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Why is metabolic labour divided in nitrification?

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Winogradsky discovered in 1890 that nitrification is carried out in two consecutive steps by two distinct groups of bacteria: ammonia-oxidizing bacteria and nitrite-oxidizing bacteria. An explanation for this division of labour is offered based on the kinetic theory of optimal design of metabolic pathways, which postulates the existence of an optimal length for a pathway that maximizes the rate of ATP production. Shortening long pathways could, therefore, increase growth rate. However, this would reduce growth yield if the shorter pathway has fewer ATP-generating steps. High yields would be advantageous when bacteria grow in clonal clusters, as is typical for biofilms. It is postulated that bacteria that completely oxidize ammonia to nitrate exist in such environments.

Nitrification as a two-step process

Nitrification is the process by which ammonia is oxidized to nitrate, which is a vital link in the biogeochemical nitrogen cycle, and is only carried out by prokaryotes [1–3]. S. Winogradsky discovered some of the bacteria responsible for nitrification in 1890 and found a division of labour between two different groups of bacteria, both of which he isolated [4]. Nitrification comprises a two-step food chain in which ammonia is first oxidized to nitrite by ammonia-oxidizing bacteria (AOB) and nitrite is then oxidized to nitrate in a second step carried out by another group of bacteria, the nitrite-oxidizing bacteria (NOB). A simplified scheme of the metabolism of the best-studied 'model' AOB and NOB and the energetics of the two steps are shown in Figure 1.

Phylogenetically, the chemolithoautotrophic ammonia oxidizers and nitrite oxidizers are not closely related. All ammonia oxidizers form a closely related monophyletic cluster within the β-subclass of Proteobacteria except *Nitrosococcus*, which forms a separate branch within the γ-subclass of Proteobacteria. The nitrite oxidizers belong to the α- and γ-subclass of Proteobacteria, apart from the distant *Nitrospina*, which belongs to a separate group with yet uncertain placement, and *Nitrospira*, which represents its own phylum [1,5,6].

Recently, the first ammonia-oxidizing archaeon, '*Nitrosopumilus maritimus*', was isolated [3] after its existence was suggested by the discovery of a unique ammonia monooxygenase gene on an archaeal-associated scaffold from the Sargasso Sea [7]. This is also the first isolated member of the abundant fraction of low-temperature marine Crenarchaeota. Interestingly, all of the previous attempts to isolate ammonia oxidizers missed ammonia-oxidizing Archaea, although environmental sequence data demonstrate the widespread occurrence of diverse and distinct communities of archaeal ammonia oxidizers in marine water columns and sediments [8] and the presence of archaeal ammonia monooxygenase sequences in grassland soil [9]. These findings highlight the fact that knowledge of the biochemistry and physiology of nitrification (Figure 1) is based on a small and unrepresentative subset of the extant diversity of nitrifying prokaryotes.

Winogradsky's discovery of the separation of nitrification into two steps carried out by distinct groups of bacteria still holds, despite extensive work on this important process [1,3,6,10–13]. This well-known fact prompts the obvious, yet unanswered, question about the reasons for this cross-feeding. In this Opinion article, we argue that the kinetic theory of optimal pathway length provides a new framework for answering this old question.

Experimental evolution of cross-feeding in *Escherichia coli*

Adams and coworkers [14,15] described the reproducible evolution of cross-feeding between an acetate-excreting and an acetate-scavenging strain from a genetically homogenous population of *Escherichia coli* growing in glucose-limited continuous culture. We argue that the existence of the two groups of nitrifying bacteria provides an example of such cross-feeding that has evolved in nature to the extreme of complete division of labour. This leads to the question of why cross-feeding evolves, which is answered in the next section.

Kinetic theory of optimal pathway length

Recently, Pfeiffer and Bonhoeffer [16] have shown that such cross-feeding relationships can arise because shorter metabolic pathways might enable a higher rate of ATP production. Their argument is based on kinetic theory [17] that makes the following general assumptions about the evolution of catabolic pathways, which are not specific but applicable to nitrification:

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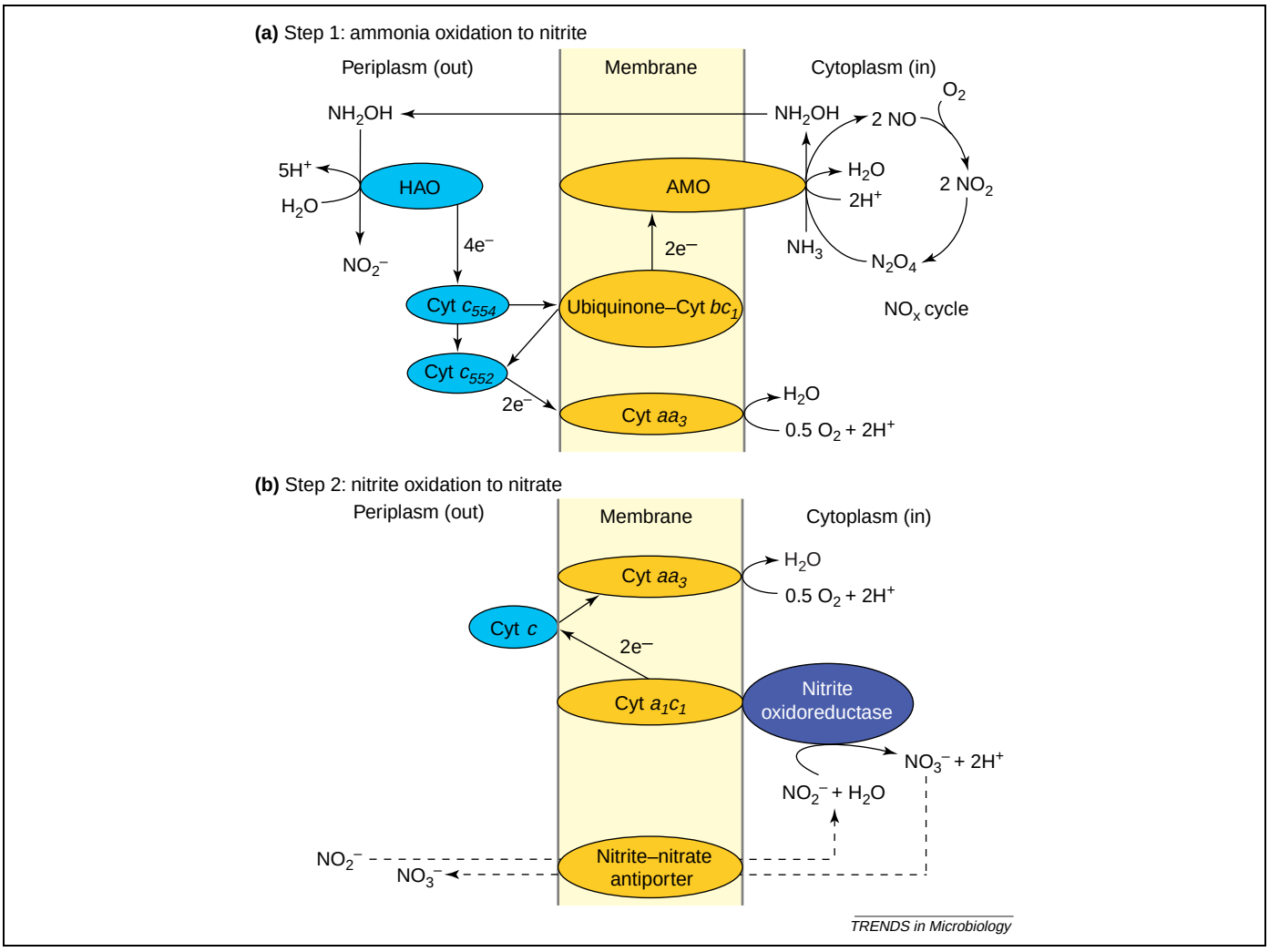

Figure 1. Simplified scheme of the aerobic energy metabolism of the best-studied 'model' nitrifying bacteria. **(a)** Step 1 by ammonia-oxidizing bacteria of the genus *Nitrosomonas*. The NO_x cycle is catalyzed by ammonia monooxygenase (AMO). The oxidation of ammonia to nitrite follows the reaction: $NH_4^+ + 1.5\ O_2 \rightarrow 2H^+ + NO_2^- + H_2O$ where $G^{o\prime} = -275$ kJ mol^{-1} N or -46 kJ mol^{-1} e^- and $E^{o\prime} = 343$ mV. **(b)** Step 2 by nitrite-oxidizing bacteria of the genus *Nitrobacter*. The oxidation of nitrite to nitrate follows the reaction: $NO_2^- + 0.5\ O_2 \rightarrow NO_3^-$ where $G^{o\prime} = -74$ kJ mol^{-1} N or -37 kJ mol^{-1} e^- and $E^{o\prime} = 434$ mV. Energetics of the two steps of nitrification were calculated from [44]. The first step is thermodynamically more favourable but the difference is less pronounced on a per-electron-acceptor basis, which is more relevant under oxygen limitation conditions. Abbreviations: Cyt, cytochrome; HAO, hydroxylamine oxidoreductase. Figure adapted from [1] and [45].

(i) Catabolic pathways are optimized by natural selection for maximal rate of ATP production, which is equivalent to maximizing growth rate if the amount of biomass formed per mole of ATP (Y_{ATP}) is approximately constant [18].

(ii) The total concentration of enzymes of the pathway is minimized because enzyme synthesis is costly or because of competition for the limiting enzyme-synthetic capacity of the cell [19].

(iii) The total concentration of intermediates is also minimized because costs due to toxicity or losses by leakage or instability increase with increasing concentrations.

(iv) The further a substrate is converted, the more ATP can be produced.

Consider extending a substrate-converting pathway by an extra step. The costs of the extra enzyme add to the total cost of enzymes, which necessitates a reduction of the concentrations of the already existing enzymes (while optimizing the distribution of enzyme concentrations). Because the intermediates are also costly, the concentrations of the intermediates that already exist must be reduced for the same reason. Because reaction rates depend on the product of enzyme and metabolite concentration, an increasing reduction in both enzyme and intermediate concentrations with increasing pathway length decreases the substrate consumption rate with the square of the pathway length (Box 1).

As a consequence of the general assumptions, an optimal pathway length exists that maximizes the rate of ATP formation (the product of ATP yield and rate of substrate consumption) provided that the increase in ATP yield is less than the decrease in substrate flux with pathway length (Box 1). In nitrification, the optimal pathway length is expected to be particularly short because of the lower-than-average increase of the ATP yield in the last step [20,21], nitrite oxidation, in contrast to methane oxidation (see [22] and references therein). This difference might explain why the methane oxidation pathway is not truncated as in nitrification (Box 2). If the intermediates are highly toxic, as in nitrification (hydroxylamine and nitrite), the higher costs caused by these

Box 1. Illustration of the kinetic theory of optimal pathway length

To illustrate the fundamental predictions of the kinetic theory about the dependence of substrate and ATP flux on pathway length (Figure I), the simplest case of a linear pathway is considered with linear, irreversible kinetics for all enzymes. For this simplistic case, it is possible to derive analytical expressions for the fluxes and the optimal length (Figure I) and optimal enzyme and intermediate concentrations so that nonlinear optimization is not needed [16].

These simplistic assumptions are obviously not met in nitrification; therefore, we checked the dependence of substrate flux on pathway length for reversible linear kinetics and for irreversible and reversible Michaelis–Menten kinetics by numerical simulation of the fluxes and optimization of enzyme concentrations. We found that all cases were similar to the case of irreversible linear kinetics shown in Figure I (E. Costa *et al.*, unpublished). The existence and position of the optimum shown in Figure I (arrows) depends on how strongly the ATP yield increases with pathway length: in nitrification, the increase of ATP yield is below linear, which tends to shift the optimum towards shorter pathways. If the ATP yield increases with the square of the pathway length, the optimum length would be infinite because the increase in ATP yield compensates for the decrease in substrate flux.

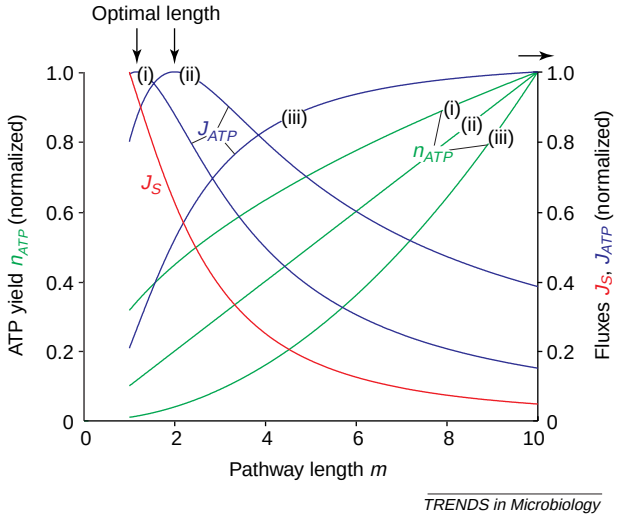

Figure I. Dependence of ATP yield, substrate and ATP flux on the length of a pathway. Note that in growing cells, the flow of metabolites through a pathway will be in a quasi-steady state because growth is a slower process than substrate turnover. Therefore, the steady state rates (called fluxes) of ammonia consumption and nitrite (nitrate) formation will be equal. For the dependence of ATP yield n_{ATP} on pathway length m, we show three hypothetical cases and indicate the position of the optimum by arrows: (i) $n_{ATP} = \sqrt{m}$ (shortest optimum); (ii) $n_{ATP} = m$; and (iii) $n_{ATP} = m^2$ (optimum at infinite length). The substrate flux J_S is the same for all three cases and monotonically decreases according to $J_S = kS\frac{E_{max}X_{max}}{X_{max}+Sm^2}$, where k is the rate constant for all enzymes and S is the substrate concentration. The total concentration of all enzymes is restricted to E_{max} and the total concentration of all intermediates is restricted to X_{max} [17]. The ATP flux J_{ATP} is the product of substrate flux and ATP yield, $J_{ATP} = n_{ATP}J_S$. If n_{ATP} is simply proportional to pathway length (case ii), the optimal length is given by $m_{opt} = \sqrt{X_{max}/S}$. In words, the optimal length is shorter the higher the substrate concentration and the higher the metabolic costs of intermediates (which restricts X_{max} to lower values). To show the shape of these functions, all parameters were set to unity apart from $S = 1/4$, therefore, $m_{opt} = 2$.

intermediates would also favour shorter pathways unless the intermediates can be kept out of the cytoplasm, which is not possible for hydroxylamine.

It is important to note that the length is optimal only in terms of the rate of ATP production, whereas the ATP yield of the shorter pathway is probably reduced as in the case of nitrification. This trade-off between rate and yield of ATP production suggests that maximizing rate might not always be the best strategy [23–25] (Box 2).

Unfortunately, the effect of pathway length on substrate affinity, which is the third key growth parameter of bacteria [26], cannot be predicted by the available kinetic theory that assumes linear rather than Michaelis–Menten kinetics for the sake of analytical treatment. Suitable extensions of the kinetic theory are currently under way.

The 'decision' to stop ammonia oxidation at the level of nitrite, or not, is the result of evolution of the fitness of the (partial or complete) ammonia oxidizer, the first organism in the food chain. The second organism cannot 'lobby' the first: what is in the interest of the first organism might or might not be in the interest of the second organism or the food chain as a whole.

If substrate turnover in the environment is lowered (e.g. low influx of substrate into a stratified sediment or a chemostat run at low dilution rate), the optimal concentration of enzymes to support the reduced flux while keeping the concentration of intermediates low enough would be reduced along with the pertinent costs [16]. The optimal concentration of intermediates of the pathway and the associated costs would also be lowered [16]. The reduced costs of intermediates and enzymes would ease the demand for truncating the pathway in low turnover habitats. This predicted complete degradation rather than cross-feeding is in line with experimental findings on the evolution of *E. coli* in a glycerol-limited chemostat at a low dilution rate, which led to the isolation of a mutant with reduced secretion of overflow metabolites such as acetate [27].

How short is the optimal pathway length in the case of nitrification?

The optimal pathway length is expected to be particularly short in nitrification because of the high toxicity of the cytoplasmic intermediate hydroxylamine and the lower-than-average increase of the ATP yield (Box 1) in the last step of nitrite oxidation, in contrast to methane oxidation (Box 2). Short pathways also exist in acetic acid bacteria, which oxidize various sugars and alcohols in merely one step in the periplasm (Box 2).

Nevertheless, the final answer can only be empirical or based on a model that includes detailed empirical knowledge of the kinetics of all relevant processes and involved costs, which is currently not feasible. However, on the level of population dynamics rather than enzyme dynamics, Noto *et al.* [28] tested the benefits of compartmentalization in nitrification by enabling the community composition of an activated sludge inoculum to change in a three-stage continuous culture with biomass retention. They found that under conditions of high ammonia load similar to conditions in wastewater treatment, the initially mixed community becomes compartmentalized. In the first two vessels, ammonia was oxidized to nitrite, albeit by different communities of ammonia oxidizers, whereas nitrite was oxidized to nitrate only in the third vessel [29]. This separation of processes increased the nitrification capacity (in terms of

Box 2. Examples of known bacteria with combined pathways

The anaerobe *Holophaga foetida* is an illustrative example for the amalgamation of two consecutive processes (that are typically catalyzed by different groups of bacteria) in a single bacterium [32–34] (Figure I). The growth yield of *H. foetida* is approximately the sum of the yields of the cross-feeding *Acetobacterium woodii* and *Pelobacter acidigallici*, and its growth rate is lower than those of the two competitors [35–37], which confirms the kinetic theory (Box 1). Moreover, *H. foetida* uses the sulfide methylation bypass (Figure I) under conditions of substrate excess, which results in a lower growth yield and higher growth rate [37], further supporting the kinetic theory. The higher abundance of *H. foetida* than *A. woodii* in all the anaerobic sediments investigated [32] could be explained by its higher yield, which is advantageous when bacteria grow in aggregates such as microcolonies or biofilms [24].

Methane oxidation, which bears many similarities to ammonia oxidation, is always carried out by one organism [46]. It seems that the oxidation of formate, which is functionally equivalent to the oxidation of nitrite, is an ideal metabolic opportunity because of its low redox potential of $E^{o\prime} = -432$ mV [44].

Long pathways are the rule for heterotrophic aerobic bacteria, which typically oxidize the organic substrate completely to CO_2. However, short pathways can be found in acetic acid bacteria that carry out incomplete oxidations, which are often only one-step oxidations in the periplasm. They have a low yield and high growth rate and thrive in rich habitats [47], as expected from kinetic theory (Box 1).

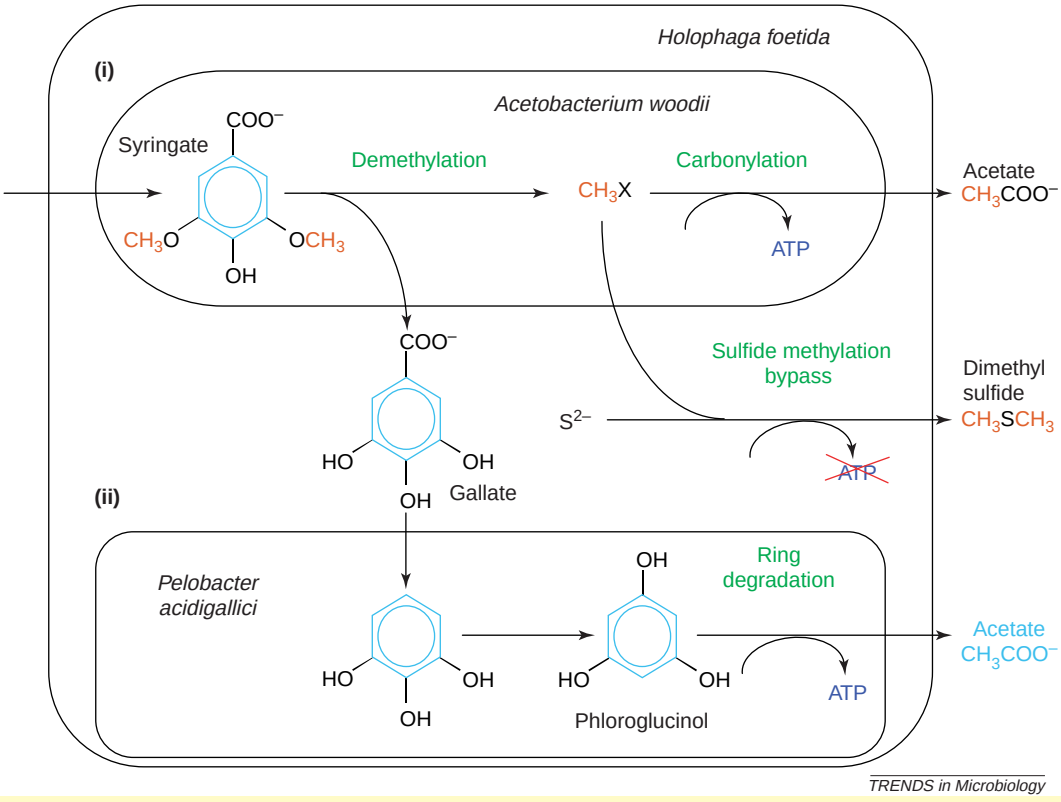

Figure I. An example of the combination of two steps of degradation in one bacterium. (i) Step 1: *Acetobacterium woodii* demethylates methylated phenolic plant compounds (e.g. syringate), followed by carbonylation of the methyl groups to acetate (acetyl-CoA or Wood pathway) and excretion of the phenolic residue (e.g. gallate). (ii) Step 2: *Pelobacter acidigallici* carries out the reductive cleavage of the aromatic ring of trihydroxybenzenes (e.g. gallate) using the phloroglucinol pathway. *Holophaga foetida* combines steps 1 and 2 to incorporate demethylation, carbonylation and ring degradation pathways in a single cell. In addition, it can bypass the acetyl-CoA pathway by methylating sulfide. This shortcut does not generate ATP and, therefore, cannot be used by *A. woodii*, in contrast to *H. foetida*, which can gain ATP downstream. The metabolism of *Parasporobacterium paucivorans* is similar to that of *H. foetida* but always uses the shortcut [48]. One might expect that short pathway competitors would be phylogenetically related to combined pathway organisms but, surprisingly, all of these bacteria are phylogenetically distant.

ammonium load) 2.5-fold compared with the single-stage process [28]. We could reproduce these results in numerical simulations based solely on standard assumptions about the growth kinetics of AOB and NOB (E. Costa *et al.*, unpublished). In conclusion, in the case of ammonia oxidation, the shortest possible length is also the optimal length for maximizing growth rate.

The longest possible nitrification pathway is, of course, the complete oxidation of ammonia to produce nitrate. Kinetic theory (Box 1) would predict that such a complete ammonia oxidizer would have (i) a lower maximal growth rate but (ii) a higher yield. In chemostats and other well-mixed systems, the faster-growing incomplete ammonia oxidizer would outcompete the complete oxidizer [24]. Conversely, in biofilms and other microbial aggregates with low mixing of substrates (diffusion gradients) and biomass (clonal clusters), a higher yield of biomass per amount of substrate consumed (which is equivalent to an economical use of resources) would benefit only the neighbourhood. In fact, economy is a primitive form of cooperation [23,30,31] and, indeed, altruism because the economical organism suffers a reduced growth rate

[24,25]. In biofilms, under a broad range of suitable conditions, an economical but slower-growing organism would have a higher fitness than a resource-wasting, fast-growing competitor [24]. This is why we postulate that such a complete ammonia oxidizer indeed exists, which we refer to as 'comammox' (*com*plete *amm*onium *ox*idation). *Holophaga foetida* is an anaerobic bacterium that combines the pathways of its cross-feeding competitors [32–34] (Box 2). In accordance with kinetic theory, it has a higher yield and a lower growth rate than its competitors [35–37]. In accordance with the predicted higher fitness of economical bacteria in biofilms [24], it outnumbers its competitors in natural sediments [32].

Lithotrophs missing in nature

This is not the first time that the existence of an unknown lithotroph has been postulated. In 1977, E. Broda postulated the existence of "two kinds of lithotrophs missing in nature": "(A) photosynthetic, anaerobic, ammonia bacteria, analogous to coloured sulfur bacteria, and (B) chemosynthetic bacteria that oxidize ammonia to nitrogen with oxygen or nitrate [or nitrite] as oxidant" [38]. He argued that because of the astonishing versatility of prokaryotes in their energy metabolism, it should be expected that anaerobic lithotrophs generating dinitrogen exist, given that these processes are thermodynamically possible [38].

One of the two types of bacteria postulated by Broda was the bacterium responsible for anaerobic ammonia oxidation (anammox), which was discovered in the early 1990s in a denitrifying pilot plant at Gist-Brocades, Delft, The Netherlands [39,40]. Anammox bacteria grow extremely slowly and are difficult to culture; in fact, pure cultures have not yet been obtained from the enrichments [39,40]. Nevertheless, they are now known to have a major role in the nitrogen cycle of low-oxygen environments such as the Black Sea [2,40,41]. Anammox bacteria are unusual because they have found many surprising solutions to the technical problems of anaerobic ammonia oxidation, such as an anammoxosome, which is an intracytoplasmic compartment bounded by a single ladderane lipid-containing membrane. All known anammox bacteria form a monophyletic branch of the Planctomycetales and are, therefore, phylogenetically completely unrelated to the aerobic ammonia oxidizers [40].

Broda's reasoning for the existence of anaerobic ammonia oxidation [38], once thought to be biochemically impossible [40], relied on the metabolic versatility of prokaryotes. Recent discoveries such as anaerobic methane oxidation (which was also thought to be biochemically impossible [40]) and ammonia-oxidizing Archaea [3] with *amoA* genes that are only distantly related to those from Proteobacteria [8] have certainly strengthened the versatility argument. Anaerobic methane oxidation is actually another example of cross-feeding because it is carried out by a syntrophic association of 'reversed' methanogens with sulfate-reducing bacteria [40]. Strous and Jetten [40] have, in fact, postulated that a single sulfate-reducing methanotroph exists. We would like to add that such a one-step process would be favoured under conditions of low substrate turnover and clustered growth.

Are ammonia and nitrite oxidation biochemically compatible?

Division of metabolic labour, or compartmentalization, might be necessary if the reactions of ammonia, hydroxylamine and nitrite oxidation are biochemically incompatible. Microscopic examinations of nitrifying reactors often show clusters of AOB and NOB touching each other [6,42,43], which suggests that intermediates of AOB metabolism (which, to some degree, diffuse out of the

Box 3. How comammox might be isolated

The following suggestions on the isolation of comammox are actually not specific for nitrification because they were deliberately derived from the optimal pathway length hypothesis, which is entirely general.

(i) Batch enrichments. The most common method of isolating bacteria is the enrichment culture, which selects for the highest growth rate under given conditions. We expect comammox to grow slower than AOB in standard enrichment cultures, which are augmented with substrate.

(ii) Chemostat enrichments. Chemostat cultures are used to avoid the high substrate concentrations of typical enrichment cultures, which often far exceed the ammonia concentrations in the environment and result in the isolation of environmentally less-important bacteria [49].

Low substrate concentrations can occur in environments with low activity and growth rates when ammonia influx is low and limiting but low concentrations might also occur as a result of high turnover rates. Although low substrate concentrations *per se* would render longer pathways more optimal (Box 1), low substrate concentrations caused by high turnover rates would imply high metabolic substrate flux, which would favour the shorter pathway of incomplete ammonia oxidation. Chemostats that run at low enough dilution rates (for practical reasons, the related retentostats should be used at low dilution rates) provide an environment with a low substrate concentration combined with a low growth rate. In this case, both low substrate concentration and low growth rate favour the longer pathway of comammox. However, because the kinetic theory developed so far does not make predictions about substrate affinity [26], the potential of chemostat cultures for the isolation of comammox (which select for the highest substrate affinity at the experimentally set growth rate) remains unclear.

(iii) Dilution culture. The direct dilution of an environmental sample, before any culturing in the laboratory, selects for those bacteria with the highest population density in a particular habitat. Note that this enabled the isolation of the complete degrader *Holophaga foetida* (Box 2). It might also enable the isolation of comammox, which might be the dominant bacterium in habitats where nitrifiers grow mainly as microcolonies or biofilms and the substrate influx is low because growth in clonal clusters favours the higher yield that comammox should have. Also, the lower rates of ammonia influx reduce the advantage of increasing ammonia oxidation rates by shortening the pathway. Dilution culture has been used for isolation of ammonia oxidizers but has never been aimed at comammox, hence, numerical dominance of comammox in the samples used is not particularly likely (see [50] and references therein).

(iv) Biofilm enrichments. Presumably the best way of enriching comammox would use biofilm or immobilized biomass systems that select for the higher growth yield of comammox while providing a macroscale gradient of turnover rates as a result of slow plug-flow type mixing. To our knowledge, this has not been tried.

Box 4. Outstanding questions

- Can genes or enzymes for ammonia oxidation and nitrite oxidation be detected in the same cell *in situ* using molecular probes? Or can both AOB- and NOB-specific genes be found in the same genome of uncultured or cultured prokaryotes? However, the similarity to known sequences might be low.
- Can comammox be isolated using biofilm enrichments (Box 3)?
- Which habitats enable comammox to dominate numerically so that it can be isolated by dilution culture?
- Can viable comammox bacteria be created by genetic engineering using genes from cultured or uncultured AOB and NOB? (This would address the biochemical compatibility question.)
- If so, are the growth parameters similar to those predicted by kinetic theory? And how do they evolve upon selection in chemostats or biofilms?
- What does kinetic theory predict about the dependence of substrate affinity [26] on pathway length?
- Can the fitness costs for enzymes and intermediates be estimated experimentally by genetic engineering? Note that changing enzyme levels will also change the concentrations of the intermediates, so combined changes of more than one enzyme concentration might be necessary.
- Is the observed niche differentiation between AOB and NOB the cause or consequence of the division of labour? Once comammox has been isolated or created, this could be studied by comparing the physiology of comammox with its competitors and by experimental evolution of comammox.

cell) do not prevent nitrite oxidation and that these reactions can occur under similar physicochemical conditions (e.g. pH, temperature, O_2 partial pressure).

In comammox, further incompatibilities might arise from the interaction of enzymes (or enzyme-bound intermediates) in physical contact. However, these undesired interactions could be prevented by evolutionary changes of the localization and surface properties of the enzymes, of the reaction mechanisms, or the binding constants. The diversity of simultaneously occurring transformations of inorganic nitrogen compounds in nitrifying bacteria supports the view that evolutionary changes can solve the technical problems of incompatible reactions.

Are there alternative explanations for the division of labour?

The division of metabolic labour opens up the chance for specialization and adaptation to somewhat different but overlapping ecological niches. A spatial separation of AOB and NOB densities on a macroscale, following environmental gradients, has often been observed [6,43]. On a microscale, however, AOB and NOB are typically juxtaposed nevertheless [6,43]. Whether this niche differentiation is the cause or the consequence of the division of labour could be studied by comparing the physiology of comammox and its competitors and by experimental evolution of comammox.

Why comammox has not yet been found and how it might be isolated

Because comammox merely combines two steps of a known process into one, rather than representing a novel link in the nitrogen cycle, its existence cannot be revealed by tracer or other geochemical studies in contrast to anaerobic methane or anaerobic ammonia oxidation. Molecular probes could be used to detect the presence of the ammonia monooxygenase and nitrite oxidoreductase enzymes or genes in the same cell but the enzymes of comammox probably differ from known AOB and NOB enzymes to various degrees, possibly to avoid cross-reactions. Nevertheless, we used BLASTn and BLASTp (NCBI; www.ncbi.nih.gov/BLAST) with the default set of databases (nr) to find regions of similarity between nucleotide and protein sequences, respectively, of all subunits of (i) ammonia monooxygenase and (ii) hydroxylamine oxidoreductase from *Nitrosomonas europaea* and (iii) nitrite oxidoreductase from *Nitrobacter winogradskyi*. No matches to AOB-specific enzymes in NOB were found or vice versa. Box 3 analyzes the potential of dissimilar techniques to isolate the postulated comammox bacteria.

Concluding remarks and future perspectives

It is surprising that no nitrifying bacterium is known that combines ammonia oxidation and nitrite oxidation similar to the combination of demethylation and ring-degradation in *H. foetida*. The potential biochemical incompatibilities that might arise upon amalgamation of ammonia and nitrite oxidation reactions in a single cell are technical problems that can probably be overcome by evolutionary optimization of the characteristics of the catabolic enzymes. Kinetic theory of optimal pathway length predicts that such a hypothetical complete ammonia oxidizer, referred to as comammox, would have a lower growth rate but a higher growth yield than its cross-feeding competitors, similar to *H. foetida*. Comammox would be competitive compared with incomplete ammonia oxidizers under conditions of slow, substrate-influx-limited growth in aggregates such as flocs, microcolonies or biofilms, which favour competitors like *H. foetida* that have a high growth yield rather than a high growth rate. We postulate that comammox does indeed exist and that it is competitive under the aforementioned conditions but its isolation has, to our knowledge, not yet been specifically attempted. We suggest that using biofilm enrichments to select for highest yield, rather than enrichment cultures to select for highest growth rate, is the most promising way to isolate comammox. As outlined in Box 4, we are only beginning to understand the ecophysiological and evolutionary pros and cons of two-step versus single-step nitrification.

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References

1 Bock, E. and Wagner, M. (2001) Oxidation of inorganic nitrogen compounds as an energy source. In *The Prokaryotes: An Evolving Electronic Resource for the Microbiological Community* (3rd edition, release 3.7) (Dworkin, M., ed), Springer-Verlag
2 Arrigo, K.R. (2005) Marine microorganisms and global nutrient cycles. *Nature* 437, 349–355
3 Könneke, M. *et al*. (2005) Isolation of an autotrophic ammonia-oxidizing marine archaeon. *Nature* 437, 543–546
4 Brock, T.D. (1998) *Milestones in Microbiology: 1546 to 1940*, ASM Press
5 Purkhold, U. *et al*. (2003) 16S rRNA and *amoA*-based phylogeny of 12 novel betaproteobacterial ammonia-oxidizing isolates: extension of the dataset and proposal of a new lineage within the nitrosomonads. *Int. J. Syst. Evol. Microbiol.* 53, 1485–1494
6 Schramm, A. (2003) *In situ* analysis of structure and activity of the nitrifying community in biofilms, aggregates, and sediments. *Geomicrobiol. J.* 20, 313–333
7 Venter, J.C. *et al*. (2004) Environmental genome shotgun sequencing of the Sargasso Sea. *Science* 304, 66–74
8 Francis, C.A. *et al*. (2005) Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean. *Proc. Natl. Acad. Sci. U. S. A.* 102, 14683–14688
9 Treusch, A.H. *et al*. (2005) Novel genes for nitrite reductase and Amo-related proteins indicate a role of uncultivated mesophilic crenarchaeota in nitrogen cycling. *Environ. Microbiol.* 7, 1985–1995
10 Belser, L.W. (1979) Population ecology of nitrifying bacteria. *Annu. Rev. Microbiol.* 33, 309–333
11 Prosser, J.I. (1989) Autotrophic nitrification in bacteria. *Adv. Microb. Physiol.* 30, 125–181
12 Koops, H.P. and Pommerening-Röser, A. (2001) Distribution and ecophysiology of the nitrifying bacteria emphasizing cultured species. *FEMS Microbiol. Ecol.* 37, 1–9
13 Kowalchuk, G.A. and Stephen, J.R. (2001) Ammonia-oxidizing bacteria: a model for molecular microbial ecology. *Annu. Rev. Microbiol.* 55, 485–529
14 Helling, R.B. *et al*. (1987) Evolution of *Escherichia coli* during growth in a constant environment. *Genetics* 116, 349–358
15 Adams, J. (2004) Microbial evolution in laboratory environments. *Res. Microbiol.* 155, 311–318
16 Pfeiffer, T. and Bonhoeffer, S. (2004) Evolution of cross-feeding in microbial populations. *Am. Nat.* 163, E126–E135
17 Heinrich, R. and Schuster, S. (1996) *The Regulation of Cellular Systems* (1st edn), Chapman & Hall
18 Stouthamer, A.H. (1979) The search for correlation between theoretical and experimental growth yields. In *International Review of Biochemistry* (Quayle, J.R., ed.), pp. 1–47, University Park Press
19 Snoep, J.L. *et al*. (1995) Protein burden in *Zymomonas mobilis*: negative flux and growth control due to overproduction of glycolytic enzymes. *Microbiology* 141, 2329–2337
20 Wetzstein, H.G. and Ferguson, S.J. (1985) Respiration-dependent proton translocation and the mechanism of protonmotive force generation in *Nitrobacter winogradskyi*. *FEMS Microbiol. Lett.* 30, 87–92
21 Whittaker, M. *et al*. (2000) Electron transfer during the oxidation of ammonia by the chemolithotrophic bacterium *Nitrosomonas europaea*. *Biochim. Biophys. Acta* 1459, 346–355
22 Vorholt, J.A. (2002) Cofactor-dependent pathways of formaldehyde oxidation in methylotrophic bacteria. *Arch. Microbiol.* 178, 239–249
23 Pfeiffer, T. *et al*. (2001) Cooperation and competition in the evolution of ATP-producing pathways. *Science* 292, 504–507
24 Kreft, J-U. (2004) Biofilms promote altruism. *Microbiology* 150, 2751–2760
25 Kreft, J-U. and Bonhoeffer, S. (2005) The evolution of groups of cooperating bacteria and the growth rate versus yield trade-off. *Microbiology* 151, 637–641
26 Button, D.K. (1991) Biochemical basis for whole-cell uptake kinetics – specific affinity, oligotrophic capacity, and the meaning of the Michaelis constant. *Appl. Environ. Microbiol.* 57, 2033–2038
27 Weikert, C. *et al*. (1997) Use of a glycerol-limited, long-term chemostat for isolation of *Escherichia coli* mutants with improved physiological properties. *Microbiology* 143, 1567–1574
28 Noto, K. *et al*. (1998) Complete oxidation of high concentration of ammonia by retaining incompatible nitrification activities in three-vessel system. *Water Res.* 32, 769–773
29 Holben, W.E. *et al*. (1998) Molecular analysis of bacterial communities in a three-compartment granular activated sludge system indicates community-level control by incompatible nitrification processes. *Appl. Environ. Microbiol.* 64, 2528–2532
30 Pfeiffer, T. and Bonhoeffer, S. (2002) Evolutionary consequences of tradeoffs between yield and rate of ATP production. *Zeitschrift für Physikalische Chemie* 216, 51–63
31 Pfeiffer, T. and Bonhoeffer, S. (2003) An evolutionary scenario for the transition to undifferentiated multicellularity. *Proc. Natl. Acad. Sci. U. S. A.* 100, 1095–1098
32 Bak, F. *et al*. (1992) Formation of dimethylsulfide and methanethiol from methoxylated aromatic compounds and inorganic sulfide by newly isolated anaerobic bacteria. *Arch. Microbiol.* 157, 529–534
33 Kreft, J-U. and Schink, B. (1993) Demethylation and degradation of phenylmethylethers by the sulfide-methylating homoacetogenic bacterium strain TMBS 4. *Arch. Microbiol.* 159, 308–315
34 Liesack, W. *et al*. (1994) *Holophaga foetida* gen. nov., sp. nov., a new, homoacetogenic bacterium degrading methoxylated aromatic compounds. *Arch. Microbiol.* 162, 85–90
35 Schink, B. and Pfennig, N. (1982) Fermentation of trihydroxybenzenes by *Pelobacter acidigallici* gen nov. sp. nov., a new strictly anaerobic, non-sporeforming bacterium. *Arch. Microbiol.* 133, 195–201
36 Kreikenbohm, R. and Pfennig, N. (1985) Anaerobic degradation of 3,4,5-trimethoxybenzoate by a defined mixed culture of *Acetobacterium woodii*, *Pelobacter acidigallici*, and *Desulfobacter postgatei*. *FEMS Microbiol. Ecol.* 31, 29–38
37 Kappler, O. *et al*. (1997) Effects of alternative methyl group acceptors on the growth energetics of the *O*-demethylating anaerobe *Holophaga foetida*. *Microbiology* 143, 1105–1114
38 Broda, E. (1977) Two kinds of lithotrophs missing in nature. *Z. Allg. Mikrobiol.* 17, 491–493
39 Strous, M. *et al*. (1999) Missing lithotroph identified as new planctomycete. *Nature* 400, 446–449
40 Strous, M. and Jetten, M.S.M. (2004) Anaerobic oxidation of methane and ammonium. *Annu. Rev. Microbiol.* 58, 99–117
41 Kuypers, M.M.M. *et al*. (2005) Massive nitrogen loss from the Benguela upwelling system through anaerobic ammonium oxidation. *Proc. Natl. Acad. Sci. U. S. A.* 102, 6478–6483
42 Mobarry, B.K. *et al*. (1996) Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. *Appl. Environ. Microbiol.* 62, 2156–2162
43 Okabe, S. *et al*. (1999) *In situ* analysis of nitrifying biofilms as determined by *in situ* hybridization and the use of microelectrodes. *Appl. Environ. Microbiol.* 65, 3182–3191
44 Thauer, R.K. *et al*. (1977) Energy conservation in chemotrophic anaerobic bacteria. *Bacteriol. Rev.* 41, 100–180
45 Schmidt, I. *et al*. (2001) Ammonia oxidation by *Nitrosomonas eutropha* with NO_2 as oxidant is not inhibited by acetylene. *Microbiology* 147, 2247–2253
46 Arp, D.J. *et al*. (2002) Molecular biology and biochemistry of ammonia oxidation by *Nitrosomonas europaea*. *Arch. Microbiol.* 178, 250–255
47 Swings, J. (1999) The genera *Acetobacter* and *Gluconobacter*. In The *Prokaryotes: An Evolving Electronic Resource for the Microbiological Community* (2nd edition, release 3.0) (Dworkin, M., ed), Springer-Verlag
48 Lomans, B.P. *et al*. (2001) Obligate sulfide-dependent degradation of methoxylated aromatic compounds and formation of methanethiol and dimethyl sulfide by a freshwater sediment isolate, *Parasporobacterium paucivorans* gen. nov., sp. nov. *Appl. Environ. Microbiol.* 67, 4017–4023
49 Bollmann, A. and Laanbroek, H.J. (2001) Continuous culture enrichments of ammonia-oxidizing bacteria at low ammonium concentrations. *FEMS Microbiol. Ecol.* 37, 211–221
50 Aakra, A. *et al*. (1999) An evaluated improvement of the extinction dilution method for isolation of ammonia-oxidizing bacteria. *J. Microbiol. Methods* 39, 23–31