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Terminal reduction reactions of nitrate and sulfate assimilation in *Streptomyces coelicolor* A3(2): identification of genes encoding nitrite and sulfite reductases

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Abstract

The model actinobacterium *Streptomyces coelicolor* A3(2) uses nitrate and sulfate as nitrogen and sulfur sources, respectively. The final step prior to assimilation into amino acids is the 6-electron reduction of the nitrite and sulfite anions, catalyzed by siroheme-dependent nitrite (NirBD) and sulfite (SirA) reductases. There are two predicted nitrite/sulfite reductases annotated in the genome of *S. coelicolor*, but it is unclear which is responsible for nitrite and which for sulfite reduction. Here we demonstrate that a knock-out in the genes SCO2487 and SCO2488 encoding NirBD prevents use of nitrite as a nitrogen source, while a knock-out in SCO6102 encoding SirA prevents sulfate assimilation. Both mutations could be phenotypically complemented by supplementation of the growth medium with ammonium or casamino acids in the case of the *nirBD* mutants or sulfur-containing amino acids in the case of the *sirA* mutants. No functional redundancy between the genes was observed and we demonstrate that NirBD is exclusively required for assimilatory nitrite (it does not detoxify nitrite) and SirA exclusively for assimilatory sulfite reduction.

Keywords: Nitrate assimilation; Sulfate assimilation; Siroheme enzymes; Nitrite reduction; Sulfite reduction

1. Introduction

Microorganisms that can assimilate the inorganic nitrogen and sulfur sources nitrate and sulfate catalyze a separate series of reactions that both culminate in a 6-electron reduction step, in which the substrate is reduced to an ammonium ion in the case of nitrate and to sulfide in the case of sulfate assimilation (see Fig. 1). These two reactions are catalyzed by nitrite and sulfite reductases, respectively, and these enzymes share a common architecture as well as a requirement for a siroheme cofactor (Crane and Getzoff, 1996; Crane et al., 1997). Siroheme is an iron-containing isobacteriochlorin cofactor that is more reduced compared with classical protoporphyrin-IX-derived macrocycles such as heme or chlorophyll. The strongly-reducing siroheme cofactor acts in conjunction with a [4Fe–4S] cluster to facilitate 6-electron reduction of the substrate. Nitrite and sulfite reductases belong to the only class of enzyme to use siroheme, and indeed, the sulfite reductase from some sources can catalyze the reduction of both sulfite and nitrite (Crane and Getzoff, 1996). Consequently, in the genomes of many bacteria, genes encoding these enzymes are often annotated as nitrite/sulfite reductase and it is not always clear from the gene context whether the respective product is functional in one pathway or the other, or indeed, in both.

Such a case arises in the genome of the obligately aerobic high-GC Gram-positive bacterium *Streptomyces coelicolor* A3(2) (Bentley et al., 2002; Hopwood, 2006), which has two sets of genes that encode putative nitrite and/or sulfite reductases (Fig. 1B). *S. coelicolor* belongs to the phylum Actinobacteria and grows forming vegetative and aerial mycelia

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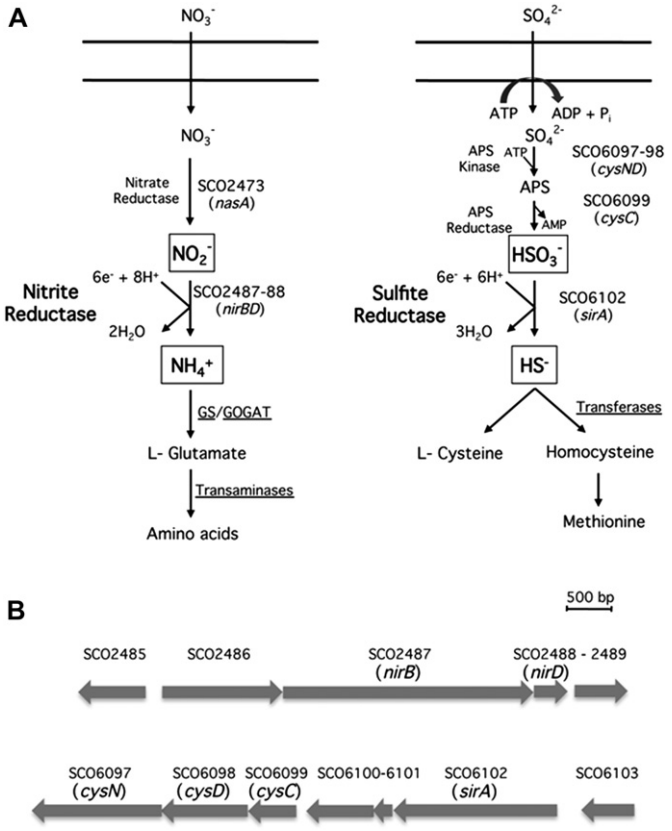

Fig. 1. The pathways of nitrate and sulfate assimilation in *Streptomyces coelicolor*. A. The key steps of nitrate and sulfate assimilation to the stage of incorporation into amino acids are shown. The double lines in the upper portion of the panel represent the cytoplasmic membrane. The 6-electron reduction reactions involving the nitrite and sulfite substrates are indicated in bold type. The genes encoding the respective enzymes are shown at the right of each reaction. B. The gene loci flanking *nirBD* and *sirA* encoding nitrite reductase and sulfite reductase, respectively, are shown.

(hyphae). *S. coelicolor* can use nitrate as a nitrogen source and sulfate as a sulfur source (Hodgson, 2000; Hopwood, 2006). In the genome of *S. coelicolor*, the genes SCO2487 and SCO2488 are predicted to encode a siroheme-dependent nitrite reductase, while SCO6102 is annotated as encoding a siroheme-dependent nitrite/sulfite reductase (http://strepdb.streptomyces.org.uk). The genome context of SCO6102, however, suggests the gene might encode a sulfite reductase because several genes adjacent to it are predicted to encode enzymes involved in early steps of sulfate activation prior to assimilation (see Fig. 1).

The allocation of an assimilatory nitrite reductase function to the SCO2487 and SCO2488 gene products is complicated by the fact that *S. coelicolor* can also catalyze respiratory nitrate reduction (Fischer et al., 2010). Theoretically, therefore, nitrite could be detoxified to ammonia, as occurs in bacteria such as *Escherichia coli*, which perform nitrate ammonification. *E. coli* does not assimilate nitrate, but nevertheless catalyzes nitrite reduction using a siroheme-dependent soluble nitrite reductase (NirBD), which shares significant amino acid sequence similarity with the SCO2487–SCO2488 gene products (Lin and Stewart, 1998; Peakman et al., 1990). Nitrite reduction to ammonia in anaerobically growing *E. coli* presumably serves to rid the cell of toxic nitrite and/or excess reducing equivalents (Gennis and Stewart, 1996).

The ability to carry out nitrate ammonification/detoxification is found amongst both low GC Gram-positive bacteria such as *Bacillus subtilis* (Hoffmann et al., 1998), as well as members of the high GC actinobacterial genus *Arthrobacter* (Eschbach et al., 2003). However, results of a recent study suggest that *S. coelicolor* does not reduce nitrite further to ammonium during respiration, at least in rich medium, because nitrate is quantitatively excreted from both spores and mycelium as nitrite (Fischer et al., 2010). A similar phenomenon has been observed for the actinobacterium *Corynebacterium glutamicum* (Nishimura et al., 2007), suggesting that some members of the Actinobacteria do not ammonify nitrate; notably, however, *C. glutamicum* does not assimilate nitrate (Takeno et al., 2007). In *Mycobacterium* species, respiratory nitrate reductase also doubles as an assimilatory enzyme (Malm et al., 2009), but it is unclear whether the bacterium also carries out ammonification, i.e. ammonia excretion. Although *S. coelicolor* has three respiratory nitrate reductases, none of these functions in nitrate assimilation (Fischer et al., 2010). Instead, *S. coelicolor* synthesizes a molybdenum cofactor-dependent assimilatory nitrate reductase encoded by *nasA* (SCO2473) (Wang and Zhao, 2009). Together, these observations suggest that the predicted NirBD enzyme of *S. coelicolor* is exclusively associated with nitrite assimilation. Tiffert et al. (2008) have also suggested that NirBD has a nitrite assimilatory function in *S. coelicolor*.

Identification of an actinobacterial assimilatory sulfite reductase, called SirA, has so far only been categorically demonstrated for *Mycobacterium tuberculosis* (Pinto et al., 2007). SirA from *M. tuberculosis* shows higher similarity to SCO6102 rather than to NirBD. Nevertheless, because of the considerable variation in nitrite assimilatory capacity, as well as the limited information available regarding sulfite reductase in Actinobacteria, this makes simple predictions of the functions of NirBD and SCO6102 difficult. Therefore, in this study, we introduced defined mutations in the respective genes in an attempt to resolve the biochemical functions of SCO2487, SCO2488 and SCO6102 in nitrite and sulfite assimilation. Furthermore, we examined whether *S. coelicolor* can indeed perform ammonification in defined minimal medium and addressed the question of potential functional redundancy of the nitrite and sulfite reductases in the nitrogen and sulfur assimilation pathways.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Media and culture conditions for *E. coli* and *S. coelicolor* were the same as those described previously (Datsenko and Wanner, 2000; Kieser et al., 2000; Fischer et al., 2010). Strains are listed in Table 1. *E. coli* DH5α (Stratagene) was used as a host for plasmid constructions. *E. coli* BW25113