**INTRODUCTION**

Molecular nitrogen (N\(_2\)) is highly abundant in air, but only diazotrophic bacteria and archaea are capable of utilizing this source of nitrogen, while non-diazotrophic prokaryotes and all eukaryotes depend on more reduced nitrogen forms like ammonium, nitrate, or organic nitrogen compounds (Dos Santos, Fang, Mason, Setubal, & Dixon, 2012; Zhang & Gladyshev, 2008; Zhang, Rump, & Gladyshev, 2011). In a process called biological nitrogen fixation, diazotrophs reduce N\(_2\) to ammonia (NH\(_3\)) by three structurally and functionally similar nitrogenase isoenzymes, each composed of two components, a catalytic dinitrogenase and a dinitrogenase reductase. All diazotrophs possess a molybdenum nitrogenase consisting of the NifDK and NifH components. Also, some diazotrophs encode either a vanadium nitrogenase (VnfDGK, VnfH) or an iron-only nitrogenase (AnfDGK, AnfH), or both.
Mo-free isoenzymes (McRose, Zhang, Kraepiel, & Morel, 2017). Since Mo-nitrogenase is more efficient than the other nitrogenases in terms of ATP consumption per N₂ reduced, diazotrophs preferentially synthesize Mo-nitrogenase when ammonium is limiting (Eady, 2003; Mus, Alleman, Pence, Seefeldt, & Peters, 2018; Schneider, Gollan, Dröttboom, Selsemeier-Voigt, & Müller, 1997; Sippel & Einsle, 2017; Thiel & Pratte, 2014). Many diazotrophs repress the production of Mo-free nitrogenases by one-component ModE regulators, which directly sense and respond to molybdate availability (reviewed by Demtröder, Narberhaus, & Masepohl, 2019). The Mo-free nitrogenases take over under Mo-limiting conditions.

Mo-nitrogenase contains a complex iron-molybdenum cofactor, FeMoco, whose synthesis requires the NifUS, NifH, NifB, NifEN, NifQ, and NfIV proteins (reviewed by Burén, Jiménez-Vicente, Echavarri-Erasun, & Rubio, 2020; Curatti & Rubio, 2014; Hu & Ribbe, 2016). The NifUS, NifB, and NfIV proteins are also involved in biosynthesis of the iron-vanadium cofactor of V-nitrogenase, FeVco, and the iron-only cofactor of Fe-nitrogenase, FeFeco (Drummond, Walmsley, & Kennedy, 1996; Hamilton et al., 2011; Hu & Ribbe, 2016; Kennedy & Dean, 1992; Sippel & Einsle, 2017; Yang, Xie, Wang, Dixon, & Wang, 2014). Electron transfer to the nitrogenases involves species-specific proteins like NifF, FdxN, RnfABCDGEH, and FixABC (Boyd, Costas, Hamilton, Mus, & Peters, 2015; Dos Santos et al., 2012; Oldroyd, 2013; Poudel et al., 2018).

In proteobacteria, expression of nif, vnf, and anf genes depends on the alternative sigma factor RpoN and the structurally and functionally related transcription activators NifA, VnfA, and AnfA, respectively (Bush & Dixon, 2012; Dixon & Kahn, 2004; Drummond et al., 1996; Fischer, 1994; Hamilton et al., 2011; Heiniger, Oda, Samanta, & Harwood, 2012; Hübner, Masepohl, Klipp, & Bickle, 1993; Joeger, Jacobson, & Bishop, 1989; Kutsche, Leimkühler, Angermüller, & Klipp, 1996; Merrick, 1993; Mus et al., 2018; Oda et al., 2005; Oliveira et al., 2012; Sarkar & Reinhold-Hurek, 2014; Souza, Pedrosa, Rigo, Machado, & Yates, 2000; Walmsley, Toukairian, & Kennedy, 1994; Zhang, Pohlmann, Ludden, & Roberts, 2000; Zou et al., 2008). These activators encompass three modules, namely an N-terminal GAF, a central AAA+, and a C-terminal HTH domain, involved in environmental sensing, ATP-dependent activation of target gene transcription, and DNA binding, respectively. Many NifA proteins contain a conserved cysteine motif in an interdomain linker connecting the central and C-terminal domains, while VnfA and AnfA proteins contain conserved cysteine motifs in their GAF domains (Figure A1 in Appendix 2). The cysteine residues are known or presumed to bind iron-sulfur clusters making these proteins sensitive to oxygen (Austin & Lambert, 1994; Fischer, 1994; Nakajima et al., 2010; Yoshimitsu, Takatani, Miura, Watanabe, & Nakajima, 2011). This prevents inappropriate induction of nitrogen fixation, a process not compatible with oxygen.

RpoN proteins bind promoters with the consensus CTGG–N₂–TTGC (N stands for any nucleotide) located at position –24/–12 upstream of the transcription start site (Buck, Miller, Drummond, & Dixon, 1986; Bush & Dixon, 2012; Fischer, 1994; Merrick, 1993; Zhang & Buck, 2015). NifA proteins bind cis-regulatory elements with the consensus TGT–N₁₀–ACA, which is typically found within a distance of 150 base-pairs upstream of the transcription start site (Buck et al., 1986; Demtröder, Pfänder, Schäkermann, Bando, & Masepohl, 2019; Fischer, 1994). NifA-binding sites are well-characterized in many α-, β-, and γ-proteobacteria (Barrios, Grande, Olvera, & Morett, 1998; Buck et al., 1986; González, Olvera, Sobero, & Morett, 1998; Guibier, 1989; Lee, Berger, & Kustu, 1993; Monteiro et al., 1999; Wang, Kolb, Cannon, & Buck, 1997), whereas VnfA- and AnfA-binding sites have been studied so far only in the γ-proteobacterium Azotobacter vinelandii, which is one of few diazotrophs having both Mo-free nitrogenases (Austin & Lambert, 1994; Drummond et al., 1996; Frise, Green, & Drummond, 1994; Woodley, Buck, & Kennedy, 1996). In this species, the sequences GTAC–N₆–GTAC and C–N–GG–N₆–GTGA have been suggested as being sites for VnFA and AnfA, respectively (Austin & Lambert, 1994; Woodley et al., 1996).

To determine AnfA-binding site requirements in a diazotroph distantly related to A. vinelandii, we examined the regulation of Fe-nitrogenase genes in the photosynthetic α-proteobacterium Rhodobacter capsulatus, which fixes nitrogen by the Mo- and Fe-nitrogenases (Schneider, Müller, Schramm, & Klipp, 1991; Schüdekopf, Hennecke, Liese, Kutsche, & Klipp, 1993; Strnad et al., 2010). Differential expression of the corresponding nif and anf genes is tightly regulated. While NifA activates the promoters upstream of multiple nif genes, AnfA activates the anfH promoter as indicated by reporter fusions and proteome profiling (Demtröder, Pfänder, et al., 2019; Figure 1a). Since the sigma factor RpoN is encoded by the nifU2–rpoN operon, NifA indirectly controls AnfA-mediated expression of the Fe-nitrogenase genes (Demtröder, Pfänder, et al., 2019).

In this study, we show that R. capsulatus AnfA binds two highly similar palindromic sites in the anfH promoter. Based on conserved sequences in various α-proteobacteria, we define a general AnfA-binding site consensus, TAC–N₄–GTA. Besides, we present evidence that the anfH promoter is the only Fe-nitrogenase-related promoter in R. capsulatus strictly depending on AnfA.

2 | MATERIALS AND METHODS

2.1 | Strains, plasmids, and growth conditions

Bacterial strains and plasmids used in this study are listed in Table A1 in Appendix 1. Rhodobacter capsulatus minimal medium V (RCV) was prepared as previously described (Demtröder, Pfänder, et al., 2019). In this medium, a fixed nitrogen source and molybdate (Mo) have been omitted. Traces of Mo arising from impurities of the chemicals used support residual Mo-nitrogenase activity but are low enough to permit the production of Fe-nitrogenase. To examine diazotrophic growth, cultures were inoculated in 3 ml
RCV medium in screw-capped 17-ml Hungate tubes before the exchange of headspace air for pure N₂ gas and incubation in the light. When required, 10 mM serine was added as a fixed nitrogen source, which (in contrast to ammonium) does not inhibit nitrogen fixation.

2.2 | Construction of *Rhodobacter capsulatus* anfH-lacZ reporter strains and β-galactosidase assays

The anfH promoter was narrowed down by nested deletions. For this, appropriate primer pairs were used to PCR-amplify promoter variants F1 to F6 (Figure 2a,b), thereby adding BamHI and HindIII sites. Corresponding BamHI-HindIII fragments were cloned into the broad-host-range vector pBBR1MCS (Kovach et al., 1995) before insertion of a *lac*TeT cassette (carrying a promoterless lacZ gene, a tetracycline resistance gene, and an oriT transfer origin) from plasmid pYP35 (Gisin et al., 2010) into the HindIII site. The resulting reporter plasmids carrying transcriptional lacZ fusions were designated pBBR_F1-lacZ to pBBR_F6-lacZ.

To generate site-directed substitution mutations in the anfH promoter (Figure 3a), plasmid pBBR_F1-lacZ served as a template. Base-pair substitutions were introduced using the QuikChange protocol (Stratagene). The resulting pBBR_F1-lacZ derivatives carrying transcriptional lacZ fusions to different promoter variants were designated pBBR_Mut1-lacZ to pBBR_Mut7-lacZ, pBBR_Mut2/6-lacZ, and pBBR_Mut2/7-lacZ.

The reporter plasmids were conjugationally transferred into the *R. capsulatus* wild-type strain B10S. Following phototrophic growth of the *R. capsulatus* reporter strains in RCV medium with 10 mM serine (no Mo added) until the late logarithmic phase, LacZ (β-galactosidase) activity was determined (Miller, 1972).

![FIGURE 1](image-url) Model of the nitrogen fixation regulon in *Rhodobacter capsulatus*. (a) Production of Mo- and Fe-nitrogenases in the wild type. In the absence of ammonium (−NH₄⁺), the superior regulator NtrC activates transcription of *nifA* and *anfA* in concert with the housekeeping sigma factor RpoD (Foster-Hartnett, Cullen, Monika, & Kranz, 1994; Kutsche et al., 1996). MopA and MopB independently repress *anfA* in the presence of molybdate (+MoO₄²⁻; Wiethaus et al., 2006). NifA and AnfA activate their target genes by partnering with the alternative sigma factor RpoN. Noteworthy, NifA indirectly controls AnfA-mediated anfHDGK expression by controlling RpoN production (Demtröder, Pfänder, et al., 2019). Involvement of NifA-activated genes in biosynthesis of the iron-molybdenum cofactor (FeMoco) of Mo-nitrogenase and the iron-only cofactor (FeFeco) of Fe-nitrogenase and electron transfer to both nitrogenases is indicated. (b) Production of active Fe-nitrogenase in a strain lacking AnfA. In this study, we constructed strain YP515-BS85 containing mutations in the *anfA* and *nifD* genes (marked by red crosses) and a chromosomal substitution of the *anfH* promoter (P*anfH*) by the *nifH* promoter (P*nifH*) thereby putting anfHDGK expression under NifA control. This strain grew under N₂-fixing conditions (Figure 4b) suggesting that AnfA is dispensable for FeFeco biosynthesis and electron supply to Fe-nitrogenase. For further details, see text.
2.3 Examination of AnfA binding to the anfH promoter

In vitro binding of the DNA-binding domain of AnfA (AnfA_{DBD}) to the anfH promoter was examined by electrophoretic mobility shift assays (EMSA) as previously described (Müller et al., 2010). To overexpress AnfA_{DBD}, appropriate primers were used to PCR-amplify a DNA fragment coding for the C-terminal 72 amino acid residues of AnfA (Figure A1 in Appendix 2) thereby adding SacI and NcoI sites. The corresponding SacI-NcoI fragment was cloned into the expression vector pASK-IBA45+ (IBA GmbH Göttingen) resulting in a hybrid plasmid pyP409. For purification of the Streptagged AnfA_{DBD}, *Escherichia coli* BL21 (DE) carrying pyP409 was cultivated with AHT induction before cell disruption and Strep-tagged AnfA_{DBD}, bound and free DNAs were separated in 6% polyacrylamide gels. Radioactive bands were detected by phosphor screen exposure.

The F1 fragment (carrying the wild-type anfH promoter) and its variants (Figure 3a) were labeled with γ-32P-ATP, and free γ-32P-ATP was removed by gel filtration using Illustra ProbeQuant G-50 Micro Columns (GE-Healthcare). After 20 min incubation of labeled promoter variants with increasing amounts of the purified AnfA_{DBD} protein, bound and free DNAs were separated in 6% polyacrylamide gels. Radioactive bands were detected by phosphor screen exposure.

2.4 Substitution of the *Rhodobacter capsulatus* anfH promoter by the nifH promoter

To replace the anfH promoter (P_{anfH}) by the nifH promoter (P_{nifH}), we exchanged the 266 bp anfA-anfH intergenic region by the 267 bp fdxD-nifH intergenic region. For this purpose, we constructed mutagenesis plasmid pyPS16 containing the 3’ end of anfA (including the translation stop codon, TGA), a gentamicin (Gm) resistance cassette, P_{nifH} and the 5’ end of anfH (starting with the translation start codon, ATG). To replace the anfH promoter and to delete the anfA gene in a single step, we constructed mutagenesis plasmid pyPS15 containing an anfA upstream fragment (but lacking the anfA coding region), a Gm cassette, P_{nifH} and the 5’ end of anfH. Plasmids pyPS16 and pyPS15 were conjugationally introduced into the *R. capsulatus* strain BS85 (ΔnifD), in which the nifD gene is disrupted by a spectinomycin (Sp) cassette. BS85 does not exhibit Mo-nitrogenase activity, and hence, any nitrogenase activity observed in this background can be assigned to Fe-nitrogenase (Demtröder, Pfänder, et al., 2019). Promoter replacement mutants were identified by selection for Gm resistance and screening for loss of vector-encoded tetracycline resistance indicating marker rescue by double cross-over events. The resulting mutants were called YP516-BS85 (P_{anfH}→ P_{nifH}, ΔanfA, ΔnifD) and YP515-BS85 (P_{anfH}→ P_{nifH}, ΔanfA, ΔnifD).

In contrast to strain YP515-BS85 (P_{anfH}→ P_{nifH}, ΔanfA, ΔnifD), strain KS94A-YP415 (ΔanfA, ΔnifD) contains the wild-type anfH promoter upstream of the anfHDGKOR3 operon. In this strain, the anfA and nifD genes are disrupted by Sp and Gm cassettes, respectively.

3 RESULTS

3.1 Localization of the *Rhodobacter capsulatus* anfH promoter by nested deletions

*Rhodobacter capsulatus* AnfA is essential for the expression of the anfHDGKOR3 operon (Demtröder, Pfänder, et al., 2019), but the anfH promoter has not been investigated. The coding regions of anfH and its upstream gene, anfA, are separated by 266 bp (Figure 2a). This intergenic region includes three conspicuous sequences, namely (a) a GC-rich inverted repeat sequence followed by a T-rich stretch likely acting as Rho-independent terminator of anfA transcription, (b) two 17 bp direct repeats each encompassing inverted repeat sequences, which are promising candidates as AnfA-binding sites, and (c) a highly conserved RpoN-binding site. For clarity, the 17 bp sequences will from now on be called distal and proximal AnfA-binding sites.

To localize the anfH promoter (P_{anfH}), we analyzed the effects of nested promoter deletions on anfH expression. For this purpose, we generated transcriptional fusions between P_{anfH} fragments, F1 to F6, and a promoterless lacZ gene (Figure 2b). *R. capsulatus* strains carrying the corresponding reporter plasmids were grown in RCV minimal medium with serine as a nitrogen source without molybdate addition, conditions compatible with the synthesis of Fe-nitrogenase (Demtröder, Pfänder, et al., 2019; Hoffmann, Wagner, et al., 2016), before determination of LacZ (β-galactosidase) activity.

Fragments F1 and F2 mediated considerable LacZ activity (Figure 2c) indicating that the anfA-anfH intergenic region and in particular the region downstream of the putative anfH transcription terminator contain all cis-regulatory elements required for P_{anfH} activation. F3-based LacZ activity was reduced to 29% of the F1 value probably due to the absence of one half-site of the distal AnfA-binding site (see below). As expected, fragments F4 to F6 resulted in only background LacZ activity consistent with the absence of both AnfA-binding sites.

3.2 Effects of AnfA-binding site mutations on anfH expression

To dissect the function of the distal and proximal AnfA-binding sites in P_{anfH}, we generated pBBR_F1-lacZ variants carrying site-directed mutations Mut1 to Mut7 (Figure 3a) and the combinations Mut2/6 and Mut2/7. Mut1 mediated clear LacZ activity (72% of the F1 value) suggesting that this sequence plays only a minor role in anfH expression consistent with our findings on nested promoter deletions (Figure 2). LacZ activity of Mut2 to Mut7 dropped to 20%-40% as compared to F1 (carrying the wild-type anfH promoter), while combined mutations Mut2/6 and Mut2/7 abolished LacZ activity (Figure 3b). These observations suggest that either of the two AnfA-binding sites mediates P_{anfH} activation to some extent and that full activation requires both sites.
3.3 | Effects of AnfA-binding site mutations on promoter binding by AnfA

The AnfA protein encompasses three domains, namely a GAF, an AAA+, and a HTH domain, involved in environmental sensing, activation of RNA polymerase, and promoter binding, respectively. To test the direct binding of AnfA to $P_{anfH}$, we performed electrophoretic mobility shift assays (EMSA). For this, the radiolabeled F1 fragment (carrying the wild-type $anfH$ promoter) and its variants (Mut1 to Mut7, Mut2/6, and Mut2/7) were incubated with increasing amounts of the Strep-tagged DNA-binding domain of AnfA (AnfA_DBD) encompassing the C-terminal 72 amino acid residues of the full-length regulator (Figure A1 in Appendix 2). AnfA_DBD was expected to bind the same target sequence as the full-length regulator, but to be more stable in solution according to findings on Azotobacter vinelandii AnfA and NifA, and Herbaspirillum seropedicae NifA (Austin & Lambert, 1994; Lee et al., 1993; Monteiro, Souza, Yates, Pedrosa, & Chubatsu, 2003).

AnfA_DBD bound the F1 fragment (carrying the wild-type $anfH$ promoter) and its variants (Mut1 to Mut7, Mut2/6, and Mut2/7) with increasing affinity, indicating that this sequence is dispensable for AnfA binding. AnfA_DBD also bound promoter variants Mut2 to Mut7 albeit less effectively than F1, and the combined Mut2/6 and Mut2/7 variants were barely shifted. These observations suggest that recognition of the distal or proximal sites by AnfA is essentially unbiased. Taken together, the in vitro binding studies (Figure 3c) are well in line with the in vivo data on $anfH$ expression (Figure 3b) supporting the hypothesis that the two 17 bp repeat sequences (Figure 2a) serve as distal and proximal AnfA-binding sites.

3.4 | NifA-driven anfHDGKOR expression restores Fe-nitrogenase activity in a strain lacking AnfA

Productive nitrogen fixation by the Fe-nitrogenase requires more than the expression of the $anfHDGKOR$ operon. Current knowledge suggests that AnfA is required only for $anfHDGKOR$ expression and has little impact on the expression of NifA-activated genes like $nifB$ and $rnfA$, which are essential for FeFeco biosynthesis and electron transfer to Fe-nitrogenase, respectively (Demtröder, Pfänder, et al., 2019; Schüdekopf et al., 1993). We, therefore, speculated that AnfA might be dispensable for the production of active (N$_2$-fixing)
Fe-nitrogenase as long as the anfHDGKOR3 operon is adequately expressed. To achieve AnfA-independent anfHDGKOR3 expression, we replaced the anfH promoter (P_{anfH}) by the NifA-activated nifH promoter (P_{nifH}). Both promoters mediate comparably strong expression of their downstream genes at least under Mo-limiting conditions (Demtröder, Pfänder, et al., 2019). If the anfHDGKOR3
operon is the only member of the AnfA regulon, nitrogen fixation by Fe-nitrogenase should become entirely NifA-dependent in the P\textsubscript{anfH} → P\textsubscript{nifH} strain (Figure 1b).

To determine the effect of P\textsubscript{anfH} → P\textsubscript{nifH} substitution on anfH-DGKOR3 expression, we generated two R. capsulatus strains, which carry the same P\textsubscript{anfH} → P\textsubscript{nifH} promoter substitution, but either in the wild-type (anfA\textsuperscript{+}) or ΔanfA background. Subsequently, we introduced transcriptional anfH-lacZ reporter fusions in these strains and, as a control, in the wild-type strain B105 by chromosomal integration of plasmid pMH187 as previously described (Demtröder, Pfänder, et al., 2019). The resulting reporter strains were grown with either serine or ammonium as a nitrogen source before LacZ activity was determined.

The wild-type control (containing the native AnfA-dependent anfH promoter) showed the expected high anfH-lacZ expression in serine cultures, while no expression was observed in ammonium cultures (Figure 4a; Demtröder, Pfänder, et al., 2019; Kutsche et al., 1996; Wiethaus, Wirsing, Narberhaus, & Masepohl, 2006). Both P\textsubscript{anfH} → P\textsubscript{nifH} strains expressed anfH-lacZ to comparable levels as the wild-type control showing that P\textsubscript{anfH} → P\textsubscript{nifH} substitution mediated effective anfH-DGKOR3 expression independent of AnfA.

To test whether nitrogen fixation by Fe-nitrogenase was entirely NifA-dependent in the P\textsubscript{anfH} → P\textsubscript{nifH} strains, we introduced a polar nifD mutation (ΔnifD) in these strains, before examination of diazotrophic growth. Regardless of the nifD mutation, these strains were expected to express all the other NifA-dependent nitrogen fixation genes involved in cofactor biosynthesis and electron transport (Figure 1b). Since ΔnifD strains lack Mo-nitrogenase, diazotrophic growth of these strains depends entirely on Fe-nitrogenase (Demtröder, Pfänder, et al., 2019). As controls, we included the wild type, the parental ΔnifD strain, and a ΔanfA-ΔnifD strain lacking both nitrogenases.

The wild-type and ΔnifD strains grew well with N\textsubscript{2} as the sole nitrogen source, while the ΔanfA-ΔnifD strain failed to grow diazotrophically (Figure 4b) consistent with earlier studies (Demtröder, Pfänder, et al., 2019; Kutsche et al., 1996). Both P\textsubscript{anfH} → P\textsubscript{nifH} strains grew almost as well as the parental ΔnifD strain suggesting that P\textsubscript{anfH} → P\textsubscript{nifH} substitution indeed decouples production of functional Fe-nitrogenase from AnfA.

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Taken together, our findings show that substitution of the AnfA-dependent anfH promoter by the NifA-activated nifH promoter restores anfH-DGKOR expression and Fe-nitrogenase activity in a strain lacking AnfA. In other words, AnfA appears to be dispensable for FeFeco biosynthesis and electron delivery to Fe-nitrogenase.

4 | DISCUSSION

Despite the wide distribution of Fe-nitrogenases (McRose et al., 2017), our knowledge of Fe-nitrogenase-related promoters was so far limited to one species, the γ-proteobacterium Azotobacter vinelandii (Austin & Lambert, 1994; Drummond et al., 1996). Here, we characterized two AnfA-binding sites in the anfH promoter in the α-proteobacterium Rhodobacter capsulatus by in vivo and in vitro studies. Each of these binding sites mediated anfH expression to some extent, but maximal expression required both AnfA-binding sites.

Sequences similar to the R. capsulatus AnfA-binding sites are conserved in α-proteobacterial promoters preceding potential anfH operons (Figure 5a; McRose et al., 2017). Sequence logo representation (based on all distal and proximal AnfA-binding sites shown in Figure 5a) revealed a conserved sequence of dyad symmetry, TAC–N7–GTA, as the AnfA-binding site consensus (Figure 5b). Binding sites of dyad symmetry are typically bound by dimeric
regulators (Kim & Little, 1992; Klose, North, Stedman, & Kustu, 1994) suggesting that AnfA proteins also bind target promoters as dimers. The AnfA-binding site consensus previously defined for A. vinelandii, C–N–GG–N₃–GGTA (Austin & Lambert, 1994), and our consensus share the strictly conserved GTA motif (Figure 5a). The A. vinelandii AnfA-binding sites, which were identified by footprint experiments, lack the TAC motif indicating that this motif is dispensable for promoter recognition by A. vinelandii AnfA. In R. capsulatus, individual TAC (Mut2, Mut5) and GTA mutations (Mut4, Mut7) reduced anfH-lacZ expression by 60%–80%, while the Mut2/Mut7 combination abolished expression (Figure 3b). Together these findings suggest that AnfA proteins in different bacteria require the GTA motif, but differ in their dependence on the TAC motif to activate their target promoters.

**Rhodobacter capsulatus** AnfA is essential for anfHDGKOR3 expression and consequently, for nitrogen fixation by Fe-nitrogenase (Demtröder, Pfänder, et al., 2019). None of these genes, however, is preceded by an obvious AnfA-binding site (or just a GTA motif) suggesting that AnfA control of these genes is indirect.

In contrast to the R. capsulatus nifB promoter, we found potential AnfA-binding sites in the nifB promoters of Rhodospirillum rubrum (TAC–N₆–GTA) and Rhodobacter capsulatus (C–N₆–GTA) suggesting direct nifB activation by AnfA in these strains. In line with the requirement of NifB for the activity of all three nitrogenases in A. vinelandii, the nifB promoter can be activated by NifA, VnfA, or AnfA, which bind to overlapping sites in the nifB promoter (Drummond et al., 1996).

Our finding that only a single Fe-nitrogenase-related target, the anfH promoter, strictly requires activation by AnfA in R. capsulatus, raises the question of why this diazotroph needs AnfA. One explanation is that AnfA contributes (indirectly) to fine regulation of NifA-dependent genes (Demtröder, Pfänder, et al., 2019). Also, Mo repression of anfA introduces a regulatory level to prevent the production of Fe-nitrogenase under Mo-replete conditions. This guarantees the exclusive activity of Mo-nitrogenase, which exhibits higher N₂-reducing activity than Fe-nitrogenase (Hoffmann, Wagner, et al., 2016; Wiethaus et al., 2006).

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CONFLICT OF INTERESTS
None declared.

AUTHOR CONTRIBUTION
Lisa Demtröder: Conceptualization (supporting); Investigation (lead); Writing-original draft (equal). Yvonne Pfänder: Investigation (supporting). Bernd Masepohl: Conceptualization (lead); Funding acquisition (lead); Writing-original draft (equal).

ETHICS STATEMENT
None required.

DATA AVAILABILITY STATEMENT
All data are provided in full in the Section 3.

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APPENDIX 1

TABLE A1  Rhodobacter capsulatus strains and plasmids

| Strain or plasmid | Relevant characteristics | Source or reference |
|-------------------|--------------------------|---------------------|
| **Strains**       |                          |                     |
| B10S              | R. capsulatus wild type; Sm⁻ | Klipp, Masepohl, and Pühler (1988) |
| BS85              | nifD::Sp mutant (ΔnifD) of B10S; Sm⁺, Sp⁺ | Hoffmann et al. (2014) |
| K944              | anfA::Sp mutant (ΔanfA) of B10S; Sm⁺, Sp⁺ | Wang, Angermüller, and Klipp (1993) |
| K944-YP415        | anfA::Sp, nifD::Gm mutant (ΔanfA, ΔnifD) of B10S; Gm⁺, Sm⁺, Sp⁺ | This study |
| YPS15-BS85        | P<sub>anfH</sub>→P<sub>nifH</sub>ΔanfA::Gm⁺, ΔnifD::Sp⁺ mutant of B10S; Gm⁺, Sm⁺, Sp⁺ | This study |
| YPS16-BS85        | P<sub>anfH</sub>→P<sub>nifH</sub>ΔanfA⁺, ΔnifD::Sp⁺ mutant of B10S; Gm⁺, Sm⁺, Sp⁺ | This study |
| **Vector plasmids** |                          |                     |
| pASK_IBA45+       | AHT-inducible expression vector; Ap⁺ | IBA GmbH Göttingen, Germany |
| pBBR1MCS          | Mobilizable broad-host-range vector; Cm⁻ | Kovach et al. (1995) |
| pUC18             | Narrow-host-range vector; Ap⁺ | Norrander, Kempe, and Messing (1983) |
| pYP35             | lacI-T cassette donor; Ap⁺, Te⁺, oriT | Gisin et al. (2010) |
| **Hybrid plasmids** |                          |                     |
| pBHR_F1-lacZ to   | pBBR1MCS derivatives carrying P<sub>anfH</sub> fragments F1 to F6 | This study |
| pBHR_F6-lacZ      | (Figure 2a,b) fused to lacZ; Cm⁺, Te⁺ |                     |
| pBHR_Mut1-lacZ to | pBHR_F1-lacZ variants carrying Mut1 to Mut7, Mut2/6, and Mut2/7 (Figure 3a); Cm⁺, Te⁺ | This study |
| pBHR_Mut2-6-lacZ  |                     |                     |
| pBHR_Mut2-7-lacZ  |                     |                     |
| pMH187            | Mobilizable narrow-host-range plasmid carrying anfH-lacZ; Te⁺, oriT | Demtröder, Pfänder, et al. (2019) |
| pYP409            | pASK_IBA45+ derivative encoding AnfA_DBDB; Ap⁺ | This study |
| pYP515            | pUC18 derivative carrying rcc00583-Gm⁺P<sub>nifH</sub>-anfH; Ap⁺, Te⁺, oriT | This study |
| pYP516            | pUC18 derivative carrying anfA-Gm⁺P<sub>nifH</sub>-anfH; Ap⁺, Te⁺, oriT | This study |

Abbreviations: Ap, ampicillin; Cm, chloramphenicol; Gm, gentamicin; Sm, streptomycin; Sp, spectinomycin; Te, tetracycline.

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### FIGURE A1  Comparison of AnfA proteins

| Protein | Sequence |
|---------|----------|
| Rcap    | MFGDQVEALELGGASQPEDEFGQCTGSETCRVLNLPPILYRLNAVISQPQPEGSLGMKLT | 60 |
| Rmar    | MFGDLQVEALELGGASQPDEFGQCTGSETCRVLNLPPILYRLNAVISQPQPEGSLGMKLT | 60 |
| Rvtr    | MVEAOFQATLSEQPESDPFVGCTGSETCRVLNLPPILYRLNAVISQPQPEGSLGMKLT | 60 |
| Rvan    | MVEAQOFQATLSEQPESDPFVGCTGSETCRVLNLPPILYRLNAVISQPQPEGSLGMKLT | 60 |
| Rubn    | MLMVYTAIADDLDDDSDYCTGCRAVLPILFIDIQISIESEDLPRLSAIILVK | 57 |
| Avin    | MVEAQOFQATLSEQPESDPFVGCTGSETCRVLNLPPILYRLNAVISQPQPEGSLGMKLT | 57 |

| Protein | Sequence |
|---------|----------|
| Rcap    | MRSSEMMEQRTVSILRERQVRVQIPGILTGRGQGIRSEGQSCQIGETQVGAETGV1AIP | 120 |
| Rmar    | MRSSEMMEQRTVSMQRQFVDFSQGISAEESESESGTTYGQVETGQARV1V1AP | 120 |
| Rvtr    | MRSSEMMEQRTVSILRERQVRVQIPGILTGRGQGIRSEGQSCQIGETQVGAETGV1AIP | 120 |
| Rvan    | MRTLEQHRQCAIYLDQIDRQESDDQGILDEQSKGQRISLQGEGTVKAVVQESKL1VVP | 117 |
| Rrubn   | MQRQRMRQGTVGSRYDGZIKFHVESGRDQDGQLAGSGQGDTCVUSVGGSKA1VVP | 117 |
| Avin    | MRSSEMMEQRTVSILRERQVRVQIPGILTGRGQGIRSEGQSCQIGETQVGAETGV1AIP | 117 |

| Protein | Sequence |
|---------|----------|
| Rcap    | RUHESPQDFLDRTDGTAGHAGEWRVRFYAVCPIMLGKVRGTVTGGCGRSYLSNLQWGQAELA | 180 |
| Rmar    | RLHNFEDFDLDTGMAKADKRRRAAQRVAFCVFLMQKVTIDGTCRQRFYV1AIP | 180 |
| Rvtr    | RUEHSPQDFLDRTDGTAGHAGEWRVRFYAVCPIMLGKVRGTVTGGCGRSYLSNLQWGQAELA | 180 |
| Rvan    | RLHEDTQFLDTFRDKHADEHRKPHKATFFCVPQGRLRVQIGAEGYRNILQFLKADQAEFLA | 177 |
| Rrubn   | EURGQTPNKRTQLQADQDSLIVFSCVPQILGKVRGTVTGGCGRSYLSNLQWGQAELA | 177 |
| Avin    | RLQEPDFIDCRDTRVSNCGKAAAACFCVPMRAQKVGCRLTAERYNPRMLQDQVEILLA | 176 |

| Protein | Sequence |
|---------|----------|
| Rcap    | A1MALIAIPAVELLYLAVANEKLELENIREALRERFRPKNIGNSKAMAKMDVY1IGK | 240 |
| Rmar    | A1MALIAIPAVELLYLAVANEKLELENIREALRERFRPKNIGNSKAMAKMDVY1IGK | 240 |
| Rvtr    | A1MALIAIPAVELLYLAVANEKLELENIREALRERFRPKNIGNSKAMAKMDVY1IGK | 240 |
| Rvan    | A1MALIAIPAVELLYLAVANEKLELENIREALRERFRPKNIGNSKAMAKMDVY1IGK | 240 |
| Rrubn   | TIASAPIAVELLYLAVANEKLELENIREALRERFRPKNIGNSKAMAKMDVY1IGK | 237 |
| Avin    | V1MALIAIPAVELLYLAVANEKLELENIREALRERFRPKNIGNSKAMAKMDVY1IGK | 235 |

| Protein | Sequence |
|---------|----------|
| Rcap    | VSXKTRATVLILLIGGKVRGGLVSAHSGDRAAIFPFYNVCAPETLAEASELFLGHEGKA | 300 |
| Rmar    | VAXKTRATVLILLIGGKVRGGLVSAHSGDRAAIFPFYNVCAPETLAEASELFLGHEGKA | 300 |
| Rvtr    | VAXKTRATVLILLIGGKVRGGLVSAHSGDRAAIFPFYNVCAPETLAEASELFLGHEGKA | 300 |
| Rvan    | VAXKTRATVLILLIGGKVRGGLVSAHSGDRAAIFPFYNVCAPETLAEASELFLGHEGKA | 300 |
| Rrubn   | VAXKTRATVLILLIGGKVRGGLVSAHSGDRAAIFPFYNVCAPETLAEASELFLGHEGKA | 296 |
| Avin    | VAXKTRATVLILLIGGKVRGGLVSAHSGDRAAIFPFYNVCAPETLAEASELFLGHEGKA | 295 |

| Protein | Sequence |
|---------|----------|
| Rcap    | FTGALAGRGLFSGAQGSTGTLFEDLGESESPQVAKLLRLVLQDGTLRQVGVSTFTQVDVGRV | 360 |
| Rmar    | FTGALAGRGLFSGAQGSTGTLFEDLGESESPQVAKLLRLVLQDGTLRQVGVSTFTQVDVGRV | 360 |
| Rvtr    | FTGALAGRGLFSGAQGSTGTLFEDLGESESPQVAKLLRLVLQDGTLRQVGVSTFTQVDVGRV | 360 |
| Rvan    | FTGALAGRGLFSGAQGSTGTLFEDLGESESPQVAKLLRLVLQDGTLRQVGVSTFTQVDVGRV | 357 |
| Rrubn   | FTGALAGRGLFSGAQGSTGTLFEDLGESESPQVAKLLRLVLQDGTLRQVGVSTFTQVDVGRV | 356 |
| Avin    | FTGALAGRGLFSGAQGSTGTLFEDLGESESPQVAKLLRLVLQDGTLRQVGVSTFTQVDVGRV | 355 |

| Protein | Sequence |
|---------|----------|
| Rcap    | IAATNRLAVRMLQGAERFREDLFYLRALVVTPTVPLRLRDGSDLILLADHVFVAKACMKEKS | 420 |
| Rmar    | IAATNRLAVRMLQGAERFREDLFYLRALVVTPTVPLRLRDGSDLILLADHVFVAKACMKEKS | 420 |
| Rvtr    | IAATNRLAVRMLQGAERFREDLFYLRALVVTPTVPLRLRDGSDLILLADHVFVAKACMKEKS | 420 |
| Rvan    | IAATNRLAVRMLQGAERFREDLFYLRALVVTPTVPLRLRDGSDLILLADHVFVAKACMKEKS | 420 |
| Rrubn   | IAATNRLAVRMLQGAERFREDLFYLRALVVTPTVPLRLRDGSDLILLADHVFVAKACMKEKS | 420 |
| Avin    | IAATNRLAVRMLQGAERFREDLFYLRALVVTPTVPLRLRDGSDLILLADHVFVAKACMKEKS | 416 |

| Protein | Sequence |
|---------|----------|
| Rcap    | VKR3STPADLNMAYHWPGVRENRVLIERAVILSDVIAHNNLPPSSQTARECSZAP4S | 480 |
| Rmar    | VKR3STPADLNMAYHWPGVRENRVLIERAVILSDVIAHNNLPPSSQTARECSZAP4S | 480 |
| Rvtr    | VKR3STPADLNMAYHWPGVRENRVLIERAVILSDVIAHNNLPPSSQTARECSZAP4S | 480 |
| Rvan    | VKR3STPADLNMAYHWPGVRENRVLIERAVILSDVIAHNNLPPSSQTARECSZAP4S | 480 |
| Rrubn   | VKR3STPADLNMAYHWPGVRENRVLIERAVILSDVIAHNNLPPSSQTARECSZAP4S | 480 |
| Avin    | VKR3STPADLNMAYHWPGVRENRVLIERAVILSDVIAHNNLPPSSQTARECSZAP4S | 477 |

| Protein | Sequence |
|---------|----------|
| Rcap    | LGLEERVKVRGLENVIAKKTQGNIQGAAELVSRSVRLGMLNMRGLIP1DHRAS | 538 |
| Rmar    | VGELEERVKVRGLENVIAKKTQGNIQGAAELVSRSVRLGMLNMRGLIP1DHRAS | 539 |
| Rvtr    | VGELEERVKVRGLENVIAKKTQGNIQGAAELVSRSVRLGMLNMRGLIP1DHRAS | 538 |
| Rvan    | VGELEERVKVRGLENVIAKKTQGNIQGAAELVSRSVRLGMLNMRGLIP1DHRAS | 538 |
| Rrubn   | VGELEERVKVRGLENVIAKKTQGNIQGAAELVSRSVRLGMLNMRGLIP1DHRAS | 537 |
| Avin    | LGLEERVKVRGLENVIAKKTQGNIQGAAELVSRSVRLGMLNMRGLIP1DHRAS | 533 |

| Protein | Sequence |
|---------|----------|
| Rvtr    | RQNGSGBRSDDKNICNG | 555 |
| Avin    | DE | 537 |
The figure shows the alignment of AnfA proteins from *Rhodobacter capsulatus* (Rcap; WP_023913804), *Rhodobacter maris* (Rmar; WP_097070194), *Rhodobacter viridis* (Rvir; WP_110806348), *Rhodomicrobium vanniellii* (Rvan; WP_013420907), *Rhodospirillum rubrum* (Rrub; WP_011389142), and *Azotobacter vinelandii* (Avin; WP_012703363). Amino acid residues identical in all sequences are marked by stars. Underlined regions in the *A. vinelandii* protein indicate conserved motifs and domains, namely a cysteine motif (residues 21–26), the GAF domain (residues 33–193), the AAA + domain (residues 218–459), and the DNA-binding helix-turn-helix domain (residues 482–522; adapted from Austin & Lambert, 1994; Joerger et al., 1989). The separated DNA-binding domain of *R. capsulatus* AnfA (AnfA_DBD) used in electrophoretic mobility shift assays (Figure 3c) is marked in yellow.