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Physiological and transcriptomic insights into the cold adaptation mechanism of a novel heterotrophic nitrifying and aerobic denitrifying-like bacterium *Pseudomonas indoloxydans* YY-1

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ABSTRACT

Development of low-temperature biological nitrogen removal processes is of scientific and engineering importance. Cold-adapted heterotrophic nitrifying and aerobic denitrifying (HNAD) bacteria have attracted increasing interest. However, the nitrogen metabolism and cold adaption mechanisms of HNAD bacteria remain unclear.

In this article, a novel cold-adapted HNAD-capable bacterium, *Pseudomonas indoloxydans* YY-1, was isolated. Analyses of draft whole-genome features indicated that strain YY-1 was capable of complete dissimilatory nitrate reduction, ammonium assimilation, and cyanate decomposition. The gene cluster of *napABCDE* and gene *norR*, which encode for the periplasmic nitrate reductase and nitric oxide reductase transcription regulator, were identified in the YY-1 genome. Adenosine triphosphate levels increased fivefold, and polysaccharide content significantly rose in the extracellular polymeric substances of strain YY-1 when temperature decreased from 25 °C to 5 °C. Comparative transcriptional profiles of the strain grown at 25 °C and 10 °C revealed that the genes involved in tricarboxylic acid cycle, cytochrome reductase, transhydrogenase, and adenosine triphosphate synthesis were overexpressed, whereas the genes that encod for nicotinamide adenine dinucleotide dehydrogenase, cytochrome reductase, and the functional proteins of nitrate assimilation were downregulated. For ammonium assimilation of strain YY-1 at 10 °C, transcriptional data revealed the overexpression of glutamate dehydrogenase and glutamate synthase genes. This study highlights the potential nitrogen metabolic diversity of HNAD bacteria and expands the understanding of physiological and transcriptional strategies of cold adaption of those bacteria.

1. Introduction

Biological nitrogen removal (BNR) from wastewater at cold temperatures has been widely recognized as a technical challenge (Liu and Li, 2015; Salvetti et al., 2006; Guo et al., 2010). Temperatures below 10 °C can deteriorate BNR performance in nitrification–denitrfication (Zhang et al., 2014) and anaerobic ammonium oxidation (Lotti et al., 2014; Gilbert et al., 2014) processes. A number of studies have examined the effects of engineered control strategies for enhancing the activity of traditional BNR functional microorganisms (Dong et al., 2016; Gilbert et al., 2014). However, implementing BNR processes below the optimal temperature remains a substantial challenge in practice. Thus, developing cold-adaptive BNR processes bears importance in maintaining a relatively stable nitrogen removal rate at low temperatures. Recently, the use of purple phototrophic bacteria (Hülsen et al., 2016), sulfur-based autotrophic denitrifiers (Capua et al., 2017), and aerobic denitrifiers (Yao et al., 2013a; Wang et al., 2013; Padhi et al., 2013), has shown considerable progress in low-temperature BNR. Heterotrophic nitrifying and aerobic denitrifying (HNAD) bacteria, which enable a novel, easily maintained, manageable, and cost-effective BNR technique, have attracted tremendous research interest and popularity in recent years (Yao et al., 2013b; Qin et al., 2016). The importance of psychrotolerant HNAD bacteria has been demonstrated, but nitrogen elimination and cold adaptation mechanisms of these microbes still requires thorough characterization (Qin et al., 2016).

Denitrification and aerobic respiratory electron transfer chains are similar and all denitrifiers are capable of aerobic respiration (Chen and Strous, 2013). However, the experimental evidence for aerobic

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denitrification is inconclusive. In general, aerobic denitrification is not a preferred pathway over aerobic respiration for typical denitrifiers (Chen and Strous, 2013). The ammonium assimilatory pathway has been overlooked in previous studies on nitrogen metabolism in aerobic denitrifiers (Gates et al., 2011). For example, at relatively high C/N ratios, the aerobic denitrifiers *Paracoccus denitrificans* and *Pseudomonas stutzeri* T13 can utilize nitrate and ammonium through assimilatory pathways instead of aerobic denitrification and heterotrophic nitrification (Gates et al., 2011; Sun et al., 2017). Nitrate assimilatory, dissimilatory, and true denitrifying pathways may also coexist in a mixed aerobic denitrification bioreactor. True denitrifying bacteria are usually outcompeted in the competition for nitrogen sources because assimilatory nitrogen reduction is a beneficial pathway for heterotrophic bacteria in absorbing the excess reductants in the reduced organic carbon pool (Nancharaiah and Venugopalan, 2011; Gates et al., 2011). Therefore, cold-adapted heterotrophic bacteria, instead of true aerobic denitrifiers, may play an important role in low-temperature nitrogen removal. Heterotrophic bacteria metabolizing nitrogen through assimilation pathways are phylogenetically widespread, particularly in marine microbial nitrogen cycles (Gates et al., 2011; Allen et al., 2001). Notably, such ammonium and nitrate assimilatory transformations can occur at low temperatures. Recently, the aerobic bacterium *Vibrio* sp. Y1-5 had been isolated from marine sediments and exhibited an efficient nitrate and ammonium assimilation performance (Li et al., 2017). Nevertheless, the principles controlling the cold response of these aerobic– heterotrophic bacteria are largely unknown.

Studies on the paradigm organisms *Escherichia coli*, *Bacillus subtilis*, and other psychrotolerant bacteria revealed that prokaryotes exhibit a range of physiological responses to cope with deceleration of cellular metabolism after temperature drop (Klein et al., 1999; Brillard et al., 2010; Bajaj and Singh, 2015). Examples of these responses include modification of fatty acid branching patterns in the cell membrane to maintain cold survival membrane fluidity (Klein et al., 1999), generation of sufficient and high intracellular ATP levels (Napolitano and Shain, 2005), mediation and depression of protoplasmic freezing point by accumulating cold-resistant compatible solutes (Davies et al., 2002; Kocharunchitt et al., 2012), and size increase when grown at cold temperatures (Forster and Hirst, 2012). The genetic transcriptional regulation of psychrotolerant bacteria also changes when the organisms are subjected to low temperatures. Researchers have characterized the cold-inducible *hutU* gene in the psychrotrophic strain *Pseudomonas syringae* (Janiyani and Ray, 2002) and identified differentially expressed genes (DEGs) in *Bacillus cereus* grown at low temperatures (Brillard et al., 2010). A similar investigation also revealed that mRNAs are involved in cold-adaptive mechanisms of *Methanosarcina mazei* (Cao et al., 2014). In recent years, high-throughput functional approaches, such as transcriptomic and proteomic analyses, have been widely used to profile gene expression as an alternative means for traditional technologies to uncover low-temperature adaptation strategies in psychrotolerant pure cultures and communities (Kocharunchitt et al., 2012; Canion et al., 2014; Qin et al., 2016). Despite these improvements, the effects of low temperatures on transcriptional profiles of certain HNAD-like bacteria and the underlying fundamental adaptation mechanisms remain unclear.

In the present study, we isolated a novel aerobic–heterotrophic bacterium *Pseudomonas indoloxydans* YY-1 from a laboratory-scale aerobic denitrification bioreactor stably operated at 5 °C (Wang et al., 2015). The identified strain showed the capacity of HNAD at room (25 °C) and low temperatures (5 °C). Batch tests were conducted to evaluate the ammonia removal performance of strain YY-1 with a temperature gradient. Then, physiological characterization and RNA-seq-based comparative transcriptomic analysis were performed to identify the physiological responses of the strain and transcript levels of cold-regulated genes when temperature decreased from 25 °C to 5 °C.

2. Materials and methods

2.1. Bacterial strain isolation and identification

In our previous study, we established a laboratory-scale aerobic denitrification process stably operated at 5 °C (Wang et al., 2015). Incubation, isolation, and identification were performed as previously described (Yao et al., 2013a). In detail, 10 mL inoculated sludge was mixed with 90 mL sterilized water and glass beads and then shaken at 155 rpm for 3 h to form a homogeneous bacterial suspension. Afterward, 1 mL bacterial suspension was diluted 10^5–10^7 times with sterilized water and spread on bromothymol blue (BTB) medium plates. The plates were incubated at 30 °C for 3–7 days until the appearance of blue colonies. Several purified bacterial colonies were isolated through subculturing on the same semisolid medium. The 16S rRNA gene of the purified bacterium with highest aerobic ammonia removal capacity was selected extracted, cleaned up, amplified with universal primers, and sequenced. A neighbor-joining phylogenetic tree was constructed after sequence alignment by MEGA 5.0, with a bootstrap value of 1000 replications.

2.2. Genome sequencing, assembly, and annotation of strain YY-1

The genomic DNA of strain YY-1 was extracted with a FastDNA SPIN Kit (MP Biomedicals, Illkirch, France). The genome of strain YY-1 was sequenced by Illumina Miseq 250 bp paired-end sequencing technology (Illumina, CA, USA). After raw sequence trimming, 7,572,680 reads were obtained, and these reads corresponded to 1,815, 892, 684 bases of total sequence information, with a 382-fold genome coverage. Trimmed reads were assembled using SOAP denovo (v2.04, http://soap.genomics.org.cn/) and generated 39 large contigs (> 1000 bp), and the assembly statistics were optimized with GapCloser (v1.12, K-mer = 31). Gene prediction was conducted with Glimmer 3.02 (http://www.cbcb.umd.edu/software/glimmer/), and gene annotation information of predicted genes was verified using BLAST (v 2.2.28+, http://blast.ncbi.nlm.nih.gov/Blast.cgi) searches against the Nr, Genes, String, and Gene Ontology (GO) protein (PN) databases. The functional categories of PNs were obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/). This whole-genome shotgun project has been deposited at the DDBJ/ENA/GenBank under the accession number PJLX00000000.

2.3. Assessment of aerobic denitrification capacity of strain YY-1 with different nitrogen sources

NH_4Cl and KNO_3 removal by strain YY-1 was assessed in 120 mL serum bottles at 25 °C and 10 °C with a nitrification medium (NM) and denitrification medium (DM). The NM and DM contained (per liter) 5.2 g of sodium succinate, 0.26 g NH_4Cl and 0.50 g KNO_3, respectively. The other components (per liter) of NM and DM included 1.50 g KH_2PO_4, 7.90 g $Na_2HPO_4{\cdot}12H_2O$, and 0.10 g $MgSO_4{\cdot}7H_2O$. In batch tests, 3 mL of pre-cultured bacterial suspension and 27 mL of inorganic NM or DM were mixed in 120 mL serum bottles. The sealed serum bottles (triplicates) were evacuated and blown with helium. Afterward, pure oxygen was used to adjust the initial oxygen content in the headspace of the serum bottles to 10% (v/v), 25% (v/v), and 40% (v/v). Gaseous N_2O and N_2 concentrations in the headspace were monitored by a roboticized incubation system containing a Varian CP 4700 micro GC and an automated gas sampling system (Molstad et al., 2007). Cell optical density (OD_{600}) and aqueous concentrations of nitrogen compounds in the medium were measured with the standard colorimetric method (Chinese NEPA, 2002). Each test was performed in triplicates. Nitrogen removal rate by assimilation was calculated using the following formula: