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Origin and phylogenetic relationships of [4Fe–4S]-containing O_2 sensors of bacteria

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Summary

The advent of environmental O_2 about 2.5 billion years ago forced microbes to metabolically adapt and to develop mechanisms for O_2 sensing. Sensing of O_2 by $[4Fe–4S]^{2+}$ to $[2Fe–2S]^{2+}$ cluster conversion represents an ancient mechanism that is used by FNR_{Ec} (*Escherichia coli*), FNR_{Bs} (*Bacillus subtilis*), $NreB_{Sa}$ (*Staphylococcus aureus*) and $WhiB3_{Mt}$ (*Mycobacterium tuberculosis*). The phylogenetic relationship of these sensors was investigated. FNR_{Ec} homologues are restricted to the proteobacteria and a few representatives from other phyla. Homologues of FNR_{Bs} and $NreB_{Sa}$ are located within the bacilli, of WhiB3 within the actinobacteria. Archaea contain no homologues. The data reveal no similarity between the FNR_{Ec}, FNR_{Bs}, $NreB_{Sa}$ and WhiB3 sensor families on the sequence and structural levels. These O_2 sensor families arose independently in phyla that were already present at the time O_2 appeared, their members were subsequently distributed by lateral gene transfer. The chemistry of [4Fe–4S] and [2Fe–2S] cluster formation and interconversion appears to be shared by the sensor protein families. The type of signal output is, however, family specific. The homologues of FNR_{Ec} and $NreB_{Sa}$ vary with regard to the number of Cys residues that coordinate the cluster. It is suggested that the variants derive from lateral gene transfer and gained other functions.

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Introduction

In modern microbes, molecular oxygen is important for many aspects of bacterial physiology, mainly catabolism (oxidation, respiration) and stress response. Cyanobacteria started producing O_2 about 2.5 billion years ago (Fischer *et al*., 2016). Prior to that time, there were only anaerobes on Earth. The appearance of an O_2-enriched atmosphere confronted microbes with the presence of a strong oxidant that could readily inactivate or otherwise poison the active site and reactive groups of ancient enzymes, mostly metalloenzymes, that we today view as oxygen sensitive (Raymond and Segre, 2006; Martin and Sousa, 2015). These forced microbes to find mechanisms of detecting and dealing with O_2, mechanisms that have persisted to the present. Access to aerobic or anaerobic niches requires extensive remodelling of energy conserving metabolic and anabolic pathways (see review by Unden and Bongaerts, 1997). Additionally, during aerobic growth reactive oxygen species such as (hydrogen)peroxide, superoxide and hydroxyl radicals are formed which require a protective response by the bacteria (for reviews see Imlay 2002; 2006).

Because of its ecological and physiological importance, most bacteria contain sensors for detecting O_2 and reactive oxygen species. Sensors for O_2 are essentially restricted to facultative anaerobic and (micro)aerobic bacteria. About 2.5×10^9 years before present, atmospheric O_2 rose to estimated levels of about 0.02–0.04 atm (Holland, 2006), close to the Pasteur point (Engelhardt, 1974) for the onset of aerobic metabolism. The Pasteur point coincides with the switch point (approx. 0.5–2% of air saturation) of O_2-regulated genes in facultative metabolism of *Escherichia coli* that respond to FNR_{Ec} and other O_2 sensors (Becker *et al*., 1996; Tseng *et al*., 1996; Becker *et al*., 1997). O_2 levels of 0.02–0.04 atm resulted in mild oxygenation of ocean surface waters, a situation that persisted for almost 2 billion years (Lenton *et al*., 2016) because deep ocean oxygenation was not completed until roughly 580–430 million years ago (Stolper and Keller, 2018). In response to environmental oxygenation, bacteria evolved a variety of O_2-sensing systems, classified as direct and indirect O_2 sensors, that recruited

different biochemical mechanisms for monitoring O_2 levels (Green and Paget, 2004; Unden *et al*., 2010).

Direct O_2 sensors use either O_2-sensitive [4Fe–4S] clusters (such as the FNR proteins of *E. coli* and *Bacillus subtilis* and NreB of *Staphylococcus carnosus*), heme B (such as FixL of *Sinorhzobium meliloti* and Dos of *E. coli*) or FAD (such as NifL of *Azotobacter vinelandii*) as O_2-reactive prosthetic groups (for review see Green and Paget, 2004; Unden *et al*., 2010). In *Pseudomonas* species, a system related to the hypoxia-inducible transcription factor (HIF) of animals has been described that employs O_2-dependent hydroxylation reactions to sense decreased O_2 availability (Scotti *et al*., 2014; Schmidt *et al*., 2016).

Indirect O_2 sensors respond to metabolites and pathway intermediates that change their cellular concentration or redox state in response to O_2 availability. The ArcAB two-component system of *E. coli* responds to the redox state of the respiratory quinones or changed quinone/quinol ratios (Malpica *et al*., 2004). The Rex transcriptional regulator of Gram-positive bacteria on the other hand measures the cellular NADH/NAD ratio, which changes as a function of O_2-reducing respiratory activity (Brekasis and Paget, 2003; Sickmier *et al*., 2005; Wang *et al*., 2008).

O_2-labile [4Fe–4S] clusters as universal cofactors for O_2 sensing

Iron–sulphur clusters are widespread and ancient metal cofactors of proteins that are composed of iron ions and sulfide. The clusters are coordinated via weak covalent bonds with cysteine thiol sidechains of the protein. Two-iron–sulfur [2Fe–2S], three-iron–sulfur [3Fe–4S] and four-iron–sulfur [4Fe–4S] clusters are common in proteins (Beinert *et al*., 1997). Proteins with [4Fe–4S] clusters are most versatile and have roles in protein-bound electron transfer in ferredoxin, fumarate reductase and many other redox or respiratory enzymes (Beinert, 1976; Malkin and Rabinowitz, 1967; Lancaster *et al*., 1999), as catalytically active sites in hydratases such as aconitase (Beinert *et al*., 1996), and in iron and redox responsive regulatory proteins (Beinert *et al*., 1996; Mettert and Kiley, 2015). All direct O_2 sensors with FeS clusters use [4Fe–4S] clusters. For this reason only [4Fe–4S] cluster binding sensors will be considered here.

In aconitase and related enzymes, the [4Fe–4S] cluster is required for binding and activation of the substrate (Beinert *et al*., 1996). In addition to its catalytic role, the cluster has a structural role in aconitase (Beinert *et al*., 1996). Thus, apo-aconitase is catalytically inactive but has a regulatory function (Beinert *et al*., 1996; Rouault *et al*., 1992). Loss of the iron–sulfur cluster causes structural rearrangements that allow binding of the apo-enzyme (iron regulatory protein; IRP) to iron-responsive elements (IREs), which are located in the 5′ region of the mRNA of iron homeostasis genes (Kaptain *et al*., 1991; Tang and Guest, 1999; Tang *et al*., 2005; Commichau and Stulke, 2008). IRE binding controls gene expression through mRNA stability.

By contrast, the transcriptional bacterial regulators FNR_{Ec}, FNR_{Bs}, NreB, WhiB3 and NsrR (Volbeda *et al*., 2017) contain [4Fe–4S] clusters that react chemically with molecular O_2 or NO, which controls the function of the sensors by cluster conversion and modification. The physiological role of FNR_{Ec}, FNR_{Bs}, and NreB is essentially that of O_2 sensors, whereas that of WhiB3 can be either that of an O_2 or NO sensor depending on physiological conditions (Singh *et al*., 2007). For each of the sensors, reaction with O_2 has been characterized in detail (see below). NsrR, in contrast, is essentially an NO sensor and the [4Fe–4S] cluster is used for NO response (Volbeda *et al*., 2017). For this reason in the present work only, FNR_{Ec}, FNR_{Bs}, NreB and WhiB3 will be discussed. The iron–sulfur clusters are surface exposed and are able to react with O_2. FNR_{Ec}, FNR_{Bs} and WhiB3 represent transcriptional regulators (Fig. 1). DNA binding and their function as transcriptional regulators is controlled by the [4Fe–4S] clusters which react directly with O_2. In the FNR proteins, the reaction of the cluster with O_2 causes cluster degradation. As a consequence FNR_{Ec} monomerizes and loses the ability for DNA binding and transcriptional activation (Lazazzera *et al*., 1996), whereas FNR_{Bs} is a permanent dimer but loses DNA binding due to conformational changes after degradation of the FeS cluster (Reents *et al*., 2006b).

In WhiB3, the [4Fe–4S] cluster is required for complex formation with the sigma factor σ^A. Degradation of the FeS cluster disassembles the complex and transcriptional activity (Kudhair *et al*., 2017). In the sensor kinase NreB, the sensory PAS domain controls the activity of the kinase domain in response to O_2 via the iron–sulfur cluster. Auto-phosphorylation of NreB leads to the phosphorylation of the response regulator NreC that activates in the phosphorylated state (NreC-P) the expression of target genes.

The Cys residues that ligate the iron–sulfur clusters in FNR_{Ec}, FNR_{Bs}, WhiB3 and NreB are located in clusters that differ in sequence, spacing and location within the sensors (Fig. 1B). In FNR_{Bs} one of the ligands of the iron–sulfur cluster is replaced by an Asp residue (Gruner *et al*., 2011). FNR_{Ec} was the first O_2 sensor of this type and represents the prototype of this type of O_2 sensors (Shaw and Guest, 1982; Shaw *et al*., 1983; Green and Paget, 2004). The crystal structure of the FNR_{Ec} type FNR_{Af} from *Aliivibrio fischeri* was solved (Volbeda *et al*., 2015). The protein consists of two domains that provide the sensory and the DNA-binding function. The N-

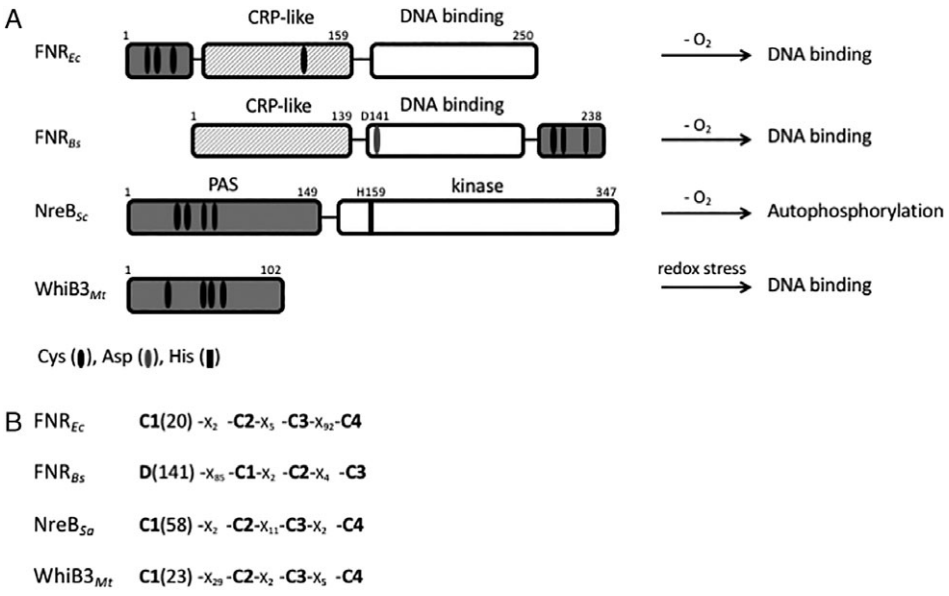

Fig. 1. Domain structure (A) and conserved cluster-binding motif (B) of the $[4Fe–4S]^{2+}$-containing sensors FNR, NreB and WhiB3.A. The cysteine-carrying sensory domains (grey), the output domains and the kind of response (DNA binding or autophosphorylation) are depicted. Approximate positions of cluster coordinating conserved cysteine and aspartate residues as well as phosphorylation residue His159 of sensor kinase NreB of *S. carnosus* are marked. B. The [Fe–S]-cluster binding sequence motifs consisting of conserved cysteine (C) and aspartate (D) residues are shown as well as their position in the protein. Variable amino acid residues between cluster ligands are indicated with x and the corresponding number. Modified after Unden and colleagues (2013).

terminal sensory domain ligates under anoxic conditions a $[4Fe–4S]^{2+}$ cluster and under oxic conditions a $[2Fe–2S]^{2+}$ cluster.

The $[4Fe–4S]^{2+}$ cluster binding sensors FNR_{Ec} and FNR_{Af} are dimers with an α-helical dimer interface, and the dimeric state is required for site specific DNA-binding (Lazazzera *et al*., 1993; Khoroshilova *et al*., 1995; Kiley and Beinert, 1998). Conversion to the $[2Fe–2S]^{2+}$ form results in a rearrangement of the dimer interface causing monomerization and loss of specific DNA-binding (Khoroshilova *et al*., 1995; Volbeda *et al*., 2015). The $[4Fe–4S]^{2+}$/$[2Fe–2S]^{2+}$ cluster conversion was identified by Mössbauer and EPR spectroscopy (Lazazzera *et al*., 1993; Khoroshilova *et al*., 1995; Lazazzera *et al*., 1996; Khoroshilova *et al*., 1997; Kiley and Beinert, 1998). Combination of visible absorbance, EPR spectroscopy and time resolved electrospray ionization mass spectrometry allowed a very detailed analysis of the reactions. The O_2 triggers a reaction in two steps (Crack *et al*., 2006; 2007; 2008):

$$\text{Step 1}: [4Fe-4S]^{2+} + O_2 \rightarrow [3Fe-4S]^{1+} + Fe^{2+} + O_2^-$$
$$\text{Step 2}: [3Fe-4S]^{1+} \rightarrow [2Fe-2S]^{2+} + Fe^{3+} + 2S^{2-}$$

Step 2 apparently involves a second oxidation by O_2 (Crack *et al*., 2017; Zhang *et al*., 2012). The two sulfide ions in step 2 are not released into aqueous solution but oxidized to sulfane (S^0) and form a persulfide with two of the Cys ligands (RS^-) of the cluster. Up to two of the Cys ligands exist then in the persulfide (RSS^-) state. The reaction of step 2 has to be reformulated accordingly (Step 2*):

$$\text{Step 2}^*: [3Fe-4S](RS)_3 + RS^- + O_2 + 4H^+ \rightarrow [2Fe-2S](RS)_2(RSS)_2 + Fe^{3+} + 2H_2O.$$

Formation of the Cys-persulfide provides a mechanism for storing the sulfur released from the iron–sulfur cluster during the [4Fe–4S]/[2Fe–2S] cluster conversion rather than releasing it to the water space. This mechanism allows reversion of the O_2-inactivated FNR and to the anaerobic $[4Fe–4S]^{2+}$ form by reduction and repair without involvement of the iron–sulfur biosynthesis machinery (Zhang *et al*., 2012; Crack *et al*., 2017).

In NreB, a $[4Fe–4S]^{2+}$/$[2Fe–2S]^{2+}$ cluster conversion is the basis for O_2 sensing (Mullner *et al*., 2008), which suggests a reaction sequence similar to FNR_{Ec}. In the anoxic form, FNR_{BS} and WhiB3 contain a $[4Fe–4S]^{2+}$ cluster as well (Jakimowicz *et al*., 2005; Reents *et al*., 2006b; Crack *et al*., 2009). It appears therefore that FNR_{Ec}, FNR_{Bs}, NreB and WhiB3 use the same cofactor for O_2 sensing and that the reactions occurring at the $[4Fe–4S]^{2+}$ during response to O_2 are similar.

Here, we investigate the phylogenetic relationships of bacterial O_2 sensors using $[4Fe–4S]^{2+}$ clusters for sensing. The protein sequences of FNR_{Ec}, FNR_{Bs}, *Staphylococcus aureus* NreB and *Mycobacterium tuberculosis* WhiB3 were used to identify homologues among gene families (clusters) generated from 1981 sequenced prokaryotic genomes and screened for retainment of the consensus Cys clusters for binding the iron–sulfur clusters. The latter represent the most characteristic feature of the proteins in order to characterize their potential for O_2 sensing and their distribution and variation among prokaryotes.

Results

Clustering and phylogenetic distribution of FNR_{Ec}, FNR_{Bs}, $NreB_{Sa}$ and WhiB3 homologues

The presence or absence of matrix in Fig. 2 summarizes the occurrence of FNR_{Ec}, FNR_{Bs}, $NreB_{Sa}$, $WhiB3_{Mt}$ and their homologues in the bacterial taxa indicated. No homologues were detected in archaea. The occurrence of CRP, a global regulator and homologue of FNR_{Ec} that lacks O_2 sensing clusters, is also indicated. Each column represents a cluster, ticks summarizing the results of BLAST searches and clustering to detect homologues (black ticks indicate presence; white ticks indicate absence) from prokaryote genomes in the RefSeq 2012 database. The clusters were generated using the standard Markov cluster algorithm (Enright *et al*., 2002) (MCL) at a 25% global identity threshold as previously described (Nelson-Sathi *et al*., 2015; Weiss *et al*., 2016). Each black tick indicates the presence of one (or more) homologous protein in the corresponding species (rows)

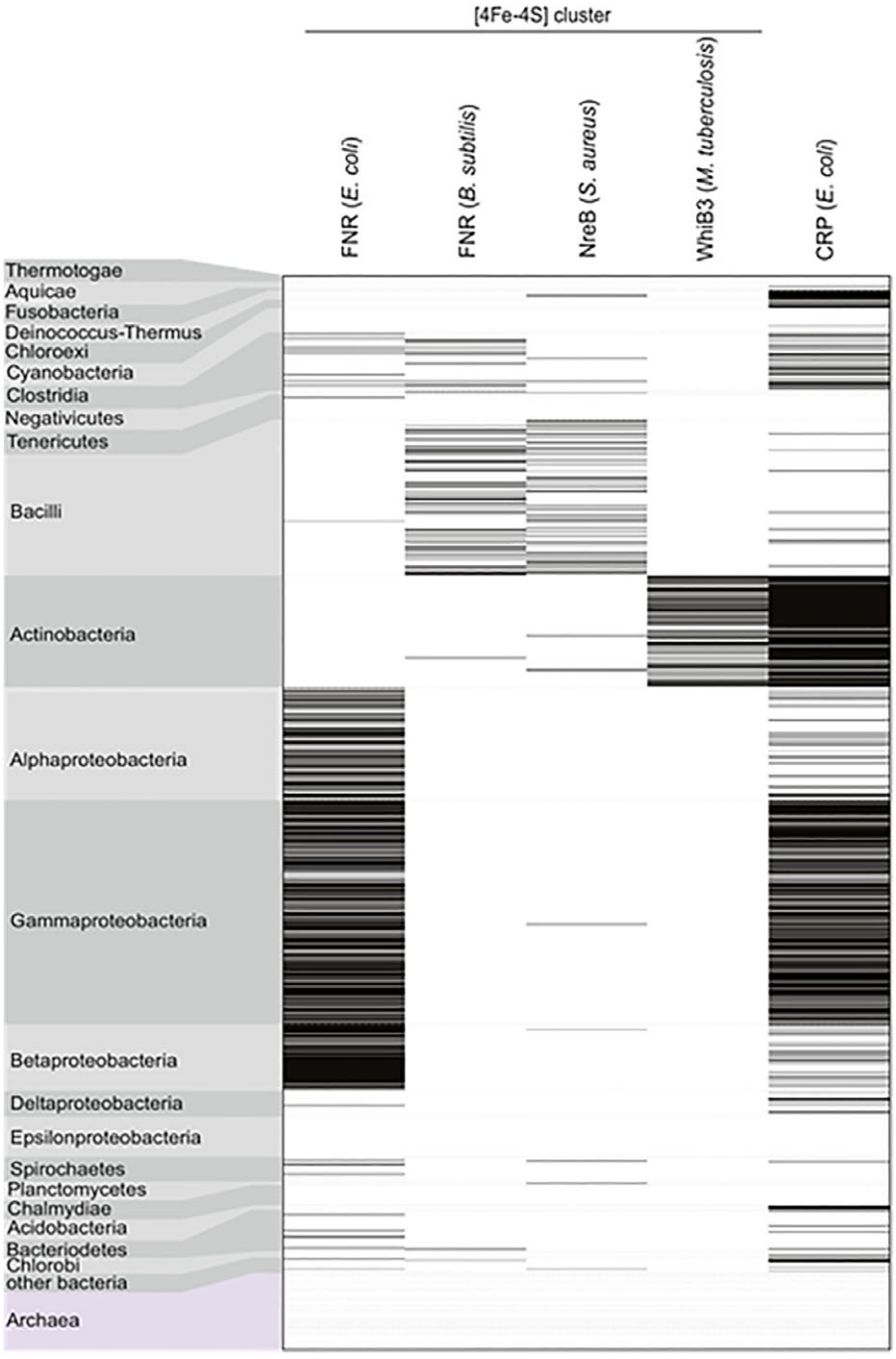

Fig. 2. Matrix of [Fe–S]-containing sensors FNR, NreB, WhiB3 and global regulator CRP.The matrix shows the distribution of the identified homologous proteins for each reference protein during BLAST search. One black line corresponds to one homologous protein in the respective bacteria. On the vertical axis, the predefined bacterial phyla (sometimes classes) are displayed.

with a BLAST hit with ≥ 25% local identity and a pairwise alignment with ≥ 25% global identity. Protein sequence similarity was scored without weighting of specific motifs. To distinguish between homologous proteins that could function as oxygen sensor and false positive sensors, the presence of the cluster-ligating Cys motif was scored. FNR homologues that lack N- or C-terminal Cys motifs were scored as CRP-type regulators due to the absence of the oxygen-responsive FeS cluster required for sensor function. The NreB-type regulators are identified by the similarity of PAS and kinase domain.

FNR of the *E. coli*-type (FNR_{Ec}) predominantly occurs among the α-, β- and γ-Proteobacteria but some homologues are found in the clostridia, spirochaetes, bacteriodetes and other phyla. By contrast, the *B. subtilis*-type FNR (FNR_{Bs}) occurs mainly within the bacilli and has no homologues in the proteobacteria. Few additional BLAST hits are found within the clostridia, negativicutes, bacteriodetes and actinobacteria. The sensor kinase NreB from *S. aureus* and its homologues are mostly restricted to the Bacilli but there are few similar proteins in other firmicutes and in the actinobacteria, proteobacteria, spirochaetes, planctomycetes as well as in the deinococcus-thermus groups. The *M. tuberculosis* WhiB3 sensor is limited to the actinobacteria. The global regulator CRP from *E. coli* that is related to FNR_{Ec} and FNR_{Bs} (Korner *et al*., 2003) has homologues in almost all bacterial phyla. Most of the CRP homologues are found within the actinobacteria and the proteobacteria whereas the bacilli that harbour most of the FNR_{Bs} proteins are mostly devoid of CRP. In general, the oxygen sensors FNR_{Ec}, FNR_{Bs}, NreB and WhiB3 are restricted to specific bacterial phyla whereas CRP occurs in most bacterial phyla. Several phyla lack specific O_2 sensors of this type (aquifex, thermotoga, fusobacteria, ε-proteobacteria, acidobacteria, chalmydiae, chlorobi, cyanobacteria and the green filamentous bacteria) or only show sporadic occurrence (bacteroides, planctomyces, tenericutes, δ-proteobacteria, negativicutes and deinococcus-thermus). Notably, the archaea, including the aerobic haloarchaea (Euryarchaeota) and the (facultatively) aerobic hyperthermophilic Crenarchaeota (*Sulfolobus*, *Acidianus*, *Pyrobaculum*, *Metallosphaera*) are devoid of FNR_{Ec}, FNR_{Bs}, NreB or WhiB3 type sensors.

The iron–sulfur clusters of FNR_{Ec}, FNR_{Bs}, $NreB_{Sc}$ and WhiB3 are co-ordinated by Cys and occasionally Asp residues. FNR_{Ec} and FNR_{Bs} are distant homologues of the CRP protein (Shaw *et al*., 1983; Korner *et al*., 2003). FNR_{Ec} contains a short N-terminal extension to CRP of about 29 AA (Fig. 1A) that are specific for FNR_{Ec} (Shaw *et al*., 1983). Three of the four Cys residues (C1–C3) of FNR_{Ec} for ligating the iron–sulfur cluster are placed in the FNR-typic extension (Fig. 1B), only the fourth residue (C122) is located in the CRP homologous region. FNR_{Bs} that is also distantly homologous to CRP carries the Cys cluster in a C-terminal extension downstream the CRP homologous region. Only the first ligand (D141) is located in the CRP homologous region. Replacement of single residues of the binding clusters by other residues generally inactivates their capacity for O_2 sensing (Melville and Gunsalus, 1990; Green *et al*., 1993; Kamps *et al*., 2004; Gruner *et al*., 2011), demonstrating the significance of the residues for the basic function of the proteins. The proteins of the searches (Fig. 2) were therefore screened for the presence of the respective Cys/Asp clusters and the presence of sensory domain. Refined overviews of FNR_{Ec}, FNR_{Bs}, $NreB_{Sc}$ and WhiB3 homologues are presented in the following sections including an evaluation of their presumptive properties in O_2 sensing.

FNR-Ec-type sensors in the prokaryotic kingdom

FNR_{Ec} is a major regulator of the aerobic/anaerobic switch in *E. coli* and responsible for the induction of genes of anaerobic and microaerobic respiration, fermentation and anaerobiosis related genes. Transcriptional activation by FNR_{Ec} depends on its dimeric state which is controlled by the [4Fe–4S]/[2Fe–2S] cluster conversion in response to O_2 presence. The matrix of Fig. 2 contains altogether 414 FNR_{Ec} homologues after deletion of redundant or closely related hits from related species. From the remaining, 95% of the homologues were located within the α-, β- and γ-proteobacteria (Fig. 3), with the γ-proteobacteria enclosing the largest number. The residual hits (5% of 414) were in diverse phyla outside the proteobacteria, that is in the clostridia (six strains), bacteriodetes (six strains), the spirochaetes (three strains) and others (Fig. 4). The homologues with the 'diverse' origin showed a closed clustering separate from the proteobacteria. For all homologues within the α-, β- and γ-proteobacteria the phylogenetic tree of the homologues agrees with that of their hosts (Supporting Information Fig. S1), suggesting that the proteins co-evolved with their phyla.

The 414 FNR-Ec type homologues were investigated for the conservation of the Cys clusters by recording the number and spacing of Cys residues. Spacing variants delineate subfamilies of FNR proteins, whereas conservation or loss of Cys residues indicates conservation of O_2 sensing. The majority of FNR_{Ec} homologues (79%) retained a 4Cys cluster (Fig. 3), and the remaining 21% were lacking one or more of the Cys residues (Fig. 3). The variants containing less than 4Cys residues of the cluster are mostly scattered in the phyla with some specific preferences (Figs 3 and 4). The 4Cys variants represent the majority of the homologues in the α-, β- and γ-proteobacteria, whereas in the other bacteria, the variants with ≤ 3Cys residues predominate (12 from

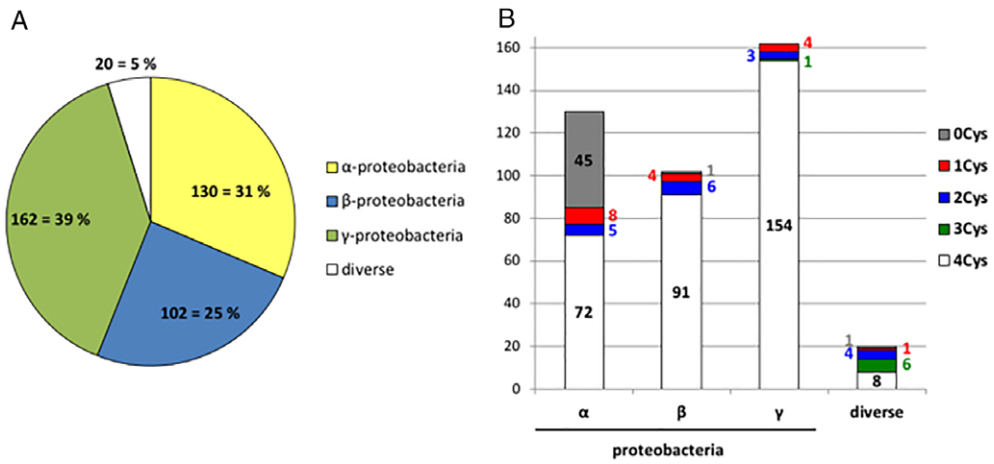

Fig. 3. Distribution of FNR_{Ec} homologues and Cys variants.The homologues (664 proteins) were identified by the BLAST search followed by the removal of close homology (> 80% global identity) in order to delete hits from closely related strains. The resulting homologues (414 proteins) represent the basis for the data in A and B. A. The distribution of the FNR_{Ec} homologues in the bacterial phyla. B. The number of Cys variants within the bacterial phyla from the total of 414 FNR_{Ec} homologues.The α-, β- and γ-proteobacteria of (A) show the same colouring as in the phylogenetic tree of Supporting Information Fig. S1. The Cys variants in B) are labelled by colour code 0Cys (grey), 1Cys (red), 2Cys (blue), 3Cys (green) and 4Cys (white).

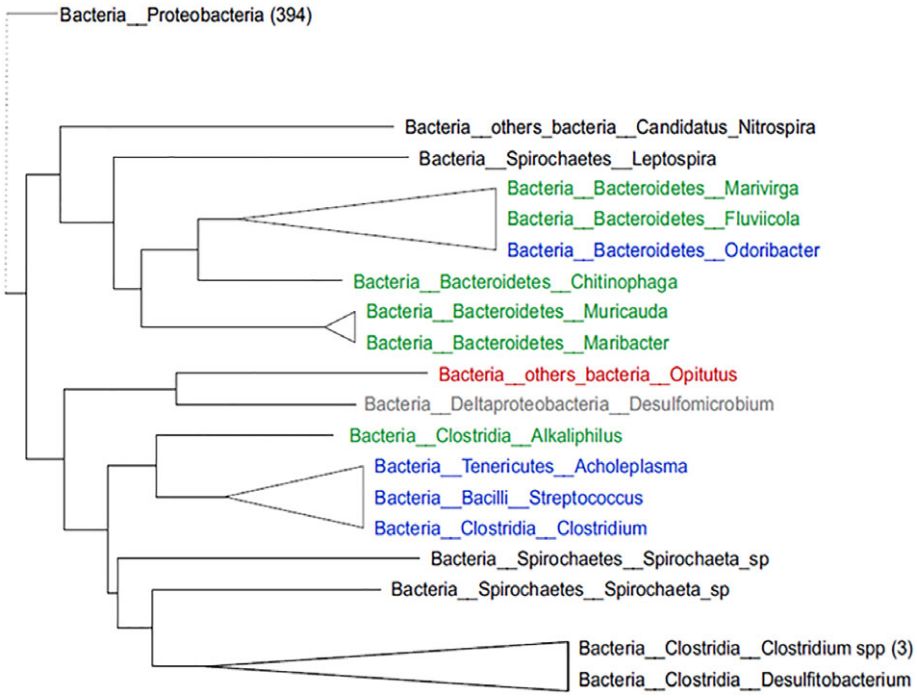

Fig. 4. FNR_{Ec} variants outside the proteobacteria phylogenetic group. Labelled in black, green, blue, red and grey are variants with four, three, two, one or zero conserved Cys residues respectively. The digits in brackets correspond to the amount of FNR homologues in the respective genus or phylum.

20 homologues). The function of these ≤ 3Cys variants has not been studied, but their prevalence suggests a modified function.

The 3Cys variants are rare and are found only in the γ-proteobacteria and the 'diverse lineages' group. The 2Cys and 1Cys variants are found in all protobacterial phyla at a frequency of ≤ 10% of phylum members each. The 0Cys variants are characteristic for the α-proteobacteria and represent 35% of the FNR_{Ec} homologues (Fig. 3). In the following, the FNR_{Ec} Cys variants will be discussed with respect to their possible function, but we note that only 4Cys and 0Cys variants of FNR_{Ec} have been characterized by experiments.

4Cys variants of FNR_{Ec}

Within the 4Cys FNR_{Ec} proteins, three types of spacing are observed. The **C1**–X_2–**C2** spacing is conserved in all types, but for the C2/C3 and C3/C4 pairs some variation can be seen (Table 1 and Supporting Information Figs S2

Table 1. Occurrence and properties of FNR_{Ec} variants in relation to their Cys clusters.

FNR_{Ec} type	Cys clusters	Bacteria (examples)	Function or regulated genes	References
FNR (4Cys)	**C1**–X_2–**C2**–X_5–**C3**–X_{92}–**C4**	γ-Proteobacteria	Transcriptional regulation in response to O_2 (anaerobic and microaerobic respirations; fermentation; related genes)	Galimand and colleagues (1991), Salmon and colleagues (2003), Sawers (1991), Shaw and Guest (1982), Spiro and Guest (1988) and Unden and Bongaerts (1997)
FNR, Anr, EtrA, HlyX, CydR, FnrA	(Alignment, Supporting Information Fig. S2)			
FNR (4Cys)	**C1**–X_2–**C2**–X_5–**C3**–X_{92}–**C4**	β-Proteobacteria	Anaerobic growth; virulence; anaerobic regulation fumarate and nitrate respiration; sugar fermentation	Bartolini and colleagues (2006), Edwards and colleagues (2010) and Wood and colleagues (1998)
Fnr, Btr	(Alignment, Supporting Information Fig. S2)	(*Bordetella pertussis*; *Neisseria meningitides*)		
FNR (4Cys)	**C1**–X_2–**C2**–X_7–**C3**–X_{87}–**C4**	α-Proteobacteria	N_2 fixation, anaerobic respiration	Anthamatten and colleagues (1992), Batut and Boistard (1994), Schluter and colleagues (1992) and Zeilstra-Ryalls and colleagues (1997)
FnrN, FnrL, AadR, $FixK_1$	(Alignment, Supporting Information Fig. S2)	(*Rhizobium* spp., *Rhodo-bacter* spp., *Bradyrhizobium* spp. and others)		
Fnr		γ-Proteobacteria [(*Pseudo-*) *Xanthomonas*]	Not characterized	Not characterized
FNR (4Cys)	**C1**–X_2–**C2**–X_{3-8}–**C3**–X_{88-98}–**C4**	Diverse bacteria	Not characterized	Not characterized
	(Alignment, Supporting Information Fig. S3)	(Clostridia, spirochaeta, leptospira)		
FNR (3Cys)	**C1**–X_2–**C2**–X_{97-98}–**C4**	γ-Proteobacteria	Not characterized	Not characterized
	(Alignment, Supporting Information Fig. S4)	(*Acidithiobacillus caldus*) Diverse bacteria (bacteriodetes, *Clostridium* spp.)		
FNR (2Cys)	**C3**–X_{92}–**C4** (most common)	Proteobacteria	Not characterized	Not characterized
	C2–X_5–**C3**	α-Proteobacteria (*Methylobacterium* spp.)		
	C2–X_{97-98}–**C4**	Diverse bacteria (bacteriodetes, clostridia, bacilli, tenericutes)		
FNR (1Cys)	**C4 (**or **C1)**	α-,β-, γ-Proteobacteria Diverse bacteria (*Opitutus*)	Not characterized	
FNR (0Cys)	No C	α-Proteobacteria (often in addition to 4Cys-FNR)	N_2 fixation (*fix* and *nif* genes), nitrate respiration	Batut and colleagues (1989) and Fischer (1994)
($FixK_2$, FixK)	(Alignment in Supporting Information Fig. S5)	*Bradyrhizobium japonicum* ($FixK_2$), *Rhizobium meliloti* (FixK)		

and S3). The prototypic spacing **C1**–X_2–**C2**–X_5–**C3**–X_{92}–**C4** (Table 1 and Supporting Information Fig. S2) from FNR_{Ec} is present in the FNR proteins of the γ- and β-proteobacteria and represent the largest group of 4Cys FNR_{Ec} proteins. FNR_{Ec} of *E. coli* represents the prototype of 4Cys FNR where all details in O_2 sensing and cluster biochemistry have been studied (Shaw and Guest, 1982; Khoroshilova *et al*., 1995; 1997; Green *et al*., 1996; Crack *et al*., 2017). In addition to FNR_{Ec}, the proteins of other γ-proteobacteria have been verified as FNR_{Ec}-type O_2 sensors, including Fnr or Anr from *Pseudomonas* spp. (Galimand *et al*., 1991; Sawers, 1991; Ibrahim *et al*., 2015), EtrA from *Shewanella* spp. (Maier and Myers, 2001; Cruz-Garcia *et al*., 2011), Fnr from *Vibrio* strains (Septer *et al*., 2010; Kado *et al*., 2017), FnrP of *Pasteurella* (Uhlich *et al*., 1999), HlyX of *Actinobacillus pleuropneumoniae* (MacInnes *et al*., 1990) and Fnr of *Klebsiella pneumoniae* (Grabbe *et al*., 2001) (Table 1). FNR proteins of the β-proteobacteria contain the same Cys spacing as the γ-proteobacteria, but the biochemistry of iron–sulfur cluster has not been studied *in vitro.* The FNR proteins of the β-proteobacteria control the expression of catabolic processes such as anaerobic respiration and fermentation (Table 1) similar to FNR from the γ-proteobacteria and are significant for the virulence of some pathogenic strains like *Bordetella*, *Neisseria* and *Burkholderia* (Bannan *et al*., 1993; Wood *et al*., 1998; Bartolini *et al*., 2006; Edwards *et al*., 2010; Sass *et al*., 2013).

The α-proteobacteria contain 4Cys variants with a slightly different spacing of the C2/C3 and the C3/C4 residues (**C1**–X_2–**C2**–X_7–**C3**–X_{87}–**C4)** (Table 1 and Supporting Information Fig. S2). For some 4Cys FNR_{Ec} variants of the α-proteobacteria, O_2 sensitivity and their capability for complementing FNR_{Ec} function *in vivo* have been verified but detailed biochemical studies on the properties of the modified Cys clusters are missing. The 4Cys FNR_{Ec} homologues of the α-proteobacteria often function as transcriptional activator for genes of anaerobic metabolism many of which are related to bacteria/plant association like nitrate and microaerobic respiration, N_2-fixation, virulence and functionally related genes (Table 1). Representatives of this class have been characterized mainly *in vivo*, such as FnrL of *Rhodobacter capsulatus* (Zeilstra-Ryalls *et al*., 1997), FnrN of *Rhizobium leguminosarum* (Schluter *et al*., 1992), SinR of *Agrobacterium tumefaciens* (Ramey *et al*., 2004), $FixK_1$ of *Bradyrhizobium japonicum* (Anthamatten *et al*., 1992) and AadR of *Rhodpseudomonas palustris (Dispensa et al.,* 1992*).* Remarkably, the 4Cys FNR of the plant pathogenic α-proteobacteria *Xanthomonas* (and *Pseudoxanthomonas*) contain the same Cys cluster indicating a relation of this type of FNR to a plant associated biotope.

The 4Cys FNR_{Ec} proteins of the 'diverse' bacteria group outside the proteobacteria (Fig. 4) differ in the 4Cys cluster slightly from that of the γ/β- and the α-proteobacteria (Table 1 and Supporting Information Fig. S3). The difference in Cys-spacing and their separate phylogenetic clustering stresses their diversification from the proteobacterial FNR_{Ec} proteins. None of the FNR_{Ec}-type proteins of this group has been tested for iron–sulfur cluster properties or function, but the presence of a modified 4Cys cluster suggests that the proteins may be O_2-sensitive transcriptional regulators similar to FNR_{Ec}. Cluster type and the protein sequence give no indication on the phylogenetic origin of this group of FNR_{Ec} proteins.

3Cys variants of FNR_{Ec}

3Cys variants of FNR_{Ec} are scarce (Fig. 3). Most variants (six) are placed within the 'diverse' group non-proteobacteria, and only one further representative is found within the (γ-)proteobacteria (Fig. 3 and Supporting Information Fig. S1). None of the variants has been characterized genetically or biochemically. In the 3Cys variants generally C3 is missing (**C1**–X_2–**C2**–$X_{97–98}$–**C4**; Table 1 and Supporting Information Fig. S4) whereas C1, C2 and C4 are conserved. In some variants, C3 is replaced by a Ser, Asn or Pro residue, or a Cys residue in a modified position. These residues could serve as the fourth ligand for the iron–sulfur cluster, with or without conservation of the function (Muraki *et al*., 2010). The 3Cys variants therefore might be functionally similar to the 4Cys FNR_{Ec} proteins, or represent precursors of other FNR variants.

2Cys variants of FNR_{Ec}

2Cys variants of FNR_{Ec} are present in small numbers (18 representatives in total) in the proteobacterial and the diverse groups (Fig. 3). The 2Cys variants uniformly lack C1. Most of the homologues contain conserved C3 and C4, but other combinations of conserved Cys residues are present as well (Table 1). Thus, in *Burkholderia* (β-proteobacteria) and *Acholeplasma* (Tenericutes), the N-terminal sequence with C1 is deleted, whereas in the variants of *Chromholaobacter* (γ-proteobacteria), *Streptococcus* (bacilli), *Clostridium cellulolyticum* and others, the Cys residues are not conserved.

None of the 2Cys variants has been functionally or biochemically characterized. The Flp proteins from lactic acid bacteria (Gostick *et al*., 1998; Scott *et al*., 2000) serve as models for the function of FNR-like proteins with two Cys residues (Table 1). FlpA from *Lactococcus lactis* is a member of the FNR/CRP family of transcriptional regulators (Korner *et al*., 2003) with low similarity (22% identity with FNR_{Ec}) and is not part of the FNR_{Ec} like protein cluster of Supporting Information Fig. S1. The Cys pair of FlpA (residues C_{15} and C_{112}) assembles in the FlpA dimer an O_2-labile [4Fe–4S] cluster that abolished DNA binding (Scott *et al*., 2000). Flp from *Lactobacillus casei* on the other hand uses the Cys pair for an intramolecular disulphide-dithiol redox switch (Gostick *et al*., 1998). The reactions of the Flp proteins *in vivo* are not clear, however. The Flp proteins respond to oxidative stress and control redox stress reactions, zinc uptake and the arginine deiminase pathway. It is feasible that the 2Cys FNR_{Ec} proteins employ similar reactions or functions.

1Cys variant of FNR-Ec

The 1Cys variants of FNR_{Ec} have high global sequence similarity to FNR_{Ec} and are located within the proteobacteria and *Opitutus terrae* from the diverse group (Fig. 3 and Supporting Information Fig. S1). *Opitutus terrae* and some proteobacteria including *Paracoccus denitrificans* contain only the 1Cys variant of FNR_{Ec}, whereas other strains carry additionally 4Cys FNR_{Ec}. The conserved Cys residue is mostly C4, whereas in some representatives C1 (*Methylobacterium*) or C3 (*O. terrae* and *Variovorax paradoxus*) is conserved. The bacteria with conserved C4 generally lack the N-terminal part of FNR_{Ec} with aa 1–25 and C1 to C3 of FNR_{Ec}.

Presence of a single redox sensitive Cys residue is reminiscent of the *B. subtilis* OhrR protein. The thiolate of Cys15 from OhrR is oxidized (reversibly) to

Cys15-SOH (sulphenic acid) by treatment with hydroperoxide. The oxidation inhibits DNA-binding of OhrR and induces expression of the organic hydroperoxidase gene (Fuangthong and Helmann, 2002). Generally, conservation of single Cys residues suggests redox regulation by reversible protein S-thiolation or thiol-based redox switches (Hillion and Antelmann, 2015; Loi *et al*., 2015).

0Cys variants of FNR-Ec

Most of the 0Cys variants of FNR_{Ec} are located in the α-proteobacteria (Fig. 3) with few examples within the δ- (*Desulfomicrobium*) and β-proteobacteria (*Burkholderia*). Some of the 0Cys variants show deletions in the N-terminal C1 to C3 region of FNR_{Ec}, whereas other cover the region without conservation of the Cys residues. The residual part of the protein is conserved which defines the proteins as FNR_{Ec} homologues.

Prototypes of the 0Cys variants are represented by $FixK_2$ of *Rhodopseudomonas japonicum* and FixK of *Rhizobium meliloti* (Batut *et al*., 1989; Fischer, 1994). $FixK_2$ and FixK are part of an O_2-regulatory cascade, which induces nitrogen fixation and nitrate respiratory genes under anoxic conditions. $FixK_2$ and FixK are no O_2 sensors on their own. Expression of the *fixK_2* and *fix* genes is, however, under the transcriptional regulation of the FixL-FixJ O_2-sensing system that stimulates expression (and function) of $FixK_2$ and FixK under anoxic conditions (for overview see Fischer (1994)). In summary, the 0Cys proteins are members of the FNR_{Ec} family but represent in the form of FixK and $FixK_2$ indirect O_2 sensors without iron–sulfur cluster.

FNR B. subtilis *(FNR_{Bs})*

The search for homologues of FNR_{Bs} yielded 96 hits, which were mostly (82%) in the bacilli phylum (Fig. 1) and among those most (63%) in the genus *Bacillus. Bacillus subtilis* FNR_{Bs} coordinates the [4Fe–4S] cluster by Asp D141 and three Cys residues. The Cys residues are located in a C-terminal domain (Gruner *et al*., 2011; Reents *et al*., 2006b) that represents an extension to the CRP homologous region (see Fig. 1). The basic function of this type of FNR in O_2 sensing has been studied for FNR_{Bs} (Gruner *et al*., 2011; Reents *et al*., a,b). FNR_{Bs} and FNR_{Bl} of *Bacillus licheniformis* (Klinger *et al*., 1998; Rey *et al*., 2004) are required for anaerobic induction of the *nar* genes coding for the nitrate respiratory system *narGHJI* and *narK* as well as for *arfM*, a fermentation regulator.

A total of 18% of the FNR_{Bs}-type proteins lack the C-terminal domain and the Cys residues for cluster binding, other variants of the Cys cluster were not identified. Most of the 0Cys variants lack also the conserved Asp residue. The function of the 0-Cys/Asp variants of FNR_{Bs} has not been analysed, but most of the strains are facultatively anaerobic and capable of fermentation or nitrate respiration (Supporting Information Table S1). The 0Cys variants of FNR_{Bs} could represent indirect, iron–sulfur deficient O_2 sensors, such as the 0Cys variants of FNR_{Ec} (compare Table 1), or other regulators of the FNR-CRP family (Korner *et al*., 2003). The FNR_{Bs} variant from aerobic *N. koreensis* has the conserved Asp residue, but lacks the C-terminal Cys cluster of FNR_{Bs}. Instead, it contains, an N-terminal Cys-cluster similar to that of FNR_{Ec}. The protein appears to be an FNR_{Bs} homologue with an FNR_{Bs}/FNR_{Ec} hybrid iron–sulfur binding 3Cys/Asp site.

Fig. 5B shows a matrix for the FNR_{Bs} proteins (80 variants) excluding 0Cys-FNR_{Bs} variants. The occurrence of the FNR_{Bs} homologues is presented in a simplified tree where strains are combined on the genus level (Fig. 5A). FNR_{Bs} is confined to the Bacilli phylum, with few exceptions of homologues in *Clostridium botulinum* (clostridia) and *Selenomonas ruminantium* (negativicutes). FNR_{Bs} is therefore restricted to the firmicutes with strong predominance in the genus *Bacillus.* Homologues are present in most of the Bacilli genera, including *Paenibacillus*, *Geobacillus*, *Exiguobacterium*, *Brevibacillus*, *Lysinibacillus*, *Macrococcus*, *Staphylococcus* and *Anoxybacillus.*

The bacteria of the matrix of Fig. 5 and the corresponding FNR_{Bs} proteins can be functionally sub-grouped by the controlled genes. (i) The typical form represented by FNR_{Bs} of *B. subtilis* and *B. licheniformis* controls anaerobic induction of the nitrate respiratory system (Cruz Ramos *et al*., 1995; Klinger *et al*., 1998; Rey *et al*., 2004; Reents *et al*., 2006a). The branch comprising 17 non-pathogenic strains with *B. subtilis*, *B. licheniformis*, *Bacillus artrophaeus* and the plant growth promoting rhizobacterium *Bacillus amyloliquefaciens* (Chen *et al*., 2007; He *et al*., 2012) and *Bacillus* sp. (Song *et al*., 2012) appears to be part of this class. FNR from the *Geobacilli* and *Bacillus megaterium* could be part of the same cluster due to the presence of nitrate respiration and colocalization with *nar* genes (Feng *et al*., 2007; Muhd Sakaff *et al*., 2012; Brumm *et al*., 2015). Interestingly, *Paenibacillus terrae*, *Paenibacillus polymyxa* and *Paenibacillus mucilaginosus* contain two FNR-like proteins each. (ii) FNR_{Bc} of the pathogenic *Bacillus cereus* modulates under anaerobic conditions glucose fermentation and other catabolic genes in a carbohydrate-dependent manner but is dispensable for nitrate respiration. FNR_{Bc} also activates the expression of enterotoxins (Zigha *et al*., 2007; Messaoudi *et al*., 2010; Esbelin *et al*., 2012). (iii) The FNR homologues of *Bacillus selenitireducens* are more distantly related and not part of the FNR_{Bs} and FNR_{Bc} branches. The bacteria are non-pathogenic and not nitrate respiring (Switzer Blum *et al*., 1998; Eppinger *et al*., 2011), suggesting that their FNR proteins

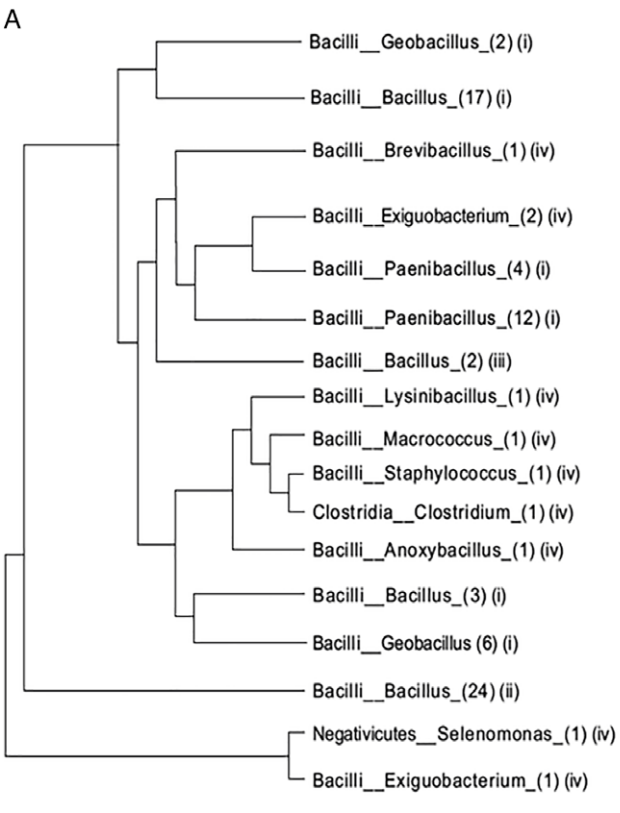

Fig. 5. Schematic tree of *B. subtilis* FNR_{Bs} (A) and matrix for strains carrying FNR_{Bs}-like proteins (B). In (B), strains are listed after removing the 0Cys/D variants (see main text), other details as for Fig. 2. In (A), the clades were collapsed on genus level. The digits in brackets correspond to the amount of FNR homologues in the respective genus.

fulfil a different function in regulation. (iv) The role of FNR_{Bs}-type proteins in bacteria outside the genus *Bacillus* is mostly unknown, e.g., in *Exiguobacterium sibiricum* and *Anoxybacillus flavithermus* (Rodrigues *et al*., 2008; Saw *et al*., 2008). FNR of *A. flavithermus* FNR might control arginine metabolism that of *Macrococcus caseolyticus* and *Staphylococcus pseudintermedius* nitrate respiration (Baba *et al*., 2009). The latter strains also contain a homologue of the NreABC system, which regulates in *Staphylococcus* nitrate respiration in response to O_2 availability (Fedtke *et al*., 2002; Schlag *et al*., 2008). The role of FNR_{Bs} in the bacteria is therefore not clear. The role of FNR_{Bs} in the pathogenic *C. botulinum* and the nonpathogenic *S. ruminantium* is also not known. Both bacteria grow anaerobically and both proteins are most similar to the FNR_{Bs} such as proteins of *S. pseudintermedius* and *Exiguobacterium* with unknown function.

NreB

For *S. aureus* NreB, 73 homologous proteins were identified in the BLAST search. Most (84%) of the homologues occur among the bacilli and of those 54% among the staphylococci, documenting predominance of NreB in bacilli and staphylococci. The sensor kinase NreB coordinates the sensory iron–sulfur cluster by four conserved Cys residues of the N-terminal PAS domain (Fig. 1; Mullner *et al*., 2008). Proteins of the matrix (12% of the homologous) that showed similarity only in the kinase domain of HisKA_3 sensor kinases (Huynh *et al*., 2010) but not in the sensory PAS domain and were deleted in the revised matrix (Fig. 6B). The revised matrix includes 65 NreB-like proteins with similarity including the PAS domain. NreB is a part of the NreABC two component system (*nreA nreB nreC* gene cluster) (Schlag *et al*., 2008). The *nreB* genes of the revised matrix were accompanied mostly by *nreA* and *nreC* genes. Among the 65 strains with NreB-like proteins, 47 encoded the complete Cys cluster for binding the iron–sulfur cluster, 12 with only one of the Cys residues and few others with two (4) or three (2) conserved Cys residues. Most of the Cys variants are found outside the staphylococci or bacilli genera and lack also the *nreA* and *nreC* genes. Figure 6 shows the schematic tree of NreB proteins after combining branches on genus level. Branches were only combined for proteins with the same amount of conserved Cys residues. The Cys residues are essential for the binding of the [4Fe–4S] cluster and function of NreB as an O_2 sensor (Kamps *et al*., 2004; Mullner *et al*., 2008). Therefore conservation of the Cys cluster was inspected as described above for FNR_{Ec}. Notably, all homologues having the complete Cys motif are grouped on one main branch of the tree whereas the variants (highlighted in

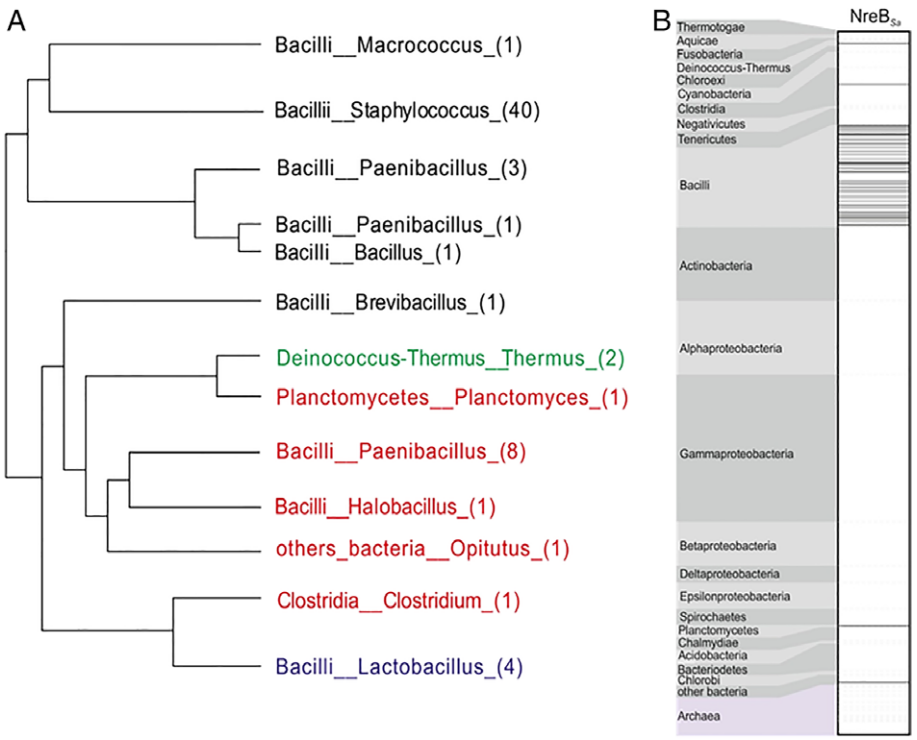

Fig. 6. Schematic tree of *S. aureus* NreB (A) and matrix of strains carrying $NreB_{Sa}$ like proteins (B). In the matrix, the 0Cys variants were deleted (compare main text). Clades were collapsed on genus level. The digits in brackets correspond to the amount of FNR homologues in the respective genus. Homologues with three conserved residues of the [Fe–S]-binding motif are highlighted in green, whereas two cysteine residues are marked in blue and only one cysteine residue is marked in red.

green, blue and red) are dispersed, with *Brevibacillus brevis* as the only exception.

4Cys variants of NreB

Most homologues of NreB (40 from 47 proteins with 4 Cys) are found in the genus *Staphylococcus.* Studies on the regulatory properties and on the biochemistry of the NreABC system were performed in the non-pathogenic *S. carnosus* and in *S. aureus* (Fedtke *et al*., 2002; Mullner *et al*., 2008; Schlag *et al*., 2008; Unden *et al*., 2013).

Some bacteria encode NreB and FNR of the Bs-type in parallel, which are responsible for anaerobic regulation of nitrate respiration in *Staphylococcus* and *Bacillus* respectively (Cruz Ramos *et al*., 1995; LaCelle *et al*., 1996; Fedtke *et al*., 2002; Schlag *et al*., 2008). The differential roles for both sensors in bacteria like *M. caseolyticus* is not known (Baba *et al*., 2009). *Bacillus clausii* encodes NreABC but lacks FNR_{Bs}. It was suggested that NreABC regulates expression of nitrate respiration in this *Bacillus* strain (Mullner *et al*., 2008). NreB homologues are found also in some paenibacilli including *Paenibacillus* sp., *P. terrae* and *P. polymyxa.* Their *nreB* gene is located near the *nar* genes. *Paenibacillus* sp. that encodes FNR of the FNR-Bs type and NreABC is capable of nitrate respiration (Mead *et al*., 2012). Gene clustering suggests that NreABC rather than FNR_{Bs} regulates expression of nitrate respiration. *Brevibacillus brevis* representing a member of the Paenibacillaceae (Chen *et al*., 2012) that also carries FNR_{Bs} and NreB, in contrast, lacks genes for the (nitrate sensory component) NreA and for dissimilatory nitrate reduction, suggesting a different role of NreB in the bacteria.

3Cys and 2Cys variants of NreB

Of the 65 NreB-like proteins, six showed variation of the Cys cluster with two or three conserved residues (Fig. 6 and Supporting Information Fig. S6). None of the Cys variants has been studied genetically or biochemically. The 3Cys-homologues of NreB contain an alternative Cys residue close to the N-terminus and may represent 4Cys variants (Supporting Information Fig. S6). The role of the 3Cys NreB of the aerobic *Thermus* species lacking anaerobic nitrate respiration (Henne *et al*., 2004) is not known.

The 2Cys variants are represented by NreB homologues in *Lactobacillus reuteri*, *Lactobacillus plantarum* and *Lactobacillus fermentum* (Fig. 6 and Supporting Information Fig. S6). In the 2Cys variants, only the C-terminal Cys pair is conserved, but the proteins share high sequence similarity with NreB PAS and kinase domains. The lactobacilli possess genes for dissimilatory nitrate reduction (Kleerebezem *et al*., 2003; Morita *et al*., 2008). *Lactobacillus fermentum* and *L. plantarum* also encode the nitrate binding NreA-like protein (Mullner *et al*., 2008; Unden *et al*., 2013). It can be speculated that

a dimer of the 2Cys variant of NreB is required for coordinating one iron–sulfur cluster as hypothesized for nitrogenase NflD subunit in *Methanocaldococcus jannaschii* (Staples *et al*., 2007) or FlpA (Scott *et al*., 2000). Alternatively, the 2Cys variants function by using a regulatory inter- or intra-molecular thiol/disulfide switch (Gostick *et al*., 1998; Scott *et al*., 2000).

1Cys variants of NreB

Twelve NreB variants with only one conserved cysteine residue are present in bacilli (*Paenibacillus* and *Halobacillus*) and *Clostridium* sp., *Planctomyces limnophilus* and *O. terrae.* Within the paenibacilli strains with 4Cys or 1Cys NreB or FNR_{Bs}-like proteins are found, indicating different functions for the variants. The 1Cys-homologues are supposed to fulfil a role in redox-sensing like the 1Cys FNR_{Ec} proteins. C1 or C2 or C4 of the Cys cluster can be conserved. In most systems NreB is not associated with NreA and NreC, which argues against involvement in transcriptional and anaerobic nitrate regulation.

WhiB3 M. tuberculosis

BLAST search identified 124 proteins that are homologues of WhiB3 of *M. tuberculosis.* The WhiB3 homologues were exclusively present in the actinobacteria (Fig. 1). Approximately one-third of the homologues were detected in the mycobacteria, and about 10% belong each to *Streptomyces* and corynebacteria. The remaining homologues are widespread in the actinobacteria including *Rhodococcus, Frankia*, *Nocardia* and *Amycolatopsis* with broad branching of the phylogenetic tree (not shown).

In general, actinobacteria contain multiple WhiB-like proteins which share similar structures including the Cys and the DNA-binding motif but have only moderate sequence similarity. Most mycobacteria possess seven WhiB-like proteins (WhiB1 to B7) which control expression of different gene clusters (Geiman *et al*., 2006; Soliveri *et al*., 2000; Saini *et al*., 2012). WhiB3 to WhiB7 respond to redox stress (O_2 and NO), WhiB1 only to NO, and the function of WhiB2 is redox-independent. WhiB5 is lacking in nonpathogenic mycobacteria (Saini *et al*., 2012). The response of WhiB3 to O_2 is very similar to that of FNR_{Ec} converting the $[4Fe–4S]^{2+}$ cluster first to $[3Fe–4S]^{1+}$ with a concomitant release of Fe^{2+} and one electron which are then used for the two-electron reduction of O_2 to H_2O_2 (Singh *et al*., 2007). In a following step, the $[3Fe–4S]^{1+}$ is converted in a nonredox reaction to $[2Fe–2S]^{2+}$. Overall, the reactions are very similar to those of Step 1 and 2 for FNR_{Ec} (see above) with an overall reaction $[4Fe–4S]^{2+} + O_2 + 2H^+ \rightarrow [2Fe–2S]^{2+} + 2Fe^{3+} + 2S^{2-} + H_2O_2$. According to Singh and colleagues (2007), the H_2O_2 may destroy the [2Fe–2S] cluster.

The structure of WhiB1, which is NO responsive, shows that WhiB1 is a four-helix bundle (Kudhair *et al*., 2017). The core of the protein is formed by three α-helices that are held together by the [4Fe–4S] cluster, which is required for formation of a complex with the major sigma factor σ^A. Reaction of the cluster with NO disassembles the complex and DNA binding as well as gene expression at target genes.

The redox sensor WhiB3 of *M. tuberculosis* controls expression of genes that are important for maintenance of intracellular redox homeostasis as well as virulence, pathogenesis and persistence (Singh *et al*., 2007; 2009). A total of 5% of the WhiB3 homologues lack the first Cys residue of the cluster-binding motif due to a N-terminal deletion of 20–30 amino acids, whereas the residual part of the protein is homologous to $WhiB3_{Mt}$, e.g., in the proteins of *Mycobacterium intracellulare*, *Mycobacterium* sp., *Streptomyces hygroscopicus*, *Amycolicicoccus subflavus* and *Thermonospora curvata.* WhiB4 of *M. intracellulare* having only three cysteine residues was proposed to take over a role in the adaptation to peroxide stress comparable to that of OxyR (Lewis and Falkinham, 2015). WhiB3 homologues with complete cluster-binding motif are found in pathogenic (*Mycobacterium bovis* and *Mycobacterium leprae*) and non-pathogenic (*Mycobacterium smegmatis*) mycobacteria. Function and regulation of WhiB3 appear to be similar in all *Mycobacterium* species.

The WhiD protein of non-pathogenic *Streptomyces coelicolor* is homologous to $WhiB3_{Mt}$ and functions in sporulation and septum formation (Molle *et al*., 2000; Jakimowicz *et al*., 2005). The degradation of native WhiD [4Fe–4S] cluster to apo-WhiD by O_2 or by peroxide stress is very slow, and no [2Fe–2S] cluster is formed as intermediate (Crack *et al*., 2009). *In vitro* the [4Fe–4S] cluster degrades similar to that of FNR_{Ec} with a [2Fe–2S] intermediate (Jakimowicz *et al*., 2005). Therefore, WhiD, such as WhiB3, acts as redox sensor but regulates different cellular responses. Nonpathogenic *Corynebacterium glutamicum* only possesses four WhiB proteins called WhcA, WhcB, WhcD and WhcE that regulate various cell functions in response to oxidative stress (Kim *et al*., 2005; Choi *et al*., 2009; Lee *et al*., 2012, 2018).

Overall, WhiB-like proteins are restricted to actinobacteria, which are all aerobic, indicating the evolution of WhiB proteins with the [4Fe–4S] cluster after accumulation of oxygen on earth.

Discussion

Origin and relation of the sensor proteins FNR_{Ec}, FNR_{Bs}, NreB and WhiB3

Sequence comparison and distribution indicate ancient origin and independent evolution of FNR_{Ec}, FNR_{Bs}, NreB and WhiB3/D. FNR_{Ec} and FNR_{Bs} proteins are derived

from the ancient carbon-regulator CRP (Saier *et al*., 1996). The global identity between FNR and CRP is low (17.9% between FNR_{Ec} and CRP_{Ec}), and the proteins fall for this reason into separate clusters and alignments. The role of CRP as the precursor and the FNR proteins as the derivatives are suggested by the broad distribution of CRP in many phyla and its full-length conservation in FNR_{Ec} and FNR_{Bs}. Korner and colleagues (2003) and Green and colleagues (2001) describe the CRP-FNR family of transcriptional regulators that includes in addition to CRP and FNR also other redox regulators like YeiI and Flp. The latter have low global identity with FNR and CRP and are not considered in the present study.

FNR_{Ec} and FNR_{Bs} are distinguished from CRP by the presence of short N- and C-terminal sequences, respectively, containing each three Cys ligands for ligation of the iron–sulfur clusters. Together with an internal fourth Cys (C4) or Asp residue, the Cys residues of the terminal extensions ligate the [4Fe–4S] cluster in FNR_{Ec} or FNR_{Bs}. In addition, FNR_{Ec} and FNR_{Bs} differ by the mode of signal output. In FNR_{Ec}, the signal output is produced by monomerization of the protein after reaction with O_2 and the oligomerization state then affects DNA binding (Lazazzera *et al*., 1993). Within the CRP/FNR family of transcriptional regulators, inactivation by monomerization is unique for FNR_{Ec} whereas other members such as CRP (Anderson *et al*., 1971; Takahashi *et al*., 1980) and FNR_{Bs} retain their dimeric state in the presence of their effector or in the active or inactive state respectively. Thus, FNR_{Bs} is a permanent dimer and DNA binding is regulated by conformational changes within FNR_{Bs} (Reents *et al*., 2006b). The broad overlap of co-occurrence of CRP with FNR_{Ec} in the γ-proteobacteria suggests the origin for FNR_{Ec} within this group.

NreB belongs to the large family of histidine sensor kinases that show broad variation in their domain composition. In NreB and related His kinases sensing occurs by an N-terminal PAS domain. The PAS domain of the closely related FixL sensor kinase binds hemeB at a position homologous to the [4Fe–4S]-binding site of NreB (Mullner *et al*., 2008; Unden *et al*., 2013). Remarkably, both NreB and FixL represent direct O_2 sensors. NreB and FixL are present, however, essentially within the Bacilli and the proteobacteria respectively. Therefore, the hemeB/[4Fe–4S]-binding sites appear to have a common origin and an occasional but rare lateral transfer to other bacterial phyla could be detected.

The WhiB3/D proteins are restricted to actinobacteria. The actinobacteria contain no FNR_{Ec}, FNR_{Bs} or NreB-type O_2 regulators but a significant variation in the WhiB3/D regulators, which take over multiple functions. Their unique presence in aerobic actinobacteria suggests development of the WhiB3/D proteins after separation or development of the group and after establishment of oxic conditions.

Recent reports for the structures of FNR_{Ec}- and Wbl-type proteins show binding and interaction of the [4Fe–4S] clusters with the respective proteins (Kudhair *et al*., 2017; Volbeda *et al*., 2015). In FNR_{Ec}, binding of the [4Fe–4S] cluster is achieved by a pocket formed from two α-helices, one β-sheet and the N-terminal loop of FNR whereas in WhiB1 proteins the [4Fe–4S] cluster is coordinated by three α-helices that are part of a four-helix bundle. WhiB1 is basically of the NO sensing type, but the overall structure is conserved in the Wbl/Whi family. NreB accommodates the cluster in a PAS domain that binds the [4Fe–4S] cluster by the α-helical PAS core and the α-helical connector which links this region to the β-scaffold and the kinase domain (Miyatake *et al*., 2000; Mullner *et al*., 2008). Therefore, the structural arrangements around the iron–sulfur clusters and their binding share no obvious similarity apart from the Cys ligands, which is again strong indication for their independent origin and evolution.

Functional diversity and diversification

Despite general restriction of FNR_{Ec}, FNR_{Bs}, NreB and WhiB3/D to diverse bacterial phyla, some bacteria contain multiple sensor proteins of these types in various combinations (Table 2). (i) Some bacteria contain multiple sensors of the same type. Thus *Pseudomonas putida* comprises three FNR-Ec type proteins that differ in their O_2 sensitivity (Ibrahim *et al*., 2015). Actinobacteria contain up to seven WhiB3/D proteins that differ in their response to O_2 but also in the target promoters for the individual WhiB3/D proteins (Molle *et al*., 2000; Steyn *et al*., 2002; Jakimowicz *et al*., 2005; Kim *et al*., 2005; Choi *et al*., 2009; Crack *et al*., 2009; Lee *et al*., 2012, 2018). There are also strains such as *P. mucilaginosus* with more than one 4Cys-FNR_{Bs} variant. (ii) Other bacteria such as *B. japonicum* and *R. meliloti* contain multiple variants of the same type of regulator, such as AadR (4Cys FNR_{Ec}, a direct O_2 sensor) and $FixK_2$ or FixK (0Cys FNR_{Ec}, an indirect O_2 regulator (Fischer, 1994) (Table 1 and Supporting Information Table S4). (iii) Other strains contain different types of predicted O_2 sensors such as 4Cys-FNR_{Bs} and 4Cys-NreB that are predicted to form functional O_2 sensors. The function and dual roles in O_2 sensing have not been verified, however. This list is extended by bacteria with 4Cys-FNR_{Bs} (or 4Cys-FNR_{Ec}) proteins that are combined with 1Cys- or 0Cys-variants of an alternative sensor (FNR_{Bs}, FNR_{Ec} and NreB). (iv) Other strains contain FNR hybrid sensors with FNR_{Ec} /FNR_{Bs} hybrid Cys clusters. Thus the FNR_{Bs} homologous protein of *Clostridium perfringens* lacks the D/Cys cluster of FNR_{Bs}-type sensors but contains an N-terminal Cys-cluster of the FNR_{Ec}-type including the central C4 residue. The spacing of the 4 Cys residues is slightly different from that of FNR_{Ec}.

Table 2. Species containing multiple forms of FNR_{Ec}, FNR_{Bs} and NreB.

Species	FNR_{Ec}	FNR_{Bs}	NreB
(i) Multiple sensors within one species			
Pseudomonas (γ)	4Cys[a] (3x)		
Paenibacillus spp. (B)		4Cys (2x)	
(ii) Multiple variants of one sensor type			
Bradyrhizobium japonicum (α)	4Cys[a], 0Cys[a]		
Rhizobium meliloti	4Cys[a], 0Cys[a]		
Rhodoferax ferrireducens (β)	4Cys[a], 1Cys		
Paenibacillus spp. (B)			4Cys[a], 1Cys
(iii) Different sensor classes in one species			
M. caseolyticus (B)	–	4Cys	4Cys[a]
Paenibacillus sp. JDR2	–	4Cys	1Cys
S. ruminantium (Neg)	–	4Cys[a]	0Cys
D. hafniense DCB2	4Cys	0Cys	–
Shewanella piezotolerans (γ)	4Cys[a]	–	0Cys
(iv) Hybrid			
C. perfringens str 13	–	No D/Cys, but FNR-Ec type N-terminal Cys cluster with variant spacing C5–x_4–C–x_3–C–x_{98}–C113	

The type of FNR or NreB (4Cys, 1Cys or 0Cys variant) is indicated. Abbreviations for the bacterial phyla in the species column: B, bacilli, Neg, negativicutes; C, clostridia; α, β or γ, α-, β- or γ-proteobacteria.
[a]Homologues of FNR or NreB that are supposed to regulate anaerobic (nitrate) respiration.

Origin of the [4Fe–4S] clusters and [4Fe–4S]/[2Fe–2S] cluster conversion in O_2 sensing: the role of the cluster biochemistry

The FNR_{Ec}, FNR_{Bs}, WhiB3/D and NreB proteins use $[4Fe–4S]^{2+}$ clusters for O_2 sensing (Khoroshilova *et al*., 1997; Jakimowicz *et al*., 2005; Reents *et al*., 2006b; Singh *et al*., 2007; Crack *et al*., 2009; 2017; Mullner *et al*., 2008; Zhang *et al*., 2012). For FNR_{Ec}, NreB and to some extent also for WhiB3/D, the response to O_2 appears to be similar by the conversion to a $[2Fe–2S]^{2+}$ cluster, despite the use of unrelated protein types and Cys clusters for ligation that differ in spacing of the Cys residues and sequence (Fig. 1). It is not known whether the similarity extends to details such as the formation of an [4Fe–3S] intermediate and of the Cys persulfides as described for FNR_{Ec} (Crack *et al*., 2017).

Cluster conversion is the basis for signal transmission to the output domains. The response triggers conversion of dimeric to monomeric FNR_{Ec} and loss of specific DNA binding (Lazazzera *et al*., 1996; Volbeda *et al*., 2015), activation of the kinase domain in NreB (Mullner *et al*., 2008; Nilkens *et al*., 2014) and loss of DNA binding by the permanent dimer FNR_{Bs} (Reents *et al*., 2006b). It appears therefore that the response of the iron–sulfur cluster depends to a large extent on the chemistry and properties of the cluster rather than on the protein, whereas the signal output is governed by the surrounding protein.

Iron–sulfur clusters can be produced in protic and aproteinogenic systems, and $[4Fe–4S]^{2+}$ and $[2Fe–2S]^{2+}$ clusters have been produced under anoxic conditions in the thiol-ligated form with various thiol ligands (for review see Venkateswara Rao and Holm, 2004). The cubane-type $[4Fe–4S]^{2+}$-thiolate clusters are very common. Formation of the $[4Fe–4S]^{2+}$ cluster represents a thermodynamic sink and the $[4Fe–4S]^{2+}$ cluster is among the most stable under anoxic conditions (Ogino *et al*., 1998; Venkateswara Rao and Holm, 2004). The clusters can be incorporated in cysteinyl peptides (Ohno *et al*., 1991; Ueyama *et al*., 1985). The [2Fe–2S] cluster, in particular the more stable $[2Fe–2S]^{2+}$ form, can also be generated in many thiolates and cysteinyl peptides (references in (Hagen *et al*., 1983; Venkateswara Rao and Holm, 2004). The $[2Fe–2S]^{1+}$ cluster is less stable, but can be produced by reduction of the $[2Fe–2S]^{2+}$ cluster. Biotin and lipoate synthases, anaerobic ribonucleotide reductase and the pyruvate formate-lyase activating enzyme contain [4Fe–4S] clusters that serve as electron donors in the radical catalysis of the enzymes (Buis and Broderick, 2005; Duin *et al*., 1997; Ollagnier-De Choudens *et al*., 2000; Ugulava *et al*., 2000; Lotierzo *et al*., 2005). The enzymes are sensitive to inactivation by O_2 that causes a side reaction with $[4Fe–4S]^{2+}$ to $[2Fe–2S]^{2+}$ conversion (compare Venkateswara Rao and Holm, 2004), resembling the reaction at the iron–sulfur cluster of FNR_{Ec}. Thus, the information from cluster (bio)chemistry is compatible with the observation that $[4Fe–4S]^{2+}$ and $[2Fe–2S]^{2+}$ clusters are present and interconverted in different protein environments. The conformational changes in cluster conversion can then be used for largely different responses, depending on the surrounding protein. The $[4Fe–4S]^{2+}$ clusters therefore represent a unique molecular device that can be modulated in evolution to bring forth O_2 sensors in different protein background and different output reactions. Remarkably, this mode of O_2 sensing that has been used for

developing independent lines of O_2 sensors in bacteria is apparently missing in archaea and eukaryotes.

Experimental procedures

Sequences

Protein sequences involved in oxygen sensing and containing a [4Fe–4S] cluster were downloaded in July 2016 from UniProt (Consortium, 2017) and GenBank (Benson *et al*., 2005): fumarate and nitrate reduction regulatory protein (FNR) from *E. coli* (UniProt: P0A9E5) and *B. subtilis* (GenBank: KIX83509), oxygen sensor histidine kinase NreB from *S. aureus* (GenBank: EFW34334), redox-responsive transcriptional regulator WhiB3 from *M. tuberculosis* (GenBank: KPU49338) and cAMP-activated global transcriptional regulator (CRP) from *E. coli* (UniProt: P0ACJ8). Furthermore, all protein sequences of 1.981 complete prokaryotic genomes were downloaded from the NCBI RefSeq database (version June 2012) and clustered into protein families as previously described (Nelson-Sathi *et al*., 2015; Weiss *et al*., 2016).

Identification of homologous protein families

Homologous sequences were identified by sequence comparisons of the five reference sequences involved in oxygen sensing, without any weighting of specific residues or motifs, with all 6.1 million proteins of the 1.981 complete prokaryotic genomes using BLASTp (Altschul *et al*., 1997) with an *E*-value threshold $\leq 10^{-10}$ and local identity cutoff $\geq 25\%$. Remaining BLAST hits with global amino acid identity not smaller than 25%, calculated with needle from EMBOSS 6.6.0 (Rice *et al*., 2000), were subsequently compared to the predefined protein families. The protein family showing the highest number of BLAST hits for the respective reference sequence was identified as the homologous protein family.

Identification of the number of cysteine residues

Conserved cysteine residues linked to the [4Fe–4S] cluster were counted manually in sequence comparisons performed with Clustal Omega v1.2.4 (Sievers *et al*., 2011) for each protein. Because of their low sequence conservation, *B. subtilis* FNR- and NreB-like proteins without conserved cysteine residues were excluded alignments used to generate trees.

Multiple sequence alignment and phylogenetic tree reconstruction

In the identified homologous protein family for FNR (*E. coli*), for all sequences showing global identity ≥ 80% on the species level, only the longest sequence was retained in the protein family. Sequences in each identified homologous protein family were aligned together with the respective reference sequence using MAFFT v7.299b (Katoh and Standley, 2013) with the options *–localpair, –maxiterate = 1000* and *–anysymbol.* Maximum likelihood trees were reconstructed using RAxML v8.2.8 (Stamatakis, 2014) using the PROTCATWAG model. The clades in the trees for NreB and FNR (*B. subtilis*) were collapsed on the genus level using Figtree v1.3.1, when the corresponding [4Fe–4S] binding motif contained the same number of cysteine residues. No schematic tree was generated for WhiB3, where all sequences of the protein family belong to the taxonomic group actinobacteria.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1.
Appendix S2.