The obligate aerobe *Streptomyces coelicolor* A3(2) synthesizes three active respiratory nitrate reductases

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Streptomyces coelicolor A3(2) synthesizes three membrane-associated respiratory nitrate reductases (Nars). During aerobic growth in liquid medium the bacterium was able to reduce 50 mM nitrate stoichiometrically to nitrite. Construction and analysis of a mutant in which all three *narGHJI* operons were deleted showed that it failed to reduce nitrate. Deletion of the gene encoding MoaA, which catalyses the first step in molybdenum cofactor biosynthesis, also prevented nitrate reduction, consistent with the Nars being molybdoenzymes. In contrast to the triple *narGHJI* mutant, the *moaA* mutant was also unable to use nitrate as sole nitrogen source, which indicates that the assimilatory nitrate reductases in *S. coelicolor* are also molybdenum-dependent. Analysis of *S. coelicolor* growth on solid medium demonstrated that Nar activity is present in both spores and mycelium (hypha). Development of a survival assay with the nitrate analogue chlorate revealed that wild-type *S. coelicolor* spores and mycelium were sensitive to chlorate after anaerobic incubation, independent of the presence of nitrate, while both the *moaA* and triple *nar* mutants were chlorate-resistant. Complementation of the triple *nar* mutant with the individual *narGHJI* operons delivered on cosmids revealed that each operon encoded an enzyme that was synthesized and active in nitrate or chlorate reduction. The data obtained from these studies allow a tentative assignment of Nar1 activity to spores, Nar2 to spores and mycelium, and Nar3 exclusively to mycelium.

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INTRODUCTION

Streptomyces coelicolor A3(2) is a soil-dwelling, obligately aerobic high-GC Gram-positive bacterium belonging to the phylum Actinobacteria (Hopwood, 2006). As is common to many genera belonging to the actinomycetes, *Streptomyces* spp. grow by filamentous tip growth, forming both vegetative and aerial mycelia (hyphae). The aerial hyphae develop into exospores, which can then re-enter the developmental cycle in the presence of appropriate nutrients. Surprisingly for an obligate aerobe, the genome of *S. coelicolor* A3(2) encodes a number of enzymes with functions that are usually ascribed to anaerobic metabolism

in facultatively and strictly anaerobic bacteria (Bentley *et al.*, 2002). Amongst these enzymes are three respiratory nitrate reductases (Nars) encoded by *narGHJI* (van Keulen *et al.*, 2003, 2005). Nars have also been identified and characterized in other Gram-positive bacteria, such as *Bacillus subtilis* (Hoffmann *et al.*, 1995) and *Mycobacterium tuberculosis* (Sohaskey & Wayne, 2003). In *B. subtilis*, Nar allows the bacterium to grow by anaerobic respiration (Hoffmann *et al.*, 1998), while in the obligate aerobe *M. tuberculosis*, Nar has been shown to be an important virulence factor that is induced upon entry into the non-replicating persistent stage 1 (NRP-1), which is induced by depletion of oxygen (Wayne & Hayes, 1998). Nar is also required for nitrate assimilation aerobically (Malm *et al.*, 2009). The fact that *S. coelicolor* cannot grow anaerobically (van Keulen *et al.*, 2007) raises the questions of whether the three Nars are synthesized in *S. coelicolor* and what the roles of these enzymes in the biology of this bacterium might be.

Respiratory Nars are membrane-associated, multi-subunit enzymes that have a heterotrimeric ($\alpha\beta\gamma$) structure, which

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Abbreviations: Apra, apramycin; bis-MGD, bis-molybdopterin guanine dinucleotide; Cm, chloramphenicol; Kan, kanamycin; MV, methyl viologen; Spc, spectinomycin; Strep, streptomycin.

A supplementary table, showing the oligonucleotides used in this study, is available with the online version of this paper.

may form a dimer *in situ* (Bertero *et al.*, 2003; Jormakka *et al.*, 2004; Richardson *et al.*, 2001; Zumft, 1997). They catalyse the reduction of nitrate to nitrite and link this process to the generation of a proton motive force, permitting nitrate to be used as an alternative electron acceptor (Richardson *et al.*, 2001). The cytoplasmically oriented ~135 kDa NarG (α) subunit contains a bis-molybdopterin guanine dinucleotide (*bis*-MGD) cofactor at its active site, as well as a [4Fe-4S] cluster. A dedicated set of proteins, abbreviated with the acronym Mo-, for the synthesis of *bis*-MGD is also required (Schwarz *et al.*, 2009). The NarH (β) subunit is approximately 60 kDa in size and binds four [4Fe-4S] clusters, and the 27 kDa membrane-integral NarI (γ) subunit has a di-*b*-type cytochrome and forms the quinol dehydrogenase component of the enzyme complex (Blasco *et al.*, 2001; Rothery *et al.*, 2001). The *narJ* gene encodes a dedicated chaperone proposed to have a variety of functions in maturation of the Nar holoenzyme (Lanciano *et al.*, 2007). The location of the active site of Nar on the cytoplasmic side of the membrane means that the nitrate anion must be transported into the cell before it can be reduced. The reaction product of Nar, nitrite, is toxic, so must be either exported or reduced in the cytoplasm to ammonium (Bancroft *et al.*, 1979). Export of nitrite is catalysed by the transporter NarK, which falls into two main functional types: a nitrate/nitrite antiporter–nitrate/proton symporter or a nitrite exporter (Goddard *et al.*, 2008; Wood *et al.*, 2002). Based on characteristic sequence motifs, the *S. coelicolor* genome encodes a NarK of each class (Bentley *et al.*, 2002).

As well as the respiratory Nar enzymes there are two further classes of nitrate reductase called Nas (nitrate assimilation) and Nap (nitrate reduction in the periplasm) (Richardson *et al.*, 2001). When present, the soluble Nas enzyme allows nitrate to be used as a nitrogen source, while the periplasmically located respiratory Nap enzyme is restricted to Gram-negative bacteria and couples nitrate reduction to quinol oxidation. The function of the Nap enzyme is probably to balance the redox potential of the electron transport chain and to provide a means of dissipating excess reducing equivalents (Ellington *et al.*, 2002). Members of all three enzyme classes are molybdoproteins.

Usually, micro-organisms that synthesize Nars can be classified as denitrifiers or ammonifiers (Richardson *et al.*, 2001; Zumft, 1997). During denitrification, nitrate or nitrite is reduced completely to dinitrogen via the intermediates nitric oxide and nitrous oxide. The reactions from the reduction of nitrite occur in the periplasm of Gram-negative bacteria. Nitrate ammonification involves the further reduction of nitrite by a soluble cytoplasmic nitrite reductase to ammonia (Richardson *et al.*, 2001). As a Gram-positive bacterium, *S. coelicolor* lacks a periplasm and therefore does not have either a Nap enzyme or the classical enzymes of denitrification (see also Bentley *et al.*, 2002). *S. coelicolor* does, however, encode a sirohaem-type soluble nitrite reductase similar to NirB from *Escherichia coli*, suggesting that, in principle, it could perform nitrate

ammonification; however, recent findings suggest that synthesis of this enzyme might occur under conditions of nitrogen limitation (Tiffert *et al.*, 2008). That nitrate can be reduced aerobically is not unprecedented, as a number of Gram-negative bacteria can carry out partial or complete denitrification aerobically (Bell *et al.*, 1990; Robertson & Kuenen, 1984); however, these bacteria probably use a Nap enzyme to catalyse the initial step of this pathway (Richardson *et al.*, 2001). There have also been reports of *Streptomyces* spp. which can perform partial denitrification, although the mechanisms remain to be clarified (Albrecht *et al.*, 1997; Chèneby *et al.*, 2000; Kumon *et al.*, 2002; Shoun *et al.*, 1998). As *S. coelicolor* has three copies of the *narGHJ* operon, this study aimed to determine whether the bacterium reduces nitrate during aerobic growth, what the product of nitrate reduction is, and whether all three enzymes are synthesized and functional.

METHODS

Bacterial strains and culture conditions. Media and culture conditions for *E. coli* and *S. coelicolor* were the same as those described elsewhere (Datsenko & Wanner, 2000; Kieser *et al.*, 2000). Strains are listed in Table 1. *E. coli* DH5 α (Stratagene) was used as a host for plasmid constructions. *E. coli* BW25113 (Datsenko & Wanner, 2000) was used to propagate the recombination plasmid pIJ790 and *S. coelicolor* cosmids (Redenbach *et al.*, 1996), and *E. coli* DH5 α carrying pCP20 (designated BT340) was used for FLP-mediated, site-specific recombination (Datsenko & Wanner, 2000; Gust *et al.*, 2003). BW25113 and BT340 were grown in super optimal broth (SOB) and Luria–Bertani (LB) medium, respectively. *E. coli* ET12567(pUZ8002) (Gust *et al.*, 2003) was the non-methylating plasmid donor strain for intergeneric conjugation with *S. coelicolor* strain M145 (Bentley *et al.*, 2002). Apramycin (Apra; 50 μ g ml⁻¹), carbenicillin (Carb; 100 μ g ml⁻¹), chloramphenicol (Cm; 25 μ g ml⁻¹), kanamycin (Kan; 50 μ g ml⁻¹) or spectinomycin (Spc; 25 μ g ml⁻¹), all from Sigma, or viomycin (Vio; 30 μ g ml⁻¹), were added to growth media when required. Vio was a kind gift from Paul Herron (University of Strathclyde). Arabinose (10 mM final concentration, Sigma) was added to SOB medium from a 1 M sterile-filtered stock solution as indicated to induce genes under the control of the P_{BAD} promoter (Guzman *et al.*, 1995).

S. coelicolor A3(2) wild-type strain M145 and mutant derivatives (Table 1) were grown on soya flour mannitol (SFM), R5 or Difco Nutrient Broth (DNB) agar media as indicated, or in liquid tryptic soy broth (TSB) containing 100 mM MOPS, pH 7.0, yeast extract/malt extract (YEME)/TSB (1:1) or DNB broth supplemented with antibiotics to maintain selection when appropriate. The minimal medium used to examine growth with nitrate as a nitrogen source was as follows: 50–100 mM MOPS, pH 7.0, 10 mM sodium/potassium phosphate, pH 7.0, 85 mM NaCl, 5 mM MgSO₄, 11 mM glucose and 1 ml l⁻¹ trace element solution SLA prepared exactly as described by Hormann & Andreesen (1994). Agarose (1%, w/v, Sigma) was used to solidify the medium. The nitrogen sources used were added to the following concentrations: 10 mM ammonium sulfate; 1 g l⁻¹ Casamino acids + 10 mM Na₂SO₄; 10 mM NaNO₃ + 10 mM Na₂SO₄; or 10 mM Na₂SO₄ when no nitrogen source was supplied. The growth medium composition and standard culture techniques used have been described elsewhere (Kieser *et al.*, 2000).

Generation of *S. coelicolor* growth curves. Cultures were generally inoculated with fresh spore stocks or with spore stocks stored at -80 °C (in 20%, w/v, glycerol), such that the viable cell count was reproducibly 2–4 $\times$ 10⁶ c.f.u. ml⁻¹. The c.f.u. of the spore

Table 1. Strains and vectors used in this study

Strain	Genotype and characteristics	Reference or source
<i>S. coelicolor</i> A3(2) strains		
M145 (wild-type)	SCP1 [−] SCP2 [−]	Kieser <i>et al.</i> (2000)
NM3 (<i>nar2</i>)	M145 ΔSCO0216–0219:: <i>aac(3)IV</i> (deletion of 6531 bp removing <i>narG2H2J2I2</i>)	This study
NM4 (<i>nar2</i>)	M145 ΔSCO0216–0219:: <i>aac(3)IV</i> (deletion of 6531 bp removing <i>narG2H2J2I2</i>)	This study
NM68 (<i>nar2 nar3</i>)	NM3 ΔSCO4947–4950:: <i>aadA</i> (deletion of 6497 bp removing <i>narG3H3J3I3</i>)	This study
NM70 (<i>nar2 nar3</i>)	NM4 ΔSCO4947–4950:: <i>aadA</i> (deletion of 6497 bp removing <i>narG3H3J3I3</i>)	This study
NM90 (<i>nar2 nar3</i>)	NM68 Apra ^R with an unmarked <i>narG3H3J3I3</i> deletion	This study
NM100 (<i>nar2 nar3</i>)	NM70 Apra ^R with an unmarked <i>narG3H3J3I3</i> deletion	This study
NM92 (<i>nar1 nar2 nar3</i>)	NM90 ΔSCO6532–6535:: <i>aadA</i> (deletion of 6209 bp removing <i>narGHJI</i>)	This study
NM102 (<i>nar1 nar2 nar3</i>)	NM100 ΔSCO6532–6535:: <i>aadA</i> (deletion of 6209 bp removing <i>narGHJI</i>)	This study
NM1821 (<i>moaA</i>)	M145 ΔSCO1821:: <i>aac(3)IV</i>	This study
NM165	NM92 with cosmid St5C7 (<i>narGHJI</i> ⁺)	This study
NM166	NM92 with cosmid St5C7Δnar1	This study
NM167	NM92 with cosmid StJ12 (<i>narG2H2J2I2</i> ⁺)	This study
NM168	NM92 with cosmid StJ12Δnar2	This study
NM169	NM92 with cosmid StK13Δnar3	This study
NM170	NM92 with cosmid StK13 (<i>narG3H3J3I3</i> ⁺)	This study
NM1821pMS82	NM1821 with integrative vector pMS82	This study
NM1821pMS1821	NM1821 with integrative vector pMS1821 (<i>moaA</i> ⁺)	This study
<i>E. coli</i> strains		
DH5α	Used for routine cloning	
ET12567(pUZ8002)	<i>dam dcm</i> ; with <i>trans</i> -mobilizing plasmid pUZ8002	MacNeil <i>et al.</i> (1992)
BW25113	K12 derivative: Δ <i>araBAD</i> , Δ <i>rhaBAD</i>	Datsenko & Wanner (2000)
BT340	DH5α/pCP20	Cherepanov & Wackernagel (1995)
Plasmids and cosmids		
pIJ790	λRED (<i>gam</i> , <i>bet</i> , <i>exo</i>), <i>cat</i> , <i>araC</i> , <i>rep101</i> ^{ts}	Gust <i>et al.</i> (2003)
pIJ773	<i>aac(3)IV</i> (Apra ^R) + <i>oriT</i>	Gust <i>et al.</i> (2003)
pIJ778	<i>aadA</i> from Ω-fragment (Spec ^R , Strep ^R) + <i>oriT</i>	Gust <i>et al.</i> (2003)
pCP20	FLP-recombination plasmid: <i>flp</i> , <i>bla</i> , <i>cat</i> , <i>rep101</i> ^{ts}	Cherepanov & Wackernagel (1995)
pMS82	ΦBT1 <i>attP-int</i> derived integration vector for the conjugal transfer of DNA from <i>E. coli</i> to <i>Streptomyces</i> (Hyg ^R)	Gregory <i>et al.</i> (2003)
pMS1821	pMS82 <i>moaA</i> ⁺	This study
St5C7	<i>narGHJI</i> ⁺	Redenbach <i>et al.</i> (1996)
St5C7Δnar1	Deletion of 6209 bp removing <i>narGHJI</i>	This study
StJ12	<i>narG2H2J2I2</i> ⁺	Redenbach <i>et al.</i> (1996)
StJ12Δnar2	Deletion of 6531 bp removing <i>narG2H2J2I2</i>	This study
StK13	<i>narG3H3J3I3</i> ⁺	Redenbach <i>et al.</i> (1996)
StK13Δnar3	Deletion of 6497 bp removing <i>narG3H3J3I3</i>	This study

*Hygromycin resistance.

stocks was regularly checked on LB agar plates according to Kieser *et al.* (2000). Standard 100 ml Erlenmeyer flasks were pre-treated with a 5% (v/v) solution of 1,1,1,3,3,3-hexamethyldisilazane (Roth) in chloroform before use (Packter & Collins, 1974). Silanized flasks containing 20–25 ml TSB medium (Oxoid) were inoculated with spores and shaken vigorously (180–220 r.p.m.) on an orbital shaker aerobically without the use of mechanical inclusions such as springs to ensure even aeration. Sterile-filtered MOPS buffer, pH 7.0, was added to the medium to a final concentration of 100 mM and pH 6.8. The medium was supplemented with the appropriate concentration of sodium nitrate, as indicated, and the culture was incubated at 30 °C.

Growth was determined by measuring the dry weight of the cultures. For each time point the bacterial contents of two flasks were collected

by vacuum filtration through a pre-weighed 2 µm pore-size filter. The flask was rinsed with 50 ml sterile water and the contents were filtered through the same filter. To ensure that no cells passed through the filter, samples were occasionally checked by passage through a 0.2 µm pore-size membrane filter. Cells and filters were dried under vacuum in a desiccator at 80 °C and allowed to cool to room temperature before weighing of the filters plus dried cells.

Construction of *narGHJI* and *moaA* knockout mutants. Single and multiple null mutations of the coding regions for the respiratory nitrate reductase operons and the molybdenum cofactor biosynthetic gene *moaA* were created using a standard PCR-targeted gene replacement technique (Gust *et al.*, 2003). The *aac(3)IV* (Apra) resistance cassette from pIJ773 (Gust *et al.*, 2003) was used to create a

deletion in the *narG2H2J2I2* operon (using oligonucleotides K2F and K2R) and the *moaA* gene (using oligonucleotides K1812F and K1812R). The *aadA* [Spec^R , streptomycin (Strep^R)] resistance cassette from pIJ778 (Gust *et al.*, 2003) was used to create individual deletions in the *narG1H1J1I1* (using oligonucleotides K1F and K1R) and *narG3H3J3I3* operons (using oligonucleotides K3F and K3R). The Spec^R -, Strep^R - and Apra^R -mutagenized cosmids St5C7 Δ nar1, StJ12 Δ nar2 and StK13 Δ nar3 (Table 1) were introduced into *E. coli* ET12567(pUZ8002) by electroporation and then transferred to *Streptomyces* by conjugation (Kieser *et al.*, 2000). The oligonucleotides used for these disruptions are given in Supplementary Table S1, and the extents of the respective deletions are indicated in Table 1.

The triple *narGHJI* deletion strains NM92 and NM102 were created by first generating the *narG2H2J2I2* mutants NM3 and NM4 (Table 1). Subsequently, the *narG3H3J3I3* operon was replaced with a Spec^R - Strep^R cassette to generate the double mutants NM68 and NM70. The Spec^R - Strep^R cassette in NM68 and NM70 was removed using a cosmid derivative in which the resistance cassette had been removed via FLP recombinase-mediated excision, as described elsewhere (Datsenko & Wanner, 2000; Gust *et al.*, 2003; see also below) to generate mutants NM90 and NM100, respectively. Finally, a derivative of cosmid St5C7 (St5C7 Δ nar1) in which the *narG1H1J1I1* genes were replaced with the Spec^R - Strep^R cassette was conjugated into NM90 and NM100 to generate the triple mutants NM92 and NM102, respectively (Table 1). Mutant cosmids used in the disruption procedure were confirmed by restriction analysis and by PCR performed with several combinations of primers upstream of, inside, and downstream of the disrupted gene. The authenticity of the deletions in the *S. coelicolor* mutant strains was confirmed by PCR, as for the cosmid mutants, and by Southern hybridization (data not shown).

In-frame deletion mediated by FLP recombinase. Electro-competent *E. coli* DH5 α /pCP20 cells were transformed with the targeted cosmid DNA; Cm^R and Apra^R or Spc^R , Strep^R transformants were selected on LB at 30 °C. After single colony purification non-selectively at 42 °C, 20–50 % of the colonies lost Apra^R (or Spc^R , Strep^R) and pCP20 (Cm^R) simultaneously, indicating successful deletion of the central part of the disruption cassette, leaving behind an 81 bp ‘scar’ sequence. Cosmid DNA containing the in-frame deletion was introduced into the *S. coelicolor* mutant carrying the marked deletion in the gene of interest by PEG-mediated transformation (Kieser *et al.*, 2000). Selecting for single cross-over events with Kan and subsequent screening for the loss of both Kan^R and the resistance derived from the marked deletion in the *S. coelicolor* mutant indicated the successful replacement of the resistance marker with an unmarked, in-frame deletion (Gust *et al.*, 2003).

Complementation of the *nar* operon and *moaA* gene mutations. The triple *nar* mutants NM92 and NM102 were complemented by introducing cosmids St5C7 (including *narG1H1J1I1*), StJ12 (including *narG2H2J2I2*) or StK13 (including *narG3H3J3I3*) (Redenbach *et al.*, 1996) via protoplast transformation (Kieser *et al.*, 2000) after first isolating the cosmid DNA from *E. coli* strain ET12567(pUZ8002). The corresponding derivatives of these cosmids used to create the original *nar* operon deletion constructs (St5C7 Δ nar1, StJ12 Δ nar2 and StK13 Δ nar3) were also introduced into NM92 and NM102 via conjugation from *E. coli* strain ET12567(pUZ8002) and acted as negative controls for the complementation analyses.

DNA corresponding to the coding region of SCO1821 (*moaA*) along with extended upstream and downstream sequences in order to include all regulatory elements was amplified by PCR using oligonucleotides 1821F and 1821R (Supplementary Table S1) and cloned into the integrating *Streptomyces* vector pMS82 (Gregory *et al.*, 2003) (Table 1 and Supplementary Table S1) to deliver pMS1821. Plasmid pMS1821 and the empty vector were introduced into

NM1821 (*moaA*) via conjugation using the complementation plasmid-containing *E. coli* strain ET12567(pUZ8002) (which mobilizes the *oriT*-containing plasmid pMS82 for conjugation *in trans*) (Table 1). Complementation was assessed using the chlorate-survival assay by comparing the phenotypes of the wild-type strain, the mutant, the mutant containing an empty plasmid or the cosmid carrying the appropriate deletion, and the mutant containing the complementing plasmid or cosmid under appropriate growth conditions.

Nitrate and nitrite determination. The concentration of nitrate in the growth medium was determined as described by Hartley & Asai (1963), with slight modifications. Cells were completely removed from the culture medium by centrifugation and filtration of the medium through a 0.25 μm pore-size filter (Sartorius). Initially, nitrite was removed from the medium by addition of amidosulfonic acid to a final concentration of 1 % (w/v). Complete removal of nitrite was confirmed by the assay used to determine nitrite (see below for details). One volume (0.5 ml) of the nitrite-free medium was mixed with 4 ml of a mixture containing equal volumes of H_2SO_4 and H_3PO_4 and mixed thoroughly. Finally, 0.5 ml 2,6-dimethylphenol (0.12 %, w/v, in concentrated acetic acid) was added and the mixture was incubated at room temperature for 15 min. The nitrophenol formed was determined by measuring A_{324} . A standard curve was prepared using a sodium nitrate solution.

Nitrite was determined using a modification of the method described by Rider & Mellon (1946). Various dilutions of cell-free culture medium (1.0 ml) were mixed with 1.0 ml of a saturated solution of sulfanilic acid prepared in 20 % (v/v) HCl. After incubation at room temperature for 5 min, 0.5 ml of a fresh aqueous solution of 0.2 % (w/v) *N*-(1-naphthyl)-ethylenediamine dihydrochloride was added and incubation was continued at room temperature for a further 10 min. The A_{540} was then determined. The concentration of nitrite present was calculated by comparison with a standard curve prepared using sodium nitrite.

Qualitative determination of nitrite production after growth in microtitre plates. Microtitre plates (24-well) were prepared with 2 ml LB agar in each well. Every third well included 85 mM sodium nitrate in the agar medium. Approximately 10^7 – 10^8 spores were added to three individual wells for each strain to be tested and incubations were performed as follows: dormant spores, which could only be tested anaerobically where no germination occurs, were added to the wells and immediately placed under a nitrogen atmosphere and incubated for 48 h at 30 °C (anaerobic spores); germlings were spores that were allowed to germinate and grow aerobically for 10 h at 30 °C, and then either tested for nitrite production immediately (aerobic germlings) or placed under a nitrogen/hydrogen (95:5) atmosphere and incubated for a further 2 days at 30 °C and then tested for nitrite production (anaerobic germlings); pre-germinated spores (germlings) were allowed to grow aerobically for a further 20 h (young aerobic vegetative mycelium) or for 72 h, after which time the plates were incubated for a further 48 h under a nitrogen/hydrogen (95:5) atmosphere prior to nitrite determination (anaerobic mycelium). After anaerobic incubation, spores were examined under a microscope to ensure that no germ tubes could be observed. Moreover, germlings were also examined microscopically to confirm that germination had indeed taken place.

Subsequent to growth, the control well was overlaid with 250 μl water, the second well was overlaid with 250 μl 85 mM nitrate and the third well, which included 85 mM sodium nitrate in the agar, was overlaid with 250 μl water. After incubation for 1 h at room temperature, 250 μl of a saturated solution of sulfanilic acid prepared in 20 % (v/v) HCl was added to each well and left to incubate for a further 1 h. Finally, the nitrite was visualized by adding 50 μl of a

fresh aqueous solution of 0.2 % (w/v) *N*-(1-naphthyl)-ethylenediamine dihydrochloride in 1.5 % (w/v) agarose as described above for the assay of nitrate reductase enzyme activity. A pink colour signified the presence of nitrite. Note that the overlay was performed anaerobically for those plates that were incubated under a nitrogen/hydrogen atmosphere.

Rapid nitrate reduction assay to test complementation. Approximately 10^4 – 10^6 spores were inoculated into tubes containing 15 ml TSB + 100 mM MOPS medium. The tubes were placed at a 45° angle and shaken at 220 r.p.m. at 30 °C for 30–50 h. The dry weight of these cultures was determined, and the mycelium was concentrated by centrifugation and resuspended in the same medium as described above to a mycelial weight of either 5 mg per 10 ml or 10 mg per 10 ml, and cultures were supplemented with 2.5 mM sodium nitrate. Aliquots (75 µl) were removed after 30 min, 1 h, 2 h, 4 h, 6 h or 14 h and the amount of nitrate reduced was determined.

To test activity in dormant spores, 10 µl of a thick spore suspension (containing minimally 10^4 spores in 20 %, w/v, glycerol) was added to 75 µl incubation buffer (50 mM MOPS) in 96-well plates, and these were immediately placed in an anaerobic atmosphere (95 % nitrogen, 5 % hydrogen) and incubated at 30 °C for up to 3 days before nitrite production was determined.

Chlorate toxicity assays. Chlorate resistance was determined using a plate-based assay analogous to the determination of anaerobic stress survival described previously (van Keulen *et al.*, 2007). The medium used was SFM containing 1.5 % (w/v) agar. Aliquots of sterile-filtered 5 M aqueous sodium chlorate (Sigma) were added to the molten agar mixture immediately prior to pouring. To test the general sensitivity of *S. coelicolor* M145 to chlorate anaerobically, resting, dormant spores (200 spores per plate) were plated onto SFM medium plates including 25 mM sodium chlorate. These plates were either put directly into a gas jar and the atmospheric oxygen then removed using AnaeroGEN (Oxoid) sachets, or the plates were incubated aerobically for 16 or 24 h (resulting in pre-germinated spores, termed mycelium) until mini-colonies could be observed under a binocular microscope prior to being placed into the gas jar (Kieser *et al.*, 2000). The plates were then incubated anaerobically for different time periods up to 14 days. Subsequent to anaerobic incubation, plates were exposed to air for a further 4 days and viable colonies counted. Data are representative (usually 6–10 repeats) of a typical experiment in which the mean result from an experiment performed in quadruplicate is shown.

To determine the survival of spores, approximately 200 resting spores were plated on each plate. Plates lacking chlorate served as a negative control and delivered the value for 100 % spore survival. Plates were placed immediately in an anaerobic jar as described above. Unless indicated otherwise, plates were incubated for 12 days at 30 °C, after which time the plates were removed and incubated for a further 4 days aerobically at 30 °C. The percentage survival was calculated by counting the surviving colonies on plates containing different concentrations of chlorate and comparing this number with the number of colonies surviving after incubation on plates lacking chlorate, which was taken as 100 % survival. The experiments were repeated at least 3–5 times and the SD determined. Initially, spore suspensions were heat-shocked for 10 min (Kieser *et al.*, 2000) prior to starting the experiment; however, no difference in the survival rate was observed and therefore this step was omitted in subsequent experiments. Determination of the survival of young mycelium in the presence of 25 mM chlorate involved using a similar approach to that described above to study spores, with the exception that prior to placing the plates in an anaerobic jar, they were first incubated for 16–20 h at 30 °C aerobically to pre-germinate the spores prior to being placed in an anaerobic jar for 12 days at 30 °C. Aerobic incubation of the plates for 4 days at 30 °C aerobically was then carried out before analysis of the survival rate.

To determine the effect of chlorate aerobically on spore and mycelium survival, the identical experiment was carried out omitting only the 12 day incubation in the anaerobic jar.

Other methods. Standard cloning methodologies carried out in *E. coli* were performed as described by Sambrook *et al.* (1989). Nitrate reductase enzyme activity was determined at 25 °C using both continuous (Jones & Garland, 1977) and discontinuous assay procedures (Showe & DeMoss, 1968). Crude cell extracts of *S. coelicolor* were prepared by sonication of mycelium in 50 mM sodium/potassium phosphate buffer, pH 7.2, containing 40 µl ml⁻¹ protease inhibitor cocktail (Roche Biochemicals) and 4 mg DNase I ml⁻¹. Resuspended cells (2–5 ml) were sonicated 5–10 times for 1 min at 30 W (pulse of 1 s on and 0.5 s off) using a Sonopuls sonifier with MS 73 probe (Bandelin). Discontinuous assay of nitrate reductase was performed by pre-incubating aliquots of crude cell extracts for 5 min at 25 °C in a 2.5 ml volume of the following reaction mixture: 0.1 M phosphate buffer (pH 7.2), 0.1 M potassium nitrate and 0.1 mM methyl viologen (MV) (Sigma). Immediately before starting the reaction, 50 mg sodium dithionite was dissolved in 10 ml 0.01 M NaOH and the solution was shaken gently to avoid oxidation of the dithionite. The reaction was started by adding 0.1 ml of the dithionite solution to the reaction mixture. After 10 min, the reaction was stopped by vortexing the tube vigorously to oxidize the remaining dithionite and reduced MV. Nitrite was determined exactly as described above. One unit of nitrate reductase activity was taken as the amount that catalysed the production of one micromole of nitrite per minute.

Continuous assay of nitrate reductase activity was determined by monitoring spectrophotometrically (Jones & Garland, 1977) the nitrate-dependent oxidation of reduced benzyl viologen (BV). One unit of nitrate reductase activity was taken as the amount that catalysed the production of one micromole of nitrite per minute. The protein concentration was determined according to Lowry *et al.* (1951) with BSA as standard. Non-denaturing PAGE was performed using 7.5 % (w/v) polyacrylamide gels, pH 8.5, and included 0.1 % (w/v) Triton X-100 in the gels (Ballantine & Boxer, 1985). Samples (50 µg protein) were incubated with 5 % (w/v) Triton X-100 prior to application to the gels. The method of Lund & DeMoss (1976) was modified to detect Nar activity in the gels after electrophoresis. Briefly, after electrophoresis, the separating gel was washed in buffer containing 50 mM MOPS, pH 7.0, and transferred to a transparent anaerobic chamber containing a nitrogen/hydrogen (95:5) atmosphere. The components of the reaction mixture were 50 mM MOPS, pH 7.0, and 10 mM MV, and to this was added a freshly prepared solution of sodium dithionite to a final concentration of 1 mM. The buffer and gel were incubated together for 15 min until the gel was uniformly stained as a blue colour due to MV reduction. The assay to detect Nar activity was started by adding 20 mM NaNO₃ to the mixture. The Nar activity was indicated as an achromatic zone in the blue gel. The processes of gel electrophoresis, equilibration and enzyme detection were carried out at room temperature.

RESULTS

S. coelicolor reduces nitrate during aerobic growth in liquid culture

As *S. coelicolor* lacks the ability to grow anaerobically, but maintains the coding capacity to synthesize three respiratory Nars, it was determined whether the bacterium could reduce nitrate during aerobic growth and how growth of strain M145 was influenced by different concentrations of nitrate. *S. coelicolor* M145 was grown in TSB-MOPS medium

without nitrate or supplemented with either 10 or 50 mM sodium nitrate in shake-flask cultures. For the first 30 h, growth of M145 was similar for all three cultures (Fig. 1). Between 30 and 40 h, the cultures with 10 and 50 mM nitrate grew more slowly than M145 without nitrate. While M145 without added nitrate entered the stationary phase after 40 h, the culture with 10 mM nitrate entered the stationary phase of growth only after approximately 60 h and the culture with 50 mM nitrate required 65 h to enter the stationary phase (Fig. 1). After 65 h of growth, both cultures reached a similar final dry weight that was equivalent to that of the culture without added nitrate (Fig. 1), indicating that although nitrate reduction slowed growth, presumably due to nitrite accumulation, all three cultures attained the same final dry weight. In the TSB culture supplemented with 10 mM nitrate, 10 mM nitrite was detected after 30 h, while after approximately 40 h, 50 mM nitrate was reduced completely to nitrite (data not shown).

Next we monitored the rate of nitrate disappearance from the culture and the rate of nitrite accumulation throughout the growth phase (Fig. 2). At the beginning of exponential growth, after approximately 15 h, nitrate began to disappear from the medium and was reduced stoichiometrically to nitrite. During the mid-exponential phase of growth (after ~24 h, Fig. 2), it was calculated that the rate of nitrate reduction by the culture was approximately $5 \mu\text{mol h}^{-1}$ ($\text{mg dry cell weight}^{-1}$). By the transition from the late-exponential into the early stationary phase (after 35 h), the nitrate was reduced completely to nitrite. Notably, all of the nitrite remained in the medium and appeared not to be reduced further to ammonia under the growth conditions tested. The pH of the culture remained relatively constant, increasing from 6.7 at the beginning of the experiment to 7.7 upon entry into stationary phase (Fig. 2).

***S. coelicolor* mutants unable to synthesize Nar enzymes do not respire nitrate**

Nar enzymes generally require molybdenum for enzyme activity, which is usually present in the form of a *bis*-MGD

cofactor (Schwarz *et al.*, 2009). As well as encoding three Nar enzymes, the *S. coelicolor* genome has the coding capacity for two soluble *bis*-MGD assimilatory nitrate reductases, termed NasA1 (SCO2473; Wang & Zhao, 2009) and NasA2 (SCO7374). In order to determine whether the Nar enzymes are exclusively responsible for the nitrate reduction activity observed for M145 (Fig. 2) and to determine whether they require molybdenum for their enzyme activity, two mutants were constructed: one mutant, represented by strains NM92 and NM102, lacked all three *narGHJI* operons; the second mutant, NM1821, carried a deletion in the gene SCO8121, which is predicted to encode the MoaA enzyme catalysing the first committed step in *bis*-MGD cofactor biosynthesis (Schwarz *et al.*, 2009). Strains NM92 and NM102 had identical genotypes and exhibited the same phenotype in all experiments described below; therefore, in the interests of clarity only data for NM92 ($\Delta nar123$) are presented.

Growth of the wild-type strain M145 as a mycelial culture in MOPS-buffered TSB liquid medium including 50 mM nitrate for a period of 4 days resulted in complete, stoichiometric reduction of nitrate to 50 mM nitrite (Fig. 2). The *S. coelicolor* mutant NM92 ($\Delta nar123$), when grown under the same conditions, did not reduce any nitrate to nitrite (data not shown). This result indicates that under the growth conditions tested the Nar enzymes are exclusively responsible for nitrate reduction. Analysis of the *moaA* mutant under the same growth conditions revealed that, like the triple *nar* operon mutant, it was unable to reduce nitrate to nitrite, indicating that a *bis*-MGD cofactor is required for the synthesis of active Nar.

The specific activity of nitrate reductase in extracts of mid-exponential phase cultures prepared from aerobically growing M145 (see Fig. 2) was determined to be $0.19 \mu\text{mol nitrate reduced min}^{-1} (\text{mg protein})^{-1}$, while the Nar specific activity in extracts generated from stationary phase cultures was $0.06 \mu\text{mol nitrate reduced min}^{-1} (\text{mg protein})^{-1}$. Extracts derived from mid-exponential phase cultures of NM92 ($\Delta nar123$) or NM1821 ($\Delta moaA$) had no measurable Nar activity.

Fig. 1. Influence of nitrate on aerobic growth of M145. M145 was grown in TSB containing 100 mM MOPS without (●) nitrate or with 10 mM (■) or 50 mM nitrate (▲), and growth over the time period indicated was measured by determining the dry weight. The experiment was performed in duplicate and error bars show the mean error.

Fig. 2. Aerobically growing *S. coelicolor* cultures reduce nitrate quantitatively to nitrite. M145 was grown in TSB containing 100 mM MOPS and 50 mM sodium nitrate, and growth over the time period indicated was measured by determining the dry weight (Δ) of cellular material derived from a 25 ml liquid culture. The concentration of nitrate ($\circ$) and nitrite ($\square$) was determined throughout the growth phase. The pH of the culture was determined and the measured values are presented below the data points. The experiment was performed in duplicate and error bars show the mean error.

Nar activity can also be visualized directly after native PAGE (Lund & DeMoss, 1976). Electrophoretic separation of detergent-treated crude extracts by 7.5% (w/v) non-denaturing PAGE, followed by staining for Nar activity, reproducibly revealed two apparent activity bands in extracts of mid-exponential phase cultures of M145 (Fig. 3, lane 1). No corresponding activity-stained bands could be detected in equivalent extracts derived from the triple *nar* mutant NM92 or the *moaA* mutant NM1821 (Fig. 3, lanes 2 and 3). Crude extracts prepared from stationary phase cultures of the wild-type M145 revealed a single activity band that migrated at a position similar to that of the faster-migrating band in exponential phase cultures (Fig. 3, lane 4).

Fig. 3. Nar enzyme activity detected after non-denaturing PAGE. Detergent-treated crude extracts (50 μ g total protein) derived from aerobically grown mycelium of *S. coelicolor* M145, NM92 ($\Delta nar123$) and NM1821 ($\Delta moaA$) were separated in a non-denaturing 7.5% (w/v) polyacrylamide gel and subsequently stained for nitrate reductase enzyme activity. The presence of Nar activity is signified by the achromatic (white) bands.

The *moaA* mutant cannot use nitrate as an inorganic nitrogen donor

The phenotypes of the *moaA* and triple *nar* mutants were analysed after growth on minimal medium plates to examine their ability to utilize different nitrogen sources. As anticipated, M145 and both mutants grew well with either ammonium or Casamino acids as nitrogen source (see Fig. 4b, d). All three strains examined grew extremely poorly when no nitrogen source was added to the growth medium (Fig. 4c); the very poor growth was presumably due to residual nitrogen in the agar, as absolutely no growth was observed in the same medium when agar was omitted (data not shown). Only M145 and the triple *nar* mutant NM92 could use nitrate as a nitrogen source, while the *moaA* mutant was unable to grow with nitrate (Fig. 4a). This result confirms that molybdenum is required for assimilatory nitrate reduction in *S. coelicolor*, and phenotypically distinguishes the *nar123* mutant NM92 from the *moaA* mutant NM1821.

Analysis of solid cultures reveals differential Nar activity in response to oxygen levels during the cell cycle

A rapid, qualitative test system was developed using 24-well microtitre plates to determine whether Nar activity during different phases of the growth cycle on solid medium could be detected and to allow the effects of anaerobiosis on development of Nar activity to be tested (Fig. 5). Each strain to be tested was inoculated as a spore suspension in three wells. The first well acted as a negative reagent control and lacked nitrate either in the growth medium or in the subsequently applied overlay (see Methods). The second well included nitrate in the overlay procedure, and in the third well nitrate was included in the growth medium from the beginning of the experiment. Cultures were then either incubated aerobically prior to testing or transferred for 2 days to anaerobic conditions subsequent to growth to a certain stage in the developmental cycle and then tested for nitrite production using a colorimetric assay (Rider &

Fig. 4. The respiratory Nars are not required for utilization of nitrite as a nitrogen source. Strains were grown aerobically on minimal medium plates with the supplements indicated, as described in Methods. M145, *S. coelicolor* wild-type; NM92, $\Delta nar123$; NM1821, $\Delta moaA$.

Mellon, 1946). The results shown in Fig. 5 present a representative example of an experiment.

Spores were only tested after exposure to anaerobiosis because under these conditions no germination occurs (van Keulen *et al.*, 2007). Nar activity was clearly observed in spores of M145 that had been incubated for 2 days anaerobically in the presence of nitrate (Fig. 5). This result indicates that at least one Nar is active in spores. Addition of nitrate at the stage of the overlay procedure did not reveal any substantial Nar activity, which suggests either that Nar activity in spores is low and that nitrite must first accumulate over a period of time before it can be detected (e.g. compare lanes 2 and 3 with spores in Fig. 5), or that nitrate is required to induce Nar enzyme activity in spores.

The negative control lacking nitrate in the overlay showed no nitrite accumulation. Moreover, spores of NM92 ($\Delta nar123$) and NM1821 ($\Delta moaA$) showed no Nar activity, even when nitrate was added to the growth medium. These latter findings indicate that the assay was specific for Nar and no chemical reduction of nitrate to nitrite by medium or assay components occurred.

A clear distinction in Nar activity could be seen between pre-germinated spores (germlings) that had been incubated anaerobically and those incubated aerobically (Fig. 5). Germlings that had been incubated anaerobically for 2 days showed strong Nar activity, based on the intensity of the pink colour, while no Nar activity could be detected in aerobically incubated germlings.

Fig. 5. Nitrate reduction occurs in spores and mycelium. *S. coelicolor* wild-type M145, the triple *nar* operon deletion mutant NM92 ($\Delta nar123$) and the molybdenum cofactor-deficient mutant NM1821 ($\Delta moaA$) were grown on rich medium in microtitre plates in the absence (wells 1 and 2) or in the presence of 85 mM sodium nitrate (well 3). Spores, pre-germinated spores (germlings) or aerobically grown mycelium (72 h mycelium) were incubated for 48 h anaerobically ($-O_2$) and then analysed for nitrite accumulation as described in Methods. Prior to the analysis for nitrite, 50 mM nitrate was added to well 2 and allowed to incubate anaerobically for 1 h. Germlings and young mycelium (20 h mycelium) were analysed directly after aerobic ($+O_2$) incubation for nitrite accumulation. The pink colour filling the wells in the plates indicates nitrite accumulation.

Mycelium grown aerobically for either 20 or 72 h (equivalent to mid-exponential and stationary phases, respectively, of liquid cultures; compare Fig. 2), then incubated in the absence of oxygen for 2 days before being overlaid under anaerobic conditions, showed the same level of nitrite accumulation when nitrate was included directly in the growth medium, and therefore data are only shown for stationary phase cultures (72 h hyphae) (Fig. 5). No Nar activity could be detected in the 2 h period after adding nitrate in the overlay assay, suggesting that Nar activity was very low or the enzymes were no longer active, that nitrate uptake was limiting under these conditions, or that the source of reducing power had become exhausted.

In contrast, aerobic mycelium that was 20 h old (mid-exponential phase) displayed Nar activity upon addition of nitrate in the overlay (Fig. 5, 20 h mycelium with O₂, lane 2). This finding correlates with the appearance of Nar activity in the liquid culture between 10 and 20 h of growth (see Fig. 2).

Nitrite accumulation was not observed at any of these growth stages in NM92 ($\Delta nar123$) or in NM1821 ($\Delta moaA$). The light pink colour observed in the vegetative mycelium of NM1821 after 72 h of aerobic growth followed by anaerobic incubation was due to production of the red antibiotic undecylprodigiosin and was observed even in the negative control to which no nitrate had been added (Fig. 5, 72 h mycelium, -O₂, *moaA* mutant).

S. coelicolor mutants defective in Nar synthesis are resistant to chlorate

Chlorate is an analogue of nitrate and functions as a substrate for respiratory Nars, with toxic chlorite as the product of reduction (Bell *et al.*, 1990, 1993). We developed an assay to determine the rate of survival after exposure of *S. coelicolor* to sodium chlorate based on a previously described anaerobic stress survival assay (van Keulen *et al.*, 2007). Spores and pre-germinated spores (germlings) of M145 were plated on SFM agar plates with or without 25 mM sodium chlorate and incubated anaerobically. After different periods of time the plates were removed and incubated for 4 days aerobically, where growth of surviving bacteria resumed and colonies could be counted (Fig. 6). It can be clearly seen that at least 80% of both spores and pre-germinated spores of M145 survived exposure to anaerobiosis for 14 days. If chlorate was added to the plates the ability of both spores and mycelium of M145 to survive decreased rapidly after a period of 7–10 days. Greater than 50% of spores and mycelium grown aerobically in the presence of 25 mM chlorate survived (data not shown).

Using this assay, and by determining the percentage of colony survival upon exposure to 25 mM chlorate anaerobically for 12 days, it could be shown that strain NM92 ($\Delta nar123$) was resistant to chlorate (Table 2). This result indicates that the Nar enzymes are exclusively

Fig. 6. Chlorate is toxic to anaerobically incubated spores and vegetative mycelium of *S. coelicolor*. Resting spores (circles, 200 spores per plate) or mycelium (squares) were plated on SFM medium plates and incubated for up to 14 days in the absence (open symbols) or the presence (closed symbols) of 25 mM sodium chlorate. To generate mycelium, the spores were allowed to germinate for 16–24 h prior to placing the plates in anaerobic conditions. After anaerobic incubation, where no growth occurred, the plates were exposed to air for a further 4 days and viable colonies counted. The value of 100% represents the number of colonies present after only aerobic incubation of the plates in the absence of sodium chlorate. The data shown are representative (10 repeats) for a typical experiment in which the mean result from an experiment performed in quadruplicate is shown.

responsible for the sensitivity of M145 towards chlorate. The *moaA* mutant NM182 also exhibited complete resistance towards chlorate, in line with the lack of synthesis of active Nar; however, survival generally under anaerobic conditions was poorer for this strain than for either M145 or NM92 ($\Delta nar123$).

Complementation of NM1821 ($\Delta moaA$) with an integrative plasmid bearing the complete *moaA* coding sequence restored sensitivity to chlorate (Table 2), indicating that the *moaA* mutation could be complemented by reintroduction of the wild-type *moaA* gene.

Using this chlorate-sensitivity assay we performed similar complementation experiments for the individual *narGHII* operons on cosmids by introducing them into NM92 ($\Delta nar123$). As a negative control for each complementation experiment the corresponding mutated cosmid used to generate the individual deletion mutations was tested. Introduction of the *narG1H1J1I1* operon on cosmid St5C7 restored partial sensitivity (~50%) of spores to chlorate (Table 2). A more moderate effect of complementation was observed with mycelium, perhaps suggesting that Nar1 is more active in spores than mycelium. Introduction of cosmid St5C7 $\Delta nar1$ bearing the mutated *narG1H1J1I1* operon failed to result in complementation of spores.

Table 2. Resistance to chlorate of *S. coelicolor* wild-type and mutant strains

ND, Not determined.

Strain and genotype/phenotype	Colony survival (%) [*]			
	Spores		Mycelium	
	No chlorate	+ 25 mM chlorate	No chlorate	+ 25 mM chlorate
M145 (wild-type)	98 % ± 9	1 %	92 % ± 5	3.5 % ± 1.5
NM92 ($\Delta nar123$)	95 % ± 10	98 % ± 8	95 % ± 12	95 % ± 9
NM166 ($\Delta nar123$ + St5C7 $\Delta nar1$)	99 % ± 9	102 % ± 8	112 % ± 15	89 % ± 8
NM165 ($\Delta nar123$ + St5C7) Nar1 ⁺	89 % ± 7	52 % ± 10	99 % ± 10	86 % ± 10
NM168 ($\Delta nar123$ + StJ12 $\Delta nar2$)	101 % ± 12	98 % ± 15	98 % ± 12	115 % ± 10
NM167 ($\Delta nar123$ + StJ12) Nar2 ⁺	110 % ± 13	52 % ± 10	97 % ± 12	18 % ± 10
NM169 ($\Delta nar123$ + StK13 $\Delta nar3$)	100 % ± 11	105 % ± 18	98 % ± 15	120 % ± 12
NM170 ($\Delta nar123$ + StK13) Nar3 ⁺	99 % ± 13	110 % ± 15	92 % ± 5	74 % ± 14
NM1821 ($\Delta moaA$)	86 % ± 15	80 % ± 7	75 % ± 12	85 % ± 16
NM1821 + pMS82	ND	ND	60 % ± 15	88.5 % ± 25
NM1821 + pMS1821 (<i>moaA</i> ⁺)	ND	ND	66 % ± 12	0 %

^{*}The mean ± SD percentage survival is shown and was determined for at least six independently performed experiments. In the case of complementation experiments, the data for at least three independently performed experiments are shown.

A strong increase in sensitivity to chlorate was observed in mycelium of NM92 ($\Delta nar123$) carrying cosmid StJ12, which includes the *narG2H2J2I2* operon (Table 1). Only approximately 20 % of the colonies survived, in contrast to NM92 ($\Delta nar123$) complemented with the mutated cosmid StJ12 $\Delta nar2$, which retained complete resistance to chlorate. Spores of NM167 (NM92 + StJ12) showed, like St5C7, an approximately 50 % restoration of chlorate resistance, indicating that Nar2 has some activity in spores but is more active in mycelium.

In contrast to the situation for Nar1 and Nar2, Nar3 appears to be mainly active in mycelium rather than in spores (Table 2). A 25 % decrease in colony survival was observed in mycelium of NM92 ($\Delta nar123$) complemented with cosmid StK13 encoding Nar3.

The same results were obtained in complementation experiments performed with the second triple *nar* operon mutant NM102 (data not shown).

Nitrite production by NM92 ($\Delta nar123$) complemented with *narGHJ* operons

A rapid assay for the reduction of nitrate to nitrite was developed for small-scale cultures, as described in Methods. Resting spores of M145 reduced 2.5 mM nitrate completely to nitrite within 10 h, but only after anaerobic incubation; no nitrate reduction was observed in spores incubated in the presence of oxygen (data not shown). In contrast, spores of NM92 ($\Delta nar123$) failed to reduce nitrate, even if incubation was continued for longer than 72 h. Spores of NM92 complemented with a cosmid encoding either Nar1 (strain NM165) or Nar2 (strain NM167) also reduced 2.5 mM nitrate completely to nitrite

within 1 h, indicating that both enzymes are active in spores. Spores of NM170 (NM92 + Nar3) were unable to reduce nitrate to nitrite, indicating that Nar3 is not active in spores. These results are in complete accord with the findings of the chlorate-sensitivity assays.

In order to examine nitrate reduction in mycelium, mycelial suspensions in TSB + 100 mM MOPS, pH 7, were prepared as described in Methods and the rate of nitrite accumulation from 2.5 mM nitrate was measured for defined amounts of mycelium in TSB-MOPS medium. After addition of 2.5 mM nitrate to cultures of M145 with 5 mg of mycelium the rate of nitrite accumulation was approximately 5 $\mu\text{mol h}^{-1}$ (per 10 ml culture), while in cultures with 10 mg of mycelium the rate was 10 $\mu\text{mol h}^{-1}$ (Fig. 7). Cultures of NM92 ($\Delta nar123$) failed to reduce nitrate to nitrite. Equally, neither NM92 ($\Delta nar123$) expressing *narG1H1J1I1* nor NM92 ($\Delta nar123$) expressing *narG3H3J3I3* reduced any nitrate under the conditions of this assay (data not shown). This result indicates that Nar1 was not active in mycelium. Prolonged incubation of NM170 (NM92 + Nar3) for 3 days also resulted in no measurable reduction of nitrate in liquid cultures. In contrast, NM92 expressing *narG2H2J2I2* reduced all of the 2.5 mM nitrate solution at exactly the same rates as M145 for the 5 mg per 10 ml and 10 mg per 10 ml suspensions of mycelium, respectively (Fig. 7).

DISCUSSION

The data presented in this study demonstrate that *S. coelicolor* has a high capacity for nitrate reduction both during aerobic growth and when the bacterium is incubated anaerobically; even low, more physiological,

Fig. 7. Nitrate reduction in liquid culture by NM92 ($\Delta nar123$) complemented with the *narG2H2J2I2* operon. Growing cultures of M145 (squares), NM92 (triangles) and NM167 ($\Delta nar123 + StJ12$, $Nar2^+$) (circles) were resuspended to 5 mg per 10 ml (open symbols) or 10 mg per 10 ml (closed symbols) in TSB+100 mM MOPS medium, and the experiment was started by addition of 2.5 mM sodium nitrate; samples were taken at different time points and the amount of nitrite in the supernatant was determined as indicated in Methods.

concentrations of nitrate in the micromolar range are very rapidly reduced to nitrite (data not shown). We measured a total Nar activity in extracts of $0.2 \mu\text{mol}$ nitrate reduced $\text{min}^{-1} (\text{mg protein})^{-1}$, which would approximately account for a rate of conversion of 1 mmol nitrate to nitrite in roughly 30 h [$28 \mu\text{mol h}^{-1} (25 \text{ ml culture})^{-1}$], which we observed during growth in liquid culture. Nitrate reduction was catalysed exclusively by the respiratory Nar enzymes in rich medium, as demonstrated by the fact that a mutant lacking all three *narGHJ* operons failed to reduce nitrate under any growth condition tested. During growth aerobically in rich medium the two assimilatory Nas enzymes, NasA1 (SCO2473) and NasA2 (SCO7374), were not active in nitrate reduction. This is in accord with the synthesis of NasA1 being under GlnR control (Wang & Zhao, 2009). That the *moaA* mutant has lost the capacity to use nitrate as sole nitrogen source, although the triple *nar* operon mutant retains the ability to use nitrate as a nitrogen source, indicates that the assimilatory NasA enzymes are Mo-dependent. These findings are in agreement with the general finding that all nitrate reductases are bis-MGD-dependent enzymes (Schwarz *et al.*, 2009).

Under the growth conditions tested nitrate was only reduced as far as nitrite. Growth experiments with and without nitrate demonstrated that in rich medium nitrate did not enhance either growth rate or growth yield. Indeed, nitrate addition to the growth medium tended to slow growth, although the same final biomass yield was attained as that of cultures grown without nitrate addition. This reduction in growth rate was probably due to accumulation of toxic nitrite in the growth medium, although this must be tested further. Earlier reports have provided evidence that *Streptomyces antibioticus* denitrifies aerobically (via classical denitrification and fungal-type co-denitrification pathways), converting nitrate to dinitrogen and nitrous oxide (Kumon *et al.*, 2002), while *Streptomyces thioluteus* performs incomplete denitrification of nitrate to N_2O (Shoun *et al.*, 1998). Indeed, Shoun *et al.* (1998) also reported that *S. coelicolor* strain JCM 4357 generates N_2O from nitrate anaerobically or under oxygen-limiting conditions. Under the growth conditions used in this study we never observed further reduction of nitrite. The

genome of *S. coelicolor* A3(2) (Bentley *et al.*, 2002) does not have the coding capacity for a classical Cu-containing or haem *cd*-type nitrite reductase, or a nitric oxide reductase, which are involved in the denitrification pathways of Gram-negative bacteria (Richardson *et al.*, 2001; Zumft, 1997). Although the genes encoding a sirohaem-type nitrite reductase (SCO2486–SCO2488) with similarity to NirB of *E. coli* are present in *S. coelicolor* A3(2) (Bentley *et al.*, 2002), the enzyme was not active in rich medium, which is consistent with *nirB* expression being controlled by GlnR (Tiffert *et al.*, 2008).

Through the development of a microtitre plate assay it was possible to show that the Nar enzymes are active in solid culture throughout the developmental cycle, including spores and mycelium. It is not possible at this stage, however, to determine directly the distribution of Nar activity among spores, vegetative mycelium and aerial hyphae. The results of the analysis of growth on solid culture also confirmed that at least one of the Nar enzymes is active in both the presence and the absence of oxygen. Notably, in contrast to the situation with anaerobically incubated young mycelium, Nar activity in aerobic young mycelium could be detected even when the cells were exposed to nitrate during the overlay stage, perhaps suggesting that the enzyme is more active under these conditions.

The results of the studies on solid cultures also indicate that nitrate uptake into the cytoplasm is not so strictly controlled by oxygen as appears to be the situation in *M. tuberculosis*, where, although Nar is synthesized constitutively, the synthesis of the nitrate transporter NarK2 is induced when the oxygen concentration is reduced (Sohaskey, 2005; Sohaskey & Modesti, 2009), or in *E. coli*, where nitrate uptake activity is inhibited by oxygen (Noji & Taniguchi, 1987).

All three Nar enzymes are active in *S. coelicolor*

The nitrate analogue chlorate was shown in the 1970s to be toxic to bacteria that synthesize Nars (MacGregor, 1975; Bancroft *et al.*, 1979). Both the triple *nar* mutant NM92 and the *moaA* mutant NM1821 were chlorate-resistant,

which demonstrates that Nar, as in other bacteria, is exclusively responsible for conferring a chlorate-sensitive phenotype. Although we could demonstrate that at least one of the Nars is active aerobically, chlorate sensitivity was manifest mainly under anaerobic conditions, not aerobically. A number of possible reasons for this can be considered: first, how chlorate enters the cell is unknown, and it is possible that chlorate and nitrate transport systems are distinct; second, growing aerobic mycelial cultures are probably not energy-limited and might be more efficient at exporting toxic chlorite than non-growing spores or mycelium under anaerobic conditions; third, although thought to function by 'bleaching' all cellular components, the actual mechanism of chlorite toxicity is unknown.

It was noted that the *moaA* mutant did not survive the 12-day period of anaerobiosis as well as either the wild-type or the triple *nar* mutant, suggesting that one or more molybdenum enzymes, some of which are of unknown function, are required for optimal anaerobic stress survival. Reduced anaerobic survival is not due to lack of Nars, as indicated by the fact that the triple *nar* mutant showed no obvious phenotype when compared with the wild-type. Moreover, neither the triple *nar* mutant nor the *moaA* mutant showed any obvious sporulation defects. This is an important consideration when discussing the possible physiological functions of these enzymes in streptomycetes (see below).

Complementation studies in which the individual *nar* operons of *S. coelicolor* were reintroduced into NM92 (Δ *nar123*) enabled us to demonstrate enhanced sensitivity to chlorate for each operon, indicating that all three enzymes are synthesized and are active. Moreover, we could demonstrate nitrate reduction by the mutant complemented with cosmids encoding Nar1 or Nar2. While NM92 synthesizing Nar1 showed increased sensitivity to chlorate and Nar activity in spores, Nar3 was active only in mycelium and only with chlorate but not in spores. Nar2 restored a high sensitivity to chlorate in mycelium, with a weaker effect in spores, as well as nitrite accumulation in both phases of development. These data suggest that the Nar enzymes do not exhibit redundancy in their roles during the life cycle of *S. coelicolor*.

What is the physiological role of Nar in an obligate aerobe?

It is intriguing that the large subunits of the three Nar enzymes of *S. coelicolor* share an amino acid identity of only around 65 %, and that of the respective NarH and NarI subunits is lower (van Keulen *et al.*, 2007). This might suggest differential substrate affinities and/or abilities to interact with alternative cellular redox complexes. The obvious likely functions of the Nars include energy conservation via proton motive force generation or redox balancing by dissipation of excess reducing equivalents generated during aerobic respiration when oxygen levels vary or reduced carbon substrates are metabolized. That

resting spores, if limited for oxygen, might use nitrate as an alternative means of maintaining a membrane potential seems plausible. However, based on our current understanding, it is unlikely that in the presence of oxygen, co-respiration of nitrate in mycelium would contribute significantly to proton motive force generation. Rather, it is possible that under these conditions, nitrate is used to dissipate excess reductant and to balance the quinone pool in a fashion similar to the proposed function of the soluble, energy-uncoupled, periplasmic Nap enzyme of *Paracoccus* sp. (Ellington *et al.*, 2002). Non-denitrifiers such as *Klebsiella pneumoniae* can also co-respire nitrate and oxygen, and it has been proposed (Robertson & Kuenen, 1984) that this phenomenon has more significance in the natural environment, where the demand for flexibility in specific growth rates is high due to the rapid adaptation necessary to compete in a constantly changing environment and an alternating mixed-carbon substrate spectrum. Having a means of balancing the quinol/quinone pool provides this metabolic flexibility, and one route to achieving this would be through the use of alternative electron acceptors such as nitrate. Because they lack a periplasm it is conceivable that Gram-positive bacteria such as *S. coelicolor* use one or more of the Nars to release excess reductant. The availability of clearly defined *narGHJ* operon mutants will greatly facilitate dissecting the biochemistry of the three Nars as well as defining their functional roles in the life cycle of *S. coelicolor*.

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