm. Environ Microbiol 18:273–287 . https://doi.org/10.1111/1462-2920.13019

Boleij M, Pabst M, Neu TR, van Loosdrecht MCM, Lin Y (2018) Identification of Glycoproteins Isolated from Extracellular Polymeric Substances of Full-Scale Anammox Granular Sludge. Environ Sci Technol 52:13127–13135 . https://doi.org/10.1021/acs.est.8b03180

Bonnington KE, Kuehn MJ (2014) Protein selection and export via outer membrane vesicles. Biochim Biophys Acta - Mol Cell Res 1843:1612–1619 . https://doi.org/10.1016/J.BBAMCR.2013.12.011

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pẽa AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R (2010) QIIME allows analysis of high-throughput community sequencing data. Nat Methods 2010 75 7:335–336 . https://doi.org/10.1038/nmeth.f.303

Cargile BJ, Bundy JL, Grunden AM, Stephenson JL (2004) Synthesis/Degradation Ratio Mass Spectrometry for Measuring Relative Dynamic Protein Turnover. Anal Chem 76:86–97 . https://doi.org/10.1021/ac034841a

Crocetti GR, Hugenholtz P, Bond PL, Schuler A, Keller J, Jenkins D, Blackall LL (2000) Identification of polyphosphate-accumulating organisms and design of 16S rRNA-directed probes for their detection and quantitation. Appl Environ Microbiol 66:1175–1182 . https://doi.org/10.1128/AEM.66.3.1175-1182.2000

Daims H, Brühl A, Amann R, Schleifer KH, Wagner M (1999) The Domain-specific Probe EUB338 is Insufficient for the Detection of all Bacteria: Development and Evaluation of a more Comprehensive Probe Set. Syst Appl Microbiol 22:434–444 . https://doi.org/10.1016/S0723-2020(99)80053-8

Doherty MK, Whitfield PD (2011) Proteomics moves from expression to turnover: update and future perspective. Expert Rev Proteomics 8:325–334 . https://doi.org/10.1586/epr.11.19

Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric Method for Determination of Sugars and Related Substances. Anal Chem 28:350–356 . https://doi.org/10.1021/AC60111A017/ASSET/AC60111A017.FP.PNG_V03

Edgar RC (2013) UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat Methods 2013 1010 10:996–998 . https://doi.org/10.1038/nmeth.2604

Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R (2011) UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27:2194–2200 . https://doi.org/10.1093/BIOINFORMATICS/BTR381

Elharar Y, Roth Z, Hermelin I, Moon A, Peretz G, Shenkerman Y, Vishkautzan M, Khalaila I, Gur E (2014) Survival of mycobacteria depends on proteasome-mediated amino acid recycling under nutrient limitation. EMBO J 33:1802–1814 . https://doi.org/10.15252/EMBJ.201387076

Felz S, Al-Zuhairy S, Aarstad OA, van Loosdrecht MCM, Lin YM (2016) Extraction of Structural Extracellular Polymeric Substances from Aerobic Granular Sludge. J Vis Exp e54534 . https://doi.org/10.3791/54534

Felz S, Vermeulen P, van Loosdrecht MCM, Lin YM (2019) Chemical characterization methods for the analysis of structural extracellular polymeric substances (EPS). Water Res 157:201–208 . https://doi.org/10.1016/J.WATRES.2019.03.068

Flemming H-C, Wingender J (2010) The biofilm matrix. Nat Rev Microbiol 8:623–633 . https://doi.org/10.1038/nrmicro2415

Goel RK, Sanhueza P, Noguera DR (2012) Evidence of Dechloromonas sp. participating in Enhanced Biological Phosphorus Removal (EBPR) in a bench-scale aerated-anoxic reactor. Proc Water Environ Fed 2005:3864–3871 . https://doi.org/10.2175/193864705783866261

Guedes da Silva L, Olavarria Gamez K, Castro Gomes J, Akkermans K, Welles L, Abbas B, van Loosdrecht MCM, Wahl SA (2020) Revealing the Metabolic Flexibility of "*Candidatus* Accumulibacter phosphatis" through Redox Cofactor Analysis and Metabolic Network Modeling. Appl Environ Microbiol 86: . https://doi.org/10.1128/AEM.00808-20

Gujer W, Henze M, Mino T, Van Loosdrecht M (1999) Activated sludge model No. 3. Water Sci Technol 39:183–193 . https://doi.org/10.1016/S0273-1223(98)00785-9

Hong KK, Hou J, Shoaie S, Nielsen J, Bordel S (2012) Dynamic ^{13}C-labeling experiments prove important differences in protein turnover rate between two *Saccharomyces cerevisiae* strains. FEMS Yeast Res 12:741–747 . https://doi.org/10.1111/j.1567-1364.2012.00823.x

Jehmlich N, Vogt C, Lünsmann V, Richnow HH, von Bergen M (2016) Protein-SIP in environmental studies. Curr Opin Biotechnol 41:26–33 . https://doi.org/10.1016/J.COPBIO.2016.04.010

Kleikamp HBC, Grouzdev D, Schaasberg P, Valderen R van, Zwaan R van der, Wijgaart R van de, Lin Y, Abbas B, Pronk M, Loosdrecht MCM van, Pabst M (2022) Comparative metaproteomics demonstrates different views on the complex granular sludge microbiome. bioRxiv 2022.03.07.483319 . https://doi.org/10.1101/2022.03.07.483319

Kleikamp HBC, Pronk M, Tugui C, Guedes da Silva L, Abbas B, Lin YM, van Loosdrecht MCM, Pabst M (2021) Database-independent de novo metaproteomics of complex microbial communities. Cell Syst 12:375-383.e5 . https://doi.org/10.1016/J.CELS.2021.04.003

Kleiner M, Kouris A, Jensen M, Liu Y, McCalder J, Strous M (2021) Ultra-sensitive Protein-SIP to quantify activity and substrate uptake in microbiomes with stable isotopes. bioRxiv 2021.03.29.437612 . https://doi.org/10.1101/2021.03.29.437612

Kong Y, Xia Y, Nielsen JL, Nielsen PH (2007) Structure and function of the microbial community in a full-scale enhanced biological phosphorus removal plant. Microbiology 153:4061–4073 . https://doi.org/10.1099/MIC.0.2007/007245-0

Lawson CE, Nuijten GHL, de Graaf RM, Jacobson TB, Pabst M, Stevenson DM, Jetten MSM, Noguera DR, McMahon KD, Amador-Noguez D, Lücker S (2021) Autotrophic and mixotrophic metabolism of an anammox bacterium revealed by in vivo ^{13}C and ^{2}H metabolic network mapping. ISME J 15:673–687 . https://doi.org/10.1038/S41396-020-00805-W

Li D, Liu CM, Luo R, Sadakane K, Lam TW (2015) MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. Bioinformatics 31:1674–1676 https://doi.org/10.1093/BIOINFORMATICS/BTV033

Liang H, DeMeester KE, Hou C-W, Parent MA, Caplan JL, Grimes CL (2017) Metabolic labelling of the carbohydrate core in bacterial peptidoglycan and its applications. Nat Commun 8:15015 . https://doi.org/10.1038/ncomms15015

Lin Y, Reino C, Carrera J, Pérez J, van Loosdrecht MCM (2018) Glycosylated amyloid-like proteins in the structural extracellular polymers of aerobic granular sludge enriched with ammonium-oxidizing bacteria. Microbiologyopen e00616 . https://doi.org/10.1002/mbo3.616

Lv XM, Shao MF, Li CL, Li J, Gao XL, Sun FY (2014) A Comparative Study of the Bacterial Community in Denitrifying and Traditional Enhanced Biological Phosphorus Removal Processes. Microbes Environ 29:261 . https://doi.org/10.1264/JSME2.ME13132

Maqbool T, Cho J, Shin KH, Hur J (2020) Using stable isotope labeling approach and two dimensional correlation spectroscopy to explore the turnover cycles of different carbon structures in extracellular polymeric substances. Water Res 170:115355 . https://doi.org/10.1016/j.watres.2019.115355

Marques R, Santos J, Nguyen H, Carvalho G, Noronha JP, Nielsen PH, Reis MAM, Oehmen A (2017) Metabolism and ecological niche of *Tetrasphaera* and *Ca.* Accumulibacter in enhanced biological phosphorus removal. Water Res 122:159–171 . https://doi.org/10.1016/J.WATRES.2017.04.072

Martin SF, Munagapati VS, Salvo-Chirnside E, Kerr LE, Le Bihan T (2012) Proteome turnover in the green alga *Ostreococcus tauri* by time course ^{15}N metabolic labeling mass spectrometry. J Proteome Res 11:476–486 . https://doi.org/10.1021/pr2009302

Matsuyama H, Katoh H, Ohkushi T, Satoh A, Kawahara K, Yumoto I (2008) *Sphingobacterium kitahiroshimense* sp. nov., isolated from soil. Int J Syst Evol Microbiol 58:1576–1579 . https://doi.org/10.1099/ijs.0.65791-0

McBride MJ (2014) The family *flavobacteriaceae*. In: The Prokaryotes: Other Major Lineages of Bacteria and The Archaea. Springer-Verlag Berlin Heidelberg, pp 643–676

Mino T, Van Loosdrecht MCM, Heijnen JJ (1998) Microbiology and biochemistry of the enhanced biological phosphate removal process. Water Res 32:3193–3207 . https://doi.org/10.1016/S0043-1354(98)00129-8

Moerdijk-Poortvliet TCW, Beauchard O, Stal LJ, Boschker HTS (2018) Production and consumption of extracellular polymeric substances in an intertidal diatom mat. Mar Ecol Prog Ser 592:77–95 . https://doi.org/10.3354/MEPS12481

Oehmen A, Yuan Z, Blackall LL, Keller J (2005) Comparison of acetate and propionate uptake by polyphosphate accumulating organisms and glycogen accumulating organisms. Biotechnol Bioeng 91:162–168 . https://doi.org/10.1002/BIT.20500

Pannard A, Pédrono J, Bormans M, Briand E, Claquin P, Lagadeuc Y (2016) Production of exopolymers (EPS) by cyanobacteria: impact on the carbon-to-nutrient ratio of the particulate organic matter. Aquat Ecol 50:29–44 . https://doi.org/10.1007/S10452-015-9550-3/FIGURES/9

Petriglieri F, Singleton C, Peces M, Petersen JF, Nierychlo M, Nielsen PH (2021) "*Candidatus* Dechloromonas phosphoritropha" and "*Ca.* D. phosphorivorans", novel polyphosphate accumulating organisms abundant in wastewater treatment systems. ISME J 2021 1512 15:3605–3614 . https://doi.org/10.1038/s41396-021-01029-2

Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO (2013) The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res 41:D590 . https://doi.org/10.1093/NAR/GKS1219

Rubio-Rincón FJ, Weissbrodt DG, Lopez-Vazquez CM, Welles L, Abbas B, Albertsen M, Nielsen PH, van Loosdrecht MCM, Brdjanovic D (2019) "*Candidatus* Accumulibacter delftensis": A clade IC novel polyphosphate-accumulating organism without denitrifying activity on nitrate. Water Res 161:136–151 . https://doi.org/10.1016/j.watres.2019.03.053

Sachsenberg T, Herbst FA, Taubert M, Kermer R, Jehmlich N, Von Bergen M, Seifert J, Kohlbacher O (2015) MetaProSIP: Automated inference of stable isotope incorporation rates in proteins for functional metaproteomics. J Proteome Res 14:619–627 . https://doi.org/10.1021/PR500245W/SUPPL_FILE/PR500245W_SI_002.PDF

Sadovskaya I, Vinogradov E, Flahaut S, Kogan G, Jabbouri S (2005) Extracellular Carbohydrate-Containing Polymers of a Model Biofilm-Producing Strain, Staphylococcus epidermidis RP62A. Infect Immun 73:3007 . https://doi.org/10.1128/IAI.73.5.3007-3017.2005

Schlame M, Xu Y, Erdjument-Bromage H, Neubert TA, Ren M (2020) Lipidome-wide ^{13}C flux analysis: A novel tool to estimate the turnover of lipids in organisms and cultures. J Lipid Res 61:95–104 . https://doi.org/10.1194/jlr.D119000318

Seviour T, Derlon N, Dueholm MS, Flemming H-C, Girbal-Neuhauser E, Horn H, Kjelleberg S, van Loosdrecht MCM, Lotti T, Malpei MF, Nerenberg R, Neu TR, Paul E, Yu H, Lin Y (2019) Extracellular polymeric substances of biofilms: Suffering from an identity crisis. Water Res 151:1–7 . https://doi.org/10.1016/J.WATRES.2018.11.020

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC (1985) Measurement of protein using bicinchoninic acid. Anal Biochem 150:76–85 . https://doi.org/10.1016/0003-2697(85)90442-7

Smolders GJF, van der Meij J, van Loosdrecht MCM, Heijnen JJ (1995) A structured metabolic model for anaerobic and aerobic stoichiometry and kinetics of the biological phosphorus removal process. Biotechnol Bioeng 47:277–287 . https://doi.org/10.1002/BIT.260470302

Smolders GJF, van der Meij J, van Loosdrecht MCM, Heijnen JJ (1994) Model of the anaerobic metabolism of the biological phosphorus removal process: Stoichiometry and pH influence. Biotechnol Bioeng 43:461–470 . https://doi.org/10.1002/BIT.260430605

Taubert M, Jehmlich N, Vogt C, Richnow HH, Schmidt F, von Bergen M, Seifert J (2011) Time resolved protein-based stable isotope probing (Protein-SIP) analysis allows quantification of induced proteins in

substrate shift experiments. Proteomics 11:2265–2274 . https://doi.org/10.1002/pmic.201000788

Tomás-Martínez S, Chen LM, Neu TR, Weissbrodt DG, van Loosdrecht MCM, Lin Y (2022) Catabolism of sialic acids in an environmental microbial community. FEMS Microbiol Ecol. https://doi.org/10.1093/FEMSEC/FIAC047

Wang ZW, Liu Y, Tay JH (2007) Biodegradability of extracellular polymeric substances produced by aerobic granules. Appl Microbiol Biotechnol 74:462–466 . https://doi.org/10.1007/S00253-006-0686-X/FIGURES/7

Weissbrodt DG, Neu TR, Kuhlicke U, Rappaz Y, Holliger C (2013) Assessment of bacterial and structural dynamics in aerobic granular biofilms. Front Microbiol 4: . https://doi.org/10.3389/fmicb.2013.00175

Welles L, Abbas B, Sorokin DY, Lopez-Vazquez CM, Hooijmans CM, van Loosdrecht MCM, Brdjanovic D (2016) Metabolic Response of "*Candidatus* Accumulibacter Phosphatis"; Clade II C to Changes in Influent P/C Ratio. Front Microbiol 7:2121 . https://doi.org/10.3389/fmicb.2016.02121

Welles L, Tian WD, Saad S, Abbas B, Lopez-Vazquez CM, Hooijmans CM, van Loosdrecht MCM, Brdjanovic D (2015) *Accumulibacter* clades Type I and II performing kinetically different glycogen-accumulating organisms metabolisms for anaerobic substrate uptake. Water Res 83:354–366 . https://doi.org/10.1016/J.WATRES.2015.06.045

Winkler MKH, Bassin JP, Kleerebezem R, de Bruin LMM, van den Brand TPH, Van Loosdrecht MCM (2011) Selective sludge removal in a segregated aerobic granular biomass system as a strategy to control PAO–GAO competition at high temperatures. Water Res 45:3291–3299 . https://doi.org/10.1016/J.WATRES.2011.03.024

Xu R, Fu Y, Xu Y, Zheng X, Huang YX, Meng F (2022) Comparing biotransformation of extracellular polymeric substances (EPS) under aerobic and anoxic conditions: Reactivities, components, and bacterial responses. Chemosphere 296:133996 . https://doi.org/10.1016/J.CHEMOSPHERE.2022.133996

Yin C, Meng F, Chen GH (2015) Spectroscopic characterization of extracellular polymeric substances from a mixed culture dominated by ammonia-oxidizing bacteria. Water Res 68:740–749 . https://doi.org/10.1016/J.WATRES.2014.10.046

Zhang P, Shen Y, Guo JS, Li C, Wang H, Chen YP, Yan P, Yang JX, Fang F (2015) Extracellular protein analysis of activated sludge and their functions in wastewater treatment plant by shotgun proteomics. Sci Reports 2015 51 5:1–11 . https://doi.org/10.1038/srep12041

Zhang X, Bishop PL (2003) Biodegradability of biofilm extracellular polymeric substances. Chemosphere 50:63–69 . https://doi.org/10.1016/S0045-6535(02)00319-3

Zhu W, Lomsadze A, Borodovsky M (2010) Ab initio gene identification in metagenomic sequences. Nucleic Acids Res 38: . https://doi.org/10.1093/NAR/GKQ275

6

Outlook

Despite all the years since the discovery of biofilms and extracellular polymeric substances (EPS), there are still many unknowns in this field of study, such as delineated in this thesis. Aiming at continuing digging into the unknown and bringing more light to the dark world of EPS in general in a more targeted approach, "*Candidatus* Accumulibacter" is used as a model system to work on in this thesis, which is a key bacterium for stable bioaggregation of granular sludge besides biological phosphoris removal. Through the chapters, we provided new views on the composition, application and synthesis/consumption of the EPS of this environmentally relevant bacterium. Although specific results were shown in the different chapters, new questions generated from them, which might be interesting to further expand the known area in EPS research and will be explored in this section.

6.1. Nonulosonic acids in non-pathogenic bacteria

Nonulosonic acids (NulOs) have been a recurrent topic throughout the chapters of this thesis. These interesting carbohydrates have been overlooked in non-pathogenic bacteria, like environmental and wastewater microorganisms. In **chapter 2**, we observed that NulOs are widely distributed through granules enriched with "*Ca.* Accumulibacter". It was shown that "*Ca.* Accumulibacter" has the potential to produce different type of NulOs as part of its EPS. In **chapter 3**, we attempted to locate these NulOs among the different molecular weight fractions of the EPS of "*Ca.* Accumulibacter". Although a final conclusion was not extracted, the results suggested that NulOs are likely present as a polymerized chain in high molecular weight glycans. Additionally, in **chapter 4**, we explored the role of NulOs in the microbial community as possible carbon source for the flanking populations.

Besides these advances, many questions remain unanswered about NulOs in "*Ca.* Accumulibacter" in particular, and in non-pathogenic bacteria in general. NulOs have been described to play important roles in pathogenesis, but this is not the case for environmental microorganisms like "*Ca.* Accumulibacter". Due to their role as recognition molecules, NulOs have been suggested to be involved in biofilm formation, which might be one of the possible functions related to NulOs in non-pathogenic bacteria. However, it can be complicated to verify this function in a mixed culture as ours, since "*Ca.* Accumulibacter" has not been yet successfully isolated and grown as single suspended cells and cannot be genetically

manipulated. To explore the involvement of NulOs in biofilm formation, it is suggested to employ NulOs-producing pure cultures, and use the following two different approaches: 1) to compare the NulO production when growing the microorganism as single cells and as biofilms; 2) to knock out the NulO biosynthetic pathway and investigate if there is change in the biofilm formation capacity.

To demonstrate where exactly NulOs are located in bioaggregates (*e.g.*, "*Ca.* Accumulibacter"), metabolic glycoengineering techniques could be applied. These techniques rely on the incorporation of reporter groups into glycans, and their subsequent visualization (Dold and Wittmann 2021). Azide- and alkene-modified hexoamines can be used as precursors for the production of NulOs, which are then incorporated into the extracellular glycans. These techniques have also been used for the isolation of glycans. In combination with the previously described fractionation steps in **chapter 3**, more precise determination of the location of NulOs in "*Ca.* Accumulibacter" might be reached.

6.2. EPS AS A SUBSTRATE FOR OTHER ORGANISMS

A second topic that we explored through this thesis is the use of the components of the EPS as substrate by the flanking populations of our enrichment. In our system, acetate (or other volatile fatty acids), which is the only carbon source, is rapidly consumed anaerobically by "*Ca.* Accumulibacter", making it unavailable for other microorganisms, suggesting that they have to grow on products originated by "*Ca.* Accumulibacter". In **chapter 4**, we explored the potential of NulOs as nutrient for these flanking populations, comparing it to the model of the gut microbiota. The results point towards the potential catabolism of NulOs in the microbial community by some microorganisms. Additionally, in **chapter 5**, we studied the turnover of the EPS in terms of polysaccharides and proteins. Although there is a higher turnover of the extracellular polymers than what is theoretically expected, this does not differ from the turnover of intracellular proteins. This indicates that, not only EPS is consumed by flanking populations, but the other components of biomass as well, so there is not a targeted consumption of the EPS.

The consumption of NulOs by the microbial community needs to be confirmed experimentally in future research. A simple way of checking this would be

performing a batch test where free NulOs are added to the biomass and the concentration of NulOs in the media is tracked. However, it could be that NulOs are just taken up and incorporated in glycans instead of being consumed as nutrient. If ^{13}C-labelled NulOs are employed, it could be possible to trace the incorporation of the label and determine which organisms used NulOs as carbon source, by analysing the incorporation of label in their proteome. However, due to the lack of chemical access to other NulOs, at present this study would be limited to the catabolism of sialic acids (*i.e.*, neuraminic acid).

Although it was found in this thesis that there is not a targeted consumption of EPS by the flanking populations, these polymers, together with intracellular products, seem to be degraded, due to the high turnover. Using the same experimental set-up as in **chapter 5**, the carbon flow in the system can be traced. Initially, ^{13}C-acetate is taken up by "*Ca.* Accumulibacter", therefore, proteins from this organism will have a faster label incorporation. To evaluate the label incorporation dynamics of each organism, the time-dependent label incorporation in the flanking populations present in the system can be analyse. This information will reveal the trophic dependency of the different microorganisms in the microbial community.

6.3. EPS AS A PRODUCT

EPS-based products are being produced and used at the moment, despite the lack of fundamental knowledge about their biosynthesis, stability and detailed molecular composition. The results from **chapter 5** indicate that the EPS of "*Ca.* Accumulibacter" is as stable as biomass, due to their similar turnover. This is positive for the development of EPS-based products, as EPS is not specifically consumed by other organisms. Additionally, during this research, we identified the presence of NulOs in the EPS. This opens the door for new applications of EPS as explored in **chapter 3**. We proposed the use of enrichments of "*Ca.* Accumulibacter" as platform for NulOs production. Additionally, we evaluated the application of EPS and the different fractions as source for the drugs of sepsis treatment.

In order to improve "*Ca.* Accumulibacter" as platform for NulOs production, NulOs content of the biomass should be increased. By changing operational conditions in the reactor, the knowledge of manipulating NulOs production can

be obtained. Further purification of NulOs needs to be developed on top of the preliminary purification and enrichment steps done in this research *via* fractionation, in order to reach to the final production of these compounds. For the application of EPS-based products in the medical field, a good reproducibility needs to be achieved to ensure a standardized production and to fulfil the acceptance requirements.

6.4. Limitations on the study of the EPS

During this thesis we have found some limiting steps that made our research more challenging. In order to answer specific questions about the composition, biosynthesis or even application of the EPS, more advances need to be made in terms of EPS extraction and analytical methods. Thanks to the research of previous PhD candidates of this group (Felz 2019; Boleij 2020; Kleikamp 2022), we could apply extraction methods and analytical techniques for our research. However, the lack of consensus in literature about extraction methods and even terminology, makes comparison between different studies complicated. Although at first it can seem less attractive, research needs to be done for the establishment of standard methodology, in order to improve the advances in the field of EPS research.

Another problem encountered during this thesis is the intracellular contaminations in the extracted EPS. During EPS extraction, there is a trade-off between getting as much EPS as possible and cell lysis, resulting in the extraction of intracellular components. However, if the aim is to study EPS, we have to ensure that we recover as much EPS as possible, otherwise, some conclusions might be irrelevant. This requires extra confirmation of findings, to ensure that the detected polymers are located in the extracellular space. For example, in this thesis, we used fluorescent labelled lectins to visualize extracellular carbohydrates. For specific proteins, antibody-based visualization techniques could be applied.

Finally, this thesis focused on the study of "*Ca.* Accumulibacter" as model organism and NulOs as one of the specific components of the EPS, rather than attempting to describe the full microbial and chemical ecology of EPS of aerobic granular sludge. Eventually, the findings from this thesis would need to be expanded in more complex system to ensure the extrapolation of our conclusions.

References

Boleij M (2020) The Anammox House. On the extracellular polymeric substances of anammox granular sludge. Dissertation. Delft University of Technology

Dold JEGA, Wittmann V (2021) Metabolic Glycoengineering with Azide- and Alkene-Modified Hexosamines: Quantification of Sialic Acid Levels. Chembiochem 22:1243 . https://doi.org/10.1002/CBIC.202000715

Felz S (2019) Structural Extracellular Polymeric Substances from Aerobic Granular Sludge. Dissertation. Delft University of Technology

Kleikamp HBC (2022) Benchmarking and developing mass-spectrometry based omics approaches for complex microbiomes. Dissertation. Delft University of Technology

Curriculum vitae
&
Acknoledgements

Curriculum Vitae

Sergio Tomás Martínez was born on the 27th of February of 1994 and grew up in Villena, Spain. Since a young age he developed an interest in science especially in biology and chemistry. In 2012, before starting university, he won the 25th Spanish Chemistry Olympiad and represented Spain in the 44th International and 17th Iberoamerican Chemistry Olympiads, obtaining bronze and silver medals, respectively.

Following his interests in biology and chemistry, he began his studies in Biotechnology in 2012 at Polytechnique University of Valencia (UPV), Spain. During his bachelor, he became passionate about industrial biotechnology and microbiology. In 2015 he started his internship and bachelor end project at Biopolis S.L, where he worked in projects involving enzymatic degradations and biofuel and enzyme production. During this time, he heard about TUDelft for the first time, and its focus on biotechnology caught his attention.

After finishing his bachelor in 2016 he obtained the "La Caixa" European postgraduate fellowship and moved to Delft to start his Master of Science in Life Science and technology at TUDelft. Thanks to the "Delft approach" he discovered a new interest in environmental microbiology and wastewater treatment. In 2017 he started his master end project, where he met the microorganism that would accompany him for the following five years: "*Candidatus* Accumulibacter". During his thesis, he focused on modelling the metabolism of "*Ca.* Accumulibacter" under cyclic environments.

In 2018, right after finishing his internship at Xendo B.V., he concluded his master and got the opportunity to continue at TUDelft with a PhD project in the Environmental Biotechnology (EBT) section. This thesis that you have in your hands (or screen) is the results of four years of successes, failures, curiosity, enthusiasm, anxiety and lots of fun.

Currently, he continues at the EBT section of TUDelft working on a postdoc project on sulfated polysaccharides from aerobic granular sludge.

List of publications

Tomás-Martínez S, Zwolsman EJ, Merlier F, Pabst M, Lin Y, van Loosdrecht MCM, Weissbrodt DG (2023) Turnover of the extracellular polymeric matrix of granules performing biological phosphate removal. Appl Microbiol Biotechnol

Tomás-Martínez S, Chen LM, Pabst M, Weissbrodt DG, van Loosdrecht MCM, Lin Y (2022) Enrichment and application of extracellular nonulosonic acids containing polymers of *Accumulibacter*. Appl Microbiol Biotechnol. https://doi.org/10.1007/s00253-022-12326-x

Tomás-Martínez S, Chen LM, Neu TR, Weissbrodt DG, van Loosdrecht MCM, Lin Y (2022) Catabolism of sialic acids in an environmental microbial community. FEMS Microbiol Ecol. https://doi.org/10.1093/FEMSEC/FIAC047

Tomás-Martínez S, Kleikamp HBC, Neu TR, Pabst M, Weissbrodt DG, van Loosdrecht MCM, Lin Y (2021) Production of nonulosonic acids in the extracellular polymeric substances of "*Candidatus* Accumulibacter phosphatis." Appl Microbiol Biotechnol. 2021 1058 105:3327–3338. https://doi.org/10.1007/s00253-021-11249-3

Guedes da Silva L, **Tomás-Martínez S**, van Loosdrecht MCM, Wahl SA (2019) The environment selects: Modeling energy allocation in microbial communities under dynamic environments. bioRxiv. https://doi.org/10.1101/689174

Conference contributions

IWA Biofilms 2022 Conference, Phuket, Thailand (2022). Turnover of the extracellular polymeric matrix of granules performing biological phosphate removal. *Oral presentation (best presentation award).*

Biofilms 10 Conference, Leipzig, Germany (2022). Stable isotope labelling to track the turnover of EPS of PAOs. *Oral presentation.*

9th IWA Microbial Ecology and Water Engeneering Specialist Confrenece (*hybrid*), Delft, The Netherlands (2021). Stable isotope labelling to track the metabolic turnover of EPS of PAOs. *Oral presentation.*

5th IWA International Conference on Ecotechnologys for Wastewater Treatment (*online*). Sialic acids in the EPS of "*Ca.* Accumulibacter". *Oral presentation.*

IWA Biofilms 2020 Virutal Conference (*online*), Notre Dame, USA (2020). Nonulosonic acids in the EPS of "*Ca.* Accumulibacter". *Oral presentation.*

8th IWA Microbial Ecology and Water Engeneering Specialist Confrenece, Hiroshima, Japan (2019). Sialic acids in the EPS of "*Ca.* Accumulibacter phosphatis". *Poster presentation (best poster award).*

Acknowledgements

Sitting alone in my studio in Hong Kong seems like a good place to disconnect from the PhD time and look back to all the people that contributed and helped me during the last years. This journey started a long time ago, as soon as I moved to Delft for my master. I would like to thank everyone who has been part of these last six years.

First of all, I would like thank my three supervisors for their help during these for years. I would like to start with **Yuemei**. Thank you for everything, for guiding me during this time, professionally and personally. Your enthusiasm towards research is contagious and helped me to go through all the moments (not few) when I was lost. You always knew how to come with research questions and stories that helped me to continue. I could not have imagined getting a better supervisor and thanks for pushing me to reach this moment.

My promotor **Mark**, thank you for all the discussions during these four years. I still remember your email after my master defense telling me that you would consider me as a candidate for this PhD position, and, well, here we are now. I really appreciate the freedom you give us to explore new things and all the guidance you provide us. I have enjoyed a lot sharing not only meetings but also conferences, drinks and social events with you.

Thanks to **David** as well. Thank you for your input and help during the project. Who would have told me that after doing an essay with you about EPS during my master, I would end up doing a PhD about EPS with you? I enjoyed a lot all the scientific discussion and participating in the courses with you as assistant. Seeing your passion towards teaching is super nice and I learned a lot from it. Thank you and lots of luck during your next step in Norway.

I would also like to thank everyone that contributed for this thesis. **Martin**, thanks for all your input in almost every chapter. You always found time to discuss about proteomics and sialic acids, and helped me to understand all the data that we generated. Thanks to **Thomas** and **Ute** for the lectin staining analysis. Thanks to you, this thesis has some nice colour inside. Also thanks to **Franck** for contributing for the analysis needed to conclude chapter 5.

Of course, this work could not have been possible without the help of all our technicians to solve all our problems. Thanks to **Ben**, **Carol**, **Dirk**, **Dita**, **Roel** and **Zita**.

Finally, thanks to all the students that participated in this project: **Lemin**, **Vlad**, **René**, **Erwin** and **Roeland**. Thanks to your hard work, this thesis has been possible. I have learned a lot from all of you, thank you for everything.

I would also like to thank the committee members for taking part of the evaluation of my thesis and for taking the time to participate in my defense.

Special thanks to my paranymphs, **David** and **Kiko**, for helping me in the organization of this defense and for being a strong support during these years. **David**, we started this journey together, and here we are, finishing together, we made it! Thank you for being there since minute one and congratulations to surviving for years of having me as officemate without killing me (special shout-out to **Ingrid** and **Lemin** for accompanying you in this process). I am going to miss a lot having you on the other side of the screen. **Kiko**, thanks for everything in these last five years, picha. I can't barely think of a moment where you haven't been as roommate, colleague, sport buddy (yes... you changed me...) and, of course, friend. Thank you for always being there in the good and the not so good moments. Muchísimas gracias a los dos, Delft me ha dado dos grandes amigos. Continuaremos dando muchos pasos juntos (con o sin muletas).

Thanks to all the friends I made during my master, before I knew Delft would be my home for the following six years. Special thanks to **Joel**, **Joan**, **Lidia**, **Marc**, **Marina**, **Miguel**, **Panos**, **Queralt**, **Sara** and **Zeynep**. You became my main support here and one of the reasons why I decided to stay.

I would have never started this project if I had not been introduced to PAOs during my master by a super enthusiastic supervisor, thank you **Leonor**. Thanks for all your guidance during my master and for helping me to reach this point. Seeing your passion for science made me decide to start this PhD.

Since I joined EBT, I felt very welcome by everyone working in this group. The environment has always been very friendly and it has been a pleasure to work here, thank you to everyone in EBT. I miss all those moments at the Botanical Garden. Thanks to **Aina**, **Chris**, **Danny**, **Hugo**, **Maria Paula**, **Marissa**,

Simon and many others for sharing that time. Special thanks to **Ingrid** and **Marta** for all the moments we have shared during coffee breaks and outside of work. Thanks to the "new" PhDs for maintaining this nice atmosphere at EBT, all the good moments and for keeping the Friday beers tradition: **Ali**, **Lemin**, **Maxim**, **Nina**, **Rodoula**, **Sam**, **Stefan**. Of course, thanks to my roommate and partner in PAO, **Timmy**, for everything. Thanks to everyone that has been part of EBT for these years: **Ahmed**, **Alba**, **Aljioscha**, **Ana Maria**, **Claudia**, **Cristian**, **Diana**, **Dimitri**, **Gerben**, **Gonçalo**, **Jelmer**, **Jitske**, **Jules**, **Jure**, **Koen**, **Karel**, **Laura**, **Luz**, **Mario**, **Michele**, **Miranda**, **Morez**, **Nienke**, **Philipp**, **Puck**, **Rebeca**, **Robbert**, **Samarpita**, **Stephen**, **Suellen**, **Viktor** and **Zejia**. There is life outside EBT, of course, and you can find amazing people there, thanks to **Alexandra**, **Flo**, **Georg** and **Mariana** for all the moments together painting, gaming, eating or partying.

I would also like to thank two special people, the last acquisition to this section. Thank you **Quentin** and **Zeger**. You have been an incredible support during these last months of the thesis. I cannot wait to keep creating moments together. Merci beaucoup, dankjewel!

También me gustaría agradecer a todos mis amigos que he dejado en España. No importa el tiempo que pase fuera, sé que siempre os tendré. Muchas gracias a todos por seguir ahí a pesar de la distancia. Me gustaría agradecer en especial a **Blopa**, **Cata** y **Dani** por hacerme sentir como si todavía estuviera ahí. Sobretodo quiero agradecer a una persona que lleva conmigo desde que decidimos matricularnos de biotecnología a saber por qué. Gracias **Teresa** por todo tu apoyo durante todos estos años. Gracias por estar ahí siempre.

Muchas gracias también a mi familia, a mis **padres**, a **Juan Antonio** y a **Marta** por todo el apoyo desde la distancia. Gracias papás por todos los sacrificios que han hecho que llegue hasta aquí.

Lastly, I don't think many people do this, but we all should. Thanks to myself, thank you for not giving up during these years and finishing this thesis.