

Microbial O₂ production in Nijmegen

Their Own Special Way

An international team, led by Mike Jetten's group in Nijmegen, discovered a novel pathway to produce oxygen from nitrate. Protagonist is a bacterium that uses the resulting O₂ to oxidize methane in an anaerobic environment.

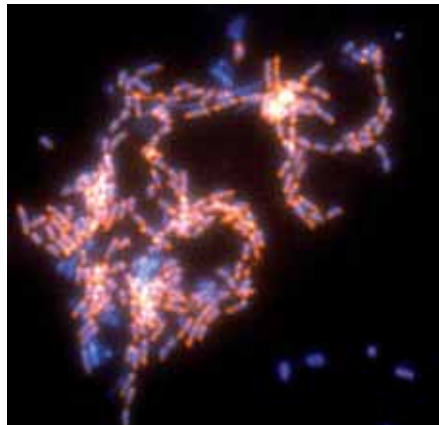
The year 2010 has been declared the International Year of Biodiversity. Yet it is generally accepted that we know only a small fraction of all the living beings that inhabit this planet. In this regard, we still have a lot to learn about microorganisms, in particular. The information we have about them is mainly based on the few species that can be grown on a plate but what about those who are reticent to traditional culturing methods?

Mike Jetten, professor in the Department of Microbiology at Radboud University Nijmegen, has been interested in those "exotic organisms" for almost two decades. "Other people said they should exist in nature and we wanted to have them in the lab," he expresses. This has not been an easy job but the effort has led to new findings about bacteria that survive by employing unexpected metabolic pathways. *Methylo-mirabilis oxyfera* is one of them: a methane consumer whose name alludes to the marvellous biochemistry behind it.

Breaking with old beliefs

Making a living by oxidizing methane was, for a long time, believed to be limited to specific bacteria living in aerobic conditions. During the last decades, however, experimental evidence has shown that this process also takes place in anoxic environments. The first anaerobic methane oxidation described was found to be mediated by methane-consuming archaea and sulphate-reducing bacteria.

In 2006, Jetten and his colleagues in Nijmegen reported that the chemical transformation was not only possible with sulphate but also with nitrate (*Nature* 440: 918-21). They took samples from anoxic freshwater sediments of ditches in The Netherlands and isolated a microbial consortium capable of oxidizing methane coupled to denitrification. The phylogenetic analysis revealed that the consortium was dominated by a bacterium belonging to a novel phylum and, in lower amounts, by an archaeon distantly related to the previously described methanotrophic Archaea. The discovery of this new microbial sink for anaerobic methane



The 'protagonist',
Methylo-mirabilis oxyfera,...

oxidation disclosed an intriguing new metabolic pathway but the best was yet to come.

Katharina Ettwig, one of the authors of the recently published article describing *M. oxyfera*'s pathway (*Nature* 464: 543-8), was in those days in the first years of her PhD. She started working with this sample with no significant results. "The culture was not really growing well. Then I saw the Archaea disappearing and I really thought that something was going wrong," she says. At that time, the only known anaerobic methane oxidation required Archaea. "It took me some time to realise that it was necessary to discard old ideas and accept that somehow this was working in a different way."

In 2008, she published an article that indeed showed that Archaea were not need-



... and the ditch in Ooijpolder where first author Katharina Ettwig collects them.

ed to oxidize the methane using nitrate. (*Environ. Microbiol.* 10: 3164-3173)

Archaea were not playing a role here, so how was the bacterium alone capable of performing anaerobic methane oxidation? In order to get further information, the complete genome of *M. oxyfera* was sequenced and assembled by Genoscope, the French National Sequencing Centre. Denitrification was clearly taking place and, therefore, this genome could be expected to include the classical pathway involved. Genes encoding most of the enzymes required were indeed found and expressed. However, the enzymes involved in the last step, in which nitrous oxide (N₂O) is converted into N₂, were almost missing completely.

Expect the unexpected

The biggest surprise, however, came when searching for the enzymes responsible for the pathway of methane oxidation. One of the initial suggestions was that anaerobic alkane enzymes were activating the compound but they were not present in the genome. "At the beginning we had very different hypotheses, which were based on other anaerobic microorganisms that do not use oxygen," says Jetten. However, finding that the genome encoded the complete pathway for aerobic methane oxidation was not one of them. Oxygen was totally absent in the culture and the presence of these genes now looked like a complicated riddle.

On the one hand, the step from N₂O to N₂ seemed to be absent in the denitrification pathway, whereas the oxygen appeared to come from somewhere. Based on this information, Ettwig *et al.* proposed a previously undescribed pathway that could explain these paradoxes: the nitrite (NO₂⁻) is first converted to nitrite oxide (NO), as it occurs in the classical denitrification process but then the two molecules of NO resulting from the reaction are converted into N₂ and O₂. This would require a hitherto unknown enzyme: a 'NO dismutase'. The proposal implied something totally unexpected and, hence, was received with scepticism – even in Jetten's group.

The genome alone doesn't assure that something is really happening. "You can have the gene but it's not expressed and you can have the inventory but not use it," says Jetten. Therefore, further experimental evidence was required. His co-workers first demonstrated that the methane oxidation activity was indeed dependent on nitrite. Another relevant question was whether N_2O was an intermediate in the process studied – and the subsequent results actually favoured the hypothesis that N_2 was not being produced by the classic denitrification pathway. The role of the enzyme involved in the first step of the aerobic oxidation, a methane monooxygenase, was also addressed and, again, the experiments supported the existence of the proposed pathway.

The idea that sounded somehow crazy at the beginning had thus gained consistent support from the genome and the sub-



Mike Jetten (front) and his Nijmegen microbiologists.

sequent experiments: *M. oxyfera* produced its own intracellular oxygen and the NO dismutase seemed to be a reasonable explanation for that.

International team

The whole project was led by Mike Jetten and Marc Strous, a former assistant professor at Radboud University Nijmegen who has recently moved to the Max Planck Institute (MPI) for Marine Microbiology in Bremen. The work, however, required the collaboration of teams from three countries: the Netherlands, France and Germany. Jetten talks about the challenges of working with different teams. "It's more complicated than working with people in your own lab, so you need a lot of communication. But it's also very necessary: all the people contribute complementary skills." The genome was sequenced at the French genome institute 'Genoscope', while the research group at the MPI in Bremen carried out the experiments for testing nitrogenous intermediates in the denitrification pathway used by *M. oxyfera*.

Ettwig agreed with the fact that, in general, this collaboration went very well. "It

always takes more time than if you go next door with your draft and discuss it. In the last phase there were even days where I had up to 50 or 60 e-mails. But it wasn't problematic at all." Mike added that it was a definite advantage to experience collaborating with other teams and, at the end, the results were very rewarding for all the participants.

Evolutionary Implications

One of the interesting questions opened up by this research is whether this pathway played a role during the evolution of metabolism on early Earth. There is evidence that in the Hadean and Archaean atmosphere, methane was a very important gas. Moreover, scientists have found isotopic signals from about 2.7 billion years ago that suggest methanotrophy was taking place. "It is rather certain that, by that

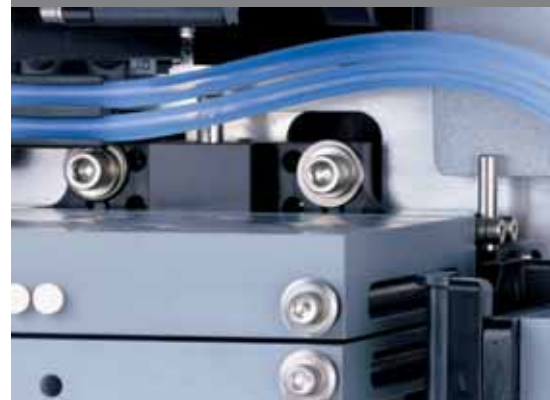
time, methane was oxidized by microorganisms but it is simply unclear at the moment what the oxidant was," Ettwig explains. On the other hand, Jetten mentions that nitrogen oxides might have been limited then but certainly present. Therefore, it cannot be excluded that this intracellular oxygen production could have preceded the Great Oxidation Event, approximately 2.4 billion years ago.

An alternative proposal is that this pathway could have evolved shortly after the oxygenation of the Earth. At that moment, more oxidizing forms of nitrogen were available and the pathway could have been a way to exploit them.

A lot of questions remain but looking for the existence and role of the putative NO dismutase could help in tracing back this pathway. It is certainly a difficult problem to address, as Jetten affirms, but unravelling the mystery looks very promising. Are there more genomes encoding the enzyme? How far is this enzyme phylogenetically distributed?

It seems that life's diversity won't stop surprising us.

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