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### A review

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## **Bioresource Technology**

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## Review Physiology of anammox adaptation to low temperatures and promising biomarkers: A review

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### HIGHLIGHTS

### GRAPHICAL ABSTRACT

- Anammox bacteria were detected in marine and freshwater ecosystems from −30 to 80 °C.
- Genomes of anammox bacteria encode cold shock proteins (Csps) and chaperones.
- At low temperature, anammox bacteria downregulate core metabolism and biosynthesis.
- Anammox adapt to low temperatures by CSDS. cryoprotectants and unique ladderanes.
- · Several promising biomarkers of anammox low temperature adaptation are suggested.

### ARTICLE INFO

Keywords: Anaerobic ammonium oxidation Temperature Adaptation Proteome Genome Kuenenia stuttgartiensis

ABSTRACT

The adaptation of bacteria involved in the anaerobic ammonium oxidation (anammox) to low temperatures in the mainstream of WWTP will unlock substantial treatment savings. However, their adaptation mechanisms have begun to be revealed only very recently. This study reviewed the state-of-the-art knowledge on these mechanisms from -omics studies, crucially including metaproteomics and metabolomics. Anammox bacteria adapt to low temperatures by synthesizing both chaperones of RNA and proteins and chemical chaperones. Furthermore, they preserve energy for the core metabolism by reducing biosynthesis in general. Thus, in this study, a number of biomarkers are proposed to help practitioners assess the extent of anammox bacteria adaptation and predict the decomposition of biofilms/granules or slower growth. The promising biomarkers also include unique ladderane lipids. Further proteomic and metabolomic studies are necessary for a more detailed understanding of anammox low-temperature adaptation, thus easing the transition to more cost-effective and sustainable wastewater treatment.

acceptor (Lotti et al., 2014).

1990 s in the Netherlands (Kuenen, 2019). The anammox bacteria oxidize ammonium to dinitrogen gas with nitrite as the final electron

### 1. Introduction

Anaerobic ammonium oxidation (anammox) was discovered in the

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The anammox pathway was shown to occur in a unique intracellular compartment termed the anammoxosome and to involve the synthesis of extremely toxic hydrazine. Another distinctive aspect of anammox bacteria is their unique membrane lipids termed ladderanes, consisting of concatenated cyclobutene rings (Peeters and van Niftrik, 2019).

Anammox bacteria are globally significant contributors to the nitrogen cycle, as they release nitrogen to the atmosphere from freshwater lakes, marine sediments, and the marine water column, including the extremely cold arctic sediments (Vipindas et al., 2020). Laboratory cultivations have suggested the most significant contributions in temperate continental shelf locations (24% and 67% of total N<sub>2</sub> production) (Dalsgaard and Thamdrup, 2002). Other studies have attributed 0.67 to 37 % of N<sub>2</sub> released from temperate forests soils and paddy soils or 3.4 to 59 % in some freshwater and marine ecosystems (Na et al., 2018; Xi et al., 2016; Yang et al., 2015; Zhu et al., 2011, 2015).

In recent decades, the anammox process has become widely applied at wastewater treatment plants for the removal of nitrogen from reject water from anaerobic digestion and other similarly warm and ammonium-rich streams (Lackner et al., 2014). There are now over 150 full-scale installations of partial nitritation-anammox under the names ANAMMOX® (Paques), AnitaMOX® (Anox Kaldnes), DEMON® (DEMON GmbH), TERRAMOX® (E&P Anlagen Bau), Anammox SBR (Eawag), DeAmmon (PURAC), ELAN (FCC Aqualia), Cleargreen (Suez), or OLAND (DeSah BV and Ghent University) on reject water or industrial streams, making the anammox technology well established.

Reject water from anaerobic digestion operates at high temperature (30-37 °C), but converts a small fraction of the N-loadto a treatment plant (roughly 15%). In contrast, the mainstream of municipal sewage converts the majority (85–90%) of the N-load to a wastewater treatment plant, but is much more diluted and has a lower temperature (10–20 °C). Such low temperatures not only promote the proliferation of undesirable nitrite oxidizing bacteria (Cao et al., 2017b), but also reduce the activity of anammox bacteria (Cao et al., 2017a; Lotti et al., 2015). For this and other reasons, implementing anammox into the mainstream of wastewater treatment plants has been lagging (Cao et al., 2017b; Qiu et al., 2021). To make mainstream anammox a reality, numerous lab- and pilot-scale systems, and even the first un-intentional mainstream installations (i.e., Singapore, Xi'an) (Yeshi et al., 2016; Yuan et al., 2021) are emerging. Some of these have provided encouraging results, such as that the low anammox activity can be alleviated by the acclimation of mesophilic biomasses to main-stream temperatures. Specifically, De Cocker et al. (2018) turned a mesophilic anammox culture into enriched cold-adapted biomass by a stepwise reduction of temperature down to 10 °C while observing a shift from "Ca. Brocadia" to "Ca. Kuenenia". Another approach is to enrich already cold-adapted anammox cultures (Hendrickx et al., 2014; Lotti and Kleerebezem et al., 2014; Park et al., 2017a).

Despite the fact that anammox bacteria have been detected in ice floe (Rysgaard and Glud, 2004) and various polar ("*Ca.* Scalindua") (Canion et al., 2014; Engström et al., 2005; Rysgaard et al., 2004; Thamdrup and Dalsgaard, 2002) and freshwater sediments ("*Ca.* Brocadia", "*Ca.* Kuenenia", "*Ca.* Jettenia") for more than a decade (Zhu et al., 2015), the adaptation mechanisms anammox bacteria use to survive in these extreme conditions have started to be unveiled only very recently due to the advent of transcriptomics (Niederdorfer et al., 2021), proteomics (Huo et al., 2020; Kouba et al., 2022; Lin et al., 2018; Wang et al., 2021a) and metabolomics (Huo et al., 2020), and systematic review of this literature is missing.

To further the understanding of anammox's adaptability to low temperatures in the mainstream of sewage, the occurrence of anammox in nature is reviewed at similarly low, but for the sake of intrigue also higher temperatures. Then, cold adaptation mechanisms for bacteria in general are introduced and the current understanding of such mechanisms for anammox bacteria is reviewed. This study reviews not only the potential physiology of anammox adaptation given by genomics, but also the early transcriptomic, metabolomic and proteomic studies that are much more relevant to the actual physiological adaptation.

### 2. Anammox adaptable to a wide temperature range

To assess anammox's adaptability to temperatures lower (Cao et al., 2017a) or higher than 30–37  $^{\circ}$ C (Vandekerckhove et al., 2020), it is intriguing to review the literature on its occurrence in the natural environment.

## 2.1. Occurrence of anammox bacteria at extreme temperatures in marine and freshwater environments

Concerning polar marine regions, a significant anamnox activity was detected in layers of ice floe of the Greenland Sea (-1.3 °C to 0.9 °C, up to 19% of N<sub>2</sub> production via <sup>14</sup>N<sup>15</sup>N dinitrogen production isotopepairing experiments) (Rysgaard and Glud, 2004), arctic marine sediments (-1.7 °C to 4 °C, 1–35% of total N<sub>2</sub> production depending on the depth of investigated sites 30–100 m water depth) (Rysgaard et al., 2004), and polar Arctic permeable sediment (-20 °C to 9 °C, 6.5% of total N<sub>2</sub> production) (Canion et al., 2014). Anammox contributed to total N<sub>2</sub> production even more (79%) in marine sediment from the Norwegian trench at the depth of 700 m (Engström et al., 2005; Thamdrup and Dalsgaard, 2002). On the other hand, anammox activity was also detected in extremely high temperatures of deep-sea hydrothermal vents (60–80 °C) in the Mid-Atlantic Ridge (Byrne et al., 2009).

In freshwater conditions, anammox activity was detected in the sediments of Songhua River (PRC) that periodically freeze in winter (-30 °C for 120 days), suggesting that anammox bacteria can at least survive these extreme temperatures. In these sediments, anammox contributed to 11-14% of total N2 production. However, the activity assays were done at ambient temperatures (Zhu et al., 2015). Anammox ("Ca. Scalindua brodae") activity was further detected in the suboxic water layer of Lake Tanganyika at 24.6 °C, contributing to total N<sub>2</sub> production by 9% and 13% in the depth of 100 and 110 m, respectively (Schubert et al., 2006). In the sediments of Thames Estuary, a relatively lower contribution of anammox to total N2 production was detected, increasing down the estuary (1-8%, 15 °C) with the increasing salinity and decreasing sediment organic content (Trimmer et al., 2003). Similar contributions to nitrogen production (3-8%) were observed at different sites in Seine Estuary (Naeher et al., 2015). Furthermore, anammox activity was detected in freshwater sediments (Bulukeyi River, Somalia), periodically exposed to temperatures over 75 °C (Zhu et al., 2015). Further, anammox biomarkers, including characteristic ladderane lipids and related genes, were detected in similarly hot California and Nevada hot springs (<60 °C) (Jaeschke et al., 2009) and geothermal subterranean oil reservoirs (55-75 °C) (Li et al., 2010). These reports show that in natural ecosystems, anammox can survive a wide temperature range of -30 °C to 80 °C, suggesting the existence of robust physiological adaptation mechanisms. Furthermore, "Ca. Scalindua" appears to be more prevalent in lower temperatures, whereas "Ca. Brocadia" and "Ca. Kuenenia" have been detected more often under moderate-to-high temperatures. To properly appreciate the value of activity assays, it is worth noting that these were done under laboratory conditions.

### 2.2. The optimum temperature of anammox bacteria

The adaptability of anammox bacteria to temperature can be inferred from the optimum temperature ( $T_{opt}$ ), indicated by the highest rate of <sup>14</sup>N<sup>15</sup>N dinitrogen production. Such  $T_{opt}$  was 12 °C in anammox bacteria in the arctic, permanently cold (-1.7 °C to 4 °C) marine sediments (Rysgaard et al., 2004) and 9 °C in permeable Arctic sediment (-20 °C to 9 °C, Svalbard, Norway) (Canion et al., 2014). In marine sediment from Skaggerak (North Sea), the  $T_{opt}$  was 15 °C (Thamdrup and Dalsgaard, 2002). Whereas in temperate sediment (annual mean temperature 11 °C, German Wadden Sea),  $T_{opt}$  was 26 °C (Canion et al., 2014). Similarly, in freshwater sediment (annual mean temperature 14 °C, Lake Kasumigaura),  $T_{opt}$  was 25 °C (Zhou et al., 2014). Even higher optimum temperatures of  $\geq$  30 °C were determined for anammox cultures operated at sewage treatment plants at 30–37 °C (Lotti et al., 2014a). These remarkable differences between temperature optima suggest the existence of crucial physiological distinctions between these anammox bacteria.

### 3. Bacteria at sub-optimal temperature

### 3.1. How do bacteria recognize sub-optimal temperature?

Multiple studies have indicated that bacteria sense sub-optimal temperature by the changes in membrane fluidity. The first sensory mechanism was reported to be membrane-bound sensory kinase (Abriata et al., 2017). The membrane-bound sensory kinase interacts with a cytoplasmic response regulator, involving the transport of phosphate moiety from the sensor to a regulator (phosphate-transfer pathway or phosphorylation), which then upregulates genes involved in the modulation of membrane fluidity and others (*i.e.*, cold shock genes) (Mendoza, 2014). Furthermore, complex multi-step signal phosphorelay

mechanisms have been discovered (Wu et al., 2013).

## 3.2. Bacterial physiology under cold exposure: Effects of cold, sensory and adaptation mechanisms

Physiologically, the decreasing temperature can (i) reduce enzyme activity, (ii) reduce the fluidity of bacterial membranes, (iii) stabilize secondary RNA structures, (iv) alter the transport of nutrients and waste products, (v) decrease the rates of transcription, translation and cell division, (vi) denature proteins, (vii) fold proteins inappropriately, and (viii) form intracellular ice (Tribelli and López, 2018).

The response of bacteria to lower than optimum temperatures is known to involve multiple mechanisms. First is the selection of coldadapted enzymes (Bhatia et al., 2021). Second is the overexpression of cold shock proteins such as nucleic-acid-binding proteins (*i.e.*, CspA), chaperones (*i.e.*, GroEL, DnaK)(Sharma et al., 2022). Related proteins can be expressed at milder sub-optimal temperatures (cold acclimation proteins or Caps) (Dasila et al., 2022). Another mechanism involves compatible solutes acting as "chemical chaperones". Thus, compatible solutes prevent denaturation and aggregation of proteins (*i.e.*, trehalose,

Table 1

To date, 14 anammox metagenomes have been published for the total of 10 species, of which only one ("Ca. Kuenenia stuttgartiensis") is closed.

Locus tag prefix	Accession to NCBI BioProject	Species	Completeness of genome(%)	GC (%)	Nr of contigs	Size (Mbp)	Nr of protein sequences	Sequencing methodology	Reference
KSMBR1	PRJEB22746	"Ca. Kuenenia stuttgartiensis" MBR1	Closed	41	1	4.2	4035	PacBio RSII Single Molecule Real-Time Sequencing (SMRT)	(Frank et al., 2018)
SCARUB	PRJNA327439	"Ca. Scalindua sp."	90*	41	121	4.59	5235	metagenomic pair-end sequencing, HiSeq 2500 (Illumina), minimum read length 150 bp	(Ali et al., 2019)
	PRJDB740	"Ca. S. japonica"	98.2*	-	47	4.81	4019	fragment and 8-kb paired-end pyrosequencing on a GS FLX titanium sequencer (Roche Diagnostics Japan, Tokyo, Japan).	(Oshiki et al., 2017)
	PRJNA228949	"Ca. Brocadia caroliniensis"	89.7*	43.3	209	3.73	3263	metagenomic Ion Torrent PGM 400 bp, Ion 316 chip	(Park et al., 2017b)
	PRJNA327439	"Ca. S. rubra"	92*	37.3	443	5.19	5235	metagenomic Ion PGM 400 bp sequencing kit. Ion 318v2 chip	(Speth et al., 2017)
BIY37	PRJNA344009	"Ca. B. sapporoensis"	93*	42.7	122	2.93	2488	metagenomic shotgun sequencing, HiSeq 500 (Illumina)	(Ali et al., 2016)
	PRJDB103	"Ca. B. sinica"	98.3+	42.41	3	4.07	3912	3-kb paired-end pyrosequencing - GS FLX titanium sequencer (Roche Diagnostics KK, Tokyo, Japan)	(Oshiki et al., 2015)
	PRJNA262561	"Ca. S. brodae"	92*	39.6	282	4.1	4016	metagenomic IonTorrent PGM 200 bp, Ion 318 chip (Life Technologies)	(2015)
BFUL	PRJEB4876	"Ca. B. fulgida"	_	-	411	-	-	metagenomic IonTorrent PGM 200 bp, Ion 318 chip (Life Technologies)	(Ferousi et al., 2013)
		"Ca. S. profunda"	-	39.1	1580	184	4756	genomic DNA: 454GS20 and 454GSFlx pyrosequencing (Roche Sciences) metagenomic DNA: 454-Tita- nium (Roche Sciences) and Sanger paired end sequencing on shoteun and fosmid libraries	(van de Vossenberg et al., 2013)
	PRJNA168041	"Ca. K. stuttgartiensis" RU1	>98†	-	1385	_	-	metagenomic high throughput sequencing GAIIx (Illumina), 35 bp reads	(Speth et al., 2012)
	PRJNA167262	"Ca. K. stuttgartiensis" CH1	>98†	-	2906	-	-	metagenomic high throughput sequencing GAIIx (Illumina), 75 bp reads	(2012)
KSU-1	PRJDB68	"Ca. Jettenia caeni" / KSU-1	-	40	4	4.1	3469	shotgun metagenomic sequencing	(Hira et al., 2012)
KUST	PRJNA16685	"Ca. K. stuttgartiensis"	98	41	5	4.2	4663	shotgun metagenomic sequencing	(Strous et al., 2006)

+estimate based on Martin et al. (2006); \*based on Parks et al. (2015); † new genes were identified in the culture originally sequenced by Strous et al. (2006).

glycine betaine, carnitine). And, antifreeze proteins and cryoprotectants create thermal hysteresis by binding ice crystals through their large complementary surface (Levy-Sakin et al., 2014). Further, maintaining membrane lipids in a suitably fluid crystalline state at low temperatures involves changes in lipid composition. Specifically, increasing the amount of alkyl moieties that are shorter, more branched, unsaturated and polyunsaturated, anteiso-branched (compared to iso-), and *cis*-un-saturated (compared to *trans*-) (Tribelli and López, 2018). But also increasing the size of polar phospholipid headgroup (*i.e.*, phosphocholine vs phosphoglycerol) is possible. Moreover, the structure of membrane proteins and non-polar carotenoids can be altered (Kahlke and Thorvaldsen, 2012).

### 4. Resolving anammox physiology at sub-optimal temperatures

## 4.1. Only closed genome available for "Ca. Kuenenia stuttgartiensis" (KSMBR1)

Due to the lack of anammox pure cultures, the complete genome has been resolved only for a single anammox species, "*Ca*. Kuenenia stuttgartiensis" (KSMBR1). This was enabled by novel 3rd generation sequencing technologies (Pacific Biosciences (PacBio) single-molecule real-time (SMRT) sequencing, Oxford Nanopore DNA sequencing), which requires no DNA amplification and generates longer reads (Frank et al., 2018). As shown in Table 1, earlier studies have succeeded in sequencing the metagenome and assembling the genome for nine other distinct anammox species, none of which have been fully closed, mainly due to the limitations of the sequencing methodologies. This study based on the complete genomic data of "*Ca.* Kuenenia stuttgartiensis" (KSMBR1). However, the draft genomes of "*Ca.* Brocadia", "*Ca.* Scalindua" and "*Ca.* Jettenia" were searched as well (Table 2, Table 3).

### 4.2. Potential physiology of low-temperature anammox: Genomics

The genome of "*Ca.* Kuenenia stuttgartiensis" (KSMBR1) (Frank et al., 2018) contains sequences identical or strongly similar to those encoding proteins typically being associated with an adaptive response to low temperature: (i) recombinase A (RecA), (ii) transcriptional regulators cold shock proteins A (CspA) and B (CspB), (iii) subunits A and B of DNA gyrase (GyrA, GyrB). Their functions are described in Table 2 and Table 3.

As depicted in Table 3, KSMBR1 also contained sequences identical or strongly similar to those encoding the chaperone complexes (i) GroEL/GroES, (ii) DnaK/DnaJ and the cofactors ClpB, DjlA, Grpe, and related protein HtpG from Hsp90 family, (iii) other proteins from Hsp90 family, (iv) Hsp20/alpha crystalline family proteins). These are typically characterized as heat shock proteins (Hsps), but can also be upregulated at low temperatures (Matz et al., 1995), UV light (Maleki et al., 2016), pH (Wan et al., 2014), salinity or osmotic stress (Wang et al., 2019).

Table 2

Sequences identical	l or strongly similar	to those encoding	cold shock	proteins in anam	mox bacteria and	the number of copies

Protein	Description	<i>"Ca</i> . Kuenenia stuttgartiensis"			"Ca. Brocadia sapporoensis"	<i>"Ca</i> . Brocadia sinica <i>"</i>	<i>"Ca</i> . Brocadia caroliniensis"	<i>"Ca</i> . Scalindua japonica"	<i>"Ca</i> . Scalindua brodae"	<i>"Ca</i> . Scalindua rubra <i>"</i>	<i>"Ca</i> . Scalindua sp."	" <i>Ca.</i> Jettenia caeni" / KSU-1	
Cold sho	ck proteins Protein recombinase A, initiation factor, involved in recombination		_										
RecA	and induction of the SOS response needed for DNA repair	1	1	1	1	1	1	1	1	1	1	1	1
CspA	70 amino acid protein encoded by the gene <i>cspA</i> , induced under major temperature reduction, is a transcriptional regulator that identifies gene promoters and turns them on, in doing so turns on the production of cold shock proteins	1	-	-	1	-	1	-	1	1	-	-	-
CspB	67 amino acid protein encoded by <i>cspB</i> , not only cold shock protein but also may function as antifreeze protein, transcriptional activator of cold shock genes by recognizing gene promoters and turns them on, thus producing cold shock proteins	1	-	-	2	-	-	-	-	1	-	-	-
GyrA	subunit A of DNA gyrase, cold shock protein, topoisomerase, catalyzes ATP-dependent negative supercoiling of double-stranded closed-circular DNA	2	1	1	2	1	1	1	1	1	1	1	1
GyrB	subunit B of DNA gyrase, cold shock protein, topoisomerase	2	1	1	2	1	1	I	1	1	1	1	1
other co	ld shock proteins (including putative)	1	3	3	-	3	4	3	6	2	2	2	6
		Frank et al (2018)†	V(CLOC)  - +- ++3	ס(בנטב) של של משפר	Strous et al 2006	Ali et al (2016)†	Oshiki et al 2015†	Park et al (2017)†	Oshiki et al 2017†	Speth et al 2015†	Speth et al 2017†	Ali et al (2019)Δ	(Hira, et al. 2012)†

### Table 3

Sequences identical or strongly similar to those encoding heat shock proteins in anammox bacteria and number of copies.

Protein	Description	"Ca. Kuenenia stuttgartiensis"			"Ca. Kuenenia stuttgartiensis"			"Ca. Kuenenia stuttgartiensis"			"Ca. Kuenenia stuttgartiensis"		"Ca. Kuenenia stuttgartiensis"		"Ca. Kuenenia stuttgartiensis"		"Ca. Kuenenia stuttgartiensis"		"Ca. Kuenenia stuttgartiensis"		"Ca. Kuenenia stuttgartiensis"		<i>"Ca</i> . Kuenenia stuttgartiensis"		"Ca. Kuenenia stuttgartiensis"		"Ca. Kuenenia stuttgartiensis"		<i>"Ca.</i> Kuenenia stuttgartiensis"		"Ca. Kuenenia stuttgartiensis"		<i>"Ca</i> . Kuenenia stuttgartiensis"		"Ca. Kuenenia stuttgartiensis"		<i>"Ca</i> . Kuenenia stuttgartiensis"		"Ca. Brocadia sinica"JPN1	<i>"Ca</i> . Brocadia caroliniensis"	<i>"Ca</i> . Scalindua japonica"	<i>"Ca</i> . Scalindua brodae"	<i>"Ca</i> . Scalindua rubra <i>"</i>	<i>"Ca</i> . Scalindua sp. <i>"</i>	" <i>Ca.</i> Jettenia caeni" / KSU-1
Heat sho	ck proteins																																												
GroEL	enzyme) GroES, assists with protein folding	4	4	4	4	4	5	3	4	4	4	4	4																																
GroES	also known as chaperonin 10 (cpn10), co-enzyme of GroEL, assists with protein folding, heat shock protein	2	2	2	1	2	2	2	2	2	2	2	2																																
DnaK	also known as Hsp70 (70 kDa), chaperone system, assist with protein folding and prevents aggregation, requires DnaJ	3	1	1	3	1	1	1	1	3	3	3	1																																
DnaJ	also known as Hsp40 (40 kDa) chaperone system, assist with protein folding and prevents aggregation, requires DnaK	3	4	4	1	1	2	1	1	4	7	7	2																																
ClpB	heat shock protein, cooperates with DnaK, DnaJ and GrpE in suppressing protein aggregation	1	1	1	-	1	1	-	1	2	4	2	1																																
DjIA	regulatory DnaK co-chaperone, direct interaction between DnaK and DjlA is needed for the induction of wcaABCDE operon, part of synthesis of colanic acid polysaccharide capsule, the capsule may help the bacteria survive stress	-	-	-	-	-	-	-	-	1	1	1	-																																
GrpE	DnaJ, DnaK and GrpE respond to the hyperosmotic and heat shock by countering the aggregation of denatured proteins. GrpE is a nucleotide exchange factor for DnaK and may function as a thermosensor. GrpE releases ADP from DnaK; ATP binding to DnaK triggers the release of the substrate protein	1	3	3	3	1	1	1	1	1	1	1	2																																
GreA	GreA is a transcriptional elongation factor that also has chaperone activity. Inhibits aggregation proteins under heat shock condition and promotes the refolding of denatured proteins. Interacts with DnaK, DnaJ, GroES, ClpX	-	-	1	-	1	-	1	1	-	-	-	-																																
GreB	Homolog of GreA, transcription elongation factor, involved in greA/greB complex chaperone activity	-	-	-	-	1	-	1	-	-	-	-	-																																
cpn10	10 kDa chaperonin	-	-	-	-	-	-	-	1	-	-	-	-																																
ppiD	peptidyl–prolyl isomerase required for folding of outer membrane proteins	-	-	-	1	-	1	1	1	1	1	1	-																																
ppiC	Homolog of ppiD	1	-	1	1	-	1	1	1	1	1	1																																	
Hsp90	90 kDa, chaperone protein, assist with protein folding and prevents aggregation	3	-	-	1	1	1	1	1	1	1	1	1																																
HtpG	Heat shock protein, chaperone, high temperature protein G, member of Hsp90 family, interacts with the DnaK/DnaJ/GrpE	3	-	-	-	1	1	1	1	1	-	-	1																																
Hsp20	Hsp20/alpha crystallin family proteins, also characterized as small heat shock proteins, chaperones, assist with protein folding and prevent aggregation	11	11	11	8	4	5	4	11	7	2	-	1																																
other heat shock proteins		1	-	-	-	-	-	-	5	2	6	6	7																																
<sup>†</sup> protein sequences extracted from UniProt database; Δproteins obtained from published annotated genomic data at https://www.ncbi.nlm.nih.gov/		Frank et al (2018)†	A(C10C) In the discussion	קנוו פו מו (בעובוש	Strous et al 2006∆	Ali et al (2016)†	Oshiki et al 2015†	Park et al (2017)†	Oshiki et al 2017†	Speth et al 2015†	Speth et al 2017†	Ali et al (2019)Δ	(Hira, et al. 2012)†																																

In Table 2 and Table 3, interesting distinctions between the draft genomes of "*Ca*. Kuenenia", "*Ca*. Brocadia", "*Ca*. Jettenia" and "*Ca*. Scalindua" were identified. First, "*Ca*. Scalindua brodae" "*Ca*. Scalindua rubra" and "*Ca*. Scalindua sp." encoded DjlA, a regulatory co-chaperone involved in encapsulating the cell into a polysaccharide capsule that may help anammox survive stress. In *Escherichia coli*, the polysaccharide comprising the capsule was colanic acid, a negatively charged polymer of glucose, galactose, fucose, and glucuronic acid (Ren et al., 2016). Second, all "*Ca*. Scalindua sp." encoded ClpB, a heat shock protein that interacts with the chaperone complex DnaJ/DnaK and cofactor GrpE to suppress protein aggregation. However ClpB was missing in one genome of "*Ca*. Kuenenia stuttgartiensis" (Raghoebarsing et al., 2006) and "*Ca*.

Brocadia caroliniensis" (Park et al., 2017b). "*Ca.* Scalindua rubra" and "*Ca.* Scalindua brodae" encoded ClpB in 7 and 4 copies compared to 1–3 copies in other genomes. Lastly, all "*Ca.* Scalindua" also encoded up to 6 cold and heat shock proteins, similarly as in a single genome of "*Ca.* Jettenia" and much more than "*Ca.* Kuenenia" and "*Ca.* Brocadia". Furthermore, all "*Ca.* Scalindua" encoded either enzymes for biosynthesis of glycine betaine cryoprotectant or its membrane transporters, while these were absent in almost all other genomes.

Thus, anammox genera and species differ in the genotype maintaining homeostasis under environmental stress. Interestingly, the genome of "*Ca.* Scalindua" appears to be exceptionally well packed with survival tools. Not to omit the similarities, the sequences encoding RecA, GyrA, GroEL/GroES, DnaK/DnaJ, and GrpE were present in all anammox species.

However, as described in Fig. 1, expressing a phenotype is quite complex, indicating that the insights from genomics carry a more uncertain contribution towards the phenotype than proteomics.

## 4.3. Early physiological evidence of anammox adaptation to low temperatures: Beyond genomics

Early studies of anammox proteome under low temperature have revealed adaptation mechanisms on the levels of biosynthesis of extracellular polymers, membrane lipids, amino acids and proteins, as well as folding of proteins and generation of energy. Huo et al. (2020) and Lin et al. (2018) cultivated anammox at 35 °C (as a control) and at lower temperatures (25, 20 and 15 °C) to study the adaptation strategies of anammox to different temperatures. Niederdorfer et al. (2021) also explored the temperature-modulated stress response to dissolved oxygen at 20 and 14 °C. Wang et al. (2021a) monitored a reactor exposed to a stepwise temperature decrease down to 15 °C. Lastly, Kouba et al. (2022) exposed one anammox enrichment to a gradual temperature decrease while two other enrichments were exposed to cold shocks. Due to the only publication of the complete genome (Frank et al., 2018), most of the studies analyzing anammox proteome under low temperatures relied on draft genomes. The following chapter discusses the findings presented in these studies.

### 4.3.1. Biosynthesis

In several studies, low temperatures induced anammox bacteria to preserve energy by downregulating the synthesis of polysaccharides, primary metabolites, cofactors and vitamins, and also reduced anabolic pathways (Huo et al., 2020; Jin et al., 2013; Wang et al., 2021a). The reduced synthesis of polysaccharides at low temperatures includes extracellular polymeric substances (EPS) (Huo et al., 2020). The anammox EPS consist in a large part of glycoproteins, sialic acids, humic acids and sulfated glycosaminoglycans (Boleij et al., 2018, 2020). The reduction in the content of these and other EPS leads to the common and easily noticeable disintegration of anammox granules (Guo et al., 2015; He et al., 2018; Morales et al., 2016). Furthermore, EPS can Huo et al. (2020) and Wang et al. (2021a) also reported downregulation of a number of proteins participating in gluconeogenesis, fatty acids biosynthesis and other growth-related processes such as tricarboxylic acid cycle, pyruvate metabolism, purine and pyrimidine metabolism, which lead to decreased growth rates. A similar effect of decoupling growth from the core metabolism has been observed in other bacteria exposed to suboptimal conditions (Haves and Low, 2009; Lempp et al., 2020; Starosta et al., 2014).

In a few very recent studies, the reduced biosynthesis at low temperatures appears to be alleviated by supplementing anammox bacteria with crucial biosynthesis intermediates (e.g., fulvic acid) or signalling molecules (e.g. N-acyl-homoserine lactones). Fulvic acid, the main constituent of humic acids, are equipped with abundant functional groups (e.g., phenols, hydroxyls, carboxyls, quinoes). It is known to



Fig. 1. Biomarkers relevant for anammox adaptation to low temperatures. The complexity of factors influencing phenotypic expression after initiation from genotype. The primary component is proteomics and metabolomics, however, other inputs include epigenomics, transcriptomics, metabolomics, environmental factors and other microbial populations. Partially . adapted from Boggess et al. (2013)

participate in biochemical reactions such as intraspecies electron transfer, tricarboxylic acid cycle, or lipid accumulation. In anammox bacteria, fulvic acid was shown to enhance the amino sugar metabolism, besides redox reactions and increasing relative abundance of genes in the central pathway (Liu, Wang, Xu, Yan, and Ji, 2022). On the other hand, N-acyl-homoserine lactones are a class of molecules involved in bacterial quorum sensing. The amendment of C6- and C8-homoserine lactones in anammox reactors increased the production of EPS and abundance of metabolism-associated genes (Liu et al., 2021; Zhang et al., 2021). These early and promising studies encourage further metabolomic studies into low-temperature anammox cultures.

Interestingly, the pentose phosphate pathway was found to be upregulated in "*Ca.* Brocadia fulgida" at 25 °C, contrary to its downregulation in "*Ca.* Jettenia caeni" at 25 °C and "*Ca.* Brocadia fulgida" at 15 °C (Huo et al., 2020; Wang et al., 2021a). This suggests that "*Ca.* Brocadia fulgida" adapts better to the decreased temperatures than "*Ca.* Jettenia caeni" and may afford to invest energy into its biosynthesis. The greater adaptability of "*Ca.* Brocadia" was further supported by differential regulation of carbon fixation pathway compared to "*Ca.* Jettenia caeni" (Huo et al., 2020), suggesting that the metabolism shift and limitations of biosynthesis may occur at different temperatures for different anammox species. The lower energy investments into such biosynthesis pathways may help anammox preserve the core metabolism, leading to increased chances of survival under cold stress.

### 4.3.2. Membranes

Under low temperatures, anammox bacteria maintain membranes in the suitable liquid crystalline state by adjusting the composition of unique ladderane and other more common lipids (Rattray et al., 2010). These adjustments appear to involve an increased content of shorter ladderane alkyls with five concatenated cyclobutane rings (C18- as opposed to C20-[5]-ladderane fatty acid) and polar headgroup also appears to play a role in the adaptation (Kouba et al., 2022). While the protein pathways involved in the ladderane biosynthesis and/or adaptation under low temperatures remain unknown, Lin et al. (2018) observed the upregulation of clusters of orthologous groups (COGs) related to cell wall/membrane biogenesis at a decreased temperature, and Huo et al. (2020) showed the upregulation of potentially relevant lipopolysaccharide biosynthesis pathway. Aside from lipids, the anammox cell wall comprises of peptidoglycan (Van Teeseling et al., 2015), whose synthesis was shown to be downregulated at 25 °C (Huo et al., 2020). The limitation of peptidoglycan biosynthesis suggests that anammox bacteria preserve energy under lower temperatures. Overall, the structure of unique lipids in anammox membranes is altered under lower temperatures which makes them especially promising as biomarkers of temperature adaptation. After all, ladderanes are often used as biomarkers of anammox bacteria for instance in the marine (Zhao et al., 2019) or river sediments (Naeher et al., 2015), so it is reasonable to assume that they could also be regularly detected in the anammox biomasses at the WWTPs and their structure could be used by process engineers for describing the degree of biomass adaptation to mainstream temperatures. It is also worth noting that bacteria can change the structure of their lipids in response to pressure which could be of interest in particular to marine microbiologists (Siliakus et al., 2017).

#### 4.3.3. Amino acids

Amino acids, specifically their biosynthesis, transport and crossfeeding, appear to be also involved in adapting anammox to low temperatures. As mentioned previously, Huo et al. (2020) reported differential adaptation of "*Ca.* Brocadia fulgida", "*Ca.* Brocadia sinica" and "*Ca.* Jettenia caeni" from 35 °C to 25 °C. While "*Ca.* Brocadia fulgida" thrived, the abundance of "*Ca.* Brocadia sinica" and "*Ca.* Jettenia caeni" decreased at this lower temperature. Interestingly, as the cultivation temperature dropped, amino acid biosynthesis and transport were upregulated in "*Ca.* Brocadia fulgida" and downregulated in "*Ca.* Jettenia caeni". More specifically, "*Ca.* Brocadia fulgida" upregulated the synthesis of glutamine, glutamic acid and glycine, whereas "*Ca.* Brocadia sinica" increased the expression of transporters of these specific amino acids. These three amino acids could theoretically decrease protein hydrophobicity, providing the protein with higher conformational flexibility and thus catalytic efficiency (*Casanueva et al.*, 2010). Since these changes in the expression profile of "*Ca.* Brocadia fulgida" were accompanied by an increase in its abundance, they may serve as an essential adaptation mechanism. In another study, "*Ca.* Brocadia fulgida" downregulated the synthesis of the two most energy demanding amino acids (arginine and histidine) at a temperature of 15 °C (Wang et al., 2021a). Therefore, it is speculated that "*Ca.* Brocadia fulgida" undergoes a similar "shutdown" of anabolism, albeit at a lower temperature than other anammox species.

Regarding protein metabolism, both Huo et al. (2020) and Lin et al. (2018) observed an accumulation of amino acids, suggesting that protein integrity was not maintained via repair of malfunctioning proteins, but rather their recycling or synthesis. This was first proposed by Lin et al. (2018), who reported an increased abundance of proteins involved in translation, ribosomal structure, protein turnover and chaperones at 20 °C and 15 °C compared to 35 °C. In the study by Huo et al. (2020), "Ca. Brocadia sinica" upregulated amino acid transporters simultaneously with upregulation of amino acids biosynthesis in "Ca. Brocadia fulgida". Therefore, the accumulation of amino acids may be advantageous, as these can be cross-fed not only between anammox (e.g., "Ca. Kuenenia") and heterotrophic bacteria (Cheng et al., 2020), but possibly among anammox genera as well. This process may help anammox further limit the energy investments into the biosynthesis and preserve it for core metabolism when the bacteria are under cold-stress conditions, therefore engaging in the division of labor (Momeni, 2018).

### 4.3.4. Protein folding, chaperones

At the level of proteins, chaperones play a crucial role in the cold adaptation of bacteria in general, but their role in the adaptation of anammox has been shown in only two studies. A first study found that unspecified Csps were upregulated in "Ca. Brocadia fulgida" by decreasing the temperature of anammox culture from 35 to 25 °C, which potentially gave this species advantage over "Ca. Brocadia sinica" and "Ca. Jettenia caeni" (Huo et al., 2020). A more recent study not only confirmed the role of cold shock proteins for low-temperature adaptation (T reduction from 30 to 15 °C) of "Ca. Kuenenia", but also described that the most efficiently upregulated were the putative cold shock proteins CspB (more specifically protein strongly similar to CspB) and TypA; however, other potential cold shock proteins with chaperone activity (e. g., ppiD, UspA, and other less characterized proteins) were associated with favorably maintained content of anammox respiration proteins and higher activity at 15 °C (Kouba et al., 2022). Csps typically act as nucleic acid chaperones, preventing the formation of secondary RNA structures, thus maintaining protein synthesis uninterrupted. Other chaperones prevent or alleviate the misfolding of proteins under reduced temperatures (Horn et al., 2007). Another study by Lin et al. (2018) exposed mixed anammox populations to temperatures 35, 20 and 15 °C and observed an upregulation of chaperone GroEL in "Ca. Brocadia" and "Ca. Jettenia" at 15 and 20 °C. Chaperonin complex GroEL/GroES, typically known as a heat shock protein, assists with protein folding, thus restoring protein structure and stability (Table 2). Thus, if confirmed by future studies, GroEL/GroES may not only be characterized as heat shock protein but because the temperature difference of 15 °C can be classified as a cold shock, it may function in anammox ("Ca. Brocadia") as a Csp.

Apart from chaperones, anammox bacteria were found to synthesize other products assisting protein folding and maintaining membrane fluidity known as cryoprotectants such as glycine betaine. The production of glycine betaine was reported at ambient temperature by two studies: Huo et al. (2020) observed the synthesis of glycine betaine in "*Ca*. Brocadia fulgida", but not in "*Ca*. Brocadia sinica" nor "*Ca*. Jettenia caeni". This may be another reason for the increased abundance of "*Ca*. Brocadia fulgida" at ambient temperature and suggests that glycine betaine plays a major role in cold adaptation. A genomic study by Cheng et al. (2020) supported this theory by detecting an increase in cross-feeding of glycine betaine at 15 °C.

While anammox bacteria appear to adapt to low temperatures by upregulating cold shock proteins, either RNA or protein chaperones, the only detailed study has been performed on "*Ca.* Kuenenia" which is highly relevant for engineering practice, as it can co-exist with or even overtake "*Ca.* Brocadia" at low temperatures (Jiang et al., 2021; Li et al., 2021; Qian et al., 2021; Wang et al., 2021b; Wu et al., 2020; Zhou et al., 2021). However, not all draft genomes of "*Ca.* Brocadia" encode putative cold shock proteins (Table 2). Therefore, studies on low-temperature adaptation, at least on this practically relevant anammox genus, are of interest.

### 4.3.5. Energy generation

Bacteria generally reduce their energy generation at lower temperatures. While anammox can be reasonably assumed to be no exception, it is notable due to the exceptionally toxic reaction intermediates such as hydrazine and the potential versatility of its metabolism (Shivaji and Prakash, 2010). The key components in anammox energy generation are the N-respiration enzymes such as hydrazine dehydrogenase (HDH), hydrazine synthase (HZS), hydroxylamine oxidoreductase (HAO), nitrite reductase (Nir), and nitrate oxidoreductase (NXR), so their up- or downregulation at decreased temperatures are of interest.

After the reduction of temperature from 35 to 25 °C, most of the enzymes related to anammox energy generation were upregulated ("Ca. Brocadia fulgida", "Ca. Brocadia sinica") (Huo et al., 2020). After a more significant temperature reduction, from 35 to 15 °C, Wang et al. (2021a) showed that NXR, HZS, HDH and HAO were all downregulated ("Ca. Brocadia fulgida"). Similarly, a metagenomic study confirmed that the temperature reduction from 35 to 15 °C resulted in a decreased abundance of hzsA and hdh genes. However, a culture of "Ca. Kuenenia" that was adapted more gradually and for a longer period (35  $^\circ$ C > 25  $^\circ$ C >15 °C) increased their abundance almost to the initial levels (Cheng et al., 2020). Another gradually adapted enrichment of "Ca. Kuenenia" maintained the level of metabolic proteins less efficiently than enrichment exposed to a more elaborate adaptation methodology (Kouba et al., 2022) which explained the increased activity of anammox bacteria as a result of this methodology in two previous studies (Kouba et al., 2018, 2021). This suggests that the downregulation of energy generation at lower temperatures could be transitory and its occurrence depends on the applied adaptive regime. In contrast, at 20 and 15 °C ("Ca. Kuenenia"), Lin et al. (2018) observed that the levels of NADH dehydrogenase-like proteins decreased, suggesting energy limitation, while the levels of HAO increased. According to Ferousi et al. (2021), HAO upregulation could counteract the accumulation of toxic nitrite and provide NO for anammox metabolism, thus alleviating energy limitation. A transcriptomic study by Niederdorfer et al. (2021) offers an adaptation mechanism to both low temperatures (14 °C) and increased levels of DO via elevated expression of hdh and reduced expression of hzs, which may assist in avoiding hydrazine accumulation.

Overall, the energy generation of anammox appears to be negatively impacted at  $\leq 15$  °C across the various anammox genera, although distinctions among the genera and species were observed. Furthermore, the reported upregulation of individual anammox enzymes suggests that the low temperatures induce imbalances in enzymatic activities leading to the accumulation of toxic nitrogenous intermediates.

### 5. Applicable biomarkers of cold-adapted anammox

To make the proposed adaptation mechanisms of anammox bacteria to low temperature useful for the operators of anammox reactors, it is necessary to convert these mechanisms into specific biomarkers that can explain and predict future process performance (Cerruti et al., 2021). Anammox process performance in response to low temperature is typically assessed either *in-situ* by the removal efficiency of nitrogenous species directly in the anammox reactor or *ex-situ* activity assays. Depending on the process configuration, both can provide a highly useful marker: relative anammox activity (=removal rate of nitrogen) and/or activation energy of the anammox bacteria. As shown in Fig. 1, a range of other markers is proposed that could also benefit operators.

The extent of the adaptation of anammox culture under lowtemperature conditions can be determined from the transcriptome, proteome, and metabolome. The biomarkers such as the relative content of i) RNA chaperones (e.g., protein strongly similar to CspB, TypA, ppiD, UspA), ii) protein chaperones (e.g., GroEL/GroES), iii) chemical chaperones/cryoprotectants synthesis proteins (e.g., synthesis of glycine betaine), iv) non-polar amino acids, v) cryoprotectants (e.g., glycine betaine), and vi) length of ladderane lipids (e.g., the relative content of C18/(C18 + C20) ladderane fatty acids) seem especially relevant as they directly relate to how anammox bacteria adapt to low temperature. The downregulation of biosynthesis, in general, could predict the reduction of growth rate and possibly even biofilm decomposition. Much remains to be done to make these proposed biomarkers applicable, one of the concerns being uniqueness for anammox bacteria. The biomarkers should be evaluated in the context of overall plant operation and other possible influences, e.g., specific industrial effluents can substantially reduce biosynthesis in anammox as well as in other bacteria. Of the reviewed mechanisms, ladderane lipids are unique for anammox and thus directly applicable, while the proteins responsible for their synthesis remain unknown. Furthermore, the protein strongly similar to CspB should be better characterized to provide a biomarker potentially unique for anammox. Cold shock proteins such as CspB, CspA, TypA and others are not only involved in the low-temperature adaptation of anammox bacteria, but also in other microbial populations. These and other less unique biomarkers could be utilized in the process streams containing anammox biomass at a high degree of enrichment, i.e., separated anammox granules. Also, this could stimulate development of methodologies for more efficient separation of anammox bacteria from other microbial populations.

Further, the potential for low-temperature adaptation can be assessed from metagenomes, such as the presence and copy numbers of genes encoding RNA and protein chaperones (*recA*, *CspA*, *CspB*, *gyrA*, *groEL*, *TypA*, *UspA*, *PpiD*,...) and cryoprotectant synthesis and transport proteins. This could be especially promising when assessing the most suitable inoculum for new low-temperature installations.

### 6. Conclusions

Anammox bacteria can adapt to low temperatures by a broad spectrum of adaptation mechanisms that, if mechanistically understood, could save costs for N-removal at WWTPs. Early -omics studies suggest that these mechanisms include the upregulation of certain chaperones, cryoprotectants, cross-feeding pathways, and alteration of the membrane composition. These chaperones, cryoprotectants as well as unique ladderane membrane lipids, or at least the corresponding genes, could find use as the biomarkers of anammox low-temperature adaptation/ adaptability. Furthermore, the amendment of crucial anammox metabolites and signalling molecules seems promising topic as well. These insights will ease the transition to more cost-efficient and sustainable WWTPs.

### **Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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#### V. Kouba et al.

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