Expression of *Bradyrhizobium japonicum* cbb₃ terminal oxidase under denitrifying conditions is subjected to redox control

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**Abstract**

*Bradyrhizobium japonicum* utilizes cytochrome cbb₃ oxidase encoded by the fixNOQP operon to support microaerobic respiration under free-living and symbiotic conditions. It has been previously shown that, under denitrifying conditions, inactivation of the cycA gene encoding cytochrome c₅₅₀, the electron donor to the Cu-containing nitrite reductase, reduces cbb₃ expression. In order to establish the role of c₅₅₀ in electron transport to the cbb₃ oxidase, in this work, we have analyzed cbb₃ expression and activity in the cycA mutant grown under microaerobic or denitrifying conditions. Under denitrifying conditions, mutation of cycA had a negative effect on cytochrome c oxidase activity, heme c (FixP and FixO) and heme b cytochromes as well as expression of a fixP'–lacZ fusion. Similarly, cbb₃ oxidase was expressed very weakly in a napC mutant lacking the c-type cytochrome, which transfers electrons to the NapAB structural subunit of the periplasmic nitrate reductase. These results suggest that a change in the electron flow through the denitrification pathway may affect the cellular redox state, leading to alterations in cbb₃ expression. In fact, levels of fixP'–lacZ expression were largely dependent on the oxidized or reduced nature of the carbon source in the medium. Maximal expression observed in cells grown under denitrifying conditions with an oxidized carbon source required the regulatory protein RegR.

**Introduction**

The cbb₃-type oxidase is a heme-copper oxidase that has been purified from several bacteria including *Paracoccus denitrificans* (de Gier et al., 1996), *Rhodobacter sphaeroides* (García-Horsman et al., 1994), *Rhodobacter capsulatus* (Gray et al., 1994) and *Bradyrhizobium japonicum* (Preisig et al., 1996). Thus far, the enzyme from *B. japonicum* is the only cytochrome cbb₃ oxidase in which substrate affinity has been measured. The experimentally determined Kₘ for dioxygen for the enzyme from *B. japonicum* is in the order of 7 nM, which is consistent with its function as a high-affinity oxidase in nitrogen-fixing bacteria (Preisig et al., 1996).

Genes encoding the cbb₃ oxidase complex were isolated initially from rhizobial species and named fixNOQP for their role in symbiotic nitrogen fixation (Preisig et al., 1993; Mandon et al., 1994). Since then, orthologous genes have been identified in other gram-negative bacteria and called ccoNOQP (for a review, see Pitcher & Watmough, 2004). The cbb₃-type oxidase is made up of three to four subunits: subunit I is encoded by ccoN and is a membrane-integral b-type cytochrome with a high-spin heme-Cu₉ binuclear center and a low-spin heme. Subunits II and III are encoded by ccoO and ccoP, respectively, and are membrane-anchored c-type cytochromes. As shown in *R. sphaeroides* and *B. japonicum*, the ccoN, ccoO and ccoP gene products are essential for both the activity and the assembly of the cbb₃ oxidase. In contrast, ccoQ, which encodes subunit IV, is sometimes missing. ccoQ is not required either for assembly or for activity of the oxidase at low O₂ concentration (Zufferey et al., 1996; Oh & Kaplan, 1999), but may play a protective, stabilizing role under oxic conditions (Oh & Kaplan, 2002). CcoNOQP has been extensively studied in *R. sphaeroides*, where it has multiple roles. It functions not only as a terminal oxidase (García-Horsman et al., 1994) but also as a redox sensor in a signal transduction pathway controlling photosynthesis gene expression (Oh & Kaplan, 2002; Kim et al., 2007).

*Bradyrhizobium japonicum* is a gram-negative bacterium found in soil and associated symbiotically with soybean
plants. Like many other anaerobic facultative bacteria, *B. japonicum* adapts to different environmental O₂ concentrations by inducing multiple terminal oxidases with different affinities for O₂ (Delgado et al., 1998). Free-living bacteria growing microaerobically and nitrogen-fixing bacteroids use a cytochrome *cbb*3-type oxidase encoded by the *fixNOQP* operon (Preisig et al., 1993, 1996). Electron transfer to this high-affinity oxidase is via the cytochrome *bc₁* complex (Thöny-Meyer et al., 1989) (Fig. 1). During aerobic free-living growth, electron transport from the cytochrome *bc₁* complex to the low oxygen affinity oxidase cytochrome *aa₃* occurs via a transmembrane cytochrome *c* (CycM, Bott et al., 1991). In *B. japonicum*, there is a second cytochrome oxidase encoded by the *cycMNOP* operon (Bott et al., 1992) that also requires electron transport via the cytochrome *bc₁* complex. In addition to cytochrome *c* oxidases, there are quinol oxidases such as the microaerobic cytochrome *aa₃* complex (Thöny-Meyer et al., 1989) (Fig. 1). During aerobic free-living growth, electron transport from the cytochrome *bc₁* complex to the low oxygen affinity oxidase cytochrome *aa₃* occurs via a transmembrane cytochrome *c* (CycM, Bott et al., 1991). In *B. japonicum*, there is a second cytochrome oxidase encoded by the *cycMNOP* operon (Bott et al., 1992) that also requires electron transport via the cytochrome *bc₁* complex. In addition to cytochrome *c* oxidases, there are quinol oxidases such as the microaerobically expressed *bbs*3-type ubiquinol oxidase encoded by *cycWXYZ* (Surpin et al., 1996).

Oxygen concentration is the primary effector of *fixNOQP* expression in *B. japonicum*. Under O₂ limitation, the FixJ protein of the FixLJ two-component regulatory system is phosphorylated by the oxygen-inhibitable, heme-based sensor kinase FixL. The only known target of FixJ in *B. japonicum* is FixK₂, whose product, FixK₂, has been shown to activate genes involved in anaerobic or microaerobic energy metabolism, including the *fixNOQP* operon (Nellen-Anthamatten et al., 1998), and the denitrification genes (Bedmar et al., 2005). Very recently, transcriptome analyses have led to a comprehensive and expanded definition of the FixJ and FixK₂ regulons (Mesa et al., 2008).

In addition to oxygen respiration, *B. japonicum* is able to obtain energy and grow from nitrate reduction to N₂ through denitrification when cultured under oxygen-limiting conditions with nitrate as the terminal electron acceptor (Vairinhos et al., 1988). The complete denitrification pathway consists of the sequential reduction of nitrate (NO₃⁻) or nitrite (NO₂⁻) to dinitrogen (N₂), through the intermediates nitric oxide (NO) and nitrous oxide (N₂O) in a four-reaction process catalyzed by nitrate, nitrite, nitric oxide and nitrous oxide reductases, respectively. Reviews covering the physiology, biochemistry and molecular genetics of denitrification have been published elsewhere (Zumft, 1997; van Spanning et al., 2005, 2007). In *B. japonicum*, denitrification reactions depend on *napEDABC* (Delgado et al., 2003), *nirK* (Velasco et al., 2001), *norCBQD* (Mesa et al., 2002) and *nosRZDYFLX* (Velasco et al., 2004) genes (Fig. 1). Similar to many other denitrifiers, expression of denitrification genes in *B. japonicum* requires both oxygen limitation and the presence of nitrate or a derived N oxide (Bedmar et al., 2005). Maximal induction of transcription from the *nap*, *nir* and *nor* promoter regions depends on the FixLJ–FixK₂–NnrR regulatory cascade (Mesa et al., 2003; Robles et al., 2006).

Very recently, it has been demonstrated that *B. japonicum* cytochrome *cbb*3 encoded by cycA, is involved in the electron transfer to the Cu-containing nitrite reductase of *B. japonicum* (Bueno et al., 2008) (Fig. 1). It was also demonstrated that inactivation of *B. japonicum* cycA gene decreases the expression of *B. japonicum* FixP and FixO heme c-stained components of the *cbb*3 oxidase, during nitrate-dependent anaerobic growth (Bueno et al., 2008). To better understand the physiological role of the cytochrome *cbb*3 in electron transport to the terminal oxidase *cbb*3 in *B. japonicum*, in this work, we have analyzed *cbb*3 expression and cytochrome *c* oxidase activity in cells incubated under low-oxygen conditions without nitrate, where only the *cbb*3 pathway is induced, and with nitrate, where *cbb*3 and denitrification pathways are induced (Fig. 1). The effect of inactivation of...
other electron carriers from the denitrification pathway such as NapC or bc1 complex and the influence of different carbon substrates on the expression of a fixP̂−lacZ reporter fusion have also been studied. Our results indicate that, in addition to oxygen concentration, the intracellular redox state might be involved in *B. japonicum* fixNOQP regulation.

**Materials and methods**

**Bacterial strains and growth conditions**

*Bradyrhizobium japonicum* USDA110 (US Department of Agriculture, Beltsville, MD), 110spc4 (Regensburger & Hennecke, 1983) wild-type (WT) strains and *cycA* mutant derivatives were used in this study. *Bradyrhizobium japonicum* strain 3604 (Zufferey et al., 1996) is a WT strain containing a fixP−lacZ fusion. In this work, plasmid pRJ3603 (Zufferey et al., 1996) containing a fixP−lacZ translational fusion was integrated by homologous recombination into the chromosome of the *cycA, napC* and *regR* mutants resulting in strains 1406, 2300 and 2200, respectively. *Bradyrhizobium japonicum* strains were grown aerobically in liquid batch cultures containing peptone–salts–yeast extract (PSY) medium (Regensburger & Hennecke, 1983) at 28°C in yeast extract mannitol (YEM) medium (Vincent, 1983) at 28°C. Microaerobic batch cultures were kept at 28°C in yeast extract mannitol (YEM) medium (Vincent, 1974) with or without 10 mM KNO3 in completely filled, rubber-stoppered serum bottles or tubes. The influence of carbon substrates on fixP−lacZ expression was investigated by growing the cells under low-oxygen conditions with nitrate in a minimum medium (Bergersen, 1977), where glycerol was substituted as the carbon substrate with either 10 mM malate as the oxidized carbon substrate or butyrate as the reduced carbon source. Antibiotics were added to *B. japonicum* cultures at the following concentrations (μg/mL−1): gentamycin, 100; spectinomycin, 200; streptomycin, 200; kanamycin, 200; and tetracycline, 100. *Escherichia coli* strains were cultured in Luria–Bertani medium (Miller, 1972) at 37°C. *Escherichia coli* DH5α (Stratagene, Heidelberg) was used as host in standard cloning procedures and *E. coli* S17–1 (Simon et al., 1983) served as the donor in conjugal plasmid transfer. The following antibiotics were used (μg/mL−1): spectinomycin, 20; kanamycin, 25; and tetracycline, 10.

**UV-visible spectroscopy**

*Bradyrhizobium japonicum* strains grown aerobically in PSY medium were centrifuged at 8000g for 10 min. The cells were then washed twice with YEM medium and incubated anaerobically with and without 10 mM KNO3. After 2 days' incubation, membrane fractions were isolated as described elsewhere (Delgado et al., 2003). After isolation, membranes were solubilized in 50 mM Tris–HCl, pH 7.5, 1 mM phenylmethylsulfonyl fluoride and 1% dodecyl maltoside (Sigma), and they were subjected to ultracentrifugation at 140 000g for 1 h at 4°C. The solubilized oxidase complex remained in the supernatant. Solubilized membrane proteins were reduced with addition of excess sodium dithionite and oxidized with ferricyanide (10 μM). Then dithionite-reduced minus ferricyanide-oxidized spectra were recorded using a Hitachi U-3310 spectrophotometer linked to a circulating BC-10 water bath (Fisher Scientific).

**Cytochrome c oxidase activity**

Cytochrome c oxidase activity was measured by monitoring the oxidation of cytochrome c by cytochrome c oxidase as a function of the decrease in A550 nm. Specifically, 10 μL of membrane fraction (3.5 mg mL−1) isolated as described elsewhere (Delgado et al., 2003) was combined with 940 μL of assay buffer (10 mM Tris–HCl at pH 7.0, 120 mM KCl). The reaction was initiated by the addition of 50 μL of reduced horse heart cytochrome c (Sigma), and the decrease in A550 nm was measured continuously for 1 min using a spectrophotometer. Reduced cytochrome c was prepared by making a solution at 218 μM in water and adding dithionite to saturation. Traces of dithionite in suspension after reduction were eliminated by filtering the solution with a PD-10 column. To assure that the level of reduction is adequate, the absorbance of a 20-fold-diluted stock was measured at both 550 and 565 nm. A ratio of A550 nm to A565 nm should be between 10 and 20 and indicates that the substrate is sufficiently reduced. Activity was quantified by the following calculation:

\[
U/\text{mL} = \left(\frac{A/\text{min}}{\text{reaction volume (mL)/volume of sample (mL)}}\right) \times 21.84
\]

where 21.84 = eMm between ferrocytochrome c and ferricytochrome c at 550 nm (Sigma protocol in reference to product code CYTO-OCX1).

**Oxygen consumption**

Cytochrome c oxidase activity was also measured in whole cells by monitoring N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD)-dependent oxygen consumption with an Apollo-4000 oxygen electrode (WPI). Cells were incubated in 2 mL of 25 mM phosphate buffer, pH 7.5, at 28°C and ascorbate (1.5 mM) and TMPD (30 μM) were added as electron donors. Succinate-dependent oxidase activity was analyzed using succinate (60 mM) instead of ascorbate–TMPD as the physiological electron donor. The time taken to consume all of the oxygen present in the system was used to calculate the rate of oxygen consumption.
Heme-c protein analysis

Cells of *B. japonicum* grown aerobically in 500 mL PSY medium were harvested by centrifugation as above, washed twice with YEM, resuspended in 1 L of the same medium supplemented or not with 10 mM KNO₃ and finally incubated under low-oxygen conditions for 2 days. Membrane preparations were performed as described earlier (Delgado *et al.*, 2003). Membrane protein aliquots (30 µg) were diluted in sample buffer [124 mM Tris-HCl, pH 7.0, 20% glycerol, 4.6% sodium dodecyl sulfate (SDS) and 50 mM 2-mercaptoethanol], and incubated at room temperature for 10 min. Membrane proteins were separated at 4°C in SDS–12% polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane and stained for heme-dependent peroxidase activity as described previously (Vargas *et al.*, 1993) using the chemiluminiscence detection kit ‘Super Signal’ (Pierce).

**β-Galactosidase assays**

Cells were grown aerobically in PSY medium, collected by centrifugation at 8000 g for 10 min at 4°C, washed twice with YEM and finally incubated microaerobically in the same medium supplemented or not with KNO₃. Cultures with an initial OD 600 nm of about 0.2 were incubated for 2 days. Activity was determined with permeabilized cells from at least three independently grown cultures as described previously (Miller, 1972). Cells removed from stoppered flasks were not kept microaerobic, but were used immediately for assays.

**Analytical methods**

The protein concentration was estimated using the Bio-Rad assay (Bio-Rad Laboratories, Richmond, CA) with a standard curve of varying bovine serum albumin concentrations.

**Results**

**Effect of cycA mutation on cytochrome c oxidase activity**

Cytochrome c-dependent oxygen consumption was measured in the WT *B. japonicum* strain 110spc4 and the cycA-deficient strain 3447 using ascorbate-reduced TMPD as a nonphysiological electron donor (Fig. 2a). The cycA mutant showed levels of TMPD oxidase activity similar to those of the WT strain when cells were incubated under microaerobic conditions without nitrate (Fig. 2a). However, when cells were incubated under microaerobic conditions with nitrate, oxygen consumption rates of cycA cells were approximately twofold lower than those of WT cells (Fig. 2a). To determine the contribution of the cytochrome cbb₃ oxidase to the total oxidase activity of WT cells, a fixN mutant was also analyzed. The oxidase activity measured was around 10% and 30% of that of the WT cells in the presence and absence of nitrate, respectively (Fig. 2a). A similar pattern of results was obtained when the cytochrome c oxidase activity was measured using reduced horse heart cytochrome c (Fig. 2b) or succinate (not shown) as electron donors.

**Effect of cycA mutation on cytochrome cbb₃ synthesis**

Synthesis of cytochrome cbb₃ can be assessed by monitoring the 32-kDa FixP protein, which is a c-type cytochrome associated with the cytochrome cbb₃ complex (Preisig *et al.*, 1993). As a control, we established that the 32-kDa heme-staining protein from membranes from *Bradyrhizobium japonicum* 110spc4 (black bar), cycA 3447 (grey bar) and fixN 3613 (white bar) strains. Cells were incubated microaerobically in YE medium with or without 10 mM KNO₃ for 2 days. Data are means ± SE from at least two different cultures, assayed in triplicate. Cytochrome c oxidase activity (b) is expressed in U mg⁻¹ protein, where 1 U is defined as the amount required to oxidize 1.0 µmol ferrocytochrome c min⁻¹ at pH 7.0 at 25°C. Each lane contains about 25 µg membrane proteins. Apparent masses of the proteins (kDa) are shown at the left margin.

![Fig. 2.](https://academic.oup.com/femsle/article-abstract/298/1/20/539408)
in the fixN mutant, a heme-stained band remains at 28 kDa, and hence, for a clear indication of the synthesis of cytochrome cbb₃, we focused on the 32-kDa FixP band. Levels of the FixP polypeptide in cells of the cycA mutant incubated under microaerobic conditions without nitrate were very similar to those detected in WT cells (Fig. 2c), but this was not the case in cells grown under microaerobic conditions with nitrate, where a decreased level of the FixP protein was observed in cycA cells (Fig. 2c).

In addition to the FixP and FixO membrane-anchored c-type cytochromes, B. japonicum cytochrome cbb₃ oxidase contains an integral-membrane b-type cytochrome corresponding to FixN (Preisig et al., 1993; Zufferey et al., 1996). Thus, the presence of heme c and heme b in the cycA mutant strain 3447 was also investigated spectroscopically. Absorption peaks characteristic of cytochrome b at 558 nm and for cytochrome c at 552 nm were detected in dithionite-reduced minus ferricyanide-oxidized difference spectra of membranes from WT cells incubated under microaerobic conditions either in the absence or in the presence of nitrate (Fig. 3a and b). In membranes prepared from cells incubated microaerobically without nitrate, no differences in cytochromes c and b composition were observed between the WT and cycA mutant strains (Fig. 3b), but membranes prepared from cycA cells incubated microaerobically with nitrate showed a significant decrease in cytochrome b and c contents compared with the parental strain (Fig. 3a). These results support the oxidase measurements and FixP heme-staining results, and, together, all three lines of evidence demonstrate that the absence of cytochrome c₅₅₀ has a strong influence on cytochrome c oxidase activity and cbb₃ production under denitrifying conditions, but not under microaerobic conditions.

**Effect of cycA and napC mutations on fixP expression**

To explore a possible effect of cycA deletion on the expression of the genes encoding cytochrome cbb₃, a chromosomal fixP–lacz reporter fusion was used. β-Galactosidase activity was comparable in WT and cycA cells grown microaerobically without nitrate, but the activity was approximately fivefold lower in cells grown microaerobically with nitrate (Fig. 4a). This pattern was similar to that observed for oxidase activity, FixP production and levels of c- and b-type cytochromes. The periplasmic location of cytochrome c₅₅₀ rules out this cytochrome as a direct transcriptional regulator of fixP, and so the results suggest that the absence of cytochrome c₅₅₀ in the cycA mutant leads to a change in the metabolic state of the cells under denitrifying conditions that, in turn, lead to a modulation of fixP expression. The most likely scenario is that this is a modulation in response to perturbation of the respiratory electron transport system.

It has been previously shown (Bueno et al., 2008) that cytochrome c₅₅₀ is the electron mediator protein between cytochrome bc₁ complex and the Cu-containing respiratory nitrite reductase in B. japonicum. It is possible that changes in the electron transfer pathway associated with the denitrification process might ultimately control cbb₃ expression. If we assume that inactivation of the c₅₅₀ electron carrier of the denitrification pathway generates a change in the redox state of the cell, we can then ask whether inactivation of other electron carriers of this pathway may have the same effect on cbb₃ expression. One such carrier is NapC, which transfers electrons to the NapAB complex of the periplasmic nitrate reductase (Delgado et al., 2003). To check this hypothesis, we examined cbb₃ expression in a B. japonicum strain mutated in the napC gene. After 2 days' incubation under microaerobic conditions with nitrate, and similarly to cycA, cells of the napC mutant, containing the fixP–lacz fusion, showed about fivefold decreased levels of β-galactosidase activity compared with those detected in WT cells containing the fixP–lacz fusion (Fig. 4a). Accordingly, very low levels of heme-c-stained FixP protein were observed in napC cells after incubation under microaerobic conditions with nitrate compared with those found in the WT strain 110spc4 (Fig. 4b).

**The effect of carbon substrate and electron transport inhibition on fixP expression**

To further explore the possibility of redox-dependent regulation of B. japonicum cbb₃ oxidase, we analyzed the effect of reduced and oxidized carbon substrates on fixP–lacz

![Image](https://academic.oup.com/femsle/article-abstract/298/1/20/539408/120539408?w=420&h=273&fit=clip&zoom=75)

Fig. 3. Visible absorption difference spectra (dithionite-reduced minus air oxidized). Membrane proteins (3 mg mL⁻¹) solubilized with dodecyl maltoside were isolated from WT Bradyrhizobium japonicum 110spc4 and cycA mutant strain 3447. Cells were incubated microaerobically in YEM medium (a) with or (b) without 10 mM KNO₃ for 2 days.
expression. Cells were grown microaerobically with nitrate as the electron acceptor in a minimum medium containing a reduced carbon source such as butyrate, or an oxidized carbon source such as malate. After 2 days’ growth, β-galactosidase activity observed in WT cells grown on butyrate was approximately threefold lower than that observed when cells were cultured on malate (Fig. 5). It is known that assimilation of reduced carbon substrates that generate more reducing equivalents than assimilation of oxidized carbon sources increases the reduction state of the UQ pool (Richardson & Ferguson, 1992). Results shown in Fig. 4 suggest that a change in the redox status of the UQ/UQH2 pool might be involved in the regulation of cbb3 expression.

Regulation of gene expression mediated by the oxidation state of the carbon substrate has been reported in Paracoccus pantotrophus (Richardson & Ferguson, 1992; Sears et al., 2000; Ellington et al., 2002, 2006) and P. denitrificans (Sears et al., 1997). In both species, expression of the nap operon, encoding the periplasmic nitrate reductase, is maximal when cells are grown aerobically with a reduced carbon source such as butyrate. The different response to carbon substrates between Paracoccus nap and B. japonicum fixNOQP genes is due to the different role of these genes. While Nap is involved in the dissipation of excess redox energy during oxidative metabolism of reduced carbon substrates, cytochrome c oxidase cbb3 supports microaerobic respiration.

We also examined the effect of the cytochrome bc1 complex-specific inhibitor myxothiazol upon cbb3 expression in the WT strain grown microaerobically in minimum medium with malate as the carbon source and nitrate as the electron acceptor. As shown in Fig. 5, fixP– lacZ expression was about threefold lower in the presence of 45 μM myxothiazol than that observed in the absence of myxothiazol. Taken together, these results suggest that interruption of the electron flow through the denitrification pathway by inactivation of the electron carriers located downstream of the UQ, such as NapC, c550 or bc1 complex, or the assimilation of highly reduced carbon substrates might increase the redox state of the UQ/UQH2 pool and consequently prevent maximal expression of cbb3. Supporting our hypothesis, it has been previously demonstrated in P. denitrificans (Otten et al., 1999, 2001) that a change of electron distribution through the respiratory network, resulting from elimination of one or more oxidase genes, changes intracellular signals that affect the activity of the cco promoter, which controls the expression of the cbb3-type cytochrome c oxidase.
In *B. japonicum*, it is rather possible that perception and transduction of redox variations may be recognized by a regulatory protein controlling *cbb*3 expression, in order to decrease cell metabolism as a response to the high electron charge. The primary environmental factor that governs the expression of the *fixNOQP* genes in *B. japonicum* is oxygen. In this work, we propose that oxygen is not the only signal involved in the regulation of *fixNOQP* genes. Also, intracellular signals derived from changes in the electron distribution, which affect the redox state of the respiratory chain associated with the denitrification process, may affect the expression of cytochrome *cbb*3 oxidase. These signals may enable *B. japonicum* to adapt its respiratory network in such a way that the network functions optimally at each given growth condition.

**The effect of regR disruption on fixP expression**

In *B. japonicum*, RegSR activates the transcription of the nitrogen fixation regulatory gene *nifA*, thus forming a RegSR-NiFA cascade, which is part of a complex regulatory network for gene regulation in response to changing oxygen concentrations. However, the nature of the signal sensed by RegS remains to be identified (Bauer et al., 1998). In order to study the involvement of *B. japonicum* RegR in the redox-dependent regulation of *fixNOQP* genes, in this work, we have analyzed the expression of the *fixP*-lacZ fusion in a *B. japonicum* regR mutant strain 2426 (Fig. 5). Only when cells were grown under denitrifying conditions with malate as the carbon source there was a significant decrease in β-galactosidase activity in the regR mutant compared with the expression levels detected in the WT strain (Fig. 5). No differences were found when cells were grown under reducing conditions. These observations suggest that *B. japonicum* *fixNOQP* operon might be under the control of RegR.

**Discussion**

The results show that disruption of *cycA*, encoding the periplasmic soluble cytochrome *c550*, affects expression of the *fixNOQP* operon encoding the cytochrome *cbb*3 oxidase, but only during growth under denitrifying conditions. It is thus possible that lack of cytochrome *c550* leads to an increase in the reduced state of the electron transport chain, and this serves as a signal for modulation of *fixNOQP* expression. This view is supported by the observations that lack of NapC, which participates in electron transport to the periplasmic nitrate reductases also leads to a modulation of *fixNOQP* expression. The ubiquinol/ubiquinone (Q-pool) is common to the cytochrome *c550* and NapC electron transport systems and is thus a likely respiratory branchpoint involved in redox signaling. This view is further supported by the effect of myxothiazol on *fixP* expression, because this inhibits the cytochrome *bc*1 complex, which will also lead to a build up of electrons in the Q-pool. Finally, growth on reduced substrates, such as butyrate, will also lead to an increased reduction of the Q-pool and we show here that this also modulates *fixP* expression. Thus, taken together, the data strongly suggest that *fixP* expression is under redox control via a system that is responsive to the redox state of the Q-pool.

Orthologs of redox-responsive proteins, members of the family of two-component regulatory systems, are present in a large number of Proteobacteria. These proteins are named RegSR in *B. japonicum*, RegBA in *R. capsulatus*, *Rhodovulum sulfidophilum* and *Roseobacter denitrificans*, PrrBA in *R. sphaeroides* and ActSR in *Sinorhizobium meliloti* (Emmerich et al., 2000) and Agrobacterium tumefaciens (Back et al., 2008). In *Rhodobacter* species, the RegBA/PrrBA systems activate expression of genes for carbon dioxide fixation and nitrogen fixation, processes that utilize reducing equivalents, and repress expression of genes for hydrogen oxidation, a process that generates reducing equivalents (Elsen et al., 2004). In *Rhodopseudomonas palustris*, RegSR represses uptake hydrogenase expression in response to intracellular redox status (Rey et al., 2006). Similarly, RegA of *R. capsulatus* has been demonstrated to affect the expression of the *cbb*3 and cytochrome *bd*-type terminal respiratory oxidases (Swem et al., 2001). In *Pseudomonas aeruginosa*, RoxR, a response regulator related to *R. sphaeroides* PrrA, activates expression of the cyanide-insensitive terminal oxidase (Comolli & Donohue, 2002). It has recently been discovered that RegB from *R. capsulatus* (Swem et al., 2005, 2006) or the ArcBA system in *E. coli* (Malpica et al., 2006) monitors the oxidation/reduction state of the UQ pool as an input signal to control kinase activity. By analogy with the well-elaborated sensing mechanisms of the orthologous two-component RegBA or the ArcBA systems, it seems attractive to speculate that the redox state of the membrane-localized Q-pool is also an important cue for *B. japonicum* RegSR.

Whole-genome transcription-profiling analyses have demonstrated that expression of almost 250 genes is dependent on RegR, a result that underscores the important contribution of RegR to oxygen- or redox-regulated gene expression in *B. japonicum* (Lindemann et al., 2007). Although, in these studies, *fixNOQP* genes were not identified as RegR targets under free-living oxic and microoxic conditions, expression of *fixNOQP* was RegR dependent at 13 days postinoculation during symbiosis (Lindemann et al., 2007). It might be possible that, as shown in this work, growth conditions producing redox potential variations in the cells are necessary to demonstrate the in vivo involvement of RegR on *cbb*3 expression under free-living conditions. Whether or not *fixNOQP* genes are direct or indirect targets of RegR is under investigation.
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References


Miller JH (1972) _Experiments in Molecular Genetics_. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.


