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Quinol Oxidase Encoded by *cyoABCD* in *Rhizobium etli* CFN42 is Regulated by ActSR and is Crucial for Growth at Low pH or Low Iron Conditions

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Quinol Oxidase Encoded by *cyoABCD* in *Rhizobium etli* CFN42 Is Regulated by ActSR and Is Crucial for Growth at Low pH or Low Iron Conditions

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Abstract: *Rhizobium etli* aerobically respires with several terminal oxidases. The quinol oxidase (Cyo) encoded by *cyoABCD* is needed for efficient adaptation to low oxygen conditions and *cyo* transcription is upregulated at low oxygen. This study sought to determine how transcription of the *cyo* operon is regulated. The 5' sequence upstream of *cyo* was analysed *in silico* and revealed putative binding sites for ActR of the ActSR two-component regulatory system. The expression of *cyo* was decreased in an *actSR* mutant regardless of the oxygen condition. As ActSR is known to be important for growth under low pH in another rhizobial species, the effect of growth medium pH on *cyo* expression was tested. As the pH of the media was incrementally

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decreased, *cyo* expression gradually increased in the WT, eventually reaching ~10-fold higher levels at low pH (4.8) compared with neutral pH (7.0) conditions. This upregulation of *cyo* under decreasing pH conditions was eliminated in the *actSR* mutant. Both the *actSR* and *cyo* mutants had severe growth defects at low pH (4.8). Lastly, the *actSR* and *cyo* mutants had severe growth defects when grown in media treated with an iron chelator. Under these conditions, *cyo* was upregulated in the WT, whereas *cyo* was not induced in the *actSR* mutant. Altogether, the results indicated *cyo* expression is largely dependent on the ActSR two-component system. This study also demonstrated additional physiological roles for Cyo in *R. etli* CFN42, in which it is the preferred oxidase for growth under acidic and low iron conditions.

Introduction

Bacteria have diverse metabolic capabilities that enable them to cope with the varying environmental conditions. One example is having branched aerobic respiratory chains that terminate at different terminal oxidases, including various cytochrome *c* oxidases and, alternatively, quinol oxidases. The paths toward these oxidases diverge at quinol. Ubiquinol-cytochrome *c* oxidoreductase (Fbc), also known as the bc_1 complex, sends electrons from quinol to cytochrome *c*. Electrons are then transferred from cytochrome *c* to oxygen via cytochrome *c* oxidases. Alternatively, electrons can flow directly from quinol to oxygen via quinol oxidases.

The quinol oxidase encoded by *cyoABCD* is widespread among proteobacteria and firmicutes. This oxidase (Cyo) is often classified as a low-affinity oxidase utilized in high oxygen based on studies in *Escherichia coli* (García-Horsman *et al.*, 1994; Morris & Schmidt, 2013). Conversely, previous work demonstrated Cyo to be important for growth and adaptation to low oxygen conditions in culture for certain rhizobial species (Surpin & Maier, 1998; Lunak & Noel, 2015). Moreover, in two rhizobial species, *cyo* genes are upregulated under low oxygen (Trzebiatowski *et al.*, 2001; Bobik *et al.*, 2006; Lunak & Noel, 2015). However, the transcriptional regulatory mechanism of this upregulation is not understood.

Oxygen is a substrate for all the terminal oxidases and is a common factor in how oxidases are regulated (Bueno *et al.*, 2012).

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Some oxidases are regulated by transcription factors that sense oxygen directly, such as Fnr proteins. Fnr becomes active at low oxygen and binds to a conserved symmetrical motif, TGGAT-N_4-ATCAA (Kiley & Beinert, 1998; Körner *et al.*, 2003). Alternatively, oxidases can be regulated by transcription factors that sense oxygen indirectly based on the redox state of certain molecules. The activity of the two-component system RegBA in *Rhodobacter capsulatus* is regulated in part by the redox state of quinone (Wu & Bauer, 2010). Homologous two-component systems (ActSR in *Sinrohizobium meliloti*, RegSR in *Bradyrhizobium japonicum*, PrrBA in *Rhodobacter sphaeroides*) can complement one another (Emmerich *et al.*, 2000a; Elsen *et al.*, 2004; Wu & Bauer, 2008). However, the DNA binding sites are not as well conserved among organisms compared with the aforementioned Fnr anaerobox. In *B. japonicum*, the RegA homologue RegR binds to an imperfect repeat NGNGNCN_{4-6}GNNNC (Emmerich *et al.*, 2000b; Lindemann *et al.*, 2007; Torres *et al.*, 2014). In this bacterium, the RegSR two-component system is important for adaptation to low oxygen during processes such as symbiosis with legumes and recently it has been implicated in denitrification (Bauer *et al.*, 1998; Torres *et al.*, 2014). In addition, the RegBA homologue in *S. meliloti* ActSR is necessary for growth and the induction of genes required for adaptation to low pH conditions (O'Hara *et al.*, 1989; Tiwari *et al.*, 1996).

Iron may be another important cue for Cyo regulation and utilization. The Fbc pathway to oxygen involves more proteins that contain iron than the Cyo respiratory pathway. Previous work showed *cyo* to be upregulated in response to low iron conditions in *S. meliloti* and *Pseudomonas aeruginosa* (Chao *et al.*, 2005; Kawakami *et al.*, 2010). It is not known how general this regulation is and what the mechanism of regulation might be.

In this study, *Rhizobium etli* was used as a model organism to determine the major transcription factor involved in regulating *cyo*. Like other rhizobia, *R. etli* resides either in the free-soil environment or as a nitrogen-fixing symbiont in root nodules of its leguminous host plant. In both environments, *R. etli* must cope with varying physiological conditions. *R. etli* CFN42 is particularly useful for studying Cyo because Cyo is the only quinol oxidase present (Lunak & Noel, 2015).

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Use was made of previously isolated mutants whose respiratory paths were constrained to end in cytochrome *c* oxidases or, conversely, only in quinol oxidases. All the aerobic respiratory chains terminating with cytochrome *c* oxidases require a functional Fbc. Therefore, an *fbc* mutant reveals the sufficiency of Cyo function under a given condition because Cyo would be the only viable oxidase option remaining in this mutant. However, a *cyo* mutant can only respire via cytochrome *c* oxidases. The inability of a *cyo* mutant to grow is evidence that the bacterium has evolved to depend on Cyo under the conditions being tested. In the analysis of *cyo* transcriptional regulation under different conditions, comparisons of an *fbc* mutant and the WT provide additional physiological insights. If there is no difference between the WT and *fbc* mutant under a particular condition, this suggests that Fbc is not normally used under that condition. Conversely, a significant increase of *cyo* transcription in the *fbc* mutant compared with the WT suggests that Fbc is preferentially utilized under that condition, but the strain can adapt to the loss of Fbc by upregulating *cyo*.

Methods

Bacterial strains and growth conditions

R. etli strains were derived from CE3, a streptomycin-resistant derivative of the CFN42 WT strain (Noel *et al.*, 1984), whose genome nucleotide sequence has been determined (González *et al.*, 2006). *R. etli* strains were grown at 30 °C on a rotating shaker in TY liquid medium [0.5% tryptone (Difco), 0.3% yeast extract (Difco) and 10 mM $CaCl_2$]. For growth under low pH, strains were grown either in TY buffered with 40 mM MES or in a low pH YGM minimal medium containing 0.4 mM $MgSO_4$, 1.25 mM K_2HPO_4, 0.11% sodium glutamate, 0.4% glucose, 4 mM NH_4Cl, 1 mM $CaCl_2$, 0.15 mM $FeCl_3$, 1 µg biotin ml^{-1}, 1 µg thiamine ml^{-1}, 1 µg pantothenic acid ml^{-1} and 40 mM MES that buffers the pH to ~4.8. For growth at neutral pH in YGM medium, the pH was adjusted to 7.0 by titration with NaOH. The pH of the medium was tested after growth to ensure the pH was maintained at ± 0.2 pH of the uninoculated medium. For growth under low iron, strains were grown in TY treated with 200 µM 2,2-dipyridl. *E. coli* strains were grown in LB liquid medium (1.0% tryptone, 0.5% yeast

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extract and 0.5% NaCl) at 37 °C on a rotating shaker (Sambrook *et al.*, 1989). Agar medium contained 1.5% Bacto agar (Difco).

To analyse growth under varying oxygen concentrations, cultures were grown as described previously (Lunak & Noel, 2015). Fully grown cultures were diluted 1 : 200 into 5 ml TY medium, resting in 60 ml serum vials. The serum vials were then capped and the headspace was flushed with nitrogen gas. Using a sterile syringe needle, ambient air (assumed 21% oxygen) was injected back into the headspace to make it 1 and 0.1% oxygen. For growth at 21% oxygen, the vials were covered with aluminium foil. Cultures were then grown at 30 °C on a rotating shaker. To follow growth, 400 µl was removed from the cultures using a sterile syringe needle and the OD_{600} was measured.

Prediction of ActR binding sites in 5′ upstream of cyo

The genomes of *R. etli* CFN42, *S. meliloti* 1021, *Rhizobium leguminosarum* bv *viciae* 3841 and *B. japonicum* USDA 110 have been determined (Finan *et al.*, 2001; Kaneko *et al.*, 2002; González *et al.*, 2006; Young *et al.*, 2006). To identify possible ActR DNA binding sites, various parts of the promoter regions were aligned with known RegR DNA binding sites from *B. japonicum* (Lindemann *et al.*, 2007; Torres *et al.*, 2014) using clustal w2 software (Thompson *et al.*, 2002).

Materials and techniques for DNA isolation

Genomic DNA for use in cloning was isolated from *R. etli* strains using a GenElute Bacterial Genomic DNA kit (Sigma). *E. coli* NEB-5α (Invitrogen) competent cells were transformed (Hanahan, 1983) and plasmids were isolated using a QIAprep Spin Miniprep kit (Qiagen). DNA was recovered from agarose gels using a Gel/PCR DNA Fragments Extraction kit (IBI Scientific) and modified with restriction enzymes purchased from New England BioLabs. Custom primers were synthesized by Eurofins MWG Operon.

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Cloning and mutagenesis

The strains used in this study are listed in Table 1. The *fbc* and *cyo* mutants were constructed in a previous study (Lunak & Noel, 2015). The *cyo* mutant (CE574) contains a kanamycin resistance cassette in the *Sal*I site (nt 39 576–39 581 of the *R. etli* CFN42 genome) of *cyoA* of the *cyoABCD* operon. The *fbc* mutant contains a Tn*5* transposon insertion in the *fbcF* gene of the *fbcFBC* operon at nt 3 178 288 of the *R. etli* CFN42 genome (E. Rosado and K. D. Noel, unpublished).

Table 1. Strains and plasmids used in study

Bacterial strain or plasmid	Description, genotype or phenotype	Reference or source
***R. etli* strains**		
CE3	WT strain, *str-1*	Noel *et al.* (1984)
CE3/pZL73	CE3 carrying pZL73, Tc^R	This study
CE119	CE3 derivative; *str-1 fbcF*::Tn*5*	Lunak & Noel (2015)
CE119/pZL73	CE119 carrying pZL73; Tc^R	This study
CE574	CE3 derivative; *str-1 cyoA*::Km	Lunak & Noel (2015)
CE574/pZL34	CE574 carrying pZL34, Tc^R	Lunak & Noel (2015)
CE605	CE3 derivative; *str-1 actS*::Km::*actR*	This study
CE605/pZL73	CE605 carrying pZL73, Tc^R	This study
CE605/pZL51	CE605 carrying pZL51, Tc^R	This study
***E. coli* strains**		
NEB-5α	Competent strain used for cloning	New England Biolabs
MT616	*pro thi endA hsdR supE44 recA-J6* pRK2013Km::Tn*9*	Finan *et al.* (1986)
Plasmids		
pBSL86	*nptII* gene cassette; Km^R	Alexeyev (1995)
pCR2.1	Ap^rKm^r, TA cloning vector for PCR products	Invitrogen
pEX18Tc	Suicide plasmid; Tc^R *ori*T *sacB*	Hoang *et al.* (1998)
pFAJ1708	Expression vector with *nptII* promoter	Dombrecht *et al.* (2001)
pMP220	Transcriptional *lacZ* fusion vector, Tc^R	Spaink *et al.* (1987)
pZL34	1.3kb *Bam*HI/*Pst*I fragment with *cyoA* in pFAJ1708	Lunak & Noel (2015)
pZL48	Km cassette replacing 1.7kb of the *actSR* operon, Km^R	This study
pZL51	3.1kb *Eco*RI/*Hnd*III fragment with *actSR* in pFAJ1708	This study
pZL73	pMP220-derived, 475bp *Kpn*I/*Xba*I fragment upstream of *cyoA* fused with *lacZ*	This study

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The *actSR* mutant (CE605) was made by deleting 1148 bp of *actS* and additionally 511 bp of *actR*. To mutate *actSR*, the initial 181 bp of *actSR* together with 566 bp of 5′ upstream sequence (nt 59 093–58 347 of the *R. etli* CFN42 genome) was amplified by PCR using ActSup-*Eco*RI and ActSup-*Xba*I primers (listed in Table S1, available in the online Supplementary Material). The PCR product was then ligated into the TA cloning vector pCR2.1 (Invitrogen) to create pZL45. In addition, the last 65 bp of *actR* together with 586 bp of 3′ downstream sequence (nt 56 614–55 964 of the *R. etli* CFN42 genome) was amplified by PCR using ActRdwn-*Xba*I and ActRdwn-*Hnd*III primers (Table S1) and ligated into pCR2.1 to create pZL46. pZL45 was then digested with *Eco*RI/*Xba*I to release the 747 bp fragment and pZL46 was digested with *Xba*I/*Hnd*III to release the 651 bp fragment. Both of these fragments were ligated into pEX18Tc (Hoang *et al.*, 1998) to create pZL47. pZL47 was then digested with *Xba*I and a kanamycin resistance cassette (Alexeyev, 1995) was inserted into this site to create plasmid pZL48. pZL48 was transferred into *R. etli* CE3 by using the plasmid-mobilizer strain MT616 on TY agar plates (Finan *et al.*, 1986; Glazebrook & Walker, 1991).

CE3 transconjugants containing pZL48 were selected and purified as described previously (Ojeda *et al.*, 2010). Double-crossover recombinants were screened on TY agar plates supplemented with 1 µg tetracycline ml^{-1}. Of the recombinants that were sensitive to 1 µg tetracycline ml^{-1} and resistant to 8 % sucrose on TY agar, it was then verified by PCR that the colonies contained only the mutant allele and that the WT allele was absent.

Complementation of actSR and cyo

The entire *actSR* operon including the flanking sequence (nt 59 093–55 964 of the *R. etli* CFN42 genome) was amplified using the primers listed in Table S1. The DNA PCR product was originally inserted into the TA cloning vector pCR2.1. The plasmid was then digested with *Xba*I/*Bam*HI to release the 3.1 kb fragment containing *actSR*, which then was ligated to pFAJ1708 (Dombrecht *et al.*, 2001). The resulting plasmid pZL51 was transferred into CE605 (*actSR* mutant) by triparental mating. Strains containing pZL51 were selected for tetracycline resistance (5 µg ml^{-1}). A similar approach was used to

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complement the *cyo* mutant CE574 and has been described previously (Lunak & Noel, 2015).

lacZ fusion and β-galactosidase measurements

To generate a *cyoA* : : *lacZ* transcriptional fusion, the promoter region of *cyoA* (nt 40 020–40 490 of the *R. etli* CFN42 genome) was amplified from *R. etli* CE3 genomic DNA by PCR using the primers listed in Table S1. The PCR product was then inserted into the TA cloning vector pCR2.1 to create plasmid pZL67. The 485 bp fragment was released from pZL67 using *Xba*I/*Kpn*I and ligated into the pMP220 plasmid (Spaink *et al.*, 1987) at the *Kpn*I/*Xba*I restriction sites to create pZL73. pZL73 was transferred into CE3 using the MT616 plasmid-mobilizer strain. The empty vector pMP220 was introduced into CE3 as a negative control. Under different oxygen (1 or 21 %) and pH (4.8–7.5) conditions, 1 ml culture was withdrawn and washed with cold Z-buffer. The β-galactosidase assay was performed and Miller units were calculated as described previously (Sambrook *et al.*, 1989).

Reverse transcription quantitative PCR (RT-qPCR)

Cultures were pelleted, immediately frozen in dry ice and stored at − 80 °C. When ready for testing, cells were thawed on ice and RNA was extracted using a NucleoSpin RNA II kit (Macherey Nagel). The RNA concentration was measured by a NanoDrop spectrophotometer and 1 µg RNA was converted into cDNA using an EasyScript cDNA synthesis kit (MidSci) with the specific reverse primer for the gene of interest. As a negative control, water was added instead of the reverse transcriptase. cDNA products were quantified by real-time PCR using EvaGreen qPCR Mastermix (MidSci), gene-specific primers and a Bio-Rad iCycler. For analysing *cyo* expression, primers were designed to detect a 118 bp fragment in *cyoB* (Table S1). Samples were initially denatured at 95 °C for 10 min followed by a 40-cycle amplification protocol (95 °C for 15 s, 60 °C for 60 s). After the PCR, a melt curve analysis was performed to ensure only one amplification product was present. The expression of the 16S RNA gene was analysed using the same approach. Results for *cyoB* expression were normalized to the expression of the 16S RNA gene.

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Results

cyo 5′ upstream sequence and prediction of regulatory elements

The genome nucleotide sequence of *R. etli* CFN42 has been determined (González *et al.*, 2006). The 5′ upstream DNA of the *cyo* operon is depicted in Fig. 1. The promoter region contained three putative ActR DNA binding sites that aligned with the consensus RegR DNA binding site in *B. japonicum* (Figs. 1 and S1). Two of the possible ActR binding sites were in succession (nt 40 164–40 130 of the *R. etli* CFN42 genome sequence). The other potential ActR DNA binding site and a potential FNR anaerobox were further upstream (nt 40 387–40 421). Potential ActR DNA binding sites were also present in the 5′ regions upstream of *cyo* in *S. meliloti*, *R. leguminosarum* and *B. japonicum* (Fig. S1).

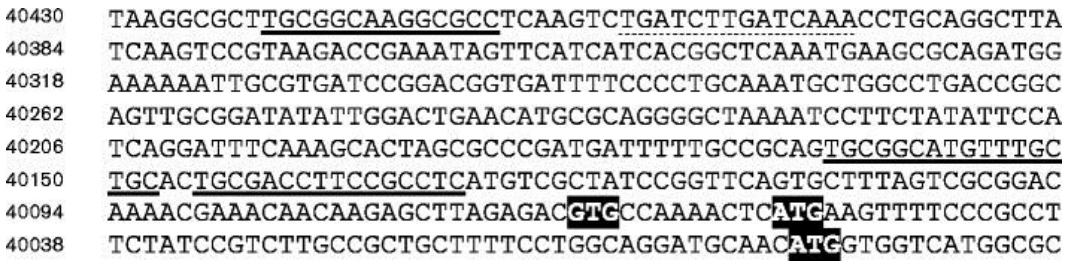

Fig. 1. The 5′ upstream promoter region of ***cyo***. The sequence contains 5′ DNA upstream of the ***cyoA*** ORF (nt 40 430–39 982 of ***R. etli*** CFN42 genome). Putative ActR DNA binding sites are underlined. A putative Crp-Fnr anaerobox is dash-underlined. Possible translation start sites are highlighted in black.

Mutagenesis of actSR and its effect on cyo expression under low oxygen

To test whether ActSR regulates *cyo* expression, mutant strain (CE605) was constructed by replacing the *actSR* operon with a kanamycin resistance cassette. The expression of *cyo* was analysed in this *actSR* mutant under various oxygen conditions using RT-qPCR and a *cyo* : : *lacZ* fusion (Fig. 2a, b). As seen previously (Lunak & Noel, 2015), the WT significantly upregulated *cyo* at 1% compared with 21% oxygen concentration. In the *actSR* mutant, *cyo* transcript levels were approximately eightfold lower compared with WT levels under

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low oxygen (1%). Under high oxygen conditions, the expression of *cyo* was decreased in the *actSR* mutant (around twofold) compared with the WT levels. The expression of *cyo* was restored to WT levels after transferring the WT copy of *actSR*, pZL51, into the *actSR* background. In addition, *cyo* promoter activity in an *fbc* mutant was assessed by use of the *cyo* : : *lacZ* fusion carried on a plasmid in mutant cells grown under 1 and 21% oxygen. Under both low and high oxygen conditions, the *fbc* mutant exhibited higher *cyo* promoter activity than the WT (Fig. 2b).

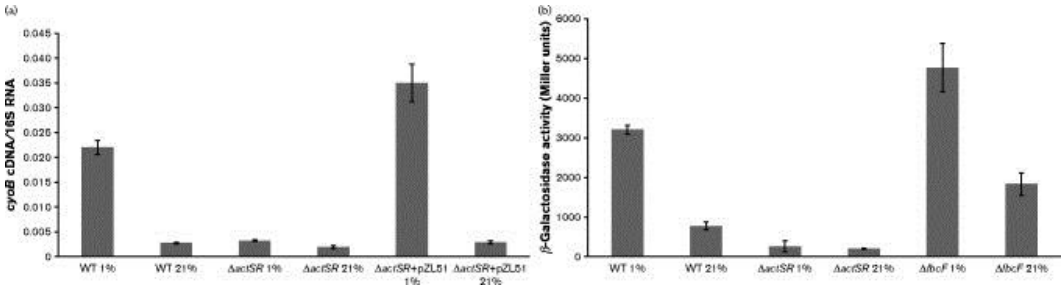

Fig. 2. *cyo* expression under 1 and 21% oxygen. Strains were grown under either 21 or 1% oxygen conditions. Protein or RNA was extracted from cells in exponential phase. (a) RT-qPCR of ***cyoB***. RNA was extracted and converted into cDNA using the reverse gene-specific primer. As described in Methods, cDNA was then quantified by qPCR. The amount of ***cyoB*** cDNA was then normalized to the amount of 16S RNA cDNA from the original RNA sample. Mean ± **sd** values were calculated from three separate qPCR assays. (b) β-Galactosidase activity of the strains carrying the transcriptional fusion plasmid pZL73 (***cyoA*** : : ***lacZ***). Mean ± **sd** values were calculated from three or more separate ***lacZ*** assays from two different cultures.

It is known that *cyo* mutants have growth defects at 0.1 and 1% oxygen. The above results suggest that *actSR* mutants should have the same defects. Therefore, growth of the *actSR* mutant was analysed under various oxygen concentrations in TY medium. The *actSR* mutant failed to grow under 0.1% oxygen and had a severe growth defect under 1.0% oxygen (Fig. 3a, b). Addition of the WT *actSR* operon (pZL51) restored growth similar to that of the WT. Under high oxygen conditions (21%), the mutant had a slight growth defect (Fig. 3c).

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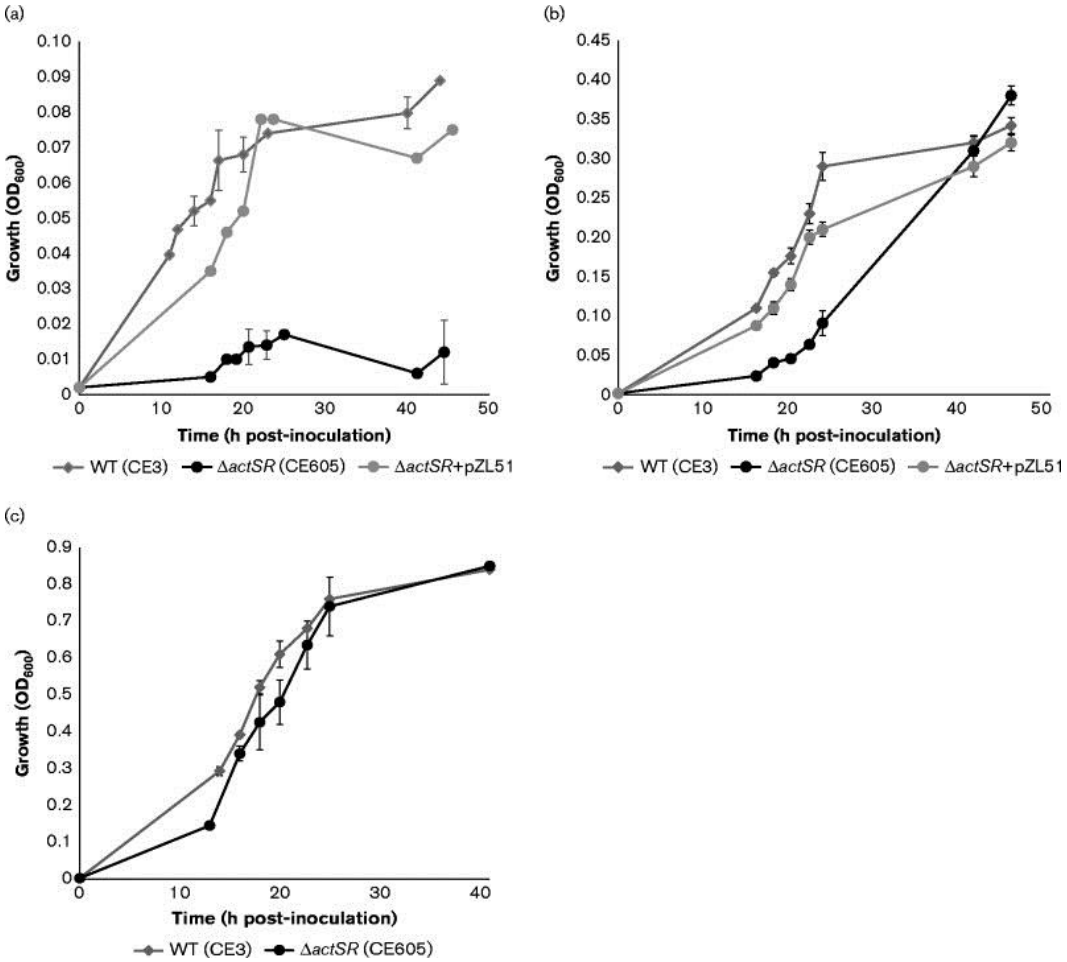

Fig. 3. Growth curves of the ***actSR*** mutant under (a) 0.1, (b) 1.0 and (c) 21% oxygen. Strains were initially grown in TY liquid under a gas phase with 21% oxygen. At full growth they were subcultured 1 : 200 into 5ml TY liquid in 60ml serum vials. As described in Methods, nitrogen and air were added to the headspaces in the vials above the liquid to give the indicated concentrations of oxygen. Growth was followed by measuring the OD_{600}. Bars, **sd** from at least three separate cultures.

cyo expression under varying pH conditions

Given ActSR's role as a global regulator in acidic conditions (O'Hara *et al.*, 1989; Tiwari *et al.*, 1996), *cyo* promoter activity was measured under various pH conditions. As the pH in the medium was incrementally lowered from 7.5 to 4.8, *cyo* promoter activity began to increase at pH < 6.5 (Fig. 4a). *cyo* expression was investigated more closely at pH 4.8 versus 7.0 using RT-qPCR in addition to the *cyo* : : *lacZ* fusion. Transcription activity was ~10-fold higher at pH 4.8 compared with pH 7.0 in the WT (Fig. 4b, c). In the *actSR* mutant, *cyo* transcript levels and promoter activity were significantly lower

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compared with WT levels under any pH condition. *cyo* expression was significantly increased after transferring the WT copy of the *actSR* operon pZL51 into the *actSR* background, but was still lower compared with WT under low pH (Fig. 4c). In the *fbc* mutant, the promoter activity was significantly higher compared with the WT when cells were grown at neutral pH (Fig. 4b). However, the promoter activities were similar between the WT and *fbc* mutant under low pH conditions.

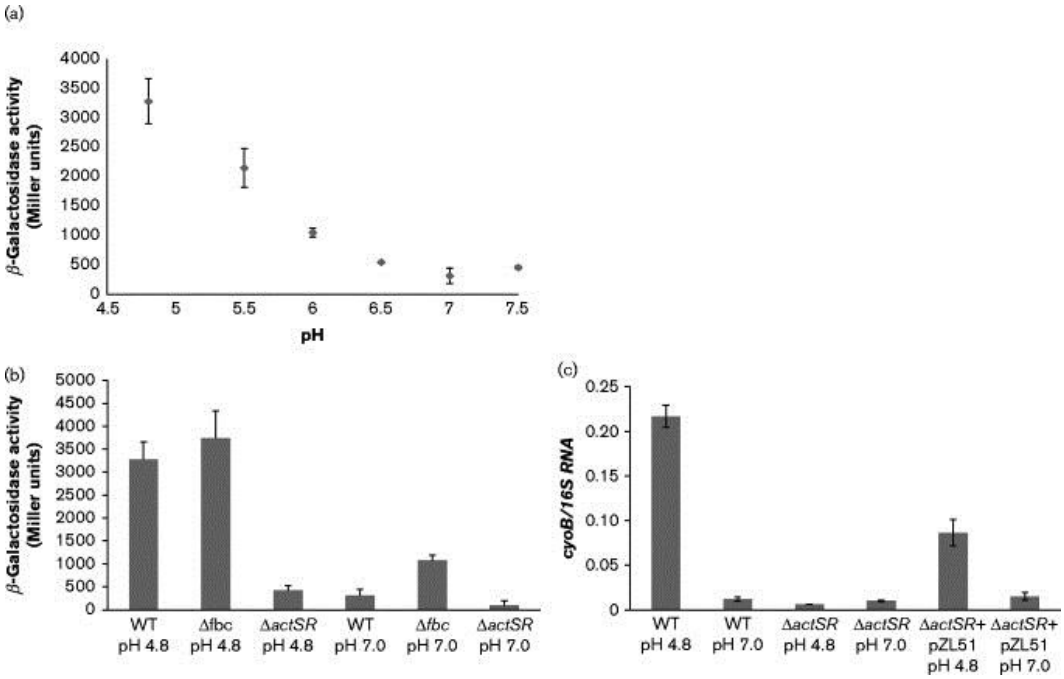

Fig. 4. *cyo* expression under varying pH conditions. (a) WT cells, carrying pZL73 (***cyo*** : : ***lacZ***), were harvested from exponentially growing cultures at different pH (4.8–7.5) in YGM media and the β-galactosidase assay was performed. Specific activity is given in Miller units. Mean ± **sd** values were calculated from three or more separate ***lacZ*** assays from two different cultures. (b) Strains, carrying pZL73 (***cyo*** : : ***lacZ***), were grown in YGM buffered with MES at pH 4.8 or 7.0. Cells were harvested in exponential phase and the β-galactosidase activity (Miller units) was determined. Mean ± **sd** values were calculated from three or more separate assays from two different cultures. (c) Strains were grown in YGM media buffered with MES at pH 4.8 or 7.0. RNA was extracted from exponential phase, and ***cyoB*** transcript levels were determined and normalized to the ***16S RNA*** levels. Mean ± **sd** values were calculated from three separate qPCR assays.

Growth under low pH

To determine if Cyo and ActSR had a significant physiological role at low pH, the growth of the mutants was analysed at pH 4.8 and 7.0 in YGM media. Both the *cyo* and *actSR* mutants had significantly

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prolonged lag phases when grown under low pH (Fig. 5a). Conversely, the *fbc* mutant grew comparably to the WT. The *cyo* and *actSR* mutants also had growth defects in TY media adjusted to pH 4.8 (Fig. 5b). The growth defects were alleviated after transferring the WT copies of *cyoA* (pZL34) and *actSR* (pZL51) into their respective mutant backgrounds (Fig. 5b, S2a). When grown under neutral pH, the *cyo* mutant grew similarly to the WT, whereas both the *actSR* and *fbc* mutants had slight growth defects (Fig. 5c).

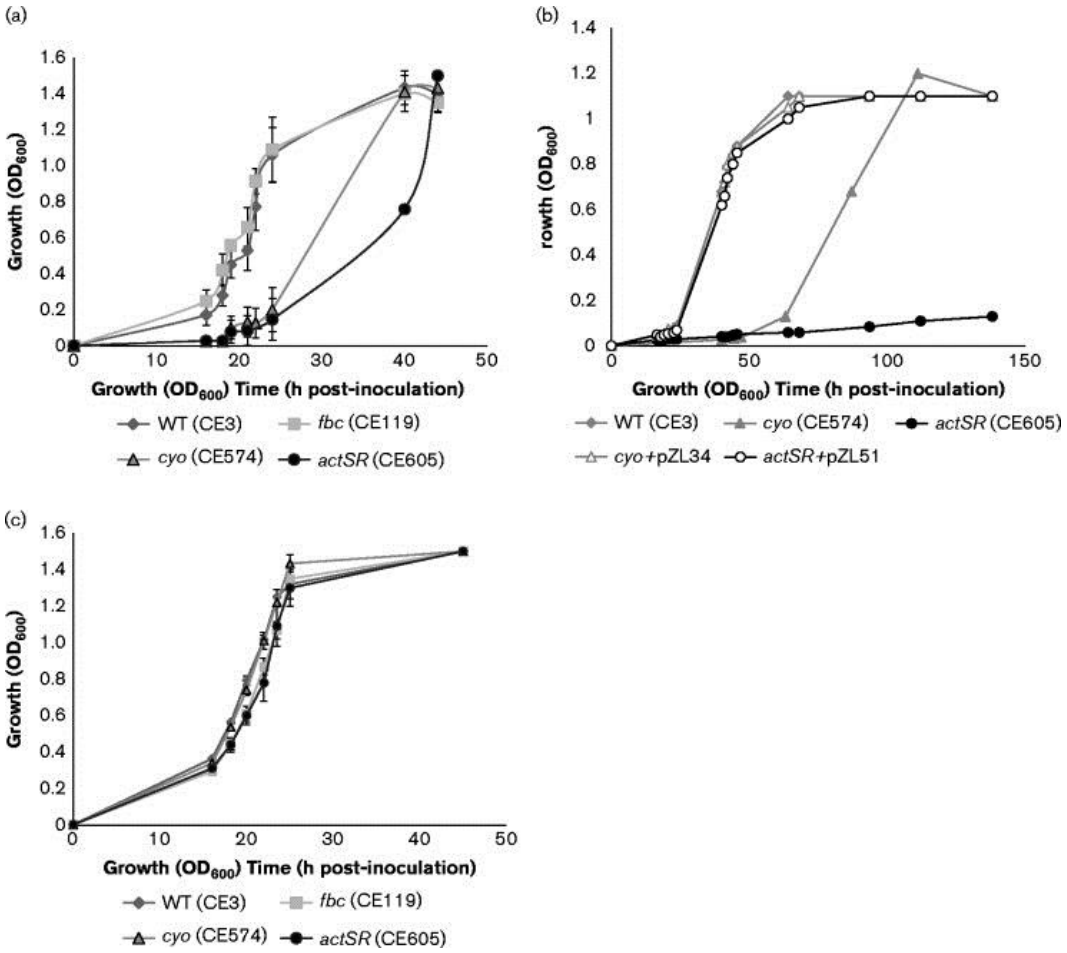

Fig. 5. Growth curves at low and neutral pH. Strains were grown aerobically in (a) YGM media buffered at pH 4.8, (b) TY media buffered at pH 4.8 (c) or YGM media buffered at pH 7.0. Growth was followed by measuring the OD_{600}. After growth, the pH was measured to ensure the pH of the culture was maintained at ± 0.2 pH of the uninoculated cultures. Bars, **sd** from at least three separate experiments.

When streaked on low pH YGM plates (pH 4.8), the *actSR* mutant did not start to form colonies until 7 days after streaking (Fig. S3), whereas the WT consistently formed isolated colonies 2–3 days

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after streaking on low pH plates. Isolated colonies were consistently observed in the *cyo* mutant 4 days after streaking. After transferring the WT copies of *cyoA* (pZL34) and *actSR* (pZL51) into their respective mutant backgrounds, the onset of isolated colonies was similar to that of the WT (2–3 days after streaking).

Growth analysis and cyo expression under low iron

To test the importance of Cyo under low iron conditions, growth and *cyo* expression were analysed in cells grown in TY medium treated with an iron chelator, 2,2-dipyridyl. Both the *cyo* and *actSR* mutants had a severe growth defect under this condition (Fig. 6a). However, growth of the *fbc* mutant was comparable to that of the WT. Addition of the WT copies (pZL34 and pZL51) to their respective mutant backgrounds alleviated the observed growth defects (Fig. S2b). In the WT, the expression of *cyo* was approximately fivefold higher in this low iron medium (Fig. 6b). Conversely, *cyo* expression was not induced under low iron concentrations in the *actSR* mutant. In the *fbc* mutant, *cyo* expression was similar to the WT under the low iron condition.

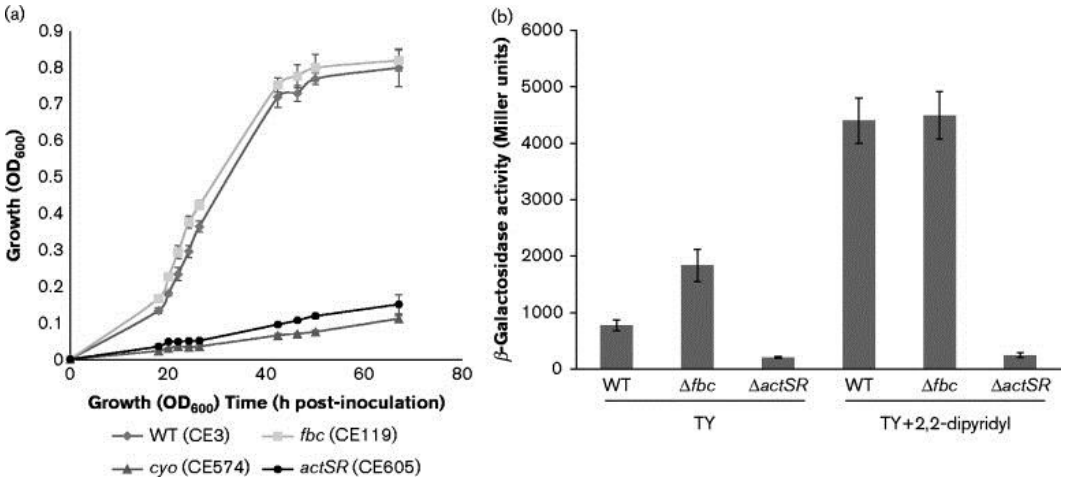

Fig. 6. Growth and ***cyo*** expression under low iron. (a) Strains were grown aerobically in TY treated with 200 µM 2,2-dipyridyl. Growth was followed by measuring the OD_{600}. Bars, **sd** from at least three separate cultures. (b) Strains carrying pZL73 were grown in 2,2-dipyridyl-treated and untreated TY media. β-Galactosidase activities were determined from cells in exponential phase. Mean ± **sd** values were calculated from three or more separate ***lacZ*** assays from two different cultures.

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Discussion

Potential ActR DNA binding sites are present in the DNA 5' to *cyoA* in strains of *R. etli*, *R. leguminosarum*, *S. meliloti* and *B. japonicum*. This suggests the existence of common mechanisms for regulating *cyo* in these species. The focus of this study was to investigate the possible role of ActSR and the genetic results imply that this two-component system is a key regulator of *cyo* expression in *R. etli* CFN42.

First, an *actSR* mutant was constructed and its *cyo* expression was tested under varying oxygen concentrations. The *actSR* mutation abrogated the strong upregulation in *cyo* expression observed in the WT under low oxygen conditions. This result correlates with a recent microarray study in *B. japonicum*, where *cyo* genes were significantly decreased under anoxic conditions in a mutant defective in the ActSR homologue RegSR (Torres *et al.*, 2014). In addition, this result may explain how *cyo* is upregulated under low oxygen in *S. meliloti*, where it had been shown that *cyo* is upregulated independently of FixJ (Bobik *et al.*, 2006).

The *actSR* mutant had a significant growth defect under low oxygen (0.1 and 1.0%) concentrations. This is consistent with findings in *B. japonicum* where it is considered a global regulator for adaptation to low oxygen conditions (Bauer *et al.*, 1998; Torres *et al.*, 2014).

The ActSR two-component system is an important global regulator under acidic conditions in *S. meliloti* (O'Hara *et al.*, 1989; Tiwari *et al.*, 1996). Therefore, it was predicted that *cyo* might be upregulated under low pH. The expression of *cyo* remained at a basal level under neutral pH but, as pH in the growth medium was lowered below 6.5, *cyo* expression rose in inverse proportion to the pH drop. Increased *cyo* expression in response to acidic conditions has been observed in a microarray study in *S. meliloti* (Hellweg *et al.*, 2009). Similar to the response to varying oxygen concentrations, deletion of *actSR* eliminated the increased *cyo* expression under low pH.

An *fbc* mutant was included in this study, because theory and all evidence to date indicate Cyo is the only viable respiratory option in an *R. etli* CFN42 mutant lacking Fbc. Based on this premise, it reveals the

capability of Cyo to function under varying conditions. For instance, this mutant was able to grow under either low or neutral pH conditions, implying that Cyo provides a useful function under a wide range of pH conditions. Moreover, Cyo seems to function at a wide range of iron concentrations, given that the *fbc* mutant can grow at both low and high iron concentrations. In conditions where *cyo* is marginally expressed in the WT, such as high oxygen or neutral pH, the *fbc* mutant had approximately threefold higher levels of *cyo* expression compared with the WT. This indicates the WT prefers the Fbc pathway under these conditions, but the mutant is still able to respond to the lack of Fbc activity by upregulating *cyo* and presumably increasing its utilization. However, under conditions where *cyo* is upregulated in the WT (low oxygen, low pH and low iron) the differences in *cyo* expression were minimal between the *fbc* mutant and the WT. These results further support that Cyo is the preferable oxidase under these conditions.

How ActSR becomes active has been characterized *in vitro* in the homologous RegBA system in *Rhodobacter capsulatus*. One study indicated autophosphorylation activity of the ActS homologue RegB can be influenced by the redox state of quinone (Wu & Bauer, 2010). Although both quinone (oxidized) and quinol (reduced) can bind to RegB with equal affinity, only quinone will promote an inactive conformation. Thus, a higher quinol : quinone ratio in the cell will lead to a more active RegBA. This may explain how *fbc* mutation leads to upregulation of *cyo* under both aerobic and neutral pH conditions. In this mutant, the Fbc pathway is non-functional, which would presumably cause a build-up of quinol, causing the quinol : quinone ratio to increase. The increase of quinol would further activate ActSR and consequently increase *cyo* expression. It is predicted that the increased *cyo* expression observed in the *fbc* mutant is fully attributable to ActSR as the *actSR*/*fbc* double mutant was unattainable in aerobic conditions.

The quinol : quinone ratio may also be affected at low pH. The premise is based on several studies in another alphaproteobacterium, *Rhodobacter sphaeroides*, where Fbc activity was optimum at pH 8.0 and dropped to a relatively low level at pH < 6.0 (Crofts *et al.*, 1999; Guergova-Kuras *et al.*, 2000; Lhee *et al.*, 2010; Zhou *et al.*, 2012). If these *in vitro* conditions are relevant to changes the pH of the growth

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medium, an acidic pH would cause lower Fbc activity leading to a higher quinol : quinone ratio and, consequently, greater ActSR activity and higher levels of *cyo* expression.

Previous work has shown *cyo* to be upregulated in response to low iron conditions in *S. meliloti* and *P. aeruginosa* (Chao *et al.*, 2005; Kawakami *et al.*, 2010). This study indicates *cyo* is not only upregulated in *R. etli*, but also necessary for efficient growth under low iron conditions as the *cyo* mutant had a severe growth defect. The results indicate ActSR is necessary for *cyo* induction under low iron similar to the other conditions tested. To our knowledge, ActSR has yet to be linked to low iron conditions. Its requirement may be that it is necessary for any high level of expression of *cyo*. Common iron regulatory elements (RirA, Irr, Fur) were not identified in the 5' promoter region of *cyo* (Rudolph *et al.*, 2006). One rationale for regulation by ActSR starts with the observation that cytochrome c_1 levels are vastly decreased in *B. japonicum* under low iron conditions (Gao & O'Brian, 2005). Theoretically, this would cause a build-up of quinol as proposed for the *fbc* mutant described above and consequently cause an increase of ActSR activity leading to higher *cyo* levels.

In summary, this study indicates that the ActSR two-component system is necessary for the transcriptional activation of *cyo* under varying physiological conditions. Knowing the involvement of this regulator led to the discovery of other physiological roles for the Cyo quinol oxidase, as the *cyo* mutant also had severe growth defects under low pH and iron conditions. Given that *cyo* was also significantly upregulated in these conditions, the implication is that Cyo is the preferred oxidase at low pH and low iron in *R. etli* CFN42.

Acknowledgements

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Abbreviations:

RT-qPCR reverse transcription quantitative PCR

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Supplementary Data

Supplementary Data

http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4635472/bin/mic-161-09-1806-s001.pdf

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Gene	Upstream Primer	Downstream Primer	Use	Source
cyoA	cyoA-XbaI GATCTAGAAGCGTGCCGGTCAGCCT	cyoA-HndIII GTAAGCTTCGGCGGTCGTCATCCAG	Mutagenesis	Lunak & Noel, 2015
ActS - upstream	ActSup-EcoRI GCGAATTCAGGAGGTACTGCAGCTT	ActSup-XbaI GTTCTAGATAGCGATCAGCGGCAAC	Mutagenesis	This work
ActR-downstream	ActRdwn-XbaI GCTCTAGACACGCCGGCTCAATATG	ActRdwn-HndIII GTAAGCTTGCGAGGAATCCGTCTGC	Mutagenesis	This work
ActSR	ActSup-EcoRI GCGAATTCAGGAGGTACTGCAGCTT	ActRdwn-HndIII GTAAGCTTGCGAGGAATCCGTCTGC	Complementation	This work
cyoA	cyoA-BamHI GCGGATCCCGGTTCAGTGCTTTAGT	cyoA-PstI TGCTGCAGTTCAGGAGGTCAGGATT	Complementation	Lunak & Noel, 2015
P*cyoA*	PCyoA-KpnI GCGGTACCTTCAAGCGACGACGA	PcyoA-XbaI GATCTAGACAGCGGCAAGACGGATA	*cyoA::lacZ* fusion	This work
cyoB	cyoBRT-Forward TGTTCGGCTATGCCTCAATG	cyoBRT-Reverse CCGAAGAAGGAATTGACGCT	RT-qPCR	Lunak & Noel, 2015
16sRNA	16sRNART-Forward TGGAGTATGGAAGAGGTGAG	16sRNART-Reverse TCAGTAATGGACCAGTGAGC	RT-qPCR	Lunak & Noel, 2015

Table 1: Primers used in study.

A) *R. etli* CFN42

TAAGGCGCTTGCGGCAAGGCGCCTCAAGTCTGATCTTGATCAAACCTGCAGGCTTATCAAGTCCGTAAGACCGAAAT
AGTTCATCATCACGGCTCAAATGAAGCGCAGATGGAAAAAATTGCGTGATCCGGACGGTGATTTTCCCCTGCAAATG
CTGGCCTGACCGGCAGTTGCGGATATATTGGACTGAACATGCGCAGGGGCTAAAATCCTTCTATATTCCATCAGGAT
TTCAAAGCACTAGCGCCCGATGATTTTTGCCGCAGTGCGGCATGTTTGCTGCACTGCGACCTTCCGCCTCATGTCGC
TATCCGGTTCAGTGCTTTAGTCGCGGACAAAACGAAACAACAAGAGCTTAGAGACGTGCCAAAACTCATGAAGTTTT
CCCGCCTTCTATCCGTCTTGCCGCTGCTTTTCCTGGCAGGATGCAACATGGTGGTCATGGCGC

B) *S. meliloti* 1021

TGTCGTCGTGAATGCGCCGCGCGTCGCGCTCCATCGAAGAGGACGACGGTCCCAGGGACTGTTGCATTGCACTGCTC
ATCAAACGCCTCCCGCGCTCGCACGGGGGCTAAGAACATGCGGTGTTCCGCCGCACCCGCTCGTACTCTCAAGATGA
GAGCGCAAGGCTCCCTTGGAGGGATCTTGACCAATCAACCGTTCATATCAAGGGGAGAAACGCGAAATAATGCGTTT
CACGTCCTCACTGTCTGCAAGCGGCGGAAGATGCTGCGTCGCAAAACGACTGCGGCGGATTGCTGCAGTGCGACCAT
CGACCTCATACCTTTCCCATCCACTCTACTTTAGTCGCAGGGCCGTCGAAACAAAATAGAGCTCCGAGCCGTGCCAG
AACTGTTGAAGTTTTCCCGCCGCCTCGCCGTTTTGCCGCTTTTCCTCGTGATGGCGGGATGCGACATGGTG

C) *R. leguminosarum* bv *viciae* 3841

GTAGCCATGAAACGCCTCCCTCGTTGAGGCGCTTTCGGCAAGGCGCCTCAACCTGATCTTGATCAAACCTGCAGGCT
TATCAAGTCCGTAAGACGCAAATAGTTCATCATCACGGCTCAAAATGAGGCGTAGACGGAAAAAATAGCGTGATCCG
GAGCGCGATTTTTCCCGGGGACGCTGGCTTGGCCGGCCCTTGCGGATATATTGGAACAAAACATGCGCTGGCGAAAG
AATCCTTCTTGATTTCATCAGGATTCCAAAGCTGTAGTGCACCATCATTTGTGCCGCAGTGCGGCATGATTGCTGCA
CTGCGACCAAACACCTCATGTCGCTATCGGGTTCAGTGCGTTAGTCGCGGAACGAACGAAACAACAAGAGCTTAGAG
ACGTGCTAAAACTGGTGAAGTTTTCCCGCCTTCTATCCGTCTTGCCGCTGCTTTTCCTGGCAGGATGCAACATGGTG

D) *B. japonicum* USDA 110

GCGCGGGAACACGATCACCGAGGCGATCGCGTAGACGAAGAAGTCGAAGAATTCCGAGGTGCGGCCGATGATGACGC
CGATGGCGATCTCGCCGGGACTGGCCTGGTCGTGGCCGTGCTCGCCCGAGTGGAGGTCTGCCATTGCAGGGGTCTGT
GCCGTCGCCATTCGTGCGCCCTTAACGTCCTGAAAACCAAAGCCGACCGCCCGGCGGGGCGCCAGGCAATCCGGGCC
TGTCTGGCCCAACATTCCGCACTGCAACATTGGACAAATTGTCCAATGTCCGGATTGCTGCGCCGCAGCTACCCGTT
GGCCCCGCAAAGGAAACTCATTCTCAAAGGCTCGGCCCGTGTCCCGTCTCAAGATCCTGGCGCTGCTACCCTTGGCA

E)

Consensus	N**G**N**G**N**C**NNNN**G**NNN**C**
R. etli 1	T**G**C**G**G**C**AAGGC**G**CCT**C**
R. etli 2	T**G**C**G**G**C**ATGTTT**G**CTG**C**
R. etli 3	T**G**C**G**A**C**CTTCC**G**CCT**C**
S. meliloti 1	T**G**C**G**C**C**GCGC**G**TCG**C**
S. meliloti 2	T**G**C**G**G**C**GGATT**G**CTG**C**
S. meliloti 3	A**G**C**G**G**C**GGAA**G**ATG**C**
S. meliloti 4	T**G**C**G**A**C**CATCGACCT**C**
R. leguminosarum 1	T**G**C**G**G**C**ATGATT**G**CTG**C**
R. leguminosarum 2	T**G**C**G**A**C**CAAACACCT**C**
B. japonicum 1	T**G**C**G**G**C**CGATGATGA**C**
B. japonicum 2	T**G**C**G**C**C**CTTAACGTC**C**
B. japonicum 3	T**G**C**G**CCGCA**G**CTA**C**

Figure S1 Nucleotide sequences 5'of potential *cyoA* orfs in **(A)** *Rhizobium etli* CFN42 (nucleotides 40,430 – 39,982) **(B)** *S. meliloti* 1021 (nucleotides 1407913-1408369) **(C)** *R. leguminosarum* bv *viciae* (nucleotides 53,510-53,971) and **(D)** *Bradyrhizobium japonicum* USDA 110 (nucleotides 144990-145374) . Putative ActR DNA binding sites are underlined (__). A putative CRP-FNR anaerobox is dashed-underline (_ _ _). Possible ATG translation start sites are highlighted in black. **(E)** Listed potential ActR DNA binding sites. In bold are the conserved residues of the consensus ActR DNA binding sites from *B. japonicum* (Lindemann *et al*., 2007, Torres *et al*., 2014; Emmerich *et al*., 2000).

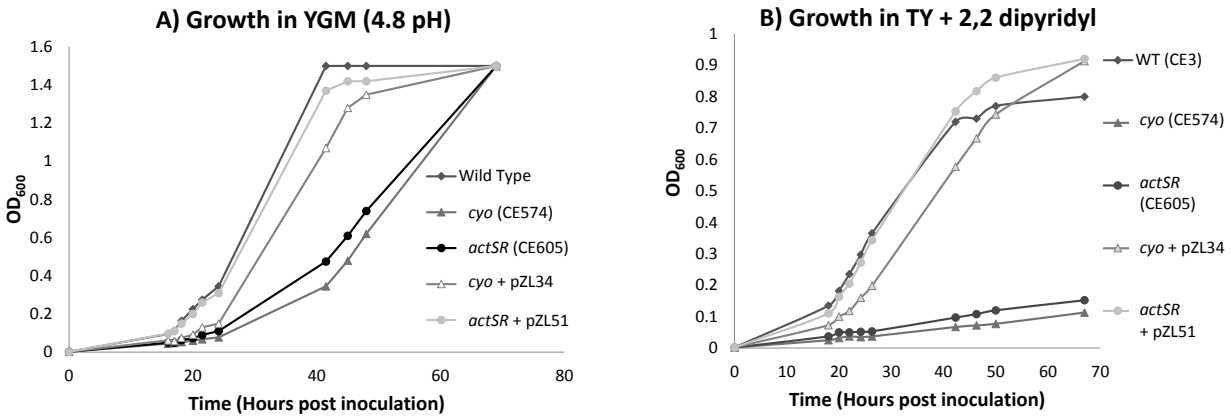

Figure S2: Complementation of *cyo* and *actSR* mutants at low pH (A) and low iron (B).

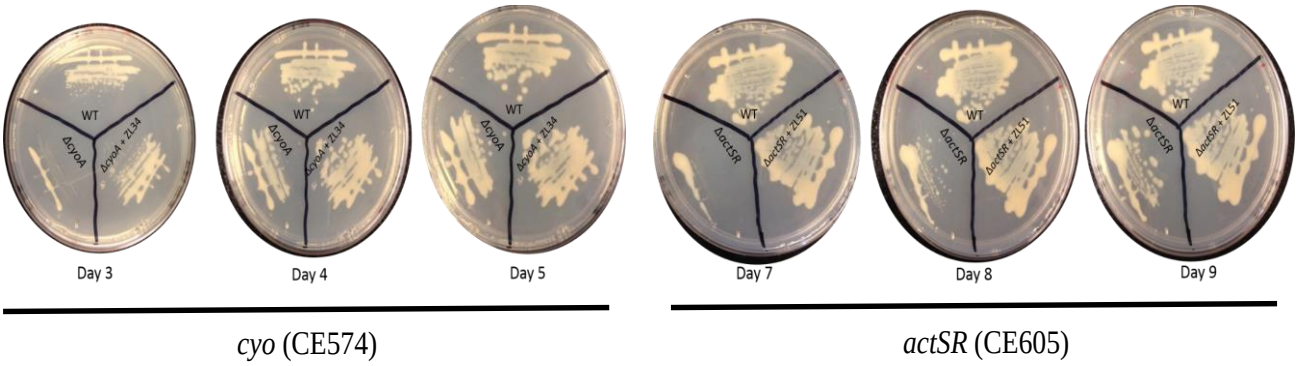

Figure S3: Growth on low pH YGM plates. Colonies were picked from TY plates and streaked on YGM agar buffered with MES at 4.8 pH. At least three different colonies for each strain were tested. Below the plates indicates the number of days the plate was incubated. The strain that was streaked is indicated in the triangle on the plates. The upper triangle is the wild type, lower left triangle is the mutant (*actSR* or *cyo*), and lower right is the mutant carrying the complemented plasmid (pZL34 or pZL51).