Investigation into the role of the truncated denitrification chain in *Rhizobium sullae* strain HCNT1

S. Casella†, J.P. Shapleigh‡, A. Toffanin‡ and M. Basaglia

†Dipartimento di Biotecnologie Agrarie, University of Padova, Agrigolis, Via dell’Università 16, 35020 Legnaro, Padova, Italy, ‡Department of Microbiology, Cornell University, 257A Wing Hall, Ithaca, NY 14853, U.S.A., and ‡Dipartimento di Biologia delle Piante Agrarie, University of Pisa, Via del Borghetto 80, 56124 Pisa, Italy

**Abstract**

Most denitrifying bacteria reduce nitrate to the inert gases nitrous oxide or nitrogen. A remarkable exception to this is *Rhizobium sullae* strain HCNT1, which catalyses only a single step in the denitrification pathway, the reduction of nitrite to the reactive molecule nitric oxide. Further study demonstrated that HCNT1 does not encode the genes for NO reductase. Prolonged incubation of HCNT1 under anoxic conditions revealed that the cells had reduced culturability but not viability when nitrite was present. This may indicate an adaptation to anoxic conditions to provide resistance to environmental stresses. A closely related strain of *R. sullae*, strain CC1335, which is unable to denitrify, was found to lose culturability but not viability irrespective of the presence of nitrite. When the gene for nitrite reductase was mobilized into CC1335, this increased culturability with or without nitrite. These results indicate that the presence of nitrite reductase can influence the long-term survival of *R. sullae* strains and may provide an explanation as to why HCNT1 possesses this unusual truncation of its denitrification electron transport chain.

Rhizobia are soil bacteria typically able to symbiotically interact with legume plants to produce nitrogen-fixing root nodules. While all rhizobia were once placed under the same genus, *Rhizobium*, nowadays the classification of rhizobia using molecular analysis has revealed a more complex relationship among strains. Several genera have been described and accepted (i.e. *Rhizobium, Allorhizobium, Azorhizobium, Mesorhizobium, Sinorhizobium* and *Bradyrhizobium*) with many species in each genus. However, within the same species, it is common to observe different strains with quite different physiological and biochemical profiles. While some traits are common among all rhizobia, selected traits such as denitrification seem to be randomly distributed among genera and species. Moreover, many species are complete denitrifiers (strains of *Bradyrhizobium japonicum*) [1] while some are only partial nitrogen oxide reducers (strains of *Rhizobium sullae*) [2]. While the evolutionary advantage deriving from the complete denitrification pathways may be easily explained, the advantage gained from expressing only a fragment of such a metabolic property has not been completely clarified.

An exceptional example of fragmentation of the denitrification pathway is given by *R. sullae*, formerly *Rhizobium* ‘hedysari’, a nitrogen-fixing bacterium that induces symbiotic nodule formation on the legume *Hedysarum coronarium* [3–6]. Some isolates belonging to this species have been shown to encode a copper-containing nitrite reductase [2,7].

Nitrite reductase reduces nitrite to nitric oxide (NO). Nitrite reductase is encoded by *nirK*, which is closely related to nitrite reductases in true denitrifiers. Expression of *nirK* is atypical in that it does not require the presence of a nitrogen oxide, but only requires a decrease in oxygen concentration (below 16.5% air saturation) [2]. Reduction of nitrite by the HCNT1 strain results in the cessation of growth. This growth inhibition is likely to be due to the accumulation of toxic levels of NO [8]. Inhibition by NO was demonstrated by measuring NO accumulation and by the observation that inactivation of *nirK* eliminated the phenotypic traits associated with NO accumulation but did not cause other obvious phenotypic changes [8]. These results indicate that there is no NO reductase activity in the cells, and this was also confirmed by a PCR-based approach using specific primers to detect the presence of *norB*, yielding no amplification product. There is no nitrate reductase activity since there is no O2 uptake inhibition if nitrate is added to the medium. No evidence consistent with the presence of an N2O reductase, such as growth on N2O, was found either. *R. sullae* strain HCNT1 is therefore an exceptional bacterium due to the radical truncation of its denitrification electron transport chain, having only one of the four terminal reductases required for complete denitrification.

The role of nitrite reductase in *R. sullae* has yet to be elucidated. These bacteria convert stable molecular nitrogen into combined forms via nitrogenase during intranodular nitrogen fixation, and at the same time they convert combined nitrogen back into gaseous molecules that are lost into the atmosphere. The most obvious advantage expected is that during free life, even in the absence of oxygen, they can
survive by anaerobic respiration of nitrite, but this hypothesis
is unsatisfactory because strain HCNT1 cannot grow as a
denitrifier. In other words, no proton translocation occurs
during the reduction of nitrite to NO. Moreover, since Nir
activity will inhibit oxygen respiration as long as nitrite is
present due to the binding of NO to terminal oxidases,
nitrite reduction also inhibits the only other means by which
cells can conserve energy. Once the available nitrite has been
reduced, O₂ consumption will resume [2]. Therefore there is
no obvious bioenergetic benefit as a consequence of nitrite
respiration.

Since nitrite reductase expression only occurs under micro-
oxic conditions, it is possible that the enzyme plays some role
within the root nodule, which is a low-oxygen environment.
For instance, it is well known that nitrite may be toxic to
nitrogenase and may bind to leghaemoglobin. Thus nitrite
reduction in HCNT1 may be as a nitrite detoxification
strategy during intranodular life [9]. This role for Nir is only
valid if the unreactive intermediate N₂O is the end-product
of this reduction. The lack of Nor means that detoxification
leads to conversion of nitrite into the even more reactive
NO. Since a more toxic compound is produced, the nitrite
detoxification hypothesis seems unlikely [10]. It has also
been observed that strains of the same species that lack Nir
(e.g. strain CC1335) can nodulate and fix nitrogen at the
same level [3]. Comparisons of nodulation efficiency, plant
growth and nitrogen fixation have not revealed any significant
differences between wild-type and nitrite reductase-deficient
strains under any of the conditions tested so far. However,
this should be further investigated because this strain was
found to gradually lose nodulation ability under laboratory
conditions. Taken together, these results do not identify
any obvious role for the truncated denitrification chain in
R. sullae.

The presence and expression of nitrite reductase has
been also investigated as a strategy to reduce the energy
content in the bacterial cell in order to induce dormancy.
Some evidence has been provided suggesting a link between
nitrite reductase activity and the viability and culturability
of the cells. In response to a number of environmental
stresses, many bacterial species, including *Vibrio vulni-
ferus*, *Sinorhizobium meliloti*, *Micrococcus luteus*, *Escherichia coli*
and *Helicobacter pylori*, enter the VBNC (viable-but-not-
culturable) status [11]. In this metabolic state, they lose their
ability to grow on media that usually sustain them and
undergo such physiological and morphological changes as
increased resistance to several physical and chemical factors,
and changes in protein and lipid content. The use of specific
fluorescent dyes such as Syto 9, CTC (5-cyano-2,3-di-4-
tolyl-tetrazolium chloride), Acidine Orange and propidium
iodide has made possible a more accurate evaluation of the
viability and the metabolic state of microbes [12]. Experiments
performed with *S. meliloti* 41, a rhizobium nodulating
*Medicago sativa*, showed that it enters VBNC status in liquid microcosms when O₂ is depleted from the
atmosphere of the incubation mixture [13,14]. Plasmid-borne,
firefly-derived, luciferase gene (*luc*) was inserted and stably
inherited in *S. meliloti* 41 (pRP4-*luc*) as a reporter gene. The strain obtained, *S. meliloti* 41 pRP4-*luc*, and its parental
strain served as a model system for VBNC experiments both
*in vitro* and, thanks to the marker gene inserted, also in soil
samples and in the plant system. This strain has been found to
recover its viability under certain conditions, but only
at a given ratio to the number of metabolically active cells
([14] and M. Basaglia, S. Povolo and S. Casella, unpublished
work). *R. sullae* strain HCNT1 enters the same VBNC status
when oxygen is limiting, but only when nitrite is present and
Nir is expressed allowing for production of NO [13]. The
hypothesis that expression of *nirK* by HCNT1 may induce
the VBNC status has been investigated through a comparison
of *S. meliloti* and *R. sullae* in order to verify the possible
connection of *nirK* with the VBNC status. It was found
that nitrite reductase activity may reduce energy content
but it was unclear whether this was an indirect consequence
of the generation of nitric oxide or a desired result that
would prolong cell viability under certain conditions. While
the energy content of *S. meliloti* decreased under anaerobic
conditions, leading the strain to the VBNC status, HCNT1
only reduced its internal energy under anaerobic conditions
when nitrite was present. NO does not accumulate at lethal
levels because differential staining of the cells during oxygen
uptake experiments in the presence of nitrite revealed that
they were still alive, although the CFU (colony forming units)
number had dramatically decreased [13]. An explanation
for this result is that NO produced by nitrite reduction
induced VBNC status, making the cells more stress-resistant.
Such a metabolic state confers to the cells the ability to
withstand stresses, including anoxic conditions, antibiotic
effects or nutrient depletion. When suitable environmental
conditions reoccur, it has been demonstrated that some
VBNC bacterial populations, including *S. meliloti*, can
recover their culturability ([14], and M. Basaglia, S. Povolo
and S. Casella, unpublished work).

To learn more about the role of Nir in *R. sullae*, we
examined the VBNC status of CC1335, which does not
possess *nirK*. Prolonged incubation of CC1335 under anaer-
bic conditions resulted in a significant decrease in CFU, but
DAP1 (4,6-diamidino-2-phenylindole) staining indicates that
the cells are still alive. Therefore, unlike HCNT1, CC1335 can
enter VBNC without Nir activity. In order to provide support
for the hypothesis that expression of *nirK* is favourable
because it induces the VBNC state, *nirK* from HCNT1 was
mobilized into CC1335, a strain of *R. sullae* that lacks *nirK*,
and the resulting phenotype was studied. The presence of *nirK*
in CC1335 resulted in a phenotype similar to that of HCNT1
under anoxic incubation: there is no anaerobic growth, but
the number of cells with the ability to form a colony does
not decrease significantly for an extended time. However,
CC1335 wild-type does not behave like HCAT2 (the Nir-
deficient strain of HCNT1). CC1335 rapidly reduces its
internal energy and the number of cells capable of forming
a colony upon re-incubation under aerobic conditions. This
suggests that strain CC1335 does not need Nir to reduce its
internal energy (culturability) under anoxic conditions. The
difference between these strains is intriguing and suggests that HCNT1 has the ability to survive for long periods without
loss of ability to form CFU, but also that the presence of Nir leads to a reduction in the internal energy to VBNC levels,
making the strain more resistant to a variety of stresses.

References

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