

Multi-Isotope Imaging Mass Spectrometry (MIMS) of a Nitrogen-Fixing Bacterium

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Biological nitrogen fixation (diazotrophy) converts molecular nitrogen (N₂), which cannot be assimilated by plants or animals, into more biologically available form of ammonium. This metabolic pathway, which occurs only in a small percentage of prokaryotic microorganisms and is absent from eukaryotes, provides a principal source of combined nitrogen for the biosphere and helps to alleviate nitrogen limitation for growth in agriculture and in many natural ecosystems. Diazotrophic prokaryotes may be found in marine and aquatic environments as well as soils. Moreover, many plants and some animals may exploit the nitrogen fixing capabilities of bacteria through specific symbiotic associations, e.g. Rhizobium species within root nodules of leguminous plants or diazotrophic bacteria proposed to occur in digestive systems of termites and within the cells of marine shipworms and diatoms. Biological nitrogen fixation provides a significant fraction of the total nitrogen required for food production by agriculture and fisheries worldwide and thus it is difficult to underestimate its importance to human health and nutrition. Here we report the heterogeneous distribution of nitrogen fixation among population of *Teredinibacter turnerae* bacteria imaged and measured with MIMS.

Teredinibacter turnerae is diazotrophic marine bacterium that can be isolated from the tissues of wood-boring marine bivalves (family Teredinidae) and grown in pure culture. Cells of *T. turnerae* were grown in sealed 16 ml Hungate tubes containing 15.5 mls of simple salt medium (SBM amended with 0.5% sucrose without combined nitrogen) allowing a headspace of 0.5 ml of atmospheric gas. After three days the headspace gas was removed by displacement with fresh medium and replaced with 0.5 ml of ¹⁵N gas (99%). Cells were then incubated at 22°C with gentle shaking for 0, 0.25, 0.5, 1, 2, 4, 8, 16, and 32 hr. After incubation, cells were sedimented by centrifugation (12,000 x g, 10 min, 4°C) and fixed by re-suspension in 500 µl 3.7% formaldehyde in seawater for 30 min. Cells were then washed by sedimentation (14,000 x g, 10 min, room temp) and re-suspension first in 50% EtOH and again in distilled water. Cells were finally resuspended in 10 µl of distilled water and 2 µl was applied to the silicon wafer and air-dried at 37°C. Cells of *E. faecalis* were prepared identically except that cells were grown in Tryptic Soy Broth medium (Bacto) at 37 °C for 2.5 hours (~ 8 doublings).

Mass images of *Teredinibacter turnerae* (nitrogen fixing bacteria) and *Enterococcus faecalis* (does not fix nitrogen) cultured for 120 hours in ¹⁵N atmosphere were recorded at mass ¹²C¹⁴N, ¹²C¹⁵N, ¹²C and ¹³C. *Teredinibacter turnerae*, a nitrogen-fixing bacterium, has a rod appearance. It is barely visible at mass ¹²C¹⁴N but it is seen as intensely labelled at mass ¹²C¹⁵N because it has used gaseous ¹⁵N to build its molecular constituents. On the contrary, *Enterococcus faecalis*, a bacterium that does not fix nitrogen, is visible as grapes of cocci at mass ¹²C¹⁴N and is barely visible at mass ¹²C¹⁵N because it does not use gaseous nitrogen and did not incorporate ¹⁵N above its natural ratio. *Teredinibacter turnerae* incorporated an enormous quantity of ¹⁵N with a ¹²C¹⁵N/¹²C¹⁴N isotope ratio at least 100 times higher than the natural ratio. The heterogeneity of nitrogen fixation among bacteria within a population of *Teredinibacter turnerae* cultured 32 hours in ¹⁵N gas is demonstrated with the Hue Saturation Intensity (HSI) images of the ¹²C¹⁵N/¹²C¹⁴N ratios. They reveal the location and the distribution of ratio values, i.e. of nitrogen fixation, among the bacteria within the analyzed field, spanning a broad range, from a maximum 19 fold higher to a minimum 43% higher than the natural ratio.

We also found large differences of ^{15}N incorporation in *Teredinibacter turnerae* (cultured 96 hours in ^{15}N atmosphere) visible and measured among a few touching bacteria, or even within a single bacterium.

In conclusion MIMS methodology is a powerful tool to study the metabolism of individual bacteria within a population and will be an invaluable tool in the investigation of diazotrophy in natural environments and in exploring the activity of diazotrophic symbionts in the tissues of plants and animals.

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