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Endogenous metabolism of anaerobic ammonium oxidizing bacteria in response to short-term anaerobic and anoxic starvation stress

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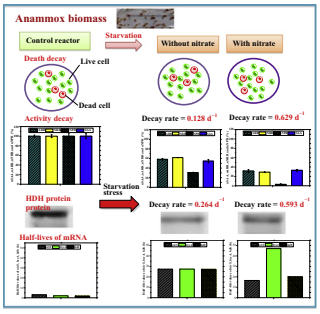
HIGHLIGHTS

- The endogenous metabolism processes of anammox sludge were studied.
- EPS and HDH protein were consumed to produce energy for the cell maintenance.
- EPS and HDH protein were used as the carbon sources of denitrification processes.
- Starvation could lead to the increase in the mRNA half-life values in AnAOB.

GRAPHICAL ABSTRACT



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ABSTRACT

Under starvation conditions, bacteria could consume intracellular macromolecules, which deteriorates the performance of the bioreactor. However, the fundamental endogenous metabolism of anaerobic ammonium oxidation (anammox) bacteria is poorly understood. Here, we describe the endogenous metabolism of anammox bacteria biomass under short-term anaerobic and anoxic starvation stress (4.5 d and 40 h, respectively) by protein, mRNA and biochemical analyses. The live/dead staining results showed negligible cell death rates. The activity decay rates (decrease in specific activity of active bacteria) were 0.128 d^{-1} and 0.629 d^{-1} under anaerobic and anoxic starvation conditions, respectively. The degradation rates of extracellular polymeric substances (EPS) and hydrazine dehydrogenase protein (HDH) were approximately 4- and 5-fold higher under anoxic starvation than under anaerobic starvation tests, suggesting the electron-donor roles for EPS and HDH in denitrification. The greater consumption of intracellular macromolecules during anoxic starvation corresponded with the greater decrease in anammox activities under this condition. Under both starvation conditions, the mRNA decay rates of *nirS*, *hzsA*, and *hdh* mRNA (representing metabolic stability) of anammox bacteria decreased from their normal levels. Such control of specific mRNAs is important for economizing protein synthesis and aids the cells' recovery after starvation stress. These identified endogenous characteristics of anammox bacteria provide new insights into starvation stress responses at the transcriptional and translational levels, and will assist the optimization of anammox processes in bioreactors, which frequently experience starvation conditions.

1. Introduction

Anaerobic ammonium oxidation (anammox) has become increasingly important in biological nitrogen removal processes worldwide [1], owing to reduced sludge production, zero organic

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carbon requirements, and substantial energy savings. Under anoxic conditions, the hypothetical metabolic pathway in anammox bacteria has been proposed: first, anammox bacteria express the cytochrome cd1 nitrite:nitric oxide oxidoreductase (NirS) with the ability to reduce nitrite to nitric oxide (NO), then the condensation of ammonium and NO into hydrazine is carried out by hydrazine synthase (HZS), and finally reaction is the oxidation of hydrazine to dinitrogen gas (N_2), which is catalyzed by hydrazine dehydrogenase (HDH) [2]. In recent years, anammox processes have been efficiently applied in the treatment of industrial wastewater with high ammonia concentration, such as landfill leachates [3] and sludge digester liquids [4].

However, the biomass in wastewater treatment systems is frequently exposed to starvation conditions imposed by the fluctuations in the flow and composition of domestic and industrial wastewater [5]. Under these conditions, which may persist for days or weeks, the biomass switches to an endogenous metabolism that consumes its own intracellular macromolecules [6]. The resultant biomass decline and cell death [7–9] decreases the specific activity of the bacteria and deteriorates the performance of the wastewater treatment system [10].

Previous studies had shown that the amount of active anammox bacteria would significant decrease during the starvation periods [11,12]. Additionally, a wide range of doubling times (2.1–25 d) has been reported for anammox bacteria [13–17]. It has been reported that when the biomass grown as free-cell suspension status, the maximum growth rate of anammox bacteria can increased to 0.21 d^{-1} (corresponding to a doubling time of 3.37 d) [14], however, most anammox cultivation systems, especially the full-scale anammox reactors, adopt granular sludge based technology [15,18], and have a slow growth rate with a doubling time of approximately 10–25 d [15,16]. Unlike heterotrophic bacteria, anammox bacteria does not contain any polyhydroxyalkanoates (PHA) synthase gene [19], which indicated that anammox bacteria could not synthetize PHA to produce energy during starvation conditions. Thus, the impact of starvation shock, killing the biomass, may be particularly problematic for anammox bacteria due to the long recovery periods needed [20–22]. Although the cell death decay and activities changes of anammox bacteria during the starvations periods had been investigated by several researchers [11,12,23,24], the endogenous metabolism processes of anammox bacteria, especially the intracellular energy sources used by anammox bacteria for the cell maintenance under starvation, have not been reported.

Starvation largely degrades the performance of biological nutrient removal in wastewater treatment plants, as it significantly alters the contents of intracellular macromolecules in the biomass [7,8]. However, under nutrient starvation, autotrophs such as ammonia-oxidizing bacteria (AOB) require stable levels of important intracellular macromolecules (e.g., proteins) for energy generation, enabling them to regain their metabolic activity when substrates again become available [25]. In one study, aerobically starved *Nitrosospira briensis* (AOB) slightly varied their soluble protein fraction from that of non-starved cells after 12 d, but regained 100% activity within 10 min after fresh ammonium was supplied [26]. This result suggested that AOB could stabilize their levels of intracellular macromolecules, such as protein, and ultimately facilitate adaptation to starvation circumstances. Although both anammox bacteria and AOB are autotrophs, whether the levels of intracellular macromolecules in anammox bacteria remain unchanged under starvation conditions (as in AOB), and the main regulatory pathways controlling the transcription and expression of specific genes in anammox bacteria's starvation response, remain to be clarified.

Recently, the regulation of mRNA degradation has been reported as an efficient response to changes in living conditions [27]. It was reported that the half-life of mRNA in *V. angustum* S14 cells, which encoding the β-subunit of the proton-translocating ATPase (atpD), increased 2 to 4-fold in carbon-starved condition, in comparison with that in the growing cells [27]. Slowing down the degradation of the available translation templates (mRNA levels) during starvation may be energy-saving than *de novo* gene expression, and could contribute to the recovery of biomass activity after starvation shock [27]. However, the mRNA stability characteristics and the corresponding regulatory mechanisms have not been confirmed in starved anammox bacteria.

Previous studies showed that in the presence of organic matters (e.g., formate, acetate and propionate), anammox bacteria can reduce nitrate to ammonium via dissimilatory nitrate and nitrite reduction, then the bacteria convert nitrite and ammonium to N_2 by normal anammox metabolism [28,29]. To prevent the occurrence of microbial sulfate reduction, nitrate was supplemented to the reactors as redox buffer during the storage periods [14,20]. However, in the presence of nitrate during anoxic starvation periods, anammox bacteria may produce energy via dissimilatory nitrate and nitrite reduction, where intracellular macromolecules may also be used as the electron donors, leading to the more serious decreased intracellular macromolecule levels (relative to those during anaerobic starvation). Therefore, the variation of intracellular macromolecule levels during anoxic starvation periods remains to be elucidated.

In this study, the endogenous metabolism of anammox bacteria was monitored over a 4.5-d anaerobic starvation period and a 40-h anoxic starvation period. Besides determining the stoichiometric parameters of the death and activity decays, we evaluated the maintenance energy transformation and the variations in extracellular and intracellular macromolecules involved in the endogenous metabolism of anammox bacteria. In particular, the mRNA levels of cytochrome cd_1-type nitric oxide/nitrite oxidoreductase (*nirS*), hydrazine synthase (*hzsA*), and hydrazine dehydrogenase (*hdh*) in anammox bacteria were quantified by reverse transcriptase quantitative polymerase chain reaction (RT–qPCR), and the hydrazine dehydrogenase protein (HDH) levels were quantified by western blotting. This study aimed to identify the endogenous metabolism and survival strategies of anammox bacteria under anaerobic and anoxic starvation conditions at the transcriptional and translational levels, and to provide implementable strategies for optimizing anammox processes in wastewater treatment plants and temporarily stored anammox sludge.

2. Materials and methods

2.1. Parent anammox reactor setup and operation

Anammox sludge was enriched in a sealed laboratory-scale anammox sequencing batch reactor (SBR) with a working volume of 20 L. The temperature was maintained at 33 ± 1 °C, and the stirring speed was 100 rpm. The SBR was operated at three 8-h cycles per day, each consisting of a 10-min filling period, a 390-min anoxic period, a 55-min sludge-settling period, a 10-min effluent decanting period, and a 15-min idle phase. During each 10-min feeding period, 10 L of synthetic wastewater was pumped into the reactor as feed substrate. The specific anammox activity (SAA) of the reactor was stabilized at approximately 0.31 ± 0.01 g N/g VSS d after cultivation for 270 d. The mixed liquid volatile suspended (MLVSS) concentration (comprising approximately 75% granules and 25% flocs) was approximately 5 g/L.

2.2. Synthetic wastewater

The synthetic wastewater contained (g/L): NH_4Cl, 1.07 (280 mg NH_4^+-N/L); $NaNO_2$, 1.77 (360 mg NO_2^--N/L); $KHCO_3$, 1.25;