Phosphate and oxygen limitation induce respiratory nitrate reductase 3 synthesis in stationary-phase mycelium of *Streptomyces coelicolor* A3(2)

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The saprophytic actinobacterium *Streptomyces coelicolor* A3(2) requires oxygen for filamentous growth. Surprisingly, the bacterium also synthesizes three active respiratory nitrate reductases (Nar), which are believed to contribute to survival, or general fitness, of the bacterium in soil when oxygen becomes limiting. In this study, we analysed Nar3 and showed that activity of the enzyme is restricted to stationary-phase mycelium of *S. coelicolor*. Phosphate limitation was shown to be necessary for induction of enzyme synthesis. Nar3 synthesis was inhibited by inclusion of 20 mM phosphate in a defined ‘switch assay’ in which highly dispersed mycelium from exponentially growing cultures was shifted to neutral MOPS-glucose buffer to induce Nar3 synthesis and activity. Quantitative assessment of *nar3* transcripts revealed a 30-fold induction of gene expression in stationary-phase mycelium. Transcript levels in stationary-phase mycelium incubated with phosphate were reduced by a little more than twofold, suggesting that the negative influence of phosphate on Nar3 synthesis was mainly at the post-transcriptional level. Furthermore, it was demonstrated that oxygen limitation was necessary to induce high levels of Nar3 activity. However, an abrupt shift from aerobic to anaerobic conditions prevented appearance of Nar3 activity. This suggests that the bacterium regulates Nar3 synthesis in response to the energy status of the mycelium. Nitrate had little impact on regulation of the Nar3 level. Together, these data identify Nar3 as a stationary-phase nitrate reductase in *S. coelicolor* and demonstrate that enzyme synthesis is induced in response to both phosphate limitation and hypoxia.

**INTRODUCTION**

Members of the genus *Streptomyces* are ubiquitous Gram-positive organoheterotrophs and are important in the global carbon cycle of soils. *Streptomyces coelicolor* A3(2) is the model species of this genus and, similar to all streptomycetes, it requires oxygen for growth. It is also characterized by a complex developmental life cycle and secondary metabolism (Hodgson, 2000; Hopwood, 2006). The developmental cycle on solid medium involves germination of spores into a vegetative substrate mycelium followed by formation of hydrophobic aerial hyphae, which develop further into chains of spores (Elliot & Flärdh, 2012). In liquid medium, *S. coelicolor* fails to carry out aerial hyphae formation or sporulation but nevertheless performs the transition from exponential-phase to stationary-phase growth. The transition into the stationary phase of growth is accompanied by the onset of secondary metabolism. This transition is also characterized by substrate limitation, where energy conservation becomes a premium (Nieselt et al., 2010). Phosphate, in particular, seems to be an important nutrient whose limitation initiates a series of events that culminate in biosynthesis of secondary metabolites such as the antibiotics actinorhodin and prodigines (Nieselt et al., 2010).

Like other actinobacteria (Bott & Niebisch, 2003; Cook et al., 2009; Sawers et al., 2016), *S. coelicolor* carries out aerobic respiration over a broad range of oxygen concentrations. In poorly aerated soils, or when cell density increases, the oxygen concentration becomes limiting and hypoxia can ensue (Fischer et al., 2014). It is likely that a bacterium with a complex secondary metabolism, such as that present in *S. coelicolor*, has contingencies in place to ensure that a

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†These authors contributed equally to this work.

**Abbreviation:** qRT-PCT, quantitative reverse transcriptase PCR.

Four supplementary figures and three supplementary tables are available with the online Supplementary Material.
membrane potential is maintained, even if the oxygen concentration decreases below the threshold necessary for effective energy conservation. Indeed, it has been shown previously that, if mycelium of *S. coelicolor* becomes severely oxygen limited, or anaerobic, it can survive for long periods, even in the absence of oxygen (van Keulen et al., 2007). One means by which *S. coelicolor* could maintain a membrane potential is by nitrate respiration (Fischer et al., 2010; van Keulen et al., 2005). While nitrate respiration per se is insufficient to allow anaerobic growth of the bacterium (Fischer et al., 2010; Sawers et al., 2016; van Keulen et al., 2005), this type of respiration could nevertheless contribute to the general fitness of *S. coelicolor* in times of respiratory stress.

*S. coelicolor* synthesizes three membrane-associated respiratory nitrate reductases (Nar), more than any other bacterium outside the streptomycetes (Fischer et al., 2010, 2013, 2014). Each enzyme is active at different stages of the life cycle. For example, Nar1 is active exclusively in spores (Fischer et al., 2013), while Nar2 is maximally active in exponentially growing mycelium (Fischer et al., 2014). Significantly, optimal conditions for synthesis of Nar2 include oxygen limitation, although nitrate reduction by both spores and intact mycelium is only achieved in the complete absence of oxygen (Fischer et al., 2013, 2014).

In contrast to Nar1 and Nar2, considerably less is known regarding synthesis of Nar3. An initial analysis of chlorate resistance phenotypes (chlorate is a nitrate analogue that generates toxic chlorite when reduced by Nar enzymes), together with complementation studies, allowed a tentative assignment of Nar3 to being mainly active in mycelium (Fischer et al., 2010). While the genes encoding Nar1 and Nar2 are encoded on the right and left arms of the *S. coelicolor* genome, respectively, the Nar3 enzyme is encoded within the core region (Bentley et al., 2002). Phylogenetic analyses demonstrated that the Nar3 catalytic subunit, NarG3, is more similar to NarG1 than NarG2 (Sawers et al., 2016). The presence of the *nar3* gene cluster is quite widespread within the genus *Streptomyces* and the enzyme is also found in the genus *Amycolatopsis*.

In the current study, we focus on the conditions that result in synthesis of active Nar3. We demonstrate that expression of the *nar3* operon is strongly induced when *S. coelicolor* enters the stationary phase. Moreover, development of a ‘switch’ assay, whereby Nar3 synthesis can be carefully controlled, allowed us to demonstrate that both oxygen and phosphate limitation are important for induction of Nar3 synthesis. To our knowledge, this is the first description of a respiratory nitrate reductase specifically induced in response to phosphate starvation in bacteria and that is active in the stationary phase.

**METHODS**

**Bacterial strains and culture conditions.** Media and culture conditions for *S. coelicolor* A3(2) were the same as those previously described (Kieser et al., 2000). *S. coelicolor* wild-type strain M145 and mutant derivatives (Table 1) were grown on SM (soya flour mannotol) medium, or DNB (Difco nutrient broth) agar medium as indicated, or in liquid TSB (tryptic soya broth) (Oxoid; full strength=30 g l⁻¹) with antibiotics to maintain selection when appropriate. The growth medium composition and standard culture techniques used have been described previously (Kieser et al., 2000). Apramycin (Apra, 25 µg ml⁻¹) or spectinomycin (Spc, 25 µg ml⁻¹), both from Sigma, was added to growth media when required.

**Culture conditions used for physiological studies.** Small-scale growth curves were performed in 24-well, cell culture plates at 30°C and 200 rpm. Individual wells were filled with 1.5 ml MOPS-buffered TSB (25% strength TSB, 100 mM MOPS, pH 7.2) and five glass beads (4 mm) to facilitate mixing and good aeration. Each well was inoculated with 15 µl of a fresh spore suspension that had an optical density of 10 at 450 nm, which was determined exactly as previously described (Kieser et al., 2000) using a Uvikon spectrophotometer. Determination of cell amount equivalents was performed using methylene blue adsorption measurements as described previously (Fischer & Sawers, 2013).

In order to perform the ‘switch’ experiments, we used highly dispersed pre-cultures as inoculum to generate a standardized 15-h-old exponential-phase culture (50 ml vials with spores inside). Mycelium from these cultures was washed twice with water and suspended to the required cell amount in 50 mM MOPS-NaOH, pH 7.2 (henceforth referred to as MOPS buffer, pH 7.2). Cell amount was determined by using methylene blue adsorption (Fischer & Sawers, 2013) and dry weight measurement (Fischer & Sawers, 2013) or by using a standard pellet size (Fischer et al., 2014). For experiments not requiring use of highly dispersed mycelium but rather homogenous mycelium pellets, the harvested, dispersed mycelium was first transferred to fresh TSB in a 100 ml flask without a spring but with a stirrer and incubated for 3–5 h with vigorous stirring to maintain optimal oxygen saturation. The resulting pellets were washed and suspended in MOPS buffer, pH 7.2, at the required cell amount as described above.

The mycelium suspensions were used as material for the incubations that varied with respect to the physiological condition being tested. For example, the concentrations of buffer, glucose or phosphate were varied as indicated in the figure legends or in Tables S1 and S2 (available in the online Supplementary Material). The amount of mycelium was also varied. In order to test the influence of other additives or supplements on the induction of Nar3 synthesis, we incubated mycelial pellets of homogeneous size (generated as described in Fischer & Sawers, 2013) aerobically in 24-well, cell culture plates without glass beads (30°C, 175 rpm). Omitting glass beads was used for standard growth conditions and prevented negative effects of excessive aeration from influencing Nar3 synthesis. To test the influence of oxygen, we used highly dispersed mycelium and incubated it as aerobic (30°C, 175 rpm) or standing-liquid cultures (30°C) in 24-well, cell culture plates (2.5 ml medium volume) or in 2 ml reaction tubes (1 ml medium volume) without shaking. The effects of phosphate and TSB on Nar3 synthesis and enzyme activity were examined using highly dispersed mycelium suspensions. These suspensions were incubated for 5–6 h in gas-tight serum bottles (these were shaken vigorously at 175 rpm and were filled with between 10% and 20% of the culture volume) by adding 1 part mycelium suspension with 1 part double-strength MOPS buffer (final concentration: 100 mM MOPS-NaOH, pH 7.2) with the indicated glucose concentration (up to 90 mM glucose) and either with or without 10 mM nitrate and the indicated concentrations of phosphate (typically 20 or 25 mM) or diluted TSB. The initial headspace volume contained air and, after 2.5 h of incubation, sterile air was added again using a sterile needle under a laminar flow hood. This allowed continuous aerobic incubation.

Excreted nitrite was determined colorimetrically as previously described (Fischer et al., 2010).
Table 1. *S. coelicolor* strains used in this study

<table>
<thead>
<tr>
<th>Strains</th>
<th>Genotype and characteristics</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td>M145 (wild-type)</td>
<td>SCP1&lt;sup&gt;−&lt;/sup&gt; SCP2&lt;sup&gt;−&lt;/sup&gt;</td>
<td>Kieser et al. (2000)</td>
</tr>
<tr>
<td>NM3 (Δnar2)</td>
<td>M145 ASC0216–SCO0219::aac(3)IV (deletion of 6531 bp removing narG2H2J2I2)</td>
<td>Fischer et al. (2010)</td>
</tr>
<tr>
<td>NM27 (Δnar3)</td>
<td>M145 ASC04947–4950::aadA (deletion of 6497 bp removing narG3H3J3I3)</td>
<td>Fischer et al. (2013)</td>
</tr>
<tr>
<td>NM29 (Δnar1 Δnar2)</td>
<td>M145 ASC06355–6532::aadA (deletion of 6209 bp removing narG1H1J1I1I), ASC0216–0219::aac(3) IV (deletion of 6531 bp removing narG2H2J2I2)</td>
<td>Fischer et al. (2013)</td>
</tr>
<tr>
<td>NM30 (Δnar1 Δnar3)</td>
<td>M145 ASC06355–6532::aadA (deletion of 6209 bp removing narG1H1J1I1I), ASC04947–4950 (unmarked deletion of 6497 bp removing narG3H3J3I3)</td>
<td>Fischer et al. (2013)</td>
</tr>
<tr>
<td>NM68 (Δnar2 Δnar3)</td>
<td>M145 ASC0216–0219::aac(3)IV (deletion of 6531 bp removing narG2H2J2I2), ASC04947–4950::aadA (deletion of 6497 bp removing narG3H3J3I3)</td>
<td>Fischer et al. (2010)</td>
</tr>
<tr>
<td>NM92 (Δnar1 Δnar2 Δnar3)</td>
<td>M145 ASC06352–6535::aadA (deletion of 6209 bp removing narG1H1J1I1I), ASC0216–0219::aac(3)IV (deletion of 6531 bp removing narG2H2J2I2), ASC04947–4950 (deletion of 6497 bp removing narG3H3J3I3)</td>
<td>Fischer et al. (2010)</td>
</tr>
<tr>
<td>NM1821 (ΔmoaA)</td>
<td>M145 ASC01821::aac(3)IV, which carries a deletion in the moaA gene</td>
<td>Fischer et al. (2010)</td>
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Antibody preparation and Western blotting. Antibodies were prepared commercially (Seqlab) against a 15 amino acid peptide (NLAE LGDAPIPTGDG – amino acid positions 1061–1075) in the NarG3 polypeptide. To minimize unspecific cross-reactions, we pre-treated antiserum raised against the NarG3 peptide using a crude extract derived from mycelium of the nar<sup>−</sup> knockout mutant NM27. The procedure for the depletion was carried out as described previously (Fischer et al., 2013). The supernatant obtained from the depletion was used as primary antibody for Western blot analysis. The treated antiserum was used in the dilution range 1:75–150.

Polyacrylamide gel electrophoresis and immunoblotting. Aliquots (45–60 µg of protein) from the indicated crude extracts were separated by SDS-PAGE using 7.5% (w/v) polyacrylamide gels (Laemmli, 1970) and transferred to nitrocellulose membranes as previously described (Towbin et al., 1979). ‘Depleted’ anti-NarG3 antiserum (dilution 1:75) was used to detect NarG3 polypeptide in crude extracts of mycelium. Secondary antibody conjugated to horseradish peroxidase was obtained from Bio-Rad. Visualization was performed by the enhanced chemiluminescence reaction (Stratagene).

RNA analyses in mycelium. RNA extraction was performed as described previously (Fischer et al., 2013). To analyse the influence of phosphate on *narG3* transcript levels, we incubated exponential-phase mycelium for 5 h aerobically at 60 rpm with glass beads after the switch to MOPS-glucose buffer (50 mM MOPS, 20 mM glucose), pH 7.2, containing 1 mM nitrate and with or without 20 mM phosphate. For quantitative reverse transcriptase PCR (qRT-PCR) analyses, 500 ng of RNA was used to synthesize cDNA using RevertAid H Minus First Strand cDNA-Synthesis Kit and random hexamer primers according to the manufacturer’s instructions (ThermoFisher Scientific). Mycelium was grown as described above in 100 mM MOPS, 80 mM glucose and 2 mM nitrate with and without 25 mM phosphate and harvested after 15 h (exponential mycelium) or 40 h (stationary phase) of growth and RNA was isolated. qRT-PCR was performed using 1.25 ng cDNA and a Quantitect SYBR Green PCR Kit (Qiagen) and analysed using a Rotor Gene 3000 cycle (Changel Cycling A Sybr, Corbett Research, Qiagen). Oligonucleotides for detection of *narG3* and *whiB* transcripts via qRT-PCR were as follows: qRT_narG3–3298rv (5′-CACCCATCTTACGTCGACCA-3′) and qRT_narG3–3298f (5′-GGACGTTCCTCCTGGTAC TCAG-3′); qRT_whiB–146f (5′-TGCAAGGACACTGCTGTC-3′) and qRT_whiB–183rv (5′-GGCTACTCTGAGGCATT-3′). The efficiency and specificity of PCR amplifications were determined by using standard curves derived from a dilution series of cDNA of the wild-type. Mean transcript levels were calculated based on values obtained from three technical replicates of at least three independent biological replicates and the levels of *whiB* transcripts (reference gene) were used as standard. After a 15 min incubation at 95°C, 45 cycles of the following programme were performed: 94°C for 15 s, 56°C for 12 s, 72°C for 11 s and 80°C for 7 s. A ramp from 60°C to 99°C was used and the final temperature was held for 90 s on the first cycle. Data are presented as standard error of the mean. The *whiB* transcript was used as a comparator because its expression varied little between exponential-phase and stationary-phase mycelium.

Other methods. Crude cell extracts of *S. coelicolor* A3(2) mycelium were prepared by sonication of mycelium in buffer (100 mM potassium phosphate, pH 7.2). Suspended mycelium (2–5 ml) was sonicated four times for 3 min at 30 W (pulses of 0.5 s on and 0.5 s off) using a Sonop lots sonifier with a sonotrode KE76 tip (Bandelin). The protein concentration was determined according to Lowry et al. (1951) using bovine serum albumin as standard.

Nitrates reductase enzyme activity in crude extracts was determined using the continuous assay procedure with reduced dithionite and benzyl viologen (0.4 mM) at 30°C as previously described (Jones & Garland, 1977). Nitrates reductase activity in whole mycelium measured nitrate production as described previously (Fischer et al., 2010).

RESULTS

Nar3 is synthesized and active in stationary-phase mycelium

Previous studies that examined nitrate reduction together with colony survival after growth in the presence of the nitrate analogue chlorate, which generates toxic chlorite when reduced by Nar-type enzymes (Azoulay et al., 1967), suggested that Nar3 was mainly active in stationary-phase mycelial cultures (Fischer et al., 2010). In order to characterize the regulation of Nar3 synthesis and activity in detail, we first aimed to define during which growth phase Nar3 was maximally active. Using quarter-strength TSB medium containing 5 mM nitrate to speed up the transition to the stationary phase, we monitored nitrate reduction and growth of defined nar deletion mutants using small-scale
liquid cultures (Figs 1 and S1). Nar3-dependent nitrate reduction occurred primarily in the stationary phase (Fig. 1a). For strains M145 (wild-type) and NM59 (Δnar1 Δnar3), nitrite production initiated after germination of the inoculated spores (approximately after 10 h) once a suitable level of oxygen-consuming mycelial biomass had been attained (compare the growth curves in Fig. S1). This nitrite production correlated with Nar2-dependent nitrate reduction (Fischer et al., 2014). Note that the standard deviation in the induction phase was high due to slight variations between cultures as to precisely when synthesis of the highly active Nar2 enzyme was induced (Fischer et al., 2014).

In contrast, strain NM29 (Δnar1 Δnar2), which only synthesizes Nar3, reduced nitrate only after a significant lag period of approximately 22 h (Fig. 1a), which correlated with entry into the stationary phase (Fig. S1). The growth curves for M145, NM59 (Δnar1 Δnar3) and NM29 (Δnar1 Δnar2) (Fig. S1) revealed a similar growth rate for all three strains; however, it was noted that the final cell amount attained was higher for strain NM29, which is possibly a consequence of the strain flocculating less than either M145 or NM59. Strain NM68 (Δnar2 Δnar3) failed to show any nitrate-reducing activity in this experiment (data not shown), which is consistent with Nar1 being exclusively a spore-specific enzyme (Fischer et al., 2013).

**Fig. 1.** Onset of Nar3-dependent nitrate reduction in *S. coelicolor* A3(2). (a) Nitrite production by wild-type M145 (squares), NM59 (Δnar1 Δnar3, circles) and NM29 (Δnar1 Δnar2, triangles) during growth in TSB (25% strength) with 5 mM nitrate. Cultures were inoculated with spores and growth was performed on a 1.5 ml liquid scale in cell culture plates (see Methods). (b) Nitrite production by strain NM29 (Δnar1 Δnar2) was monitored during cultivation in different strengths of TSB medium (10%, filled squares; 25%, filled triangles; 50%, open triangles; 100% TSB, open squares) supplemented with 5 mM nitrate. Cultivation was initiated using a standardized inoculum of 2 mg ml⁻¹ dry weight (homogeneous mycelial pellets from an exponential-phase mycelial pre-culture). Furthermore, nitrite production was monitored in a control with MOPS-NaOH buffer, pH 7.2 (filled circles). The data points represent the average values for three technical replicates.

In order to determine whether Nar3 enzyme activity correlated with appearance of Nar3 polypeptide during the growth phase, we used an antiserum raised against a peptide specific for the catalytic subunit (NarG3) of the Nar3 enzyme in Western blots (Fig. 2). Crude extracts derived from strain NM29 (Δnar1 Δnar2) grown for 15 h (exponential-phase mycelium) and 40 h (stationary-phase mycelium) were separated by SDS-PAGE and challenged with anti-NarG3 peptide antiserum after transfer to a nitrocellulose membrane. The results clearly show that, while no signal was observed in exponential-phase mycelium (Fig. 2a), a strong signal could be detected in stationary-phase mycelium that migrated at approximately 130 kDa, which is close to the deduced molecular mass of NarG3 (135 kDa) (Fig. 2b). Various control strains were grown under the same conditions and analysed for the appearance of the polypeptide. No polypeptide was observed in stationary-phase mycelium of strain NM27 (Δnar1 Δnar3), NM92 (Δnar1 Δnar2 Δnar3) or NM1821 (ΔmoaA). Like all other Nar enzyme, Nar3 is dependent on the molybdenum cofactor biosynthetic machinery for activity and the enzyme is degraded in strains lacking MoaA, which catalyses an initial step in cofactor synthesis (Fischer et al., 2010). These results demonstrate that the NarG3 polypeptide is only detectable in stationary-phase mycelium and this correlates with the appearance of Nar3 enzyme activity (see Fig. 1a).

**Nar3 activity is not regulated in response to limitation of carbon source**

To examine the effects of different concentrations of TSB medium on the appearance of Nar3 enzyme activity, we sub-cultured aliquots of mycelial mini-pellets (2 mg ml⁻¹) (see Methods for details) of strain NM29 (Δnar1 Δnar2), which had not yet induced synthesis of Nar3 activity, in TSB medium of different strengths. Cultures were also supplemented with 5 mM nitrate. Nitrite release from the mycelium into the culture medium was then monitored for a further 20 h of growth (Fig. 1b). The results revealed that the more the initiation of Nar3-dependent nitrite production was delayed, the higher the TSB concentration in the medium was (Fig. 1b). This result suggests that substrate limitation was the signal for induction of Nar3 activity.

Nar3 activity could also be induced in MOPS-NaOH buffer, pH 7.2, which included 20 mM glucose as carbon source (Fig. 1b). This finding strongly suggests that carbon limitation was not the induction signal for Nar3 synthesis.

**Controlled induction of Nar3 achieved in a defined ‘switch’ system**

In order to identify the parameters required to induce Nar3 enzyme activity, strain NM29 (Δnar1 Δnar2) was grown to exponential phase in TSB medium and aliquots of the mycelium were transferred into MOPS-NaOH buffer (pH 7.2). By the addition of 10 mM nitrate along with different carbon, nitrogen or phosphate sources, as well as varying
the availability of oxygen, the conditions resulting in Nar3-dependent nitrite production by intact mycelium after a 7.5 h incubation period could be empirically determined (Tables S1 and S2). The results revealed that incubation with buffer alone, regardless of oxygen availability, was insufficient to induce Nar3 activity. However, addition of carbon sources such as glucose, mannose, galactose, fructose, cellobiose, glycerol, xylose or mannitol (Table S1), when added at various concentrations, resulted in release of nitrite by the mycelium. This result indicates that an electron donor is required for appearance of Nar3 activity and confirms that carbon limitation is not involved in enzyme induction.

Highly reproducible induction of Nar3 enzyme activity was achieved by 'switching' mycelium from full-strength TSB to MOPS-NaOH buffer (pH 7.2) supplemented with up to 50 mM glucose (Table S1) and by incubating this suspension aerobically with shaking for a period of 7.5 h (Fig. 3). Induction of Nar3 activity could be inhibited by addition of 200 µg ml⁻¹ chloramphenicol to the buffer at the onset of the switch. Addition of chloramphenicol at this concentration prevents protein synthesis in S. coelicolor but it was shown previously to have no effect on the levels of spore-specific Nar1 because the enzyme is constitutively present in spores (Fischer et al., 2013, 2014).

Incubation of the mycelium for 2.5 h aerobically followed by a shift to incubation as a standing-liquid culture, in which shaking was stopped, resulted in severely oxygen-limiting or anaerobic conditions within a few minutes (Fischer & Sawers, 2013; van Keulen et al., 2003; Fig. S2). This procedure resulted in an approximate fivefold increase in Nar3-dependent nitrite production (Fig. 3). This result suggested that, while anaerobiosis is necessary for high Nar3 activity to be measured (Fischer et al., 2010), residual oxygen acting as an electron acceptor (presumably allowing ATP synthesis) is initially required for induction of Nar3.

Fig. 2. Synthesis of NarG3 polypeptides is induced in stationary-phase mycelium. Crude extracts derived from exponential-phase (a) and stationary-phase (b) mycelia were analysed by Western blotting for the presence of the NarG3 polypeptide. Polypeptides in aliquots (45 µg of protein) of the indicated crude extracts were separated by SDS-PAGE in a 7.5 % (w/v) polyacrylamide gel. The catalytic subunit NarG3 has a deduced molecular mass of 135 kDa and was detected by using peptide-specific antibodies (1 : 75; the migration position of NarG3 is indicated by an arrow). The asterisk signifies an unspecific cross-reacting polypeptide, which acted as a loading control.

Fig. 3. Effects of oxygen limitation on Nar3-dependent nitrate reduction in intact mycelium. The amount of nitrite produced by intact mycelium of NM29 (Δnar1 Δnar2) after transfer of exponential-phase mycelium to MOPS-glucose buffer, pH 7.2, with subsequent incubation under the indicated conditions was determined. Mycelium from the same initial TSB-grown culture was aliquoted and after suspension in buffer, incubation was continued for the times indicated under aerobic conditions (AE) or under standing-liquid conditions (SL) or under a combination of both conditions. Samples were incubated in the absence or presence of 0.2 mg ml⁻¹ chloramphenicol (CLM) as indicated, and when applied, chloramphenicol was added immediately after the switch to MOPS-glucose buffer.
synthesis to occur. In line with this hypothesis, we noted that immediate transfer of the mycelium from aerobic to anaerobic conditions did not result in active Nar3 enzyme, regardless of whether glucose was present (Fig. 3); however, after 25 h of incubation, a low level of nitrite could be detected in the culture medium (Fig. S3). This result supports the notion that a minimal level of oxygen, as well as concomitantly energy, is necessary to activate synthesis of active Nar3.

**Phosphate inhibits Nar3 synthesis**

While establishing the conditions that switched on Nar3 activity, it was noted that addition of 20 mM phosphate to the MOPS-glucose buffer, pH 7.2, prevented detectable nitrite release by intact mycelium of strain NM29 (∆nar1 ∆nar2) (Table S2). To verify this negative influence of phosphate, we measured the Nar3 enzyme activity in crude extracts derived from mycelium of NM29 harvested 5–6 h after the shift from TSB medium into MOPS-glucose buffer, pH 7.2; the effect of adding 20 mM sodium phosphate was also tested (Fig. 4a). Nar3 enzyme activity was approximately 10-fold higher in extracts of mycelium that had been incubated without phosphate compared to the activity in extracts from mycelium that had been incubated with 20 mM sodium phosphate (Fig. 4a). No Nar3 enzyme activity was detectable in extracts derived from the TSB-grown, exponential-phase mycelium prior to the shift to MOPS-glucose buffer (Fig. 4a, lane 1). The negative effect of phosphate on Nar3 enzyme activity was observed regardless of whether sodium or potassium phosphate was used (Table S2), clearly demonstrating that it was phosphate and not the monovalent cation that prevented the detection of Nar3 activity. The presence of 10 mM nitrate in the incubation buffer did not have a significant effect either on Nar3 activity or on polypeptide levels (Fig. 4).

To determine whether the reduction in Nar3 enzyme activity was caused by regulation of enzyme activity or whether it was due to reduced Nar3 synthesis, we used a Western blot using anti-NarG3 peptide antiserum (see Methods) (Fig. 4b). Analysis of mycelial crude extracts derived from NM29 (∆nar1 ∆nar2) revealed that the levels of NarG3, the catalytic subunit of Nar3, were significantly reduced when mycelium was incubated with phosphate. A similar result was observed for the wild-type strain M145 (Fig. S4). Exogenous addition of nitrate to the incubation buffer had no influence on the level of the NarG3 polypeptide. Interestingly, in the control samples taken from TSB-grown mycelium, there was also virtually no detectable full-length NarG3 polypeptide that migrated at 135 kDa (Fig. 4b). As expected, no NarG3 polypeptide was found in samples of extracts derived from the nar3 mutant NM27 (Fig. 4b, right-hand lane), which acted as a control.

Supplementation of the MOPS-glucose buffer with either 2 % (v/v) or 4 % (v/v) TSB also induced Nar3 enzyme activity, but only when exogenously added phosphate was omitted from the incubation (Fig. 4a). It was noted that, upon addition of 4 % (v/v) TSB, Nar3 activity was reduced by

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**Fig. 4.** The influence of phosphate on the specific activity of Nar3 in mycelial crude extracts. (a) Nar3 enzyme activity was determined in crude extracts of mycelium derived from strain NM29 (∆nar1 ∆nar2) after aerobic incubation in MOPS-NaOH-glucose buffer without TSB or containing 2 % (v/v) strength TSB or 4 % (v/v) strength TSB and with or without 20 mM phosphate (indicated by + or − sign, respectively) and nitrate (no nitrate, black histogram; 10 mM nitrate, white histogram). The specific activity of Nar3 was determined by monitoring the nitrate-dependent oxidation of reduced benzyl viologen. The histogram labelled with 15 h TSB shows the Nar3 enzyme activity in the mycelium used prior to transfer to the MOPS-glucose buffer. (b) Analysis of mycelial crude extracts from (a) for the presence of the NarG3 polypeptide via Western blot. Polypeptides in aliquots (60 µg of protein) of the indicated crude extracts were separated in a 7.5 % (w/v) SDS polyacrylamide gel. The catalytic subunit NarG3 migrates with a molecular mass of 135 kDa and was detected by using peptide-specific antibodies (1 : 75; the migration position of NarG3 is indicated by an arrow). A crude extract derived from mycelium of strain NM27 (∆nar3) acted as a negative control and was electrophoresed in the right-hand lane in the figure. The asterisk signifies an unspecific cross-reacting polypeptide, which acted as a loading control.
approximately 20–30% compared to when 2% (v/v) TSB was added. Standard TSB medium contains 14.3 mM phosphate, explaining why increasing amounts of TSB caused a reduction in Nar3 activity. Analysis of these extracts by Western blotting demonstrated that they indeed contained less NarG3 polypeptide compared with extracts derived from mycelium incubated in MOPS-glucose buffer alone (Fig. 4b). Extracts of mycelium incubated with 4% (v/v) TSB plus phosphate showed the same results as for incubation with 2% (v/v) TSB plus phosphate (data not shown).

**Regulation of nar3 transcript levels indicates transcriptional activation in the stationary phase and suggests post-transcriptional control in response to phosphate**

In a previous proteomic study (Thomas et al., 2012), it was reported that Nar3 synthesis was detected at similar levels in both the wild-type strain M145 and in a phoP mutant, indicating that phosphate has a limited influence on the transcription of the narG3 operon. To address the question of nar3 operon regulation directly, we determined quantitatively the difference in narG3 transcript levels between exponential- and stationary-phase mycelium of strain M145 and with respect to the influence of phosphate (Fig. 5). The essentially constitutive transcript level of whiB mRNA (Soliveri et al., 1992) was used as a comparator (see Table S3). Only very low levels of narG3 transcript were detectable in exponential-phase mycelium. In contrast, narG3 transcript levels increased more than 30-fold in stationary-phase mycelium (Fig. 5). These data indicate strong transcriptional up-regulation of nar3 operon transcription in the stationary phase. As a control, no narG3 transcripts could be detected under any condition for the strain NM92 (Δnar1 Δnar2 Δnar3).

Incubation of the stationary-phase mycelium with 25 mM phosphate reduced transcript levels a little more than twofold. Thus, while this result suggests that some transcriptional repression in response to phosphate might occur, it would appear that phosphate principally regulates Nar3 enzyme levels at the post-transcriptional (translational control) or post-translational (control of Nar3 turnover) levels.

**DISCUSSION**

Despite early evidence indicating that the Nar3 enzyme was active during the stationary phase of growth in batch cultures of *S. coelicolor* (Fischer et al., 2010), it was unclear at what level control of enzyme synthesis and activity occurred. We demonstrate in this study that transcription of the nar3 operon is induced in stationary-phase mycelium and that this correlates with appearance of the catalytic subunit (NarG3), as determined by Western blotting, as well as with Nar3 enzyme activity. Moreover, we show that control of Nar3 synthesis underlies at least two levels of regulation. Firstly, Nar3 synthesis appears to be initiated only when phosphate levels in the culture become limiting. Phosphate limitation has been shown to be a key signal for the onset of secondary metabolism in streptomycetes and *Serratia* species (Nieselt et al., 2010; Slater et al., 2003) and has an important role in regulating nitrogen metabolism (Rodríguez-Garcia et al., 2009). The presence of phosphate led to a roughly 60% reduction in the narG3 transcript level in stationary-phase mycelium. This transcript level is nevertheless 15-fold higher than in exponential-phase mycelium (Fig. 5). In stationary-phase mycelium grown in the presence of phosphate, both NarG3 polypeptide and Nar3 enzyme activity were barely detectable, which suggests that a further level of regulation controls Nar3 enzyme levels in response to phosphate. One possibility is that the enzyme is rapidly degraded in the presence of phosphate. The alternative possibility is that translation of the nar3 transcript is under post-transcriptional control. An increase in Nar3 polypeptides during the growth phase has been observed in a recent proteomic study and those authors also demonstrated that similar, if not slightly increased, amounts of the polypeptides were detectable in a phoP mutant (Thomas et al., 2012). These data strongly suggest that the PhoPR two-component system does not regulate nar3 operon expression at the transcriptional level. The mechanism underlying how phosphate limitation might control translation of nar3 operon transcripts, or enhanced turnover of the Nar3 protein, is currently unclear and will require further experimentation.

The second level of regulation involves induction of Nar3 enzyme synthesis in response to oxygen limitation. This regulation might be linked to energy status and the need to maintain a proton gradient in the stationary phase when
oxygen becomes limiting. This can be achieved by inducing Nar3 as an alternative respiratory enzyme allowing use of nitrate as an alternative electron acceptor. Whether this regulation is somehow linked to the effect of phosphate limitation is unclear. However, phosphate limitation has been shown to signal energy limitation in other bacteria, such as *Escherichia coli* (Schumacher et al., 2014), suggesting that some regulatory link is conceivable.

We could show that, while the abrupt shift of exponentially growing mycelium to anaerobic conditions prevented induction of Nar3 activity (Fig. 3), a gradual shift from aerobic to oxygen-limited conditions resulted in maximal levels of Nar3 activity. A formally similar regulatory control by hypoxia has been recently demonstrated for Nar2 synthesis in *S. coelicolor*, although Nar2 is active in exponential-phase mycelium (Fischer et al., 2014). It is notable that, in both *Corynebacterium* and *Myco bacterium* species, hypoxic regulation of nitrate reductase synthesis/activity has also been observed (Boot & Niebisch, 2003; Cook et al., 2009; Nishimura et al., 2007; Sohaskey & Wayne, 2003; Takeno et al., 2007), perhaps suggesting that this might be a trait characteristic of nitrate-reducing actinobacteria. Induction of Nar3 synthesis during oxygen limitation would ensure that respiratory metabolism could still be retained in the presence of nitrate and would nevertheless aid in maintenance of a membrane potential during the stationary phase although inadequate for growth. The abrupt shift to severe oxygen limitation/anaerobiosis in stationary-phase mycelium would be prohibitive to transcription and enzyme synthesis due to their associated high energetic demands. Carbon limitation is clearly not the signal that induces nar3 operon expression in the stationary phase because glucose in high concentration proved to be required for Nar3 synthesis to occur. In contrast, high glucose concentrations increased Nar3 activity in the stationary phase, presumably by acting as an electron donor.

Exogenously supplied nitrate (millimolar concentrations) does not significantly affect Nar enzyme synthesis in *S. coelicolor*, and this is also in agreement with recent studies by our group and others (Fischer et al., 2013, 2014; Thomas et al., 2012). Thus, nitrate regulation of Nar enzyme synthesis as observed in *E. coli* (Stewart, 2003) or *Bacillus subtilis* (Hoffmann et al., 1998) can be excluded for *S. coelicolor*.

Despite Nar3 synthesis requiring oxygen, it must be stressed that activity of the Nar3 enzyme, like that of Nar1 in intact spores (Fischer et al., 2013) and Nar2 in intact exponentially growing mycelium (Fischer et al., 2014), only occurs when oxygen is absent or at very low concentrations and insufficient to support growth. Because the transporter involved in Nar3-dependent nitrate reduction is currently unknown, we do not know whether the nitrate transport process itself is subject to oxygen regulation or whether, thermodynamically, the presence of oxygen is sufficient to divert electron flow through the respiratory chain to one of the terminal oxidases in the bacterium. Future studies with defined oxidase and nitrate-transport mutants will be required to answer this question.

Finally, the control of Nar3 synthesis by phosphate and hypoxia offers the perspective of using this MOPS-based switch assay to characterize the complex regulatory processes occurring during the transition from exponential to stationary phase. Further variations in supplements and analysis of defined mutants will allow us to dissect the metabolic and genetic processes controlling the regulation of Nar3 synthesis.

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