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 N₂ 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 (Stremińska 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), flavohemebdoxin (NorVW), cytochrome c₅₃₄ (CycA; in nitrifiers), cytochrome cα-beta (CytS) and cytochrome cα-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 bacteria 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 & Kroncke, 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 Cu₅₉ site 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, Anaeromyzobacter dehalogenans and Wolinella succinigenes. 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 & Kroncke, 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 (Bokranz 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 1740T) 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 (Kröger 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. The 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 (Finnzymes) 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'-GGGAGATCCAGAGGATTTGAGG-3' and 5'-CCGGATCTTACCCGCTTCTTGTTAAATTGGCATTAC-3' for amplifying the upstream fragment, and 5'-CCGGATGCGAACCTTCTTCTCCATAGATGTAGTGATGTTG-3' for the downstream fragment (black bars in Fig. 1). Primers carried EcoRI, BamHI or HindIII restriction sites (underlined) for cloning. Both fragments and the cat fragment were consecutively inserted into plasmid pPR-IBAI (IBA Lifesciences) using *E. coli* XL-1 Blue as cloning host, grown in LB medium at 37 °C. PCR analysis was used to confirm that the plasmid contained cat in the same orientation as its surrounding regions. Transformation of *W. succinogenes* DSM 1740 cells was performed by electroporation as described previously (Simon et al., 1998, 2000). Transformants of *W. succinogenes* were selected in the presence of chloramphenicol (12.5 mg l⁻¹) and the desired gene deletion was confirmed by PCR. The integrity of DNA stretches involved in recombination events was confirmed by sequencing suitable PCR products.

To construct *W. succinogenes kompₚΔnosZ*, the nosZ gene lacking IS1302 was restored on the genome of *W. succinogenes* ΔnosZ upon integration of plasmid pkomppₚΔnosZ kan (Fig. 1) This plasmid contained an intact copy of nosZ surrounded by the upstream and downstream cat-flanking fragments of pΔnosZ cat. In addition, a kanamycin resistance gene cassette (kan), obtained by BamHI excision from pUC4K, was inserted between nosZ and the downstream flanking fragment. Finally, a nos promoter element was inserted downstream of kan to ensure transcription of the residual nos operon (Fig. 1). In a first step to create pkomppₚΔnosZ kan, the cat gene was replaced by kan in pΔnosZ cat, resulting in pΔnosZ cat. PCR analysis was used to confirm that the plasmid contained kan in the reverse orientation relative to the nos sequence. The intact nosZ gene was amplified using the primer pair 5'-ATGCGAAGATTACTAAAGACATCTTTGGTGTG-3' and 5'-TTAGAAGAGACCCGCTTCTCATC-3' using genomic DNA of wild-type cells as template (as template that a small percentage of the cells transmissibly lack the copy of IS1302 within nosZ). To avoid any adverse effects of adding additional restriction sites between the native nos promoter and the nosZ gene, the amplified gene was blunt-end ligated with a linear plasmid fragment obtained by PCR from pΔnosZ kan with the primer pair 5'-GGGAGATCCAGAGGATTTGAGG-3' and 5'-CCGGATCTTACCCGCTTCTTGTTAAATTGGCATTAC-3', resulting in pkomppₚΔnosZ kan. This corresponding mutant, *W. succinogenes kompₚnosZ kan*, was constructed by transforming *W. succinogenes* ΔnosZ cat with pkomppₚnosZ kan. Transformants were selected in the presence of kanamycin (25 mg l⁻¹). The desired double homologous recombination was verified by PCR and the integrity of DNA stretches involved in recombination events as well as the entire nosZ gene was confirmed by sequencing suitable PCR products.

**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 ΔNosZ. To overcome the problem of genetic variability, mutants *W. succinogenes* ΔnosZ and *W. succinogenes* kompₚnosZ 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 kompₚnosZ 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 kompₚnosZ was routinely examined by PCR and confirmed that the mutant was genetically stable. The presence of the ΔnosZ protein was shown in cells of mutant kompₚnosZ 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) BHI. 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 be quickly converted to ammonium by cytochrome c nitrite reductase, NirA, as described previously (Bokranz et al., 1983, Lorenzen et al., 1993; Simon 2002). Production of the gaseous nitrogen...
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.

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 haem 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.

Fig. 4. 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 komp⁺nosZ 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 ΔnosZ and NrfA proteins were detectable in cells of W. succinogenes komp⁺nosZ (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). 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 komp⁺nosZ converted N₂O stoichiometrically to N₂ (replicate experiments are shown in Figs S4 and S5). In nitrate-limited medium, cells of the komp⁺nosZ 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 ΔnosZ detectable in cells grown
in nitrate-limited medium (Fig. 2). Additional experiments with 10% acetylene in the headspace demonstrated efficient acetylene inhibition of N₂ formation by strain komp_PnosHosZ (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 komp_PnosHosZ 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 komp_PnosHosZ and the measured OD (converted to cell dry weight vial⁻¹ assuming 0.4 mg cell dry weight ml⁻¹ at an OD₅₇₈ 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* (Gilberthorpe & 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 oxyanions (Poole, 2005). N₂O and ammonium are typical NO detoxification products when oxygen is absent. In the case of _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 _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 N₂O by NrfA proteins from _Desulfovibrio desulfuricans_, _W. succinogenes_ and _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 N₂O (Saraiva _et al._, 2004). However, this reaction has not been demonstrated for _W. succinogenes_ Fdp since the protein has not been purified. Further possible NO reductases in _W. succinogenes_ are the hybrid cluster protein (Hcp) and a homologue (57% identity) of the HP0013 protein from _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 N₂O production capacity in future experiments.

The ability of nitrate-ammonifying cells to produce N₂O in pure culture has been reported before (Bleakley & Tiedje, 1982; Schumacher & Kroneck, 1992; Rowley _et al._, 2012; Streminińska _et al._, 2012). In _Salmonella enterica_ serovar Typhimurium, 20% of nitrate-N was released as N₂O 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 N₂O. Streminińska _et al._ (2012) demonstrated that soil isolates belonging to the genera _Bacillus_ or _Citrobacter_ formed N₂O under nitrate-sufficient conditions (low C-to-NO₃⁻ ratio) and found that a strain of _Citrobacter_ sp. reduced up to 5% of nitrate-N to N₂O. Kaspar & Tiedje (1981) reported that the nitrate-ammonifying rumen microbiota accumulated up to 0.3% of the added nitrate-N as N₂O and consequently stated that denitrification is absent from the rumen. In the present study, exponentially growing _W. succinogenes_ cells converted about 0.15% of nitrate-N to N₂O under the nitrate-sufficient conditions tested (Fig. 3). These percentages, however, should be compared with caution as _W. succinogenes_ is not a dominant bacterium in cattle rumen.
In nitrate-sufficient medium, *W. succinogenes* cells continued to produce N₂O 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 & Kronteck, 1992).

**Consumption of N₂O**

*W. succinogenes* cells were previously reported to grow by ‘N₂O respiration’ using N₂O as sole electron acceptor of anaerobic respiration (Yoshinari, 1980). In these experiments the cells were gassed with pure N₂O and grew to a modest optical density with formate as electron donor. In contrast, our experiments describe N₂O turnover during respiratory nitrate ammonification. The results demonstrate that the *W. succinogenes* cytochrome c nitrous oxide reductase (cNosZ) is able to convert N₂O to N₂ 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, N₂O 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 N₂O 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 N₂O reduction.

The specific rates of N₂O reduction (Fig. 7) are very low compared to those of regular denitrifying bacteria. In *Paracoccus denitrificans*, for instance, the maximum specific rate of N₂O reduction was found to be 2.5 fmol N₂O cell⁻¹ h⁻¹ (Bergaust et al., 2012), which is equivalent to 12.5 mmol N₂O (g cell dry weight)⁻¹ h⁻¹ (dry weight per cell = 2 x 10⁻¹³ g). This is two orders of magnitude higher than the maximum specific rates estimated for *W. succinogenes* kompₚₚₚₚₑₚₑₚₑₚₑ₂Z. This could be taken to suggest that *W. succinogenes* is an insignificant sink for N₂O in the environment. It remains to be explored, however, to what extent the N₂O 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 N₂ 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 N₂O 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 N₂O formation in *W. succinogenes* resulted from NO detoxification and that N₂O serves as an additional electron sink during growth by respiratory nitrate ammonification.

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