

# Proof Delivery Form

**Journal and Article number:** BST 353

**BST reference:** BST2005/799

**Number of colour figures:** Nil

**Number of pages (not including this page):** 3

---

## Biochemical Society Transactions

Please print out your proof, mark any corrections needed, and return it, together with the offprint order form, by FAX to +44 (0)20 7280 4169 as soon as possible (no later than 3 days after receipt). Queries raised by the sub-editor are listed below; the text to which the queries refer is flagged in the margins of the proof. Please ensure that you answer these queries.

- You are responsible for correcting your proofs! Errors not found may appear in the published journal.
- The proof is sent to you for correction of typographical errors only. Revision of the substance of the text is not permitted.
- Please answer carefully any queries from the sub-editor.
- A new copy of a figure must be provided if correction is required.

### Notes:

1. The quality of half-tone and colour will be checked by the editorial office.
  2. If you have any queries, please telephone the editorial office/by email ([editorial@portlandpress.com](mailto:editorial@portlandpress.com)) or by telephone on 020 7280 4110 (+44 20 7280 4110 from outside the UK).
- 

**Author queries:**

---

**Typesetter queries:**

---

**Non-printed material:**

# Order for Offprints for *Biochemical Society Transactions*: Individual Articles

Ref. number

No. of pages

If you require offprints please complete and return this form with the proofs of your article to  
***Biochemical Society Transactions* Editorial Office, Portland Press Ltd, Third Floor, Eagle House, 16 Procter Street, London WC1V 6NX, UK**

Offprints must be ordered using this form. If an official order form from your organization is required, it may be sent on later (please quote the reference number above), but it will be assumed that any such order received is not an additional order. Free offprints are not available (with the exception of Medal Lectures) and offprints cannot be supplied after publication.

Offprints are despatched by surface mail about 2 weeks after publication (allow up to 6 months for delivery). If you wish them to be despatched by air mail please mark section 3 below and add 40% to the indicated price.

Title of paper

- 1** I do not wish to order offprints of my article
- 2** I wish to purchase  (minimum 50) offprints of my article at the price indicated overleaf
- 3** Please send my offprints by air mail (add 40% to cost)

We accept payment in:

- pound sterling cheques drawn on UK banks
- US dollar cheques drawn on US banks
- Euro cheques drawn on any European bank
- most credit and debit cards (not Diners Club)

For payment in \$US or Euros, please convert the charges shown over leaf (add 40% for air mail, if requested) at the current rate of exchange and add £15 for bank charges.

- 4** I enclose a remittance £/\$/€ (delete as appropriate) for  payable to Portland Press Limited
- Please invoice me at the address in section 5 (offprints will not be despatched until payment is received).
- I wish to pay by credit card.  
Payments may be made by Access/American Express/Eurocard/Mastercard/Visa/Visa Delta/Switch (delete as appropriate).

Account number

Expires \_\_\_\_\_ Start date \_\_\_\_\_ Issue Number (Switch only) \_\_\_\_\_

Cardholder's name   
*(as on card)*

Cardholder's address

Signature























**5. Name and address**   
*to which offprints should be sent*



















Number of pages	Number required [price in Pounds sterling (£)]				
	50	100	200	300	400
1-2	96	131	190	250	301
3-4	120	159	240	327	372
5-8	160	212	336	458	565
9-12	249	315	522	730	918

- Notes:**
1. For quantities not listed, please ask for a quotation
  2. For offprints containing colour printing, add £50 per 50 copies
  3. Prices revised November 2005

## Proof correction

Please indicate to the Editorial Office on the enclosed proof what needs to be corrected. The Editorial Office staff will then make the corrections on the separate proof that they will send to the Printers. It is therefore important to make clear to the Editorial Office what correction is needed, but it is not important how this is done. Corrections may be made in the text and margin in any of the commonly used systems (Continental European or American as well as British). The main points of the British system are given below.

Correction required	Marginal mark	Corresponding mark in text
Insert new material	Inserted material, followed by caret, 	
Delete		(Cross out what is not wanted)
Substitute new material for something typeset	Write new material followed by 	(Cross out material to be replaced)
Leave as printed		..... under material to remain
Insert space		 where space is required
Take out space (close up)		 around material to be closed up
Insert hyphen		 where hyphen is required
Change letter(s) to capital(s)		 under letter(s) required
Change letter(s) to small capital(s)		 under letter(s) required
Change to small letter(s)		(Encircle required letter(s))
Change to bold type		 under letter(s) to be altered
Change to italic type		 under letter(s) to be altered
Begin a new paragraph		 before first word of new paragraph

Correction required	Marginal mark	Corresponding mark in text
No new paragraph		 between paragraphs
Inferior (subscript) letter, e.g. x		 if to be inserted; cross out incorrect letter if a replacement
Superior (superscript) letter, e.g. p		 if to be inserted; cross out incorrect letter if a replacement
Transpose letters or words		 between letters or words
Indicate letters set upside-down		(Encircle letters to be altered)
Broken letter; replace by similar but undamaged character		(Encircle letters to be altered)
Insert parentheses		 where parentheses required
Insert square brackets		 where square brackets required
Insert apostrophe		 where apostrophe required
Insert quotation marks		 where quotation marks required

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

S. Casella<sup>\*1</sup>, J.P. Shapleigh<sup>†</sup>, A. Toffanin<sup>‡</sup> and M. Basaglia<sup>\*</sup>

<sup>\*</sup>Dipartimento di Biotecnologie Agrarie, University of Padova, Agripolis, Viale dell'Università 16, 35020 Legnaro, Padova, Italy, <sup>†</sup>Department of Microbiology, Cornell University, 257A Wing Hall, Ithaca, NY 14853, U.S.A., and <sup>‡</sup>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 sulae* 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. sulae*, strain CC1335, 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. sulae* 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 sulae*) [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. sulae*, 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 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 it 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 O<sub>2</sub> uptake inhibition if nitrate is added to the medium. No evidence consistent with the presence of N<sub>2</sub>O reductase, such as growth on N<sub>2</sub>O, was found as well. *R. sulae* 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. sulae* 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

**Key words:** bacteria, denitrification, electron transport chain, HCNT1, nitric oxide, *Rhizobium sulae*.

**Abbreviations used:** CFU, colony forming units; VBNC, viable-but-not-culturable.

<sup>1</sup>To whom correspondence should be addressed (email [sergio.casella@unipd.it](mailto:sergio.casella@unipd.it)).

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, O<sub>2</sub> 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 N<sub>2</sub>O 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. sultae*.

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 vulnificus*, *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), Acridine 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 O<sub>2</sub> 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. Povo and S. Casella, unpublished work). *R. sultae* 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. sultae* 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 anoxic conditions, leading the strain to the VBNC status, HCNT1 only reduced its internal energy under anoxic 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 [14,15].

To learn more about the role of Nir in *R. sultae*, we examined the VBNC status of CC1335, which does not possess *nirK*. Prolonged incubation of CC1335 under anaerobic conditions resulted in a significant decrease in CFU but DAPI (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. sultae* 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

- 1 Bedmar, E.J., Robles, E.F. and Delgado, M.J. (2005) *Biochem. Soc. Trans.* **33**, 141–144
- 2 Casella, S., Shapleigh, J.P. and Payne, W.J. (1986) *Arch. Microbiol.* **146**, 233–238
- 3 Casella, S., Gault, R.R., Reynolds, K.C., Dyson, J.R. and Brockwell, J. (1984) *FEMS Microbiol. Lett.* **22**, 37–45
- 4 Squartini, A., Dazzo, F.B., Casella, S. and Nuti, M.P. (1993) *Symbiosis* **15**, 227–238
- 5 Struffi, P., Corich, V., Giacomini, A., Benguedouar, A., Squartini, A., Casella, S. and Nuti, M.P. (1998) *J. Appl. Bacteriol.* **84**, 81–89
- 6 Squartini, A., Struffi, P., Doering, H., Selenska-Pobell, S., Tola, E., Giacobini, A., Vendramin, E., Velasquez, E., Mateos, P., Martinez-Molina, E. et al. (2002) *Int. J. Syst. Evol. Microbiol.* **52**, 1267–1276
- 7 Casella, S. (1988) in *Physiological Limitation and the Genetic Improvement of Symbiotic Nitrogen Fixation* (O’Gara, F., Manian, S. and Dervon, J.J., eds.), pp. 117–125, Kluwer Academic, Dordrecht
- 8 Toffanin, A., Wu, Q., Maskus, M., Casella, S., Abruña, H.D. and Shapleigh, J.P. (1996) *Appl. Environ. Microbiol.* **62**, 4019–4025
- 9 Casella, S., Shapleigh, J.P., Lupi, F. and Payne, W.J. (1988) *Arch. Microbiol.* **149**, 384–388
- 10 Basaglia, M., Toffanin, A., Shapleigh, J.P., Baldan, E. and Casella, S. (2004) in *COST Action 856: Ecological Aspects of Denitrification with Emphasis on Agriculture*, 25–28 March, Marburg, Germany, p. 56
- 11 McDougald, D., Scott, A.R., Weichert, D. and Kjelleberg, S. (1998) *FEMS Microbiol. Ecol.* **25**, 1–9
- 12 Basaglia, M., Toffanin, A., Corich, V., Vian, P., Concheri, G., Giacomini, A., Squartini, A., Nuti, M.P. and Casella, S. (1997) in *IMPACT 2 Contractors Meeting*, Granada, Spain, pp. 19–20
- 13 Toffanin, A., Basaglia, M., Ciardi, C., Vian, P., Povolo, S. and Casella, S. (2000) *Biol. Fertil. Soils* **31**, 484–488
- 14 Casella, S., Fontana, F., Povolo, S. and Basaglia, M. (2001) *ISME-9, 9th International Symposium on Microbial Ecology*, Amsterdam, The Netherlands, p. 191

---

Received 27 September 2005