Production and consumption of nitrous oxide in nitrate-ammonifying *Wolinella succinogenes* cells

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Global warming is moving more and more into the public consciousness. Besides the commonly mentioned carbon dioxide and methane, nitrous oxide (N2O) is a powerful greenhouse gas in addition to its contribution to depletion of stratospheric ozone. The increasing concern about N2O emission has focused interest on underlying microbial energy-converting processes and organisms harbouring N2O reductase (NosZ), such as denitrifiers and ammonifiers of nitrate and nitrite. Here, the epsilonproteobacterial model organism *Wolinella succinogenes* is investigated with regard to its capacity to produce and consume N2O during growth by anaerobic nitrate ammonification. This organism synthesizes an unconventional cytochrome c nitrous oxide reductase (cNosZ), which is encoded by the first gene of an atypical nos gene cluster. However, *W. succinogenes* lacks a nitric oxide (NO)-producing nitrite reductase of the NirS- or NirK-type as well as an NO reductase of the Nor-type. Using a robotized incubation system, the wild-type strain and suitable mutants of *W. succinogenes* that either produced or lacked cNosZ were analysed as to their production of NO, N2O and N2 in both nitrate-sufficient and nitrate-limited growth medium using formate as electron donor. It was found that cells growing in nitrate-sufficient medium produced small amounts of N2O, which derived from nitrite and, most likely, from the presence of NO. Furthermore, cells employing cNosZ were able to reduce N2O to N2. This reaction, which was fully inhibited by acetylene, was also observed after adding N2O to the culture headspace. The results indicate that *W. succinogenes* cells are competent in N2O and N2 production despite being correctly grouped as respiratory nitrate ammonifiers. N2O production is assumed to result from NO detoxification and nitrosative stress defence, while N2O serves as a terminal electron acceptor in anaerobic respiration. The ecological implications of these findings are discussed.

INTRODUCTION

Nitrous oxide (N2O, also known as laughing gas) is a chemically rather inert trace gas in Earth's atmosphere whose level has been consistently increasing over the last decades (Smith, 2010), largely driven by high input of reactive nitrogen in agriculture (Reay et al., 2012). N2O is involved in stratospheric ozone depletion and, moreover, it is a strong greenhouse gas. Its capacity to absorb infrared radiation is about 300 times greater than that of CO2 and therefore anthropogenic N2O emissions, unless balanced by N2O-consuming processes, contribute significantly to climate change. This is even more important in the light of the fact that an ever increasing amount of synthetic nitrogen-based fertilizers is used to raise agricultural efficiency.

For a long time N2O has been known as a crucial intermediate in the biogeochemical nitrogen cycle, and the majority of atmospheric N2O is of microbial origin (for recent reviews see Richardson et al., 2009 and Thomson et al., 2012). The most important processes in biological N2O production and turnover are thought to be denitrification [reduction of nitrate or nitrite to N2 via nitric oxide (NO) and N2O as intermediates] and nitrification (oxidation of ammonium or nitrite to nitrate with N2O being a side product under aerobic conditions in a process...
referred to as nitrifier denitrification) (Stein, 2011). Notably, some denitrifying organisms lack N₂O reductase activity and release N₂O as an end product. Recently, nitrate- (or nitrite-) ammonifying cells have also been reported to release N₂O (Stromsæt et al., 2012; Giles et al., 2012). In bacterial denitrification and in denitrifying nitrifiers, NO is reduced by one of various membrane-bound respiratory NO reductases (predominantly cNor and qNor enzymes) (Simon & Klotz, 2013). Since NO is a highly toxic compound that exerts nitrosative stress on cells and organisms, it needs to be detoxified (Poole, 2005). It is therefore not surprising that N₂O production from NO has been described for numerous non-respiratory enzymes, including flavodiiron proteins (Fdp), flavohydrin (NorVW), cytochrome c₃₄₅₆ (CycA; in nitrifiers), cytochrome c₄₃₅₆- (Cyts) and cytochrome c₇₅₆-alpha (CytP) (Simon & Klotz, 2013). In these cases, NO reduction is thought to serve predominantly in NO detoxification, i.e. in nitrosative stress defence.

In many bacterial and archaeal prokaryotes, N₂O is further reduced to N₂ by a homodimeric N₂O reductase (NosZ), which is a copper-containing enzyme located on the outside of the cytoplasmic membrane (i.e. the periplasm in Gram-negative bacteria) (Zumft, 1997; Zumft & Kronkeck, 2007; Pomowski et al., 2011; Wüst et al., 2012; Pauleta et al., 2013). Various NosZ enzymes carry a C-terminal extension that forms a monohaem cytochrome c domain, which was first described for non-denitrifying Epsilonproteobacteria (Teraguchi & Hollocher, 1989; Simon et al., 2004). The cytochrome c domain of such cNosZ enzymes is thought to donate electrons to the Cu₅₆₆₇₈₉₉₉₉ domain of NosZ (Dell’Acqua et al., 2011; Simon & Klotz, 2013). Recently, so-called atypical NosZ proteins (including cNosZ enzymes) and atypical nos gene clusters from non-denitrifying bacteria and archaea were found to be widespread in terrestrial environments, where they form an evolutionarily distinct clade of N₂O reductases (Sanford et al., 2012; Jones et al., 2013). Based on the abundance of these genes in soils, it was suggested that the corresponding organisms could be a significant sink for N₂O (Jones et al., 2013). Furthermore, genome sequences suggest that ammonification and N₂ production from nitrate/nitrite may coexist in some bacteria (Sanford et al., 2012; Mania et al., 2014).

The metabolism of N₂O in nitrate- or nitrite-ammonifying organisms is poorly understood. The respective organisms reduce nitrate to nitrite using a membrane-bound nitrate reductase (Nar) and/or a periplasmic nitrate reductase (Nap) (Richardson et al., 2001; Kern & Simon, 2009; Simon & Klotz, 2013). Prominent examples are Gamma-, Delta- and Epsilonproteobacteria such as Escherichia coli, Salmonella enterica serovar Typhimurium, Shewanella oneidensis, Anaeromyxobacter dehalogenans and Wolinella succinogenes. Subsequently, nitrite is reduced to ammonium by cytochrome c nitrite reductase (NrfA), which obtains electrons from the quinone/quinol pool through several different electron transport redox enzyme systems, depending on the organism (Simon, 2002; Kern & Simon, 2009; Simon & Klotz, 2013). Many NrfA-containing ammonifiers that also reduce N₂O encode cNosZ, for example W. succinogenes, Campylobacter fetus, Desulfitobacterium dehalogenans and A. dehalogenans (Payne et al., 1982; Kern & Simon, 2009; Sanford et al., 2012).

The epsilonproteobacterium W. succinogenes is a versatile respiratory rumen bacterium that typically uses hydrogen gas or formate as electron donor combined with a multitude of electron donor substrates such as nitrate, nitrite, sulfite, polysulfide and fumarate (Kröger et al., 2002; Kern & Simon, 2009; Kern et al., 2011a; Simon & Kronkeck, 2013). The cells stoichiometrically produce ammonium from both nitrate and nitrite and the corresponding cell yields (normalized on the basis of formate consumption) were found to be similar using either of these terminal electron acceptors (Bokrnan et al., 1983). W. succinogenes employs the Nap and Nrf systems for respiratory nitrate ammonification but lacks NO-producing nitrite reductases of the NirK- (copper nitrite reductase) or NirS-type (cytochrome cd, nitrite reductase) known from denitrifiers as well as a respiratory NO reductase of the cNor and qNor families (Kern & Simon, 2009). In addition to its function as terminal reductase in respiratory nitrite ammonification, NrfA was recently shown to function in nitrosative stress defence, implying that NrfA efficiently reduces NO to ammonium (Kern & Simon, 2009; Kern et al., 2011b; Simon & Klotz, 2013).

W. succinogenes cells were previously reported to grow with N₂O as sole electron acceptor using formate as electron donor although growth parameters have not been reported (Yoshinari, 1980; Schumacher et al., 1992). The W. succinogenes cNosZ enzyme has been purified from N₂O-grown cells and shown to contain N₂O reductase activity using reduced benzyl viologen as electron donor (Teraguchi & Hollocher, 1989). When the genome of the W. succinogenes type strain (DSM 17405T) was sequenced, an atypical nos gene cluster was found whose first gene encodes the cNosZ enzyme (Baar et al., 2003; Simon et al., 2004). Interestingly, this gene was found to be disrupted in the genome sequence by a copy of insertion element IS1302 (Simon & Kröger, 1998), thus raising the question whether this strain was comparable to the strains used in previous studies. Here, we report the construction of mutants that either lack nosZ or contain a genetically stable nos gene cluster. The capacity of these mutants and of the wild-type strain to produce and to consume N₂O during growth by nitrate ammonification was examined using nitrate-sufficient or nitrate-limited medium containing formate as electron donor. Using a robotized cell incubation system, the concentrations of nitrate, nitrite, NO, N₂O and N₂ were quantified with high sensitivity. The applied growth conditions for nitrate respiration are regarded to be more physiologically relevant than previously conducted growth experiments in the presence of externally added N₂O serving as sole electron acceptor.

**METHODS**

**Organisms and liquid media.** W. succinogenes cells used in this study are described in Fig. 1. Cultures were routinely grown in liquid
medium at 37 °C containing formate as sole electron donor and either fumarate (Kroger et al., 1994) or nitrate as terminal electron acceptor. Nitrate-sufficient (80 mM sodium formate/50 mM potassium nitrate) or nitrate-limited (80 mM sodium formate/10 mM potassium nitrate) medium was used. In addition to formate and nitrate, each medium (pH 7.5) contained 1 mM K2HPO4, 5 mM fumaric acid, 5 mM ammonium sulfate, 1 mM MgCl2, 0.2 mM CaCl2, 0.68 mM glutamic acid, 0.57 mM cysteine and 0.2 ml trace element solution l-1 as described by Pfennig & Trüper (1981). Media were degassed and flushed with either N2 gas or helium several times to reduce the oxygen content. Brain heart infusion broth (BHIB; 0.5 %, w/v), kanamycin (25 mg l-1) and/or chloramphenicol (12.5 mg l-1) were added to the medium as appropriate.

Growth conditions, determination of cytochrome c and nitrogen compounds. Standard growth curves were generated by measuring the OD578 of cultures (50 ml) inoculated with overnight pre-cultures at an initial OD578 of about 0.05. Nitrate and nitrite concentrations in cell supernatants were determined using colorimetric assays based on the formation of spectrophotometrically detectable compounds. Nitrate was converted to 4-nitro-2,6-dimethylphenol under acidic conditions (Hartley & Asai, 1963). For nitrite determination, diazotization with sulfanilic acid and subsequent coupling with N-(1-naphthyl)ethylenediamine was performed (Rider & Mellon, 1946).

Detection of cytochromes c in SDS-polyacrylamide gels was performed by a haem staining procedure using 3,3'–dimethoxybenzidine as described previously (Kern et al., 2011a).

For monitoring gas kinetics 120 ml serum vials (50 ml medium) equipped with a triangular magnetic stirring bar were used. The vials were tightly sealed with rubber septa and aluminium caps. Prior to inoculation, the air was replaced by helium gas (six cycles of evacuation and helium-filling) using a semi-automated system (Molstad et al., 2007). During this process, the medium was stirred at 950 r.p.m. to ensure sufficient gas exchange between the liquid and the gas phase. Excess pressure was relieved using a water-filled syringe. The vials were incubated at a constant temperature of 37 °C in a robotized incubation system designed for measuring gas kinetics (Molstad et al., 2007; Bergaust et al., 2008). In short, this system monitors NO, N2O and N2 by frequent sampling with a syringe through the septum using a peristaltic pump coupled to the inlet of a gas chromatograph and a chemiluminescence NO analyser. After each sampling, the pump is reversed and pumps back helium to ensure near-constant pressure (1 atm) during the entire incubation period. The sampling thus dilutes the headspace with helium. This dilution was 3.4 % per sampling in the original system (Molstad et al., 2007), but only 0.9 % in the new system used for most of the experiments in the present study. There is also a marginal but constant leakage of traces of N2 and O2 with each sampling (14 nmol O2 and 42 nmol N2 per sampling). Dilution by sampling and the minor leakage of N2 into...
the vials are taken into account when estimating gas transformation rates for each time increment between two samplings (see Molsdad et al., 2007 for details), and when calculating cumulative N₂ production (reported N₂ is invariably cumulative N₂ production, corrected for leakage and sampling dilution). Several batch cultivation experiments were run with a variety of treatments designed to characterize the gas kinetics in the various cultures. Replication was secured by repeating entire experiments, rather than including replicate vials in each run.

**Construction of W. succinogenes mutants.** Standard genetic procedures were used (Sambrook et al., 1989). Genomic DNA was isolated from *W. succinogenes* using the DNeasy Tissue kit (Qiagen). PCR was carried out using Phusion High Fidelity DNA polymerase (Finzymes) for cloning procedures or Biotaq Red DNA polymerase (Bioline) for mutant and plasmid screening with standard amplification protocols.

*W. succinogenes* ΔnosZ was constructed through double homologous recombination of the wild-type (strain DSM 1740) genome with a deletion plasmid (pΔnosZ cat) designed to replace the entire nosZ gene in the nos operon by a chloramphenicol resistance gene cartridge (cat). For homologous recombination, the respective deletion plasmid contained cat flanked by two DNA segments obtained by PCR that were identical to appropriate regions in the *W. succinogenes* genome (Fig. 1). The two PCR fragments were synthesized using the following primer pairs: 5'-GGGATTCCTTGAGAGGATATGGG-3' and 5'-GGGATTCCTTGAGAGGATATGGG-3' amplifying the upstream fragment, and 5'-GGGATTCCTTGAGAGGATATGGG-3' and 5'-GGGATTCCTTGAGAGGATATGGG-3' for amplifying the downstream fragment. The desired double homologous recombination was verified by PCR (Fig. 1). The ΔnosZ deletion mutant lacks the entire nosZ gene, which is replaced by a chloramphenicol resistance gene cartridge (cat), while mutant kompPΔnosZ possesses an intact nosZ gene, a kanamycin resistance gene cartridge (kan) and an additional nos promoter to ensure transcription of accessory nos genes (Fig. 1). The absence of IS1302 in the nosZ gene of mutant kompPΔnosZ was routinely examined by PCR and confirmed that the mutant was genetically stable. The presence of the nosZ protein was shown in cells of mutant kompPΔnosZ by haem staining, whereas it proved undetectable in cells of the wild-type and of mutant *W. succinogenes* ΔnosZ (Fig. 2).

All three *W. succinogenes* organisms were grown in anoxic batch cultures with formate and nitrate as energy sources in either formate-limited/nitrate-sufficient (80 mM formate/50 mM nitrate) or formate-sufficient/nitrate-limited (80 mM formate/10 mM nitrate) medium, each supplemented with 0.5% (w/v) BHIB. In both media, the cells showed comparable growth behaviour (Fig. 3). The stationary growth phase was reached after 7–8 h in nitrate-sufficient and 5–6 h in nitrate-limited medium and similar doubling times of about 1.6 h were determined. However, in nitrate-sufficient cultures large amounts of nitrite (30 to 40 mM) accumulated (Fig. 3a), whereas in nitrate-limited medium nitrite was only transiently produced at a maximal concentration of about 5 mM (Fig. 3b). Under the latter conditions, accumulating nitrite is assumed to rapidly converted to ammonium by cytochrome c nitrite reductase, NrfA, as described previously (Bokranz et al., 1983, Lorenzen et al., 1993; Simon 2002). Production of the gaseous nitrogen

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**RESULTS**

**Construction of *W. succinogenes* mutants and characterization of cultures**

As mentioned in the Introduction, the ΔnosZ-encoding gene from *W. succinogenes* wild-type cells (strain DSM 1740) was found to be disrupted by a copy of insertion element IS1302. Therefore, it was assumed that the vast majority of these cells were unable to produce functional NosZ. To overcome the problem of genetic variability, mutants *W. succinogenes* ΔnosZ and *W. succinogenes* kompPΔnosZ were constructed through double homologous recombination using suitable plasmids as described in Methods (Fig. 1). The ΔnosZ deletion mutant lacks the entire nosZ gene, which is replaced by a chloramphenicol resistance gene cartridge (cat), while mutant kompPΔnosZ possesses an intact nosZ gene, a kanamycin resistance gene cartridge (kan) and an additional nos promoter to ensure transcription of accessory nos genes (Fig. 1). The absence of IS1302 in the nosZ gene of mutant kompPΔnosZ was routinely examined by PCR and confirmed that the mutant was genetically stable. The presence of the nosZ protein was shown in cells of mutant kompPΔnosZ by haem staining, whereas it proved undetectable in cells of the wild-type and of mutant *W. succinogenes* ΔnosZ (Fig. 2).
compounds NO, N₂O and N₂ was determined in a series of experiments with the three strains grown in either nitrate-sufficient or nitrate-limited medium. Fig. 4 shows a representative result for the three strains (single vial data) grown in nitrate-sufficient medium. The wild-type and mutant _W. succinogenes_ ΔnosZ accumulated low amounts of N₂O during the exponential growth phase (up to 1.7 μmol vial⁻¹ after 15 h of incubation), whereas N₂ was very close to the detection limit. The final cumulative N₂ production by these cultures was about 0.3 μmol. This was most likely due to chemical reactions between nitrite and organic compounds in the medium, as discussed below. In contrast, the _kompPnosnosZ_ mutant accumulated N₂ but lacked N₂O, indicating the presence of active nitrous oxide reductase.

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**Fig. 2.** Detection of cNosZ and NrfA in different _W. succinogenes_ cells grown in either formate-limited/nitrate-sufficient (80 mM formate/50 mM nitrate, designated NS) or formate-sufficient/nitrate-limited (80 mM formate/10 mM nitrate, designated NL) medium (see text for details). Cells were harvested in the late exponential growth phase and whole-cell extracts were subjected to denaturing SDS-PAGE (100 μg protein was applied to each lane). Subsequently, cytochrome c was detected by hem staining. Arrows indicate cNosZ and NrfA proteins (Kern et al., 2010). M, size marker proteins.

**Fig. 3.** Growth of _W. succinogenes_ cells and concomitant nitrate and nitrite concentrations in the medium. Cells were grown at 37 °C in sealed 120 ml vials (50 ml medium). (a) Formate-limited, nitrate-sufficient medium (80 mM formate and 50 mM nitrate). (b) Formate-sufficient, nitrate-limited medium (80 mM formate and 10 mM nitrate). Representative experiments are shown (performed in triplicate; deviation less than 5%). ▲, Optical density; ●, nitrate concentration; ■, nitrite concentration.
These differences between the cultures regarding N2O and N2 accumulation were also observed in two further replicate experiments (Figs S2 and S3, available in the online Supplementary Material). Supplementary experiments were run with 10% acetylene (an inhibitor of nitrous oxide reductase) in the headspace, demonstrating that the reduction of N2O to N2 by mutant kompPnosZ was effectively inhibited by this treatment (Figs S9 and S10). For all three organisms significant accumulation of NO was detected, but only during the late exponential and stationary growth phases (Fig. 4). NO accumulation initiating during the late exponential/stationary phase was also observed in several experiments, shown in Figs S9 and S10.

In nitrate-limited medium, the concentrations of NO invariably remained below 1 nmol vial−1 and N2O below 0.1 μmol vial−1 and the production of N2 was below the detection limit (~0.5 μmol for cumulative N2 production over a 15 h incubation period) (not shown). Nonetheless, the ñNosZ and NrfA proteins were detectable in cells of W. succinogenes kompPnosZ (Fig. 2). The very low amounts of gaseous nitrogen compounds in nitrate-limited medium suggest that the appearance of both NO and N2O depended on nitrite accumulation in nitrate-sufficient medium. Therefore, control experiments were conducted in which varying amounts of nitrite (10, 20 or 30 mM) were added to sterile nitrate-sufficient medium (Fig. 5). In addition, vials with medium lacking BHB were also analysed. N2O production could not be detected in any of the treatments. On the other hand, NO was found to accumulate in the medium with BHB at a rate largely proportional to the nitrite concentration but the rate declined with time [initial rate of NO formation ~10−4 mol NO (mol nitrite)−1 h−1; see Fig. S1 for a closer inspection of the kinetics]. In the medium without BHB, however, NO production was marginal (~5% of that in the presence of BHB). The production of N2 was also significant and independent of BHB. The rate of N2 production was a linear function of nitrite concentration [~10−4 mol N2 (mol nitrite)−1 h−1; see Fig. S1]. These results imply that NO is formed by chemical reactions between nitrite and BHB compounds, whereas the chemical N2 formation appears to be due to reactions between nitrite and compounds within the basal medium. In theory, one could use the observed kinetics of chemical N2 formation from nitrite to correct the observed N2 formation in the cultures, thus estimating the enzymic production of N2. However, this turned out to be problematic since the measured N2 accumulation in the wild-type and in mutant W. succinogenes ΔnosZ was lower than that predicted (Fig. 4). In fact, these two organisms accumulated only about 0.3 μmol N2 vial−1, which was much lower than predicted by the observed reaction rate between nitrite and fresh sterile medium. Assuming that the mean concentration of nitrite was 35 mM for a time span of 7 h, the predicted chemical N2 formation would amount to 1.2 μmol N2 vial−1. Thus, N2 formation by nitrite reacting with ‘spent medium’ (i.e. after 7 h of growth) appears to be much lower than with fresh medium.

The fact that NO concentrations during the first 8–10 h of the batch cultivations were invariably lower than those predicted by the chemical decomposition of the accumulating nitrite suggests that W. succinogenes cells are able to efficiently reduce NO in order to maintain non-toxic levels. The enzymic inventory putatively serving in the underlying NO detoxification processes is considered in the Discussion.

**Addition of N2O and NO to W. succinogenes cultures**

Additional experiments analogous to that shown in Fig. 4 were conducted, in which 1% N2O was added to the headspace of cultures grown in nitrate-sufficient medium (Fig. 6). Under these conditions, only cells of W. succinogenes kompPnosΔZ converted N2O stoichiometrically to N2 (replicate experiments are shown in Figs S4 and S5). In nitrate-limited medium, cells of the kompPnosΔZ mutant also reduced added N2O to N2, but at a considerably lower rate (about 30%) compared with nitrate-sufficient medium (Figs S4–S6). Conceivably, this might be due to the apparently lower amount of ñNosZ detectable in cells grown in nitrate-limited medium, cells of the kompPnosZ mutant were able to efficiently reduce NO in order to maintain non-toxic levels. The enzymic inventory putatively serving in the underlying NO detoxification processes is considered in the Discussion.

**Fig. 4.** Measured NO, N2O and N2 during growth of three W. succinogenes organisms in nitrate-sufficient medium. N2 is the cumulative N2 production (corrected for leaks and sampling dilution). Representative experiments are shown (performed in triplicate; deviation less than 5%). ᵃ, NO (nmol vial−1); △, N2O (μmol vial−1); □, N2 (μmol vial−1).
in nitrate-limited medium (Fig. 2). Additional experiments with 10% acetylene in the headspace demonstrated efficient acetylene inhibition of N2 formation by strain komppnosZ (Fig. S11). Finally, we conducted an experiment where a bolus of NO (~1 µmol NO vial$^{-1}$) was injected at the onset of the incubations. All three organisms reduced NO to N2O within the first 4 h but only cells of mutant komppnosZ reduced this N2O further to N2 (Figs S7 and S8).

**Estimated specific cellular N2O reduction rates**

The data for N2O reduction or N2 production rates in the various batch cultivations of mutant komppnosZ and the measured OD (converted to cell dry weight vial$^{-1}$ assuming 0.4 mg cell dry weight ml$^{-1}$ at an OD$_{578}$ of 1) were used to estimate the specific N2 production rate throughout the incubations. The result is shown in Fig. 7, where the rate expressed as µmol N2 (g cell dry weight)$^{-1}$ h$^{-1}$ is plotted against time for the different treatments. In vials without NO or N2O injected, low and relatively constant specific rates were recorded throughout the initial 8–15 h. In vials with NO injected, high rates were recorded initially, coinciding with a rapid reduction of the entire injected NO, which caused a transient peak in N2O during the first 4 h (see Fig. S8). In vials with excess N2O in the headspace, the specific rates increased gradually during the first 10 h, and declined rapidly after 12 to 15 h. The N2O concentration in the headspace was only reduced by about 15%; thus the sharp decline in N2 production after 15 h was not due to substrate depletion. It is currently unknown whether the presence of NO enhances the production of cNosZ in nitrate-grown W. succinogenes cells.

**DISCUSSION**

The results demonstrate that nitrate-ammonifying cells of the W. succinogenes cultures tested catalyse both N2O-producing and N2O-consuming reactions that eventually result in N2 formation. Nonetheless, W. succinogenes cannot be regarded as a denitrifier since the vast majority of nitrate has previously been shown to end up in the respiratory ammonification pathway using the respiratory Nap and Nrf systems (see Introduction). The production of N2O seems to be mainly associated with the detoxification of the NO generated by chemical reactions between nitrite and components of the medium, whereas N2O consumption is envisaged as an additional mode of anaerobic respiration, as discussed in the following sections.

**Production of NO and N2O**

In nitrate-sufficient cultures of W. succinogenes, the accumulation of nitrite causes the emergence of small amounts of NO. This reaction is chemically obvious and apparently facilitated by BHIB ingredients, presumably involving nitrosation and decomposition of nitroso compounds. It has not been elucidated to date whether W. succinogenes synthesizes an enzyme that produces NO from nitrite (assuming that NrfA is unlikely to release NO as a by-product). The formation of NO from nitrite was reported for membrane-bound nitrate reductase (NarGHI complex) from Salmonella enterica serovar Typhimurium, which, however, has no counterpart in W. succinogenes (Gilbertherpe & Poole, 2008; Rowley et al., 2012). In contrast, there is no report that a periplasmic nitrate reductase of the Nap-type is able to generate NO.
It is widely accepted that bacteria thriving in different habitats need to combat environmental NO derived from nitrogen oxynions (Poole, 2005). $\text{N}_2\text{O}$ and ammonium are typical NO detoxification products when oxygen is absent. In the case of \textit{W. succinogenes} it has been reported that at least two proteins are involved in nitrosative stress defence (Kern et al., 2011b). These are periplasmic cytochrome $c$ nitrite reductase (NrfA) and a cytoplasmic flavodiiron protein (Fdp). NrfA is induced in the presence of nitrate and efficiently catalyses the reduction of nitrite, NO and hydroxylamine to ammonium in the periplasm (Simon, 2002; Einsle, 2011). This reaction is thought to make \textit{W. succinogenes} cells tolerant to high nitrite concentrations, in line with the fact that nitrite does not need to be transported into the cytoplasm during nitrate ammonification. Reduction of NO to $\text{N}_2\text{O}$ by NrfA proteins from \textit{Desulfovibrio desulfuricans}, \textit{W. succinogenes} and \textit{E. coli} was also reported, but the physiological significance of this reaction is questionable (Costa et al., 1990; Simon et al., 2011). Fdp proteins, on the other hand, are known to reduce NO to $\text{N}_2\text{O}$ (Saraiva et al., 2004). However, this reaction has not been demonstrated for \textit{W. succinogenes} Fdp since the protein has not been purified. Further possible NO reductases in \textit{W. succinogenes} are the hybrid cluster protein (Hcp) and a homologue (57\% identity) of the HP0013 protein from \textit{Helicobacter pylori} 26695 (WS1903, NP_908012) (Justino et al., 2012). Fdp, Hcp and WS1903 are all predicted to be cytoplasmic proteins. Gene deletion strains lacking either nrfA, fdp or hcp are available and will be examined as regards their $\text{N}_2\text{O}$ production capacity in future experiments.

The ability of nitrate-ammonifying cells to produce $\text{N}_2\text{O}$ in pure culture has been reported before (Bleakley & Tiedje, 1982; Schumacher & Kroneck, 1992; Rowley et al., 2012; Stremińska et al., 2012). In \textit{Salmonella enterica} serovar Typhimurium, 20\% of nitrate-N was released as $\text{N}_2\text{O}$ under nitrate-sufficient conditions when nitrite had accumulated to millimolar levels (Rowley et al., 2012). This phenotype is envisaged to result from NO production from nitrite by the Nar-type nitrate reductase (see above) and subsequent anoxic NO detoxification to $\text{N}_2\text{O}$. Stremińska et al. (2012) demonstrated that soil isolates belonging to the genera \textit{Bacillus} or \textit{Citrobacter} formed $\text{N}_2\text{O}$ under nitrate-sufficient conditions (low C-to-$\text{NO}_3$ ratio) and found that a strain of \textit{Citrobacter} sp. reduced up to 5\% of nitrate-N to $\text{N}_2\text{O}$. Kaspar & Tiedje (1981) reported that the nitrate-ammonifying rumen microbiota accumulated up to 0.3\% of the added nitrate-N as $\text{N}_2\text{O}$ and consequently stated that denitrification is absent from the rumen. In the present study, exponentially growing \textit{W. succinogenes} cells converted about 0.15\% of nitrate-N to $\text{N}_2\text{O}$ under the nitrate-sufficient conditions tested (Fig. 3). These percentages, however, should be compared with caution as \textit{W. succinogenes} is not a dominant bacterium in cattle rumen.

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Fig. 6. Concentrations of $\text{N}_2\text{O}$ and $\text{N}_2$ in \textit{W. succinogenes} cultures with $\text{N}_2\text{O}$ added to the headspace. The experiment corresponds to that shown in Fig. 4 but 1\% $\text{N}_2\text{O}$ was injected into the headspace prior to inoculation. Representative experiments are shown (performed in triplicate). (○, red) Wild-type in nitrate-sufficient medium, (■, red) wild-type in nitrate-limited medium, (▲, green) mutant ΔnosZ in nitrate-sufficient medium, (●, green) mutant ΔnosZ in nitrate-limited medium, (△, blue) mutant komp$_{\text{nosnosZ}}$ in nitrate-sufficient medium, (▲, blue) mutant komp$_{\text{nosnosZ}}$ in nitrate-limited medium.

Fig. 7. Estimated specific cellular rates of $\text{N}_2$ production throughout the batch cultivation of \textit{W. succinogenes} komp$_{\text{nosnosZ}}$ in nitrate-sufficient medium with or without either NO or $\text{N}_2\text{O}$ in the headspace at the onset of cultivation. (●, ■, red) NO added (two individual vials), (▲, ▼, blue) $\text{N}_2\text{O}$ added (three individual vials), (●, green), no NO or $\text{N}_2\text{O}$ added (for clarity only one representative vial, out of three, is shown). Details of the experiments with added NO are shown in Figs S7 and S8. dw, Dry weight.
In nitrate-sufficient medium, *W. succinogenes* cells continued to produce N$_2$O after the transition from the exponential to the stationary growth phase, indicating that nitrite-dependent NO production as well as NO detoxification continued even after cell growth had ceased. This phenomenon was also reported for other nitrate ammonifiers (Bleakley & Tiedje, 1982; Schumacher & Kroneck, 1992).

**Consumption of N$_2$O**

*W. succinogenes* cells were previously reported to grow by ‘N$_2$O respiration’ using N$_2$O as sole electron acceptor of anaerobic respiration (Yoshinari, 1980). In these experiments the cells were gassed with pure N$_2$O and grew to a modest optical density with formate as electron donor. In contrast, our experiments describe N$_2$O turnover during respiratory nitrate ammonification. The results demonstrate that the *W. succinogenes* cytochrome *c* nitrous oxide reductase (cNosZ) is able to convert N$_2$O to N$_2$ under the applied conditions. This reaction is thought to play a minor role in menaquinone replenishment since menaquinone is required for formate oxidation, which is catalysed by an electrogenic, i.e. proton-motive force (PMF)-generating, membrane-bound formate dehydrogenase complex (Kern & Simon, 2009; Simon & Kern, 2008; Simon & Klotz, 2013; Simon et al., 2008). In this way, N$_2$O reduction is equivalent to formate-dependent nitrate and nitrite reduction performed by the respiratory Nap and Nrf systems. In each case, menaquinol oxidation seemingly does not contribute to the generation of a PMF in *W. succinogenes* (Kern & Simon, 2009; Simon & Klotz, 2013). The precise architecture of the electron transport chain that catalyses N$_2$O reduction by menaquinol is not known, but a menaquinol dehydrogenase of the NapGH-type (named NosGH) might be involved in order to direct electrons from the membrane to the periplasmic space (Simon et al., 2004; Simon & Klotz, 2013). Subsequently, electrons are likely to be transferred either directly or indirectly via the cytochrome *c* domain of cNosZ to the copper-containing catalytic site of N$_2$O reduction.

The specific rates of N$_2$O reduction (Fig. 7) are very low compared to those of regular denitrifying bacteria. In *Paracoccus denitrificans*, for instance, the maximum specific rate of N$_2$O reduction was found to be 2.5 fmol N$_2$O cell$^{-1}$ h$^{-1}$ (Bergaust et al., 2012), which is equivalent to 12.5 mmol N$_2$O (g cell dry weight)$^{-1}$ h$^{-1}$ (dry weight per cell=2×10$^{-13}$ g). This is two orders of magnitude higher than the maximum specific rates estimated for *W. succinogenes* komp$_{Pna}$nosZ. This could be taken to suggest that *W. succinogenes* is an insignificant sink for N$_2$O in the environment. It remains to be explored, however, to what extent the N$_2$O reductase activity of the organism could be enhanced by conditions other than those tested in the present study.

**CONCLUSION**

In recent years the view has emerged that individual bacterial cells are capable of simultaneously producing ammonium and N$_2$ from nitrate, albeit to different extents (Sanford et al., 2012; Heylen & Keltjens, 2012; Mania et al., 2014). It remains to be established, however, whether such cells indeed perform both types of anaerobic respiration or if one reaction mainly results from detoxification. In this context, the discrimination between detoxifying and respiratory processes is not trivial, especially given the fact that quinol oxidation by nitrate, nitrite and N$_2$O by the respective Nap, Nrf and cNos systems is thought to be electroneutral and therefore not directly involved in the generation of a PMF and hence ATP production (Simon & Klotz, 2013). The results presented here suggest that N$_2$O formation in *W. succinogenes* resulted from NO detoxification and that N$_2$O serves as an additional electron sink during growth by respiratory nitrate ammonification.

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