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Clarifying the regulation of NO/N_2O production in *Nitrosomonas europaea* during anoxic–oxic transition via flux balance analysis of a metabolic network model

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ABSTRACT

The metabolic mechanism regulating the production of nitric and nitrous oxide (NO, N_2O) in ammonia oxidizing bacteria (AOB) was characterized by flux balance analysis (FBA) of a stoichiometric metabolic network (SMN) model. The SMN model was created using 51 reactions and 44 metabolites of the energy metabolism in *Nitrosomonas europaea*, a widely studied AOB. FBA of model simulations provided estimates for reaction rates and yield ratios of intermediate metabolites, substrates, and products. These estimates matched well, deviating on average by 15% from values for 17 M yield ratios reported for non-limiting oxygen and ammonium concentrations. A sensitivity analysis indicated that the reactions catalysed by cytochromes aa3 and P460 principally regulate the pathways of NO and N_2O production (hydroxylamine oxidoreductase mediated and nitrifier denitrification). FBA of simulated *N. europaea* exposure to oxic–anoxic–oxic transition indicated that NO and N_2O production primarily resulted from an intracellular imbalance between the production and consumption of electron equivalents during NH_3 oxidation, and that NO and N_2O are emitted when the sum of their production rates is greater than half the rate of NO oxidation by cytochrome P460.

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1. Introduction

Ammonia oxidizing bacteria (AOB) produce nitric and nitrous oxide (NO and N_2O) during nitrification and autotrophic denitrification in wastewater treatment processes (Ahn et al., 2010; Foley et al., 2010; Kampschreur et al., 2009). A survey of wastewater treatment plants in the US showed that aerobic zones generally contributed more to N_2O fluxes than anoxic zones from BNR reactors (Ahn et al., 2010); the N_2O emission fraction ranges between 0 and 25% of the influent N-load according to the International Panel for Climate Change (IPCC) (Kampschreur et al., 2009). N_2O emissions contribute to ozone depletion and its global warming potential is ~300 times higher than that of CO_2 on a per molecule basis (Ravishankara et al., 2009; Wuebbles, 2009). The operational conditions of wastewater treatment processes that lead to NO and N_2O production by AOB are related to changes in the concentration of electron donors (NH_4^+ and NH_2OH) and acceptors (O_2 and NO_2^-) (Chandran et al., 2011; Kampschreur et al., 2008). However, despite the availability of extensive information on nitrogen respiration and energy production in AOB, the metabolic triggers and regulatory mechanisms controlling NO and N_2O production are not well understood.

NO/N_2O production in AOB occurs via two pathways: (i) the aerobic hydroxylamine oxidation pathway mediated by hydroxylamine oxidoreductase (HAO) and (ii) the nitrifier denitrification pathway mediated by nitrite reductase (NIR) and nitric oxide reductase (NOR) enzymes (Cabail and Pacheco, 2003; Stein, 2010, 2011; Wunderlin et al., 2012). From pure cultures of *Nitrosomonas europaea*, a model AOB species that has been abundantly detected in full scale nitrification processes (Wagner et al., 2002), it is known that these two pathways are part of nitrogen respiration, electron transport chain, and energy generation mechanisms of AOB (Whittaker et al., 2000; Yu et al., 2010). As a result, activation of the HAO mediated pathway leads to generation of electron equivalents and activation of the NIR mediated pathways implies a consumption of electron equivalents. However, details of how the availability of electron donors (NH_4^+ or NH_2OH) and acceptors (O_2 or NO_2^-) influences the activity of these pathways remain unclear.

Activated Sludge (ASM) models have been modified to dynamically predict NO and N_2O production under different environmental conditions by linking NO and N_2O production to the respiratory activity and the responsible metabolic pathway (Kampschreur et al., 2007; Ni et al., 2011, 2013; Pan et al., 2013; Yu et al., 2010). This approach however does not clarify why these gases are produced, as NO and N_2O production has largely been described as being decoupled from the cell's energy metabolism. Furthermore, different routes for production or consumption of electron equivalents are ignored. A different approach taken by Wunderlin et al. (2013) involving the use of isotope signatures of N_2O produced by mixed microbial populations could quantify the contribution of each of the two N_2O production pathways under different environmental conditions. However, the method provides no explanation for what activated the dominant pathway. Added to this, the difficulty and expense of such isotopic signature based experiments could limit their wide adoption in understanding the behaviour of laboratory or full scale systems.

Stoichiometric metabolic network (SMN) modelling and flux balance analysis (FBA) are emerging techniques in systems biology that could be used to quantify the rate of reactions within the network formed by chemical compounds and sequenced chemical reactions in cells' metabolism (Durot et al., 2009; Oberhardt et al., 2009; Orth et al., 2010). FBA provides a 'snapshot' estimation of reaction rates in the metabolic network at a specific metabolic steady state (Orth et al., 2010), and we use it to quantify the simultaneous activity in the hydroxylamine mediated and nitrifier denitrification pathways during NO and N_2O production by AOB. In this study *Nitrosomonas europaea* served as a model AOB as its metabolism as well as the pathways for ammonium oxidation and production of energy, NO and N_2O, are known (Poughon et al., 2001; Sayavedra-Soto and Arp, 2011; Stein, 2010). Furthermore, its genome has been sequenced, which allows the reconstruction of its entire complement of metabolic pathways (Chain et al., 2003). We construct a SMN model based on *Nitrosomonas europaea* energy production metabolism and enzymology, and use it in combination with FBA to quantify the metabolic rates of NO and N_2O production pathways during oxic–anoxic–oxic transitions of *N. europaea* cultures. The obtained metabolic rates are used to infer the physiological mechanisms responsible for regulation of pathways leading to the production of these gases.

2. Materials and methods

2.1. Metabolic network development

A metabolic network model for biochemical reactions and metabolites formed during *N. europaea* energy production metabolism was constructed by following the procedure described by Thiele and Palsson (2010) and using organism-specific genomic and biochemical information from literature (references in Table S1 of Supporting Information (SI)) and the metabolic pathway databases KEGG and MetaCyc (respectively accessible at http://www.genome.jp/kegg/ and http://metacyc.org/). The network consists of mass and charge balanced stoichiometric biochemical reactions that are classified as either reversible or irreversible (Savinell and Palsson, 1992; Thiele and Palsson, 2010).

The reactions for energy production in *N. europaea* cells are modelled as occurring in three cell compartments: extracellular, periplasmic and cytoplasmic spaces (Chain et al., 2003); labels [e], [p] and [c] have respectively been assigned to metabolic compounds to indicate their occurrence in extracellular, periplasmic and cytoplasmic compartments (Fig. 1A). The metabolite NH_4^+[e] is thereby differentiated from NH_4^+[p] and the exchange between extracellular and periplasmic spaces can be simulated as NH_4^+[e] $\leftrightarrow$ NH_4^+[p]. Further information about network compartmentalization can be found in Thiele and Palsson (2010). The constructed AOB-SMN model consists of 44 metabolites and 49 stoichiometric reactions categorized as follows: 11 exchange reactions representing the flow of metabolic compounds in and out of the cell (IDs of