Alkane degradation under anoxic conditions by a nitrate-reducing bacterium with possible involvement of the electron acceptor in substrate activation

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Summary
Microorganisms can degrade saturated hydrocarbons (alkanes) not only under oxic but also under anoxic conditions. Three denitrifying isolates (strains HxN1, OcN1, HdN1) able to grow under anoxic conditions by coupling alkane oxidation to CO2 with NO3⁻ reduction to N2 were compared with respect to their alkane metabolism. Strains HxN1 and OcN1, which are both Betaproteobacteria, utilized n-alkanes from C6 to C8 and C8 to C12 respectively. Both activate alkanes anaerobically in a fumarate-dependent reaction yielding alkylsuccinates, as suggested by present and previous metabolite and gene analyses. However, strain HdN1 was unique in several respects. It belongs to the Gammaproteobacteria and was more versatile towards alkanes, utilizing the range from C6 to C30. Neither analysis of metabolites nor analysis of genes in the complete genome sequence of strain HdN1 hinted at fumarate-dependent alkane activation. Moreover, whereas strains HxN1 and OcN1 grew with alkanes and NO3⁻, NO2⁻ or N2O added to the medium, strain HdN1 oxidized alkanes only with NO3⁻ or NO2⁻ but not with added N2O; but N2O was readily used for growth with long-chain alcohols or fatty acids. Results suggest that NO2⁻ or a subsequently formed nitrogen compound other than N2O is needed for alkane activation in strain HdN1. From an energetic point of view, nitrogen–oxygen species are generally rather strong oxidants. They may enable enzymatic mechanisms that are not possible under conditions of sulfate reduction or methanogenesis and thus allow a special mode of alkane activation.

Introduction
Saturated hydrocarbons (alkanes) as major constituents of petroleum (Tissot and Welte, 1984) enter the environment via natural seeps or accidental spills, or due to the use of refined petroleum products. Furthermore, alkanes are widespread products of living organisms (Birch and Bachofen, 1988). Aerobic alkane biodegradation, in particular the initial O2-dependent activation by monooxygenases, has been studied since many decades (Rojo, 2009). In recent years, alkanes were also shown to be degraded anaerobically with nitrate (Ehrenreich et al., 2000; Bonin et al., 2004; Grossi et al., 2008; Callaghan et al., 2009) or sulfate (Aeckersberg et al., 1991; 1998; So and Young, 1999; Cravo-Laureau et al., 2004; Davidova et al., 2006; Kniemeyer et al., 2007; Higashioka et al., 2009) as electron acceptor, or under conditions of methanogenesis (Zengler et al., 1999; Anderson and Lovley, 2000; Jones et al., 2008). The only established mechanism for anaerobic activation of alkanes to date is the radical-catalysed addition to fumarate yielding alkylsuccinates, as suggested by present and previous metabolite and gene analyses. However, strain HdN1 was unique in several respects. It belongs to the Gammaproteobacteria and was more versatile towards alkanes, utilizing the range from C6 to C30. Neither analysis of metabolites nor analysis of genes in the complete genome sequence of strain HdN1 hinted at fumarate-dependent alkane activation. Moreover, whereas strains HxN1 and OcN1 grew with...
Of three denitrifying strains, HxN1, OcN1 and HdN1, that were isolated with \(n\)-hexane, \(n\)-octane and \(n\)-hexadecane, respectively (Ehrenreich et al., 2000), only the first one has been formerly studied with respect to its alkane metabolism (Rabus et al., 2001; Wilkes et al., 2002; Grundmann et al., 2008). A subsequent comparative study including the two other strains revealed that also strain OcN1 formed alkylsuccinates during growth with alkanes and harboured a gene apparently encoding the responsible enzyme. In contrast, alkylsuccinates were not detectable in strain HdN1, and its complete genome sequence did not reveal any gene likely to encode (1-methylalkyl)succinate or alkylsuccinate synthase. A unique physiological characteristic of strain HdN1 was that it did not grow with alkanes if \(N_2O\) was added instead of \(NO_3^-\), whereas growth with alcohols and fatty acids readily occurred with \(N_2O\). In contrast, strains HxN1 and OcN1 grew well with \(N_2O\) and alkanes. These findings suggest that alkane activation in strain HdN1 differs principally from alkane activation in strains HxN1 and OcN1 and requires an \(NO_3^-\)-derived compound other than \(N_2O\).

**Results and discussion**

*Cultivation, phylogenetic relationships, morphology and purity control*

The focus of this study is on strain HdN1 and its apparently unusual physiology with respect to \(n\)-alkane utilization. The isolation of strain HdN1 along with that of strains OcN1 and HxN1, a few substrate tests, and the capacity for complete alkane oxidation in anoxic medium with \(NO_3^-\) have been documented previously (Ehrenreich et al., 2000). Unless indicated otherwise, the strains were grown in conventional \(HCO_3^-/CO_2\)-buffered defined medium (Rabus and Widdel, 1995) with alkanes as the only organic substrates.

The study of anaerobic microbial hydrocarbon utilization requires the strict exclusion of any traces of \(O_2\) from air which through monoxygenases could lead to hydroxyl compounds (which can be further degraded anaerobically). Hence, in addition to physical exclusion of air (Widdel and Bak, 1992), the presence of a reductant (‘redox buffer’) is advisable. Unlike sulfate-reducing bacteria that form a chemical reducing agent, sulfide, nitrate-reducing bacteria do not produce a reductant. Addition of sulfide (or other reducing sulfur compounds) is inappropriate because it is easily oxidized in by-reactions of the ‘high-potential’ nitrate reduction pathway, or because it can inhibit denitrifiers (F. Widdel, unpubl. results). We therefore added ascorbate (4 mM) as a mild reductant (Rabus and Widdel, 1995; Ehrenreich et al., 2000; Widdel, 2009). Ascorbate did not serve as substrate for growth and nitrate reduction, as revealed in control incubations with ascorbate alone. We also verified that ascorbate in our medium did not scavenge nitrite, the intermediate of nitrate reduction, by chemical reaction. If sterile medium with ascorbate (\(pH\) 7.2) and \(NaNO_2\) (2 mM) was incubated for 10 days and analysed by ion chromatography (Rabus and Widdel, 1995), there was no noticeable decrease of the nitrite concentration. At low \(pH\), reduction of the protonated form (\(HNO_3\)) by ascorbic acid to yield nitric oxide can be significant (Yamasaki, 2000). Furthermore, tests were carried out to exclude an adverse physiological effect of ascorbate. Strain HdN1 was grown with \(n\)-tetradecane and \(NO_3^-\) or \(NO_2^-\) in ascorbate-containing medium as well as in ascorbate-free medium deoxygenated by vigorous sparging with \(N_2\). The cultures with and without ascorbate grew equally well.

Strain HdN1 affiliates with the *Gammaproteobacteria*, whereas strain OcN1 and HxN1 are members of the *Betaproteobacteria* (Fig. 1). Most nitrate-reducing bacteria enriched and isolated with various aromatic or saturated petroleum hydrocarbons are *Betaproteobacteria* (Widdel et al., 2009). Some denitrifying strains that degrade petroleum hydrocarbons are *Gammaproteobacteria*; these also include alkane degraders affiliating with *Marinobacter* sp. (Bonin et al., 2004) and *Pseudomonas balearica* (Grossi et al., 2008).

The cell shape of strain HdN1 was unusually variable and significantly influenced by the organic growth substrate (Ehrenreich et al., 2000). In particular long-chain alkanes caused swelling of a large fraction of the cells. In such cells, spacious inclusions resembling storage compounds could be seen at high magnification (Fig. 2A). However, polyhydroxyalkanoates were not detectable (A. Steinbüchel, pers. comm.) by gas chromatography following acidic hydrolysis and methylation of freeze-dried cells (Steinbüchel and Wiese, 1992). Cells in alkane cultures tended to grow in close contact with the overlying insoluble hydrocarbon phase. The bulk of alkane-grown cells was buoyant, possibly due to association with or storage of alkane droplets. Alkane storage and buoyancy is a phenomenon known from aerobic alkane degraders (Scott and Finnerty, 1976). This behaviour rendered harvesting by centrifugation difficult. A minor fraction of the cells was motile.

Thorough purity tests excluded that cell shape heterogeneity in cultures of strain HdN1 was due to accompanying microorganisms. First, repeated aerobic and anaerobic (with \(NO_3^-\)) liquid dilution series (according to the most probable number technique) were carried out separately with \(n\)-tetradecane or \(n\)-valerate (\(n\)-pentanoate). All cultures derived from the highest positive dilution tubes were microscopically indistinguishable and always able to use both, tetradecane and valerate. Second, cultures were streaked on agar plates containing valerate and yeast
extract and incubated in a jar under air with 3% CO₂. All
well-separated valerate-grown colonies transferred to
anoxic liquid media grew again with tetradecane, and
cultures had the microscopic appearance as before. Third,
strain HdN1 was mixed with strain OcN1, and a specific
16S rRNA-targeting fluorescent oligonucleotide probe
(Appendix S1) was applied. Whereas in the pure culture all
cells exhibited the specific hybridization signal (Fig. 2B),
the mixed culture contained in addition the expected non-
hybridizing cells that exhibited only the general fluorescent
stain (Fig. 2C). Hence, strain HdN1 is in principle distin-
guishable from contaminants by specific probing.

Anaerobic growth tests with alkanes and alkanoates
The capability of strain HdN1 for complete hexadecane
oxidation with nitrate according to
\[ \text{C}_{16} \text{H}_{34} + 98 \text{NO}_3^- + 18 \text{H}^+ \rightarrow 80 \text{HCO}_3^- + 49 \text{N}_2 + 54 \text{H}_2\text{O} \]
has been verified formerly with small, precisely quantifiable amounts of alkane (Ehrenreich et al., 2000). In all subsequent exper-
iments, significantly higher amounts of alkanes were added
than could be oxidized by the electron acceptor (10 mM
\text{NO}_3^-). In this way, a large contact area between the
insoluble hydrocarbon and the aqueous phase was pro-
vided which favoured growth (Widdel, 2009). In further
growth tests, alkanes with carbon chains \( \leq \text{C}_{10} \) were pro-
vided as solutions in 2,2,4,4,6,8,8-heptamethylnonane
(HMN) as an inert carrier phase to avoid toxic effects
(Appendix S1). Tests revealed that strain HdN1 utilized
\( n \)-alkanes from \( \text{C}_6 \) (\( n \)-hexane) to \( \text{C}_{30} \) (\( n \)-triacontane) as
carbon sources and electron donors (\( \text{C}_6 \) to \( \text{C}_{20} \), \( \text{C}_{24} \),
\( \text{C}_{26} \), \( \text{C}_{28} \), \( \text{C}_{30} \) and \( \text{C}_{40} \) tested). Fastest growth was observed
in the range from \( \text{C}_{14} \) (tetradecane) to \( \text{C}_{18} \) (octadecane).
With an inoculum size of 1% (v/v), full growth and complete
\text{NO}_3^- consumption occurred within 7 days. A doubling time
of 11–13 h during early growth was estimated from an
analysis of the nitrate consumption curve (see also Ehren-
reich et al., 2000). (Inhomogeneous growth and alkane
droplets prevented measurement of the optical density as
a growth parameter.) Growth with alkanes of shorter or
longer chains was slower (two- to threefold time required
for full growth and \text{NO}_3^- consumption). The other strains,
HxN1 and OcN1, utilized a significantly narrower range of
alkanes, which was from \( \text{C}_6 \) to \( \text{C}_8 \) (\( n \)-octane) and \( \text{C}_8 \) to

Fig. 1. Phylogenetic (16S rRNA-based) affiliation of strain HdN1 with selected Beta- and Gammaproteobacteria including other strains able to degrade aromatic or saturated petroleum hydrocarbons with nitrate (*). Strains able to degrade \( n \)-alkanes anaerobically are highlighted in bold; occurrence of (1-methylalkyl)succinate formation for alkane activation is also indicated (**). Bootstrap values (%; only \( > 60 \)% shown) were obtained after 1000 resamplings. Scale bar, 10% estimated sequence divergence.
C12 (n-dodecane) respectively. Also alkane-utilizing sulfate-reducing bacteria utilized a narrower range (Rueter et al., 1994; Aeckersberg et al., 1998).

Strain HdN1 utilized monocarboxylic acids (sodium salts; method of preparation and addition given by Widdel and Bak, 1992; see also Appendix S1) from acetate to stearate (C2–C18; higher fatty acids not tested), with best growth (roughly twice as fast as with alkanes) with valerate (C5) and with fatty acids from n-decanoate (C10) to stearate. Some primary linear alcohols were also tested (C8 and C10 provided as solutions in HMN; C14 and C16 added as solid compounds). Strain HdN1 grew well with 1-decanol, 1-tetradecanol and 1-hexadecanol; growth with 1-octanol was poor, and no growth occurred with ethanol.

Growth tests with different electron acceptors

All three strains grew also aerobically with alkanes. Examination of strain HdN1 in more detail revealed that almost the same range of n-alkanes (and fatty acids) was oxidized with O2 as in anaerobic cultures with NO3-. Only n-hexane was not utilized so far with O2. Another slight difference between aerobic and anaerobic alkane utilization was observed if cultures grown with hexadecane were transferred to medium with tridecane (C13) or dodecane (C12). Whereas aerobic cultures grew immediately with the lighter alkanes, anaerobic cultures exhibited a lag-phase of >10 days.

The transient formation by strain HdN1 of NO2- (≤1.5 mM; not shown) and N2O (Fig. 3A and B, lower curves) at low concentration during NO3- reduction and the detection of N2 in all cultures grown under an argon atmosphere indicated the common denitrification pathway. To further examine the capability for efficient use of NO2- and N2O, these electron acceptors were tested individually in the absence of NO3-.

Growth with alkanes also occurred with added NO2- (instead of NO3-), but was slightly slowed down if more than 5 mM NO2- was added. Furthermore, a lag-phase of c. 2 days was sometimes observed after inoculation of new medium with NO2-. Hence, several mM may be somewhat inhibitory.

Surprisingly, strain HdN1 did not grow with alkanes in the growth tests with N2O. In accordance with the lack of growth, N2O was not consumed (Fig. 3A, upper curve), and N2 (Fig. 3C) or CO2 (Fig. 3E) were not formed. In contrast, growth with 1-tetradecanol, 1-hexadecanol or fatty acids was possible with added N2O, and consumption of N2O (Fig. 3B) as well as formation of N2 (Fig. 3D) and CO2 (Fig. 3F) was obvious. A minor formation of N2 from N2O during incubation with hexadecane can be explained by reduction with an endogenous electron source in the inoculum. The formation of N2 from N2O
Fig. 3. Time-courses of the formation of N₂O (A and B), N₂ (C and D) and CO₂ (E and F) in anaerobic cultures of strain HdN1 with n-hexadecane (A, C and E) or palmitate (B, D and F). The electron acceptors were added in stoichiometrically limiting amounts (100 μmol of NO₃⁻; c. 250 μmol of N₂O) relative to the electron donor (171 μmol of hexadecane, advantage of large excess explained in text; 10 μmol of palmitate). Results show that alkane oxidation to CO₂ was not possible with N₂O, but readily occurred with NO₃⁻. The functionalized compound, palmitate, was oxidized with N₂O. Duplicates yielded the same results (not shown). Culture volumes of 10 ml (phosphate-buffered medium, pH ≥ 7.1, without addition of NaHCO₃; Appendix S1) were incubated in 165 ml serum bottles under an argon headspace. N₂O was injected as pure O₂-free gas. Cultures were very gently shaken for a few minutes per day. Vigorous shaking had to be avoided because it impeded growth. Samples from the headspace were analysed with a gas chromatograph employing argon as carrier gas and a thermal conductivity detector. The calculated dissolved amounts of gases were added so as to obtain the total amounts in the bottles. Calculation was based on literature data (Wilhelm et al., 1977; Stumm and Morgan, 1995), assuming equilibrium (which may not have been fully reached due to limited agitation) and considering pH in the case of CO₂.
requires only 2 e\textsuperscript{−}, whereas formation of N\textsubscript{2} from NO\textsubscript{3} requires 10 e\textsuperscript{−} from an electron donor. The lack of alkane utilization with N\textsubscript{2}O was not due to specific inhibition. The same amount of N\textsubscript{2}O added to a culture with hexadecane and NO\textsubscript{3} did not inhibit growth. For physiological comparison, strains HxN1 and OcN1 were also incubated with N\textsubscript{2}O as the only electron acceptor and utilizable alkanes (n-hexane and n-octane respectively). These strains were able to grow with N\textsubscript{2}O and alkanes. Results are summarized in Fig. 4. The inability for coupling alkane utilization to N\textsubscript{2}O reduction is apparently unique for strain HdN1.

Other electron acceptors tested (concentrations in mM) but not utilized were sulfate (15), thiosulfate (5), sulfur (added as slurry), fumarate (10) and perchlorate (10). Toxic effects were excluded in controls containing in addition NO\textsubscript{3}. In contrast, chlorate was toxic.

Search for metabolites and genes involved in alkane degradation

Investigation of metabolites and genes involved in alkane degradation via addition to fumarate have been reported for strain HxN1 (Rabus et al., 2001; Wilkes et al., 2002; Grundmann et al., 2008). The presently performed metabolite analysis of strain OcN1 upon growth with n-octane and NO\textsubscript{3} revealed (1-methylheptyl)succinate (extraction, methylation and analysis as in Rabus et al., 2001; Wilkes et al., 2003), again indicating an activation via addition to fumarate. In contrast, alkyl-substituted succinates were never detectable in cultures and cells of strain HdN1. Another product searched for [by gas chromatography-mass spectrometry of extracts silylated with N\textsubscript{2}O-bis(trimethylsilyl)acetamide; Appendix S1] in anaerobic n-hexadecane cultures of strain HdN1 was 1-hexadecanol. If air was strictly excluded and if the anaerobically grown culture was exposed to air for 20–30 min (data not shown), Such 1-alkanol formation is a long-known indicator of alkane monooxygenase activity (Britton, 1984). Metabolite analysis in anaerobic alkane degraders with facultative aerobic metabolism thus requires careful avoidance of artefacts due to reaction with O\textsubscript{2} from air.

The gene possibly encoding the alkane-activating enzyme in strain OcN1 was retrieved via polymerase chain reaction with degenerate primers for mas and ass genes, generation of a probe and screening of a genomic library, similar as described for strain HxN1 (Grundmann et al., 2008). The derived amino acid sequence (Accession No. FN675935) revealed close relationships (Fig. S1) to the orthologue from strain HxN1 (Grundmann et al., 2008) and a sulfate-reducing bacterium (Callaghan et al., 2008). Attempts to amplify in an analogous manner mas- or ass-like genes from strain HdN1 failed. Therefore, a shotgun genomic library of strain HdN1 was established. This allowed assemblage of the complete genome sequence (4 587 455 bp; 3762 coding sequences; Accession No. FP929140; for some more details see Table S1). But neither this revealed mas- or ass-like genes (Table S2).
These findings suggested that the mechanism for alkane activation in strain HdN1, which has to involve the cleavage of a strong, apolar C–H bond, differs basically from the mechanism with formate as co-substrate in the two other strains.

**Linkage of alkane activation in strain HdN1 to the nitrate reduction pathway?**

The distinctive results of the incubation experiments with either alkanes or functionalized (O-group-containing) substrates and \( \text{N}_2\text{O} \) may offer a clue as to how strain HdN1 could initiate alkane degradation under anoxic conditions. The electron acceptor tests with functionalized electron donors as well as identified genes (Table S3) indicate that strain HdN1 employs the common reduction sequence \( \text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NO} \rightarrow \text{N}_2\text{O} \rightarrow \text{N}_2 \), viz. is in principle able to readily reduce \( \text{N}_2\text{O} \). Also during growth with alkanes as organic substrates and \( \text{NO}_3^- \) or \( \text{NO}_2^- \) as electron acceptors, \( \text{N}_2\text{O} \) must have been a regular intermediate because \( \text{N}_2 \) rather than \( \text{N}_2\text{O} \) was the end-product. However, \( \text{N}_2\text{O} \) added alone did not allow growth with alkanes. An early reaction during alkane utilization must thus depend on a nitrogen–oxygen (N–O) species other than \( \text{NO}_3^- \). The early reaction could be the biochemically crucial activation of the alkane. The required N–O species cannot be \( \text{NO}_3^- \), because growth with alkanes was also possible if \( \text{NO}_2^- \) was added instead of \( \text{NO}_3^- \). Hence, \( \text{NO}_2^- \) or \( \text{NO} \) (or a so far unknown product from \( \text{NO}_2^- \) reduction) may be essential for alkane activation. The basic hypothesis is depicted in Fig. 5. In further experiments, added \( \text{NO} \) (prepared from acidified \( \text{NaNO}_2 \) and \( \text{KI} \); Schreiber et al., 2008) turned out to be very toxic so that application of amounts theoretically sufficient to achieve measurable growth was not possible; \( \text{NO} \) at a partial pressure of c. 75 Pa [0.0775% (v/v) in gas mixture of ambient pressure] in the headspace completely inhibited growth with \( \text{NO}_3^- \). At a partial pressure of 50 Pa (0.05%) \( \text{NO} \) did not completely inhibit growth with \( \text{NO}_3^- \), albeit growth was retarded. To test whether such still tolerated \( \text{NO} \) concentration is sufficient to initiate alkane degradation and in this way allow growth, 50 Pa \( \text{NO} \) was provided together with \( \text{N}_2\text{O} \) (17 mmol l\(^{-1}\)), the latter serving as main electron acceptor for anaerobic respiration. However, growth was not observed unless \( \text{NO}_3^- \) was added. It thus remains elusive whether \( \text{NO}_2^- \) or \( \text{NO} \) (or an unknown \( \text{NO}_2^- \)-derived species) is actually required to initiate alkane degradation.

From a thermodynamic point of view, an involvement of N–O species in alkane activation under anoxic conditions is an appealing hypothesis. N–O species other than \( \text{NO}_3^- \) (Fig. 6A) are all metastable (Garrels and Christ, 1965; Thauer et al., 1977) and represent or can provide strong potential oxidants; this property may be enzymatically exploited to achieve alkane activation. An indirect use to form another reactive compound as well as a direct use of an N–O species can be envisaged.

One mode of indirect use of \( \text{NO}_2^- \) and \( \text{NO} \) could be their dismutation (formally an ‘internal’ reduction of \( \text{N} \) and oxidation of \( \text{O} \)) leading to \( \text{O}_2 \), according to the following equations:

\[
\begin{align*}
4 \text{NO}_3^- + 4 \text{H}^+ &\rightarrow 2 \text{N}_2 + 3 \text{O}_2 + 2 \text{H}_2\text{O} \quad \Delta G^{\circ} = -55.2 \text{kJ (mol O}_2^- \text{)}^{-1} \\
2 \text{NO} &\rightarrow \text{N}_2 + \text{O}_2 \quad \Delta G^{\circ} = -173.1 \text{kJ (mol O}_2^- \text{)}^{-1}
\end{align*}
\]

\( \text{O}_2 \) could then be used for an alkane monoxygenase reaction (alkane hydroxylation). Also \( \text{N}_2\text{O} \) can in principle lead to \( \text{O}_2 \) [\( 2 \text{N}_2\text{O} \rightarrow 2 \text{N}_2 + \text{O}_2; \Delta G^{\circ} = -208.4 \text{kJ (mol O}_2^- \text{)}^{-1} \)], but the present results exclude its use for an initiation of alkane degradation. There is indeed evidence for \( \text{O}_2 \) formation at very low concentration during \( \text{NO}_2^- \) reduction in a methane-utilizing enrichment culture dominated by ‘Candidatus Methylomirabilis oxyfera’. The enrichment grew under exclusion of air and depended on \( \text{NO}_2^- \) addition (Ettwig et al., 2010). \( ^{18}\text{O}_2 \) formation from \( \text{N}^{18}\text{O}_2^- \) (indirectly labelled through \( \text{H}_2^{18}\text{O} \)) became detectable upon specific inhibition of methane monoxygenase.

![Fig. 5. Hypothetical involvement of denitrification intermediates in alkane activation.](https://example.com/fig5.png)
NO dismutation (Eq. 2) was suggested as the underlying mechanism. Neither was NO$_3^-$ or N$_2$O reduced, nor did the genome of the dominant bacterium harbour typical N$_2$O-reductase genes. Results therefore suggested that NO dismutation was a main reaction during NO$_2^-$ reduction in ‘Candidatus M. oxyfera’. An earlier example of a metastable inorganic oxo-compound enabling biodegradative reactions through O$_2$ formation is chlorite, an intermediate of microbial chlorate reduction (ClO$_2^-$ → Cl$^-$ + O$_2$; $\Delta G^{\circ}$ = -148.4 kJ mol$^{-1}$; Ginkel et al., 1996; Chakraborty and Coates, 2004; Tan et al., 2006; Weelink et al., 2008; Mehboob et al., 2009a,b). The presently investigated alkane-degrading strain HdN1 differs metabolically from ‘M. oxyfera’ in several respects. Strain HdN1 does not utilize methane, grows with NO$_3^-$ and obviously involves the conventional reduction sequence via N$_2$O to N$_2$. If strain HdnN would employ NO$_2^-$ or NO-derived O$_2$, the demand per hydrocarbon molecule utilized would be much lower than in ‘Candidatus M. oxyfera’. Long-chain alkane activation would require only a minor withdrawal of NO$_2^-$ or NO from the respiratory path that mainly leads to N$_2$ through N$_2$O. For instance, n-hexadecanol resulting from oxygenation of n-hexadecane (C$_{16}$H$_{32}$CH$_3$ + O$_2$ + 2 [H] → C$_{16}$H$_{31}$CH$_2$OH + H$_2$O) yields as many as 96 [H] (C$_{16}$H$_{32}$CH$_3$OH + 31 H$_2$O → 16 CO$_2$ + 96 [H]) per substrate molecule. With 2 [H] consumed for activation, each oxygenation event thus leaves 94 [H] per C$_{16}$H$_{32}$ for respiratory energy conservation. In contrast, each oxygenation event in methane utilization provides only 4 [H] per CH$_4$ for respiration. According to genomic data, strain HdnN may form a di-iron monoxygenase, a P450-type monoxygenase and possibly a third type of
monooxygenase (Table S4). Multiple monooxygenases are not uncommon in aerobic alkane degraders (Rojo, 2009).

O$_2$ formation could not be detected so far in strain HdN1. We mixed a culture of strain HdN1 with a culture of luminous bacteria (isolated from herring using glycerol-peptone medium; Farmer and Hickman-Brenner, 2006) as sensitive O$_2$ indicators (Chance et al., 1978); both cultures had been adapted to brackish water (180 mM NaCl and 20 mM MgSO$_4$) medium. After extinction of luminescence due to oxygen consumption, neither addition of NO$_3$- nor of NO$_2$- or NO-saturated water caused the luminescent reaction to resume (whereas air did immediately). Neither was oxygen detectable by means of an O$_2$-microelectrode (lower detection limit, 1 μM; Revsbech, 1989) in cultures supplied with NO$_3$- or NO. Nevertheless, results do not rule out O$_2$ as an intermediate. A very low production rate and effective scavenging by alkane monooxygenase and competing respiratory enzymes (if present under anoxic conditions) such as high-affinity cbb$_3$-type oxidases (Pitcher and Watmough, 2004; predicted for strain HdN1; Table S5) could maintain the O$_2$ concentration below detection level. Also, the produced alcohol may be consumed effectively by the subsequent reaction. Only upon sudden exposure to air, the anaerobically grown cells accumulated detectable n-hexadecanol (see above).

The slight differences between the growth tests under oxic and anoxic conditions with alkanes of various chain lengths (see growth tests with different electron acceptors) do not necessarily contradict the hypothesis that monooxygenases are used under oxic as well as under anoxic cultivation conditions for alkane activation. There might be slight differences with respect to chain length specificity between the monooxygenase(s) formed in aerobic and denitrifying cultures.

Still, also other modes of an indirect use of N–O species for alkane activation can be envisaged. For instance, they may serve as high-potential (strongly oxidizing) electron acceptors for anaerobic respiration. An intermediate formed during NO$_3$- or NO$_2$- reduction or directly from NH$_4$+ oxidation) should not be considered merely as electron acceptors for anaerobic respiration. An intermediate formed during NO$_3$- or NO$_2$- reduction may provide as co-reactant for the biochemical activation of various hydrocarbons or even of other chemically unreactive compounds. NO$_3$- or NO$_2$- in anoxic habitats could, in principle, promote or enable the degradation of certain organic fractions which tend to be refractory under conditions of sulfate reduction or methanogenesis.

Concluding remarks

In conclusion, results suggest a mechanistic alternative to the fumarate-dependent reaction for anaerobic alkane activation. Also a sulfate-reducing bacterium, strain Hxd3, metabolized long-chain n-alkanes obviously via an initial reaction different from that in other anaerobic alkane degraders (Ackersberg et al., 1998; So et al., 2003; Callaghan et al., 2006). This raises the question whether nitrate-reducing strain HdN1 and sulfate-reducing strain Hxd3 employ basically the same reaction or different reactions to initiate alkane degradation. If they would employ essentially the same fumarate-independent activation reaction, strain HdN1 cannot employ an N–O species or derived O$_2$ directly in the mechanism because they are excluded in strain Hxd3; the sulfate reducer was grown without nitrate. Also other ways to generate O$_2$ are essentially excluded in sulfate reducers; sulfate and its metabolites are all thermodynamically very stable and represent very weak oxidants. Hence, alkane activation by the same basic mechanism in strains HdN1 and Hxd3 would imply that the denitrifier uses an N–O species or O$_2$ only indirectly to generate an alkane-activating factor, whereas the sulfate reducer would generate the same type of factor in a different manner. If strains HdN1 and Hxd3 use different mechanisms for alkane activation, which is more appealing to assume, the reaction in strain HdN1 would represent a third type of alkane activation under anoxic conditions, besides the fumarate-dependent mechanism and the speculative mechanism in strain Hxd3. More refined physiological experiments (preceded by an improved method for harvesting the buoyant cells associated with alkane) are needed to provide further hints as to the alkane activation mechanism in strain HdN1, with consideration of its apparently diverse monooxygenases.

Finally, the present results as well as the oxidation of methane with NO$_2$- (Ettwig et al., 2010) indicate that NO$_3$- or NO$_2$- (either from NO$_3$- reduction or directly from NH$_4$+ oxidation) should not be considered merely as electron acceptors for anaerobic respiration. An intermediate formed during NO$_3$- or NO$_2$- reduction may provide as a co-reactant for the biochemical activation of various hydrocarbons or even of other chemically unreactive compounds. NO$_3$- or NO$_2$- in anoxic habitats could, in principle, promote or enable the degradation of certain organic fractions which tend to be refractory under conditions of sulfate reduction or methanogenesis.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Relationship of the assumed catalytic (large) subunit (MasD) of the n-alkane-activating enzyme in strain OcN1 to other enzymes activating hydrocarbons by addition to fumurate.

Table S1. General genome features of strain HdN1.

Table S2. Genome-based search for genes (bss4 and homologues) that may encode fumurate-dependent glycol radical enzymes for anaerobic alkane activation.

Table S3. Genome-based prediction of enzymes for the denitrification pathway in strain HdN1.

Table S4. Genome-based prediction of alkane monooxygenases (alkane hydroxylases) in strain HdN1.

Table S5. Genome-based prediction of cbb3-type oxidases (oxidases with high O2 affinity) in strain HdN1.

Appendix S1. Cultivation of strains, physiological experiments.

Appendix S2. Formal derivation of the average redox potential.

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