Doctoral Thesis

The cbb3- and bd-type oxidases of the soybean symbiont Bradyrhizobium japonicum

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The \textit{cbb}_3- and \textit{bd}-type oxidases of the soybean symbiont

\textit{Bradyrhizobium japonicum}

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Abstract

The present work describes the analysis of biochemical properties of the *Bradyrhizobium japonicum* cbb$\text{\textsubscript{3}}$-type oxidase and the discovery of a gene region, called the *cyd* locus, which encodes a *bd*-type quinol oxidase. The *cbb$_3$*-type terminal oxidase supports microaerobic respiration and is essential for enzymatic N$_2$ fixation in endosymbiosis with the soybean *Glycine max*. The *cbb$_3$*-type oxidase, encoded by the *fixNOQP* genes, is a heme-copper oxidase that consists of four subunits. Three of these subunits are redox active. The largest subunit is a di-heme cytochrome *b* (FixN), with low- and high-spin B hemes and the Cu$_B$ centre. It is a classical membrane protein with 12 transmembrane helices. Multiple alignments show a 20% identity to subunit I of other heme-copper oxidases. FixO and FixP are membrane-anchored. FixO is a mono-heme c-type cytochrome, and FixP is a di-heme c-type cytochrome. These two subunits share no similarity with the subunits II and III of the mitochondrial or bacterial *aa$_3$*-type oxidases. The smallest subunit, FixQ, is also membrane-anchored and forms part of the complex. In *B. japonicum*, no role was found for this subunit.

We investigated the proton-pumping ability of the *cbb$_3$*-type oxidase. For that purpose, the purified enzyme complex was re-integrated into lipid vesicles (reconstitution). The detergent, which holds the membrane protein in solution, was exchanged by phospholipids. The *cbb$_3$*-type oxidase of *B. japonicum* was solubilized and purified in the presence of the non-ionic detergent laurylmaltoside. This detergent was hardly dialyzable, and therefore the method of co-dialysis was used for successful reconstitution. In our case, co-dialysis was performed with Na-cholate, which is a dialyzable anionic detergent.

The proton-pumping assay was performed with a pre-steady-state pulse experiment in a vessel with a sensitive pH probe. The proton-pump efficiency for the *cbb$_3$* oxidase was determined to be between 0.2-0.5 H$^+$/e$, whereas the H$_2$O formation occurred with 1H$^+$/e$, as expected. This efficiency concurs with estimates that were obtained for *cbb$_3$*-type oxidases from other bacteria (Chapter II).

We wondered how the electrons might flow through the *cbb$_3$*-type oxidase, as electrons are transferred passively from a centre with lower midpoint potential to higher potential. Especially, it was of interest to see which subunit (FixO, FixP) might be the acceptor for the electrons coming from reduced cytochrome *c*. The midpoint potentials for the c-type cytochrome subunits were determined to be $-90$ mV, $+90$ mV and $+330$ mV, and...
Abstract

tentatively assigned to the c-type cytochromes FixP (-90mV and +90mV) and FixO (+330mV). The redox potentials for the b-type cytochrome in the oxidase were +380mV for the low-spin and -60mV for the high-spin heme B. A potential route of electrons is suggested that runs from FixP to FixO and finally to FixN (Chapter III).

A heterologous expression system was developed to study the individual c-type cytochrome subunits of the cbb3 oxidase (FixO and FixP). Escherichia coli was used as the host for expression. Since c-type cytochromes are localized without exception in the periplasm, they are prone to degradation by proteases and dependent on a translocation and maturation system. The constitutive expression of the ccmABCDEFGH (cytochrome c maturation complex) genes of E. coli and the utilization of a degP-mutant E. coli strain led to the maturation of 1.4 mg and 0.4 mg FixOsol and FixPsol, respectively, per liter culture. Carbon monoxide reactivity was assigned to FixPsol (Chapter IV).

The genes coding for the first and second subunit of a bd-type quinol oxidase (cydAB) were discovered incidentally (Chapter V). Upstream of cydA, a gene called mqo (malate:quinone oxidoreductase) was found. A deletion-insertion mutant of cydA was found to be unaffected both in growth and in symbiosis in the conditions tested. A very low expression of a cydA'-lacZ gene fusion was measured. Heterologous expression of the B. japonicum cydAB genes in an E. coli strain that was depleted in its own three terminal oxidases, revealed the spectrophotometric features of a bd-type oxidase. Microaerobic growth of this E. coli strain, when complemented with cydAB of B. japonicum, was stimulated. This result underlined the functionality of cydAB in E. coli.

However, the benefit of cydAB for the B. japonicum could not be demonstrated. With the discovery of cytochrome bd, the respiratory chain of B. japonicum was extended by one quinol branch to a total of five terminal oxidases, similarly as in Azorhizobium caulinodans.
Kurzfassung


Die Protonenpumpaktivität wurde mit einem sogenannten Vor-Gleichgewicht-Puls-Experiment in einem Glasbehälter mit einer sensitiven pH-Sonde detektiert. Die Protonenpumpaktivität wurde mit 0.2-0.5 H^+/e⁻ bestimmt. Der Wasserbildungsquotient war mit 1H^+/e⁻ gleich gross wie der Quotient anderer Häm-Kupfer Oxidasen. Die Effizienz entspricht den Bestimmungen, welche mit cbb^−-Typ Oxidasen aus anderen Bakterien gemacht wurden (Kapitel II).
Kurzfassung

Weil die Elektronen passiv von einem Zentrum mit tieferem Mittelpunktspotential zu Zentren mit höheren Potentialen fließen, interessierten wir uns für die Mittelpunktspotentiale, welche den Elektronenfluss durch die Oxidase beschreiben könnten. Im speziellen waren wir daran interessiert, welche Untereinheit (FixO oder FixP) der Elektronenakzeptor vom reduziertem Cytochrom c ist. Die Mittelpunktspotentiale für die c-Typ Cytochrome wurden bestimmt, und sie sind mit $-90\text{mV}$, $+90\text{mV}$ und $+330\text{mV}$ dem FixP ($-90\text{mV}$ und $+90\text{mV}$) und dem FixO ($+330\text{mV}$) zugeordnet worden. Die Mittelpunktspotentiale für das b-Typ Cytochrom wurden mit $+380\text{mV}$ dem "low-spin" Häm B und $-60\text{mV}$ dem "high-spin" Häm B zugeordnet. Daraus ergab sich ein möglicher Elektronentransferweg über FixP zu FixO und schliesslich zu FixN (Kapitel III).

Ein Expressionssystem zur Untersuchung für c-Typ Cytochrome (FixO und FixP) der cbb$_3$-Typ Oxidase wurde entwickelt. *Escherichia coli* wurde als Expressionsorganismus verwendet. Weil c-Typ Cytochrome ausschliesslich im Periplasma vorkommen, sind sie den lokalen Proteasen ausgesetzt und ihre Synthese ist vom Translokations- und Cytochrom-c-Reifungsapparat abhängig. Die konstitutive Expression der Cytochrom c Reifungsgene *ccmABCDEFGH* aus *E. coli* und der Gebrauch einer degP-Mutante Stammes führte zur Reifung von 1.4 mg FixO löslich beziehungsweise 0.4 mg FixP löslich pro Liter Kultur. Reaktivität mit Kohlenmonoxid konnte für FixP löslich zugewiesen werden (Kapitel IV).

CHAPTER I

Introduction
I. 1. Aerobic respiration in mitochondria and prokaryotes

Energy-rich substrates are oxidized during respiration. In general, the term respiration is used for the reduction of an exogenous electron acceptor, preferably O\textsubscript{2}, whereas the electrons come mainly from reduced C sources. These substrates are first oxidized 'step-by-step' during glycolysis, in the Krebs cycle or in fatty acid metabolism. At the same time the electrons are transferred to the universal electron carrier, such as NAD\textsuperscript{+}, during these oxidation processes. This reduced mediator NADH\textsubscript{2} then transports the electrons to the respiratory chain. The substrate becomes eventually end-oxidized to CO\textsubscript{2}. Specialized enzyme complexes catalyze the entrance into the respiratory chain. The electrons, which are on NADH\textsubscript{2} with the redox potential of -320 mV, are passed through the respiratory complexes towards O\textsubscript{2}, with a redox potential of +820 mV. O\textsubscript{2} finally becomes reduced to H\textsubscript{2}O. Apart from the transfer of electrons, protons are translocated through the respiratory complexes. This process leads to the generation of an electrochemical proton gradient, which in turn is used by the ATP synthase for the generation of ATP (Saraste, 1999).

Fig. 1. Mitochondrial respiration in the eukaryotic cell. The abbreviations im and im stand for intermembrane space and innermembrane, respectively. The letter p and n stand for positive and negative side of the im, respectively. The four complexes are depicted along with their inhibitors. The entrance of electrons occurs in complexes I and II. Whereas complexes I, III and IV are proton pumps, complex II is not. The ATP synthase, also called complex V, uses the electrochemical proton gradient, which is formed by proton-pumping complexes.

In the following the different enzyme complexes are briefly described regarding their cofactors and enzymatic functions.
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The electron entrance depends upon which carrier transports it to complex I or II. In the case of NADH$_2$ they enter complex I (Walker, 1992; Weiss et al., 1991). This complex is called NADH:UQ oxidoreductase. It is a large enzyme complex (880 kDa) consisting of at least 34 subunits in mitochondria. This complex harbors several cofactors including FMN and Fe-S centres. The two electrons of NADH$_2$ are transferred to FMN, which in turn becomes FMNH$_2$. The electrons pass through the Fe-S centres and are finally passed to UQ (ubiquinone) that becomes UQH$_2$ (ubiquinol), while at the same time complex I pumps protons to the intermembranous space. Ubiquinone and -quinol, which are strongly hydrophobic molecules, transfer the electrons through the membrane bilayer to complex III. Another entry site for electrons is complex II, which is enzymatically a FADH:UQ oxidoreductase. This complex consists of 4 subunits, and it has a molecular mass of 140 kDa. The cofactors are Fe-S centres and FAD$^+$. This enzyme complex attaches the Krebs cycle to a membranous process. The substrate is succinate, which is further oxidized to fumarate. Due to this enzymatic reaction, complex II is also called succinate:fumarate oxidoreductase. This complex does not pump protons during the passage of electrons. The same ubiquitous molecule as by complex I, i.e. ubiquinone, becomes reduced (ubiquinol) and then delivers the electrons to complex III.

Complex III is also called the ubiquinol:cytochrome c oxidoreductase. The crystal structure of the bovine heart $bc_1$ complex has been solved. It shows that the complex consists of 11 subunits. Three of them are redox active subunits. The complex has a molecular mass of 240 kDa (monomer). The crystal structure further demonstrates a dimeric structure of this complex (Iwata et al., 1998; Trumpower, 1990a). The largest subunit harbors two b-type cytochromes, which are distinguished by the subscripts H and L. These letters refer to a high- and low potential b-type cytochrome ($b_H$ and $b_L$). Another subunit carries an Fe-S-centre. The third subunit of this enzyme complex is a membrane-bound cytochrome c, which is called $c_1$. The electrons from ubiquinol enter the complex through the Fe-S-centre for bifurcation. They are distributed in two directions: one goes directly to the $c_1$ centre and then to cytochrome c, which transports it to complex IV (see later). This oxidation of ubiquinol leads to a semi quinone anion (Q$^\cdot$). This moiety passes its electron to $b_L$, and becomes ubiquinone. The electron subsequently passes to $b_H$. The reduced $b_H$ centre transfers its electron to a bound ubiquinone, which becomes reduced by one electron and a new semi quinone anion is
Chapter I

built. At this stage a new ubiquinol enters the cycle at the Fe-S centre and the oxidation procedure happens once again. However, this time the reduced \( b_{11} \) gives its electron to the semi quinone anion (\( Q\bullet^- \)) to complete the cycle. This cycle of electrons and the simultaneous translocation of protons is called the Q-cycle (Trumpower, 1990b). The Q-cycle allows changing from two-electron carriers (\( QH_2, NADH_2 \) and \( FADH_2 \)) to a one-electron carrier, like cytochrome \( c \). Cytochrome \( c \) is a soluble protein in the mitochondrial intermembrane space that transports the electron to the terminal cytochrome \( c \) oxidase, which is also called complex IV.

Mitochondrial complex IV consists of 13 subunits and harbors two \( a \)-type cytochromes and the \( Cu_A \) and \( Cu_B \) atoms as cofactors (Tsukihara et al., 1995; Tsukihara et al., 1996; Tsukihara and Yoshikawa, 1998). Genes that are localized in the mitochondrial genome encode 3 of the 13 subunits. These three subunits SUI, SUII and SUIII are also used for diagnostic division and comparison of the different types of prokaryotic terminal heme-copper oxidases (Garcia-Horsman et al., 1994a). Genes from the nucleus of the eukaryotic cell encode the remaining 10 subunits. The mitochondrial terminal oxidase is considered to be the classical respiratory oxidase and is called the \( aa_3 \) type oxidase corresponding to heme types. The electrons are transferred to \( O_2 \) in the binuclear centre that consists of a heme \( A \) and a \( Cu_B \) (\( a_3-Cu_B \)); \( O_2 \) becomes reduced to \( H_2O \), and protons are simultaneously pumped to the intermembranous space (Babcock and Wikström, 1992).

The proton gradient, which is generated by passing electrons through each complex (complexes I, III and IV), is used by the ATP synthase to synthesize ATP. This energy is used for many different cellular processes such as biosyntheses, flagellar rotation and active transports. The entire step-by-step oxidation of a carbon substrate all the way down to ATP synthesis is called oxidative phosphorylation (Saraste, 1990). In the following, the respiratory chains will be drawn in a simpler way. The different membrane complexes will be depicted as boxes, like in Figure 2 on page 10. The nomenclature that was used for the mitochondrial respiration will be also used for prokaryotic respiratory chains.

Figure 2 on page 10 summarizes a general respiratory chain often occurring in a prokaryotic organism as it is exemplified in \textit{Paracoccus denitrificans}. In contrast to the
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mitochondrial respiratory chain, the prokaryotic respiratory chains are branched, either at the entry site of electrons into the quinone pool, or at the site where the transfer of electrons to O₂ occurs. The terminal oxidases are divided into two classes. One class, which becomes reduced by electrons from cytochromes, are cytochrome c oxidases (class I). The other class (class II) of oxidases is reduced directly by ubiquinol. Thus, they are ubiquinol oxidases. In Table 1, bacterial oxidases of classes I and II along with their heme cofactors are listed (Thöny-Meyer, 1997). Based on the sequence similarity to subunit I of the mitochondrial terminal oxidase, further division might be possible in addition to the organization into classes I and II. The presence of a subunit that is homologous to subunit I of the mitochondrial oxidase, and the presence of a unique bimetallic centre (e.g. Cu₅₋₆), allows an assignment of the oxidases that exhibit these characteristics into the superfamily of heme-copper oxidases. Especially the six strictly conserved histidines that build the ligands to the cofactors heme A, heme A₃ and Cu₅ are indicative of a heme-copper oxidase (Garcia-Horsman et al., 1994a).

Table 1. Different terminal oxidases of class I and II. The italic letters represent the heme types of the oxidases. Subscript 3 indicates the heme that is in the binuclear centre (historical reasons).

<table>
<thead>
<tr>
<th>Class I</th>
<th>Class II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome c oxidases</td>
<td>Ubiquinol oxidases</td>
</tr>
<tr>
<td>aa₃</td>
<td>ba₃</td>
</tr>
<tr>
<td>ba₃</td>
<td>bb₃</td>
</tr>
<tr>
<td>baa₃</td>
<td>bo₃</td>
</tr>
<tr>
<td>caa₃</td>
<td>d</td>
</tr>
<tr>
<td>cbb₃</td>
<td>bd</td>
</tr>
<tr>
<td>cao₃</td>
<td>aa₃</td>
</tr>
<tr>
<td>co₃</td>
<td></td>
</tr>
</tbody>
</table>

As a general example the branched respiratory chain of the Gram-negative soil bacterium Paracoccus denitrificans is depicted in Figure 2. The complex I and complex II homologues are often indicated as general dehydrogenases. In P. denitrificans the complex I homologue has been biochemically characterized, and no evidence for a second complex I homologue was found (Yagi, 1991). The complex II homologue has been purified and extensively characterized. It shows similar features as its mitochondrial counterpart (Albracht et al., 1980; Waldeck et al., 1997). The X-ray
structure of the first complex II (of *Wolinella succinogenes*) has been determined (Lancaster and Kröger, 2000; Lancaster *et al.*, 1999). This is the first crystal structure of a membrane protein-complex that directly oxidizes a carbon substrate (succinate) and thereby transfers the electrons into the respiratory chain.

**Fig. 2.** A general scheme of a branched prokaryotic respiratory chain, as it is known in *Paracoccus denitrificans* (de Gier *et al.*, 1996). The branching point is either on the side of the terminal oxidases or on the side of the dehydrogenases. Electrons are delivered either by NADH or succinate from the Krebs cycle into the respiratory chain. An alternative way to feed the respiratory chain is through the fatty acid oxidation (not shown). A third possibility feeding the respiratory chain is through alternative dehydrogenases (not shown). CI, CII, CIII and CIV stand for complexes I, II, III and IV. On the right the heme types of the oxidases are indicated (*aa*$_3$, *cbb*$_3$ and *bb*$_3$).

The aerobic respiratory chain of the soil bacterium *P. denitrificans* is terminated by a classical heme-copper oxidase of the *aa*$_3$ type (Iwata *et al*., 1995; Ludwig, 1980; Ludwig and Schatz, 1980). A further terminal cytochrome *c* oxidase is a *cbb*$_3$-type oxidase (de Gier *et al*., 1996). In addition to the cytochrome *c* oxidases, *P. denitrificans* has one quinol oxidase. This oxidase is a *bb*$_3$-type oxidase (de Gier *et al*., 1996). Thus, *P. denitrificans* terminates its respiration aerobically using at least three distinct oxidases.

*Escherichia coli* also terminates its aerobic respiration with three different oxidases but all of them are quinol oxidases (Anraku and Gennis, 1987; Dassa *et al*., 1991; Kiyoshi *et al*., 1984). Under normal aeration the *bo*$_3$-type oxidase is highly expressed, whereas under low aeration a *bd*-type oxidase is expressed. This *bd*-type oxidase has a high
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affinity for oxygen (D'Mello et al., 1996). The physiological role of the third oxidase has remained unknown, but it is also a bd-type oxidase as inferred from the heme cofactors (Sturr et al., 1996). Only the bo3-type oxidase belongs to the superfamily of heme-copper oxidases (Garcia-Horsman et al., 1994a). In contrast to the aa3 type oxidase, however, the bo3 type oxidase of E. coli does not have a CuA cofactor (Wilmanns et al., 1995). A complex III homologue does not exist in E. coli.

I. 2. Aerobic respiration in diazotrophic prokaryotes

Air consists to 78% of molecular nitrogen. N2 is used exclusively by diazotrophic prokaryotes to synthesize NH4+. Ammonia is integrated into C compounds, and amino acids are synthesized. The enzymatic reduction of N2 to NH4+, which is called N2 fixation, is a reaction that is restricted only to bacteria and archaea. The nitrogenase complex, which performs this reduction, consists of two large components. One component, which is a dinitrogenase reductase (Fe-protein), hydrolizes ATP and becomes itself reduced by reductases such as ferredoxin. The other subunit, which is a dinitrogenase, harbors the Mo-Fe cofactors that are, like the Fe cofactors, highly sensitive to O2 (Burris, 2000). Nitrogen fixation is an energy-consuming process. In order to transform 1 mol N2 into NH4+, at least 16 mol ATP are needed (Hill, 1992). This reduction of the N2 molecule is performed at near anaerobic conditions (Hennecke, 1998). In spite of this requirement oxidative phosphorylation must be possible in strict aerobes, in order to meet the high energy demand of nitrogen fixation. The need to consume oxygen and to simultaneously exclude it from nitrogenase is often called the ‘oxygen paradoxon’ (Appleby, 1984). Strictly aerobic diazotrophic bacteria use different strategies to overcome this problem. In general, N2 fixation occurs under microaerobic conditions. Disparate solutions are known by which aerobic diazotrophs reconcile N2 fixation with oxygen: (i) strategies that take advantage of physical barriers against oxygen diffusion, like heterocysts in cyanobacteria and (ii) strategies that make use of special physiological and biochemical adaptations. Respiratory protection is an example of adaptation elaborated in Azotobacter species.

An elegant solution of the oxygen paradox is realized in the case of the nitrogen-fixing root nodule symbiosis between rhizobia and legumes. Here, we encounter on the one hand the host plant’s strategy of building up a physical oxygen diffusion barrier and on
Chapter I

the other hand the physiological and biochemical strategies in both the plant and the bacteria by which they respond to the nearly anoxic conditions inside the nodule (Appleby, 1984; Witty and Minchin, 1990). Advancements made in the analysis of the composition and function of rhizobial respiratory chains, and also of respiratory chains in non-symbiotic aerobic bacteria, have led to a better understanding of how microaerobic respiration is carried out. The so-called high-affinity terminal oxidases are of utmost importance in this context.

Another important strategy used by diazotrophs is to allow nitrogenase synthesis only when the oxygen concentration is permissive for N₂ fixation. Sensor proteins in the membrane (FixL) and in the cytoplasm (NifA) detect the actual O₂ concentration. During aerobic conditions the regulators for activation or derepression are either not expressed or they are inactive. If the concentration of O₂ drops below a certain threshold level the genes for the activators are expressed, or the regulators become active and then activate the transcription of other genes that are needed for N₂ fixation (Fischer, 1994). Since the dinitrogenase is highly O₂ sensitive, it makes sense that the sets of genes for the two sub-complexes are only expressed in microaerobic conditions (Fischer, 1994).

I. 2.1. Non-symbiotic diazotrophs

The non-symbiotic diazotrophs utilize energy generated by the oxidation of organic compounds or by photosynthesis (Hill, 1992). The group of non-symbiotic diazotrophic organisms is quite diverse. In general, one possibility is to divide them into non-phototrophic and phototrophic diazotrophs. In this sub-chapter, only the respiratory chains of these organisms are surveyed. These bacteria are assigned, according to their 16S rRNA-sequences, to different subdivisions among the proteobacteria (Table 2).

<table>
<thead>
<tr>
<th>Organism</th>
<th>Subdivision</th>
<th>Phototroph (no/yes)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Azotobacter vinelandii</em></td>
<td>γ</td>
<td>no</td>
</tr>
<tr>
<td><em>Rhodobacter sphaeroides</em></td>
<td>α</td>
<td>yes</td>
</tr>
<tr>
<td><em>Rhodobacter capsulatus</em></td>
<td>α</td>
<td>yes</td>
</tr>
</tbody>
</table>
Most knowledge in the group of asymbiotic diazotrophs has been accumulated for *A. vinelandii*, which is an obligate aerobic organism. To protect the nitrogenase complex from oxygen damage, this organism uses three strategies. Like in all known diazotrophs, transcriptional regulation is used to initiate the transcription of genes that are needed for N$_2$ fixation. Conformational protection involves complex formation of a small [2Fe-2S] protein (Shetna protein) with the Mo-Fe protein (dinitrogenase) and the iron protein (dinitrogenase reductase). This leads to a more stable complex that is resistant against oxygen damage (Kolonay *et al.*, 1994). The third strategy is based on the properties of the terminal oxidase. *A. vinelandii* has three different terminal oxidases (Kelly *et al.*, 1990). The respiratory chain is branched, like in all known prokaryotic organisms. Under non-fixing conditions, two o-type cytochrome oxidases are present (coupled to ATP production); under N$_2$-fixing conditions a high-turnover bd-type oxidase is present in the cytoplasmic membrane (respiratory protection, less coupled). The action of cytochrome bd makes the cytoplasm of this bacterium nearly anaerobic, and hence, the Mo-Fe cofactors of the dinitrogenase and the Fe cofactor of the dinitrogenase reductase are not irreversibly oxidized. Considering the entire respiratory chain, the respiration (when N$_2$ is fixed) is less coupled (1H$^+/e^-$) as by respiration (no N$_2$ fixation) with the o-type oxidases (5H$^+/e^-$. Therefore, respiration under non-fixing conditions (NADH-I=2H$^+/e^-$ and cytc$_4$ or c$_5$ and o-type oxidase=3H$^+/e^-$) leads to a coupled respiration with
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5H⁺/e⁻. Oxygen scavenging is independent from pumping protons. It was demonstrated, in an in vitro system, that this bd-type oxidase is a proton pump with a pump efficiency of 1H⁺/e⁻ (Kolonay and Maier, 1997), and that respiration occurs through NADH-II (uncoupled), therefore leading to a less coupled (1H⁺/e⁻) respiratory chain. Nevertheless, in the work of Oelze, (2000) the hypothesis that the nitrogenase is protected from oxygen damage by a respiratory protection oxidase is questioned. The author describes that changing the C/N-ratio in a chemostat to higher ratios results in a higher respiratory activity. This observation confirms the respiratory protection hypothesis. However, this behavior holds true only at a certain O₂ concentration (70 µM O₂), which is one third of the O₂ concentration up to which N₂ fixation can occur in this organism (230 µM O₂). Increasing the C/N-ratio ends in a constant turnover rate of the bd oxidase, which is contradictory to the respiratory protection hypothesis. The author interprets this observation as follows: Nitrogen is directly assimilated into biomass, whereas carbon is dissimilated into biomass and NADH₂ and CO₂. Further increase of the ratio leads to higher amounts of NADH₂ and in turn ATP. At a certain C/N-ratio, nitrogen becomes limiting and the nitrogenase genes are transcribed and translated, which ends in the detection of the nitrogenase activity in the cytoplasm. Nitrogenase activity needs high amounts of NADH₂ and ATP. Consumption of much NADH₂ leads to a low redox state in the cytoplasm. If this is primarily consumed by the nitrogenase, less NADH₂ remains for the respiratory chain (NADH-I and NADH-II). This would lead to less ATP, because less H⁺ are pumped (less coupled). It is assumed that under such conditions the electrons are transferred by the coupled respiratory chain (5H⁺/e⁻, more ATP). The fact that a bd-minus strain results in a nitrogen fixation minus phenotype is explained as follows by the author: The cytochrome bd-minus strain is no more able to adapt its energy metabolism to the level required for allowing the nitrogenase to work properly. An alternative hypothesis may be derived, therefore, from evidence suggesting that the nitrogenase activity depends on the maintenance of a sufficiently low redox state (NADH₂/NAD⁺ = low). It may be speculated that, depending on the cellular demands for ATP, electron flow within the coupled and less coupled branches of the respiratory chain is not static, but rather dynamically regulated. This means that the dependence of the efficiency of ATP regeneration and, consequently, the activity of the nitrogenase on the actual rate of O₂ consumption is less than assumed in light of the hypothesis of respiratory protection. It appears further likely that a mechanism depending on the
energy metabolism does not only provide protection of nitrogenase from O₂ inactivation but also controls nitrogenase synthesis in the presence of O₂ (Oelze, 2000).

*R. sphaeroides* and *R. capsulatus* are phototrophic diazotrophic bacteria. Some knowledge has been accumulated about their branched respiratory chain. The photosynthetic reaction is anoxygenic and occurs under anaerobic conditions and in light (Oh et al., 2000).

*R. sphaeroides* has two cytochrome c oxidases with the cofactors that are diagnostic of an *aa₃*-type oxidase and a *cbb₃*-type oxidase (Fig. 4). The branching point for the electrons is in the complex III homologue, the *bc₁* complex. Furthermore, there are two ubiquinol oxidases. One quinol oxidase is a putative *bd* oxidase, whereas the other quinol oxidase type is not known regarding the cofactors (Oh and Kaplan, 1999). Under aerobic growth conditions, this organism expresses a classical *aa₃*-type terminal oxidase; under anaerobic-to-microaerobic conditions the *cbb₃* oxidase is mainly expressed and the *aa₃* type oxidase is repressed. Phototrophic growth is only possible under anaerobic conditions and in the light. The *cbb₃*-type oxidase, which is expressed under microaerobic conditions, is assumed to be an oxidase with high affinity for oxygen. However, this assumption was only deduced from fact that the *cbb₃*-type oxidase of *Bradyrhizobium japonicum* has a high affinity for O₂ (Preisig, 1994; Preisig et al., 1996b).

An intriguing result was brought about for the role of the *cbb₃*-type oxidase of *R. sphaeroides*. It was demonstrated that this oxidase fulfills two tasks. The classical one is energy conservation by proton pumping (Toledo-Cuevas et al., 1998), while the other is implemented to work as a redox sensor. It was known that genes that are needed for photosynthetic (PS) growth are derepressed under anaerobic/microaerobic conditions, in order to allow photosynthetic growth (Oh et al., 2000; Oh and Kaplan, 1999). Activation of PS gene expression by a so-called PrrBA two-component system results from an interruption of an inhibitory signal evolving from the *cbb₃*-type oxidase. The transmitter protein between the *cbb₃*-type oxidase subunit CcoQ and the sensor PrrB is a protein called PrrC. A deletion in *prrC* shows the same phenotype as *ccoQ*-mutant: derepression of PS genes (*puf, puc, crt, beh* and *puhA*) even under aerobic conditions. It is assumed that the sensing of the reductant flow ("redox sensing") is performed by the *cbb₃*-type oxidase (Eraso and Kaplan, 2000). The more aerobic the environment for the
cells is, the more intensive is the reductant flow, resulting in a stronger inhibitory signal for transcription. Is the cell exposed to a more anaerobic environment, the outcome is less reductant flow and a decreased inhibition signal for the transcription. This cascade results in a higher derepression of the PS gene, hence, the expression of \( puf, puc, crt, bch \) and \( puhA \). Especially CcoQ and PrrC are crucial for signal transduction (Eraso and Kaplan, 2000).

Fig. 4. The branched respiratory chain of \( R. \) sphaeroides. Only one type of quinol oxidase is known, regarding the heme types. It is a \( bd \)-type oxidase. The cofactor type of the other quinol oxidase is not known. Anoxygenic phototrophy is possible by reaction centre (RC), which becomes reduced by \( e_2 \). Two cytochrome \( c \) oxidases are depicted. These oxidases are, regarding their cofactors, an \( aa_3 \)- and \( cbb_3 \)-type oxidase. The \( c_y \) cytochrome can transfer electrons either to the \( aa_3 \) or \( cbb_3 \)-type oxidase.

The respiratory chain of \( R. \) capsulatus is also branched and is terminated by one cytochrome \( c \) oxidase and one quinol oxidase (Fig. 5). The cytochrome \( c \) oxidase is, regarding the cofactors, a \( cbb_3 \)-type oxidase. The genes coding for the \( cbb_3 \) oxidase (\( ccoNOQP \)) were ‘knocked out’ and nitrogen fixation capability was still found (Thöny-Meyer et al., 1994a). This result was contrary to symbiotic diazotroph \( Bradyrhizobium japonicum \) (Preisig et al., 1993). Instead, the \( R. \) capsulatus \( cbb_3 \)-type oxidase is needed for efficient aerobic growth and it was shown that it is mainly expressed under aerobic conditions (Thöny-Meyer et al., 1994a). Anaerobic, chemotrophic growth is possible. Phototrophic growth is also possible under anaerobic conditions.

The \( cbb_3 \)-type oxidase belongs to the superfamily of heme-copper oxidases, but it lacks the Cu\(_A\) centre (Garcia-Horsman et al., 1994b) as the electron acceptor from cytochrome \( c \). \( R. \) capsulatus can also express a quinol oxidase, preferably under low-
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oxygen conditions. The heme cofactors are most probably of type b and d (F. Daldal, personal communication). However, up to now only b-types cytochromes have been detected.

![Diagram of the branched respiratory chain of R. capsulatus](image)

**Fig. 5.** The branched respiratory chain of *R. capsulatus* is terminated by at least two oxidases. RC indicates the photosynthetic reaction centre. Also, in this branched respiratory chain $c_2$ is responsible for the anoxygenic phototrophic growth. The $c_1$ is believed to be the electron donor for the cytochrome oxidase.

The dependency of the assembly of the $cbb_3$-type oxidase from the gene products of *ccoGHIS* was systematically investigated in *R. capsulatus*. The *ccoGHIS* genes are located downstream of the *ccoNOQP* operon. It was previously demonstrated in *B. japonicum* that a fixGHI-deletion mutant (deleted genes corresponding to *ccoGHI*) shows little amount of $cbb_3$ activity (in vivo TMPD oxidase activity) when cells were grown under anaerobic conditions. Residual N$_2$ fixation activity was about 5% like in a fixNOQP mutant (Preisig et al., 1996a). The subunits (FixN, FixO and FixP) were hardly detectable in the membranes of the mutant. These results suggested a role for fixGHIS in the assembly of $cbb_3$-type oxidase of *B. japonicum*. Sequence alignments revealed a putative Cu$^{2+}$ transport function for FixI. It was concluded that the binuclear Cu$_B$ centre is not built in the mutant, and in turn an active and stable subunit composition of FixNOP cannot be achieved in the membranes (Preisig et al., 1996a).

The new insights, which came out from research with *R. capsulatus* concerning the assembly of the $cbb_3$ oxidase, are as follows. Genetic analysis of *ccoGHIS* demonstrated that *ccoG, ccoH, ccoI* and *ccoS* are expressed independently of each other and do not form a single operon. In-frame deletion mutations in each gene were investigated.
Absence of CcoG, which has a putative 4Fe-4S cluster binding motif, does not significantly affect \( cbb_3 \) oxidase activity. The absence of either CcoH or CcoI influences the steady-state amount of \( cbb_3 \) oxidase in the membranes. CcoI appears to be involved in the acquisition of \( Cu^{2+} \) for the \( cbb_3 \) oxidase because a CcoI-minus mutant phenotype could be mimicked by \( Cu^{2+} \) starvation of a wild-type strain. The function of the smallest protein CcoS is supposed to be the functional integrity of the redox-active prosthetic groups, low-spin and high-spin heme B and \( Cu^{2+} \), into the \( cbb_3 \) oxidase. Thus, the \( ccoGHIS \) gene products are involved in several steps during the maturation of the cytochrome \( cbb_3 \) oxidase in \( R. \ capsulatus \) (Koch et al., 2000).

I. 2.2. Symbiotic diazotrophs

Symbiotic diazotrophs are bacteria which live in mutual symbiosis with the host plant. They are Gram-negative soil bacteria, collectively called rhizobia, that are able to establish a root nodule symbiosis with legumes (Appleby, 1984). \( B. \ japonicum \) (Jordan, 1982), \( Azorhizobium caulinodans \) and \( Sinorhizobium meliloti \) are bacteria that belong to the \( \alpha \)-subdivision of the proteobacteria. They can live either free-living in soil, or as true endosymbionts (bacteroids) within the cytoplasm of the plant’s cell. The oxygen partial pressure in the infected nodule cells is about 10'000-times less than in oxygen-saturated aqueous solution. Rhizobia are able to cope with these extreme differences in oxygen partial pressure by expressing different respiratory oxidases with different affinities for oxygen. A high-affinity oxidase is needed to keep an efficient energy metabolism in symbiosis, where the concentration of free oxygen drops down to the range of about 5-25 nM (Hennecke, 1998; Layzell et al., 1990). During endosymbiosis, the plants benefit from fixed nitrogen, and in turn the bacteria are fed with \( C_4 \) compounds from the plant. A lot of symbiotic diazotrophs are known. In most cases, except \( B. \ japonicum \) and \( A. \ caulinodans \), not much is known about the respiratory system of these organisms. In addition, little knowledge has been accumulated concerning the protection of the nitrogenase complex.
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I. 2.2.1. Respiration in *Bradyrhizobium japonicum*

The respiratory chain of *B. japonicum* is branched like in all other bacteria (Fig. 6). On the reducing side (e.g. succinate dehydrogenase, hydrogenase, and short chain fatty-acyl-CoA dehydrogenases) less is known than on the oxidizing side. The electrons are delivered via the dehydrogenases into the quinone pool, which becomes reduced to quinol. The quinol pool can be oxidized by two means in this bacterium. Under aerobic free-living conditions the electrons are passed to the $b_{c_1}$-complex (Thöny-Meyer et al., 1989). The electrons are subsequently passed to CycM, which is a membrane-anchored mono-heme cytochrome c (Bott et al., 1991). This cytochrome serves as the mediator protein for transferring electrons from the $b_{c_1}$-complex to the cytochrome c oxidase, which is – with regard to the cofactors – an aa$_3$-type heme-copper oxidase (Bott et al., 1990). Another oxidase was found, which represents the features of a cytochrome c oxidase as deduced from sequences (Bott et al., 1992). However, only the genes (coxMNOP) are known, and no expression conditions with translational fusion constructs were found for this oxidase (O. Preisig, personal communication). The physiological benefit of this oxidase for the cell remains enigmatic. Under endosymbiotic conditions (microaerobic) a different oxidase is expressed (Preisig et al., 1993). Regarding the cofactors, it is assigned as a $cbb_3$-type oxidase (Preisig et al., 1996b), and it belongs to the superfamily of heme-copper oxidases (Garcia-Horsman et al., 1994a). This oxidase consists of three redox-active subunits (FixN, FixO and FixP) and a fourth small subunit FixQ (Zufferey et al., 1996a). The largest subunit, FixN (subunit I), harbors the six diagnostic histidines for liganding low- and high-spin hemes B and Cu$_B$. The high-spin heme B and Cu$_B$ build the unique bimetallic centre, which is also called the binuclear centre. The oxygen reduction to water occurs in this centre by an enzymatic transfer of electrons and protons (scalar) on oxygen, whereas protons (vectorial) are presumably pumped into the periplasm to establish an electrochemical proton gradient. FixO and FixP are mono- and diheme cytochromes c, respectively (Zufferey et al., 1997). These subunits do not show any similarity to classical aa$_3$ type oxidase subunits II and III. An additional difference is that there is no Cu$_A$ centre (Garcia-Horsman et al., 1994a). This oxidase has a high affinity for oxygen and is thus essential for symbiotical nitrogen fixation (Preisig et al., 1993; Preisig et al., 1996b). It was known that each gene product by itself could have an influence on the assembly of the oxidase. Therefore a detailed study of the assembly of this oxidase, which is
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encoded by the \textit{fixNOQP} operon, was performed. The most important result that came out of this investigation was that FixNO could build a stable precomplex in the membranes, whereas FixP was absent (in a \textit{fixP} deletion mutant). This means that FixP is not needed for the assembly of the complex. Precomplex formation is FixP independent (Zufferey \textit{et al.}, 1996a). Furthermore, all 12 conserved histidines – six are cofactor ligands (Garcia-Horsman \textit{et al.}, 1994a) and the other six are conserved in alignment comparisons among FixN homologues in the FixN subunit – were mutated, and their influence on enzyme activity and nitrogen fixation were examined. The histidines were substituted by valine or alanine. The H131A mutant enzyme is defective in assembly and function. The H226, H246, H333, and H457 are not essential for activity and assembly. By contrast, H331, H410, and H418 were required for activity and stability of the enzyme. The last group of mutant enzymes, H420A, H280A, H330A, and H316V, were assembled but not functional (Zufferey \textit{et al.}, 1998).

The fact that a \textit{bc} \textsubscript{1} mutant still shows growth under aerobic conditions led to the proposal that there must be at least one quinol oxidizing terminal oxidase (Thöny-Meyer \textit{et al.}, 1989). Surpin \textit{et al.}, (1994) published the sequence of genes for the putative subunits of a quinol oxidase. The genes were named \textit{coxWXYZ}, and it was demonstrated by the same group that the gene products encode a \textit{bb}\textsubscript{3}-type quinol oxidase (Surpin \textit{et al.}, 1996; Surpin and Maier, 1999). The gene nomenclature \textit{coxWXYZ} is inappropriate. Historically, \textit{cox} stands for cytochrome oxidase, but this oxidase is a quinol oxidase. In Figure 6 (next page) the branched respiratory chain of \textit{B. japonicum} is depicted as it was known at the beginning of this Ph.D-thesis. An updated version of the branched respiratory chain of \textit{B. japonicum}, which is extended by one cytochrome \textit{bd}-containing quinol branch, will be shown in chapters V and VI as one result of this thesis. Therefore, a brief general introduction into \textit{bd}-type quinol oxidases is relevant here.

The \textit{bd}-type oxidases do not belong to the super family of heme copper oxidases (Horsmann \textit{et al.}, 1994a), but regarding the electron donor they are also quinol oxidases, like for example the \textit{bb}\textsubscript{3} or \textit{bo}\textsubscript{3} oxidase (Surpin and Meier, 1999; Puustinen and Wikström, 1999). They are not considered as real proton pumps, in terms of channeling protons through them. This means during oxidation of quinol at the oxidase, protons are released at the periplasmic side of the protein complex without pumping, leading to a electrochemical proton gradient. A stoichiometry of \(1\text{H}^+/e^-\) can be
accounted for by transmembrane movement of electrons combined with the formation of a proton gradient by substrate protons, i.e., the release of two protons per quinol molecule oxidized on the periplasmic side without invoking a proton pump (Puustinen et al., 1991).

Fig. 6. The branched respiratory chain of *B. japonicum*. Each oxidase is indicated as a separate box. There is little information known about the reducing part of the quinone pool. More is known about the oxidizing part of this pool. CycM is a membrane bound cytochrome *c*, which donates the electrons to the *aa*$_3$ oxidase. There are genes known for four different terminal oxidases. Only the CoxMNOP oxidase is not characterized regarding its cofactors.

The importance of these oxidases is best exemplified for *E. coli* (Anraku and Gennis, 1987) and *A. vinelandii* (Kelly et al., 1990). In *E. coli*, this oxidase (*bd-I*) is a high-affinity oxidase, which is expressed under low oxygen concentrations (D’Mello et al., 1996). Under low oxygen concentrations it allows *E. coli* to synthesize ATP by oxidative phosphorylation. In addition, there is a second *bd*-type quinol oxidase in *E. coli* (Sturr et al., 1996) (*bd-II*); however its benefit for growth until now is not known. In *A. vinelandii*, which is a diazotrophic organism, this oxidase is a protective oxidase, in terms that it shows high turnover rates during asymbiotic nitrogen fixation. This observation has also led to the term ‘O$_2$-scavenging oxidase’ (see page 13). *Azorhizobium caulinodans*, as a facultative symbiotic diazotroph, needs two oxidases
(bd- and cbb$_3$-type oxidases) for efficient N$_2$ fixation (Kaminski et al., 1996; Kitts and Ludwig, 1994).

*B. japonicum* can grow anaerobically with NO$_3^-$ as electron acceptor (Daniel and Appleby, 1972). There is biochemical evidence for the presence of two membranous NO$_3^-$ reductases (Fernandez-Lopez et al., 1989; Fernandez-Lopez et al., 1996). Soluble cytochrome c$_{550}$ (cycA) is indispensable for anaerobic growth with nitrate (Bott et al., 1995). There are two further soluble cytochromes characterized, c$_{552}$ (cycB) (Rossbach et al., 1991) and c$_{555}$ (cycC) (Tully et al., 1991; Appleby et al., 1991; Bott et al., 1995). These cytochromes do not seem to be involved in the denitrification pathway. A triple mutant (cycA, cycB, cycC) was tested for its symbiotic phenotype, because the initial idea was that one of these could donate the electrons to the cbb$_3$-type oxidase. However, it turned out that none of these cytochromes are involved as the native electron donor for the cbb$_3$-type oxidase. Anaerobic growth with a sole C-source as the energy source was not demonstrated with this organism, which leads to the assumption that *B. japonicum* cannot grow in a fermentative way. Aerobic growth with different sole C-sources in minimal-salts medium is possible (Dunn, 1998; Poole and Allaway, 2000).

I. 2.2.2. Respiration in *Azorhizobium caulinodans*

*A. caulinodans* uses up to four terminal oxidases for its aerobic and microaerobic growth. Two of them are cytochrome c oxidases; regarding their cofactors they are aa$_3$, and cbb$_3$ oxidases. The remaining two others are quinol oxidases of the bo$_3$ and bd type (Kaminski et al., 1996; Kitts and Ludwig, 1994). It is interesting that in this organism the cbb$_3$- and the bd-type oxidases are needed simultaneously for an effective nitrogen fixation. In fact, just like a cydAB-minus mutant (bd-type oxidase), a cytNO-minus mutant (cbb$_3$-minus) shows only 50% of nitrogen fixation activity. Only a double mutant (cydAB-minus and cytNO-minus) shows no nitrogen fixation activity (Kaminski et al., 1996; Kitts and Ludwig, 1994). This leads to the assumption that each of these oxidases has the property of high affinity for oxygen (Hennecke, 1998). Besides symbiotic N$_2$-fixation, this organism can also fix N$_2$ asymbiotically (Kaminski et al., 1996).
I. 3. Proton pumping by terminal oxidases

Terminal oxidases perform three reactions simultaneously. This is based on the fact that they are localized in biological membranes, and thus between two cellular compartments. This topology leads to the fact that one side is directed towards the intermembrane space (or periplasm in bacteria), whereas the other is towards the cytoplasm or matrix (in mitochondria) (Saraste, 1990; Saraste, 1999). Two reactions are oxidation of the electron donor and the reduction of \( \text{O}_2 \) to \( \text{H}_2\text{O} \). Simultaneously, protons are translocated out of the cytoplasm to the periplasm, as the third reaction. This generates an electrochemical proton potential, which is subsequently used by ATP synthase for the synthesis of ATP.

Acidic or polar amino acid residues in subunit I are believed to be involved in proton translocation. This finding is based on the observation that acidic and polar residues in bacteriorhodopsin (Dickopf et al., 1995; Mollaaghababa et al., 2000; Moltke et al., 1995) and the bacterial photosynthetic reaction centre are also involved (Krebs et al., 1993; Krebs et al., 1991; Krebs and Khorana, 1993). The mitochondrial terminal oxidase (complex IV) is a proton pump (Babcock and Wikström, 1992). The X-ray structures of the mitochondrial bovine-heart cytochrome \( \text{c} \) oxidase and of a bacterial \( \text{aa}_3 \)-type terminal oxidase from \( \text{P. denitrificans} \) have been solved (Iwata et al., 1995; Tsukihara et al., 1995; Tsukihara et al., 1996). The polypeptide of subunit I spans the biological membrane with 12 transmembrane helices. In the \( \text{P. denitrificans} \text{ aa}_3 \)-type oxidase, two channels are believed to act in \( \text{H}^+ \)-transport through subunit I. The K-channel (named after K354) consists of S291, K354, T351 and Y280 and is believed to be the route of protons to the binuclear centre. The D-channel (named after D124) consists of D124, N113, N131, N199, Y35, S134, S193 and E278, and directs \( \text{H}^+ \) through the subunit I for the electrochemical proton gradient (Iwata et al., 1995; Pfitzner et al., 2000). D124 (\( \text{E. coli} \) numbering D135 in cytochrome \( \text{bo}_3 \)) and E278 (\( \text{E. coli} \) numbering E286 in cytochrome \( \text{bo}_3 \)) are crucial for proton pumping activity in the \( \text{bo}_3 \) oxidase of \( \text{E. coli} \), which is also a heme-copper oxidase (Puustinen and Wikström, 1999; Thomas et al., 1993; Verkhovskaya et al., 1997). Despite these results, the mechanism of proton translocation is controversially discussed in these days (Michel, 1998; Michel, 1999a; Michel, 1999b; Wikström, 2000a; Wikström, 2000b; Wikström et al., 2000).
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The polar residues are absent in the terminal oxidases of the archaeon *Sulfolobus acidocaldarius* (Lübben et al., 1992), or also in the two oxidases of *Thermus thermophilus* (*ba* and *ca*3) (Mather et al., 1993). However, recently obtained results demonstrate that the discussed residues are not needed for proton translocation in the other oxidases (Gleissner et al., 1994; Schäfer et al., 1999). Terminal *ba*3-type oxidase of *T. thermophilus* is a proton pump, however, with a proton pump stoichiometry of 0.5 \( \text{H}^+/\text{e}^- \) (Kannt et al., 1998). It was demonstrated in the reconstituted *ca*3-type oxidase of *Rhodothrumus marinus* that it works as a proton pump with an efficiency of 1 \( \text{H}^+/\text{e}^- \), in spite of the fact that the key glutamic acid E278 is missing in the D-channel. It has been discussed that a tyrosin residue Y256 nearby (R. marinus numbering) acts as functional substitute (Backgren et al., 2000; Pereira et al., 2000b).

Like in the archaeal terminal oxidases, polar residues are not present in subunit I of the *cbb*3-type oxidases. However, de Gier et al. (1996) demonstrated for the first time, although by indirect means, that the *cbb*3 oxidase of *P. denitrificans* pumps protons, but with less stoichiometry than the classical *a*3-type oxidase in the same organism. Experiments with *R. sphaeroides* cells, impaired in all oxidases except the *cbb*3 oxidase, revealed 1\( \text{H}^+/\text{e}^- \) pump efficiency (Toledo-Cuevas et al., 1998). These experiments were made with an oxygen pulse in an anaerobic medium. *In vitro* (reconstituted), it was demonstrated for the first time that the *cbb*3-type oxidase of *Helicobacter pylori* is a proton pump with less proton pump efficiency. The authors published 0.5 \( \text{H}^+/\text{e}^- \) (Tsukita et al., 1999). It will be demonstrated, as a result of this thesis, that the *cbb*3-type oxidase of *B. japonicum* is also a proton pump with less proton pump efficiency in comparison to the classical *a*3-type oxidase of *P. denitrificans* (Arslan et al., 2000). It turns out, that in the vast variety of terminal oxidases the proton pump efficiencies vary substantially. Whether or not this reflects functional purposes and the energy requirements of the cell is not known.

1.4. Aim of the work

An improved purification protocol for the *cbb*3-type oxidase of *B. japonicum* had to be established. The yield and the purification factor were to be increased by enlarging the copy number of the genes coding for the *cbb*3-type oxidase (*fixNhisOQP*) on a plasmid. Heme-copper cytochrome *c* oxidases are usually proton pumps (see above). The application of a classical pre-steady-state cytochrome *c* pulse experiment, in order to
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demonstrate proton-pumping activity of the $cbb_3$-type oxidase, was to be developed (Chapter II).

The redox potential of each cofactor in $cbb_3$-type oxidase was to be determined and to be compared with $cbb_3$ cytochrome $c$ oxidases of other organisms (Chapter III).

Since *E. coli* is not able to express cytochrome $c$ aerobically, an expression system for FixO$_{sol}$ and FixP$_{sol}$ (c-type cytochromes) was to be developed (Chapter IV).

The genes for subunits of a putative $bd$-type quinol oxidase were found. It was therefore needed to deliver proof that these genes encode a $bd$-type quinol oxidase, and to investigate its potential importance for symbiotic N$_2$ fixation (Chapter V).
CHAPTER II

The symbiotically essential $cbb_3$-type oxidase of *Bradyrhizobium japonicum* is a proton pump

Chapter II

The symbiotically essential cbb3-type oxidase of *Bradyrhizobium japonicum* is a proton pump

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II. 1. Abstract

Purified cbb\textsubscript{3}-type oxidase of Bradyrhizobium japonicum was reconstituted into phospholipid vesicles. Tight vesicles were obtained as shown by the disturbance of ΔpH with CCCP and the membrane potential with valinomycin, which led to a six-fold increase in cytochrome c oxidase activity. The vesicles were thus suitable for proton translocation experiments. In the presence of valinomycin, a pulse with reduced cytochrome c caused an acidification with a subsequent alkalinization, whereas the same pulse caused only an alkalinization in the presence of valinomycin plus CCCP. We conclude that the cbb\textsubscript{3}-type oxidase of B. japonicum is a proton pump.

*Key words*: Bradyrhizobium japonicum; cbb\textsubscript{3}-type oxidase; cytochrome c oxidase; nitrogen fixation, proton pumping

*Abbreviations*: CCCP, carbonyl cyanide m-chlorophenylhydrazine; Covs, cytochrome c oxidase vesicles, PMSF, phenylmethylsulfonyl fluoride; RCR, respiratory control ratio.
Chapter II

II. 2. Introduction

The $cbb_3$-type oxidase of the soybean symbiont *Bradyrhizobium japonicum* is essential for respiration when the bacterial cells live in endosymbiotic, nitrogen-fixing conditions within the root nodule (Preisig *et al.*, 1993; Preisig *et al.*, 1996b; Zufferey *et al.*, 1996a). It belongs to the superfamily of heme-copper oxidases (Saraste, 1990) and is thought to transfer electrons from the cytochrome $bc_1$ complex to molecular oxygen. Such an electron transfer is usually coupled to the net translocation of protons from the cytoplasm to the periplasm. The resulting proton gradient is then used for the synthesis of ATP by ATP synthase (Nicholls and Ferguson, 1992; Saraste, 1999).

Seven years ago, the $cbb_3$-type oxidase was discovered as a novel type of a cytochrome $c$ oxidase as far as subunit composition and content of cofactors are concerned (Garcia-Horsman *et al.*, 1994a; Garcia-Horsman *et al.*, 1994b; Gray *et al.*, 1994; Preisig *et al.*, 1993). The membrane-integral FixN subunit (also called CcoN) resembles subunit I of classical heme-copper oxidases in having a low-spin heme and a binuclear centre composed of a high-spin heme and CuB, where $O_2$ reduction to $H_2O$ occurs. The heme and copper cofactors are liganded by six strictly conserved histidines. The other redox-active, membrane-anchored subunits FixO (CcoO) and FixP (CcoP) are mono- and diheme $c$-type cytochromes, respectively. A fourth, hydrophobic subunit (FixQ, CcoQ) is firmly attached to the oxidase but has no obvious function (Zufferey *et al.*, 1998). All four proteins are encoded by the fixNOQP operon (Preisig *et al.*, 1993).

With a $K_M$ of 7 nM, the $cbb_3$-type oxidase has an extremely high affinity for oxygen (Preisig *et al.*, 1996b), allowing *B. japonicum* to respire in the micro-oxic environment within the root nodule. Although this species possesses a branched respiratory chain with at least four terminal oxidases (Bott *et al.*, 1990; Bott *et al.*, 1992; Preisig *et al.*, 1996b; Surpin *et al.*, 1996), the $cbb_3$-type oxidase is the only one that supports symbiotic nitrogen fixation. This oxidase’s function is either to scavenge oxygen in order to protect nitrogenase from irreversible oxidative damage, or to act as a proton pump which – coupled with oxidative phosphorylation – would help meet the high ATP demands of the nitrogenase reaction (Hill, 1992). Here, we report the *in vitro* reconstitution of purified $cbb_3$-type oxidase in the form of proteoliposomes and...
demonstrate proton pumping activity. Hence, this oxidase plays a pivotal role in energy conservation for nitrogen fixation in symbiosis.
II. 3. Materials and Methods

3.1. Overexpression of the B. japonicum cbb₃ oxidase

To enhance expression of the cbb₃-type oxidase, the fixN\textsuperscript{fix}OQP genes were cloned into the broad host-range plasmid pPP375Ω-l (Ramseier et al., 1991; Sambrook, 1989) derived from pRK290. This plasmid was linearised with BanHI, and the 5' overhangs were filled with T4 DNA polymerase. A KpnI linker was ligated to this blunt-end site (pRJ4662). The fixN\textsuperscript{fix}OQP operon was excised with KpnI from pRJ4620 (Sambrook, 1989; Zufferey et al., 1998) and ligated into KpnI-digested pRJ4662, resulting in pRJ4639. This plasmid was conjugated into B. japonicum strain BJ4621 (Sambrook, 1989; Zufferey et al., 1998), yielding strain Bj4639.

3.2. Enzyme preparation and activity measurement

The Paracoccus denitrificans aa₃-type oxidase was purified as described (Kleymann et al., 1995). The cbb₃-type oxidase was isolated from membranes of B. japonicum cells grown anaerobically with nitrate as described previously (Zufferey et al., 1996a) except for the following modifications: the membranes were isolated from cells disrupted in the presence of 0.15 mg lysozyme per ml and washed in 20 mM Tris-HCl, pH 8, 1 mM PMSF at 4°C. Chromatographic columns were equilibrated with the same buffer as described previously (Zufferey et al., 1998), but with 10% (v/v) glycerol added. The entire purification procedure was performed at 4°C. Buffer exchange against the reconstitution buffer (100 mM Hepes-KOH, 10 mM KCl, pH 7.3) was performed on a gel filtration column. The enzyme preparation was concentrated by ultrafiltration with 100-kDa cut-off Amicon centricons up to a concentration of 190 μM (24 mg/ml). Oxidase concentration was measured spectrophotometrically by recording the absorption difference between Na-dithionite-reduced and air-oxidized samples at 551 nm (Zufferey et al., 1998), using an extinction coefficient of 19 cm\(^{-1}\) mM\(^{-1}\) for one heme C. Enzyme activity was calculated by measuring the change in absorbance at 550 nm of reduced horse heart cytochrome c during its oxidation by the cbb₃-oxidase at 25°C in 10 mM Hepes-KOH, 40 mM KCl, 40 mM sucrose, 0.01% (w/v) dodecylmaltoside. The preparation was stored at 4°C.
3.3. Preparation of proteoliposomes

L-α-phosphatidylcholine (type IVS) from soybean was purified and stored as a stock-solution (140 mg/ml) in chloroform at -20°C (Kagawa and Racker, 1966). Before preparation of the vesicles, L-α-phosphatidylcholine was dried at 4°C for 1h under a stream of argon (Ludwig and Schatz, 1980). The dried fraction was then solubilized in reconstitution buffer containing 1.5% (w/v) cholate, to give a final concentration of 62.5 mg/ml, by gentle stirring at 4°C for 2h under argon. The suspension was sonicated with 30-sec pulses (max. 40 Watt), using a Branson tip-sonifier B12, followed by 30-sec incubations at 4°C until clarity was reached. The sonicated liposomes were centrifugated at 20,000g and 4°C for 20 min. The cbbs3-type oxidase was pre-incubated in 1.5% (w/v) cholate for 15 min at 4°C before it was added to the liposomes to give a final concentration of 4 μM, which corresponds to 1:125 protein:l lipid ratio (Solioz et al., 1982). The mixture was then dialysed for 5h against 500 volumes of the reconstitution buffer without cholate, two times (12 h each) against 500 volumes of 10 mM Hepes-KOH, 50 mM KCl, 50 mM sucrose, pH 7.3, and finally two times (12 h each) against 500 volumes of 10 μM Hepes-KOH, 55 mM KCl, 55 mM sucrose, pH 7.3. Dialysis was performed in Pierce Slide-A-Lyzer® cassette, with a 10-kDa cut-off at 4°C. The proteoliposomes were centrifuged at 15,000g at 4°C. The supernatant was used for RCR (respiratory control ratio) estimations, right-side-out determinations and for proton pump experiments.

3.4. RCR estimations and right-side-out determination

The RCR estimation (Casey, 1986; Müller et al., 1986) was performed in 1 ml of the second dialysis buffer (see above) at 25°C. The change of absorbance of reduced horse heart cytochrome c was recorded at 550 nm in a Hitachi U3000 double-beam UV-VIS scanning spectrophotometer. In the presence of 50 μM reduced horse heart cytochrome c, a baseline for auto-oxidation was recorded. After 10 sec, 20 μl of a 1:50 diluted proteoliposome solution was added. The activities in the coupled and uncoupled forms, i.e., without and with valinomycin/nigericin and CCCP, respectively, were expressed as a quotient to give the RCR values (Casey, 1986; Müller et al., 1986). The final
concentrations in the assay were 10 μM of CCCP and 5 μM of either valinomycin or nigericin, each added from ethanol stock solutions (Brown and Cooper, 1995). Addition of identical amounts of ethanol did not alter the activity. The fraction of right-side-out vesicles was determined by the detergent method (Carroll and Racker, 1977) with 0.1 % (w/v) dodecylmaltoside.

3.5. Proton pump experiment

The proton pump experiment was performed in a thermostated 2-ml glas vessel at 25°C. The pH change was measured with an Inlab® 410 pH-electrode from Mettler-Toledo and a Knick 766 Calimatic® pH-meter. The pH was recorded with a Philips PM8261 Xt-recorder. The concentration of proteoliposomes was 2 μM in the experimental setup. After incubation with 5 μM valinomycin (Sarti et al., 1985) for 3 min, the pH was adjusted to 7.3 with traces of 10 mM KOH or 10 mM HCl. The reduced horse heart cytochrome c solution was also adjusted to the same pH. Calibration was done by addition of known amounts of HCl. After recording of a base line, proteoliposomes were pulsed with reduced horse heart cytochrome c (16 nmol). Upon complete uncoupling of the proteoliposomes with 10 μM CCCP (Sarti et al., 1985), a new calibration with KOH was performed, and the pH was again adjusted with traces of KOH and HCl. The proteoliposomes were then pulsed again with the same amount of reduced horse heart cytochrome c. To determine the proton pump and water formation activities the signals were compared with the calibration bars (Solioz et al., 1982).
II. 4. Results and discussion

In order to perform proton-pumping experiments with proteoliposomes it was necessary to prepare cbb\textsubscript{3}-type oxidase from B. japonicum in mg quantity. This was achieved by expression of a modified fixNOQP plasmid that led to synthesis of an oxidase carrying the histidine-tagged FixN. Subsequent purification included Ni-NTA affinity chromatography, which proved to be effective for increasing the amount of oxidase that was solubilized from membranes. Figure 1 shows a silver-stained SDS-polyacrylamide gel (Laemmli, 1970) of the purified oxidase. FixN, the largest subunit migrates at ~45 kDa (Zufferey et al., 1998).

\begin{table}[h]
\centering
\begin{tabular}{c c}
\hline
kDa & cbb\textsubscript{3} \\
\hline
175 & \\
83 & \\
62 & \\
48 & FixN \\
33 & FixP \\
25 & FixO \\
17 & \\
7 & \\
\hline
\end{tabular}
\caption{SDS-PAGE of the purified cbb\textsubscript{3}-type oxidase (10\,\mu g). Protein subunits were visualized by silver stain. The apparent molecular masses of marker proteins are indicated on the left.}
\end{table}

The diheme c-type cytochrome FixP and the monoheme c-type cytochrome FixO are detected at the 32-kDa and 28-kDa positions, respectively. FixO and FixP were also detected by heme staining (not shown). With the improved oxidase expression and purification system the yield of oxidase was doubled (2.7 mg oxidase from 6 g wet cells) and oxidase activity with reduced horse heart cytochrome c as substrate (90 to 100 e/s; Table 1, see next page) was four times higher as compared with previously obtained values (Zufferey et al., 1998). The reduced cbb\textsubscript{3}-type oxidase (Fig. 2, see next page) showed absorption maxima in the α region characteristic for c- and b-type cytochromes (Zufferey et al., 1998).
Table 1. Characteristics of reconstituted cytochrome c oxidases

<table>
<thead>
<tr>
<th></th>
<th>B. japonicum cbb₃</th>
<th>P. denitrificans aa₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>Turnoverᵃ</td>
<td>90-100 e⁻/s</td>
<td>320 e⁻/s (Kleymann et al., 1995)</td>
</tr>
<tr>
<td>RCR</td>
<td>5.5 - 6</td>
<td>4.3 - 4.5</td>
</tr>
<tr>
<td>H⁺ pumping</td>
<td>0.2 - 0.4 H⁺/e⁻</td>
<td>0.5 - 0.6 H⁺/e⁻</td>
</tr>
<tr>
<td>H⁺ consumptionᵇ</td>
<td>0.9 - 1 H⁺/e⁻</td>
<td>0.9 - 1 H⁺/e⁻</td>
</tr>
</tbody>
</table>

ᵃOxidase solubilised in non-ionic detergent
ᵇWater formation

Fig. 2. Visible absorption spectra of 10µg air-oxidized (thin line) and dithionite-reduced (thick line) cbb₃-type oxidase.

Based on established procedures worked out with the aa₃-type cytochrome c oxidase of P. denitrificans (Solioz et al., 1982), we developed a reconstitution procedure for the B. japonicum cbb₃-type oxidase. Estimates of the proportion of right-side-out vesicles revealed that 70-80% of the vesicles contained the embedded oxidase with the cytochrome c binding site facing outwards. As illustrated in Fig. 3a, the oxidase activities of coupled versus uncoupled proteoliposomes differed by a factor of 5.5 to 6 (RCR value).
For comparison, and to control our experimental system, the RCR value of proteoliposomes containing *P. denitrificans* *aa*<sub>3</sub>-type oxidase was also determined and shown to be 4.3 to 4.5 (Table 1).

![Diagram](image)

Fig. 3. Oxidation of horse heart cytochrome c by the cbb<sub>3</sub>-type oxidase reconstituted in proteoliposomes. The reaction was started by addition of Covs containing 1.6 μmol oxidase. (a) The coupled state was monitored in the presence of 0.2% (v/v) ethanol. The uncoupled state was monitored after addition of 5 μM valinomycin (Val) and 10 μM CCCP, each dissolved in ethanol. (b) The same experimental setup as in panel a was used (without 0.2% (v/v) ethanol). After 30 sec of oxidation the established ΔpH across the membrane was abolished by addition of 10 μM CCCP. After another 20 sec, the membrane potential was disrupted completely with 5 μM valinomycin.

Next, we investigated the contribution of the ΔpH and the membrane potential to the coupled activity. Figure 3b shows that abolishing of the ΔpH with a protonophor (CCCP) after 30 sec of oxidation led to a two-fold increased activity. After disruption of the membrane potential with valinomycin, a further 2.5- to 3-fold increase in activity was observed. From these experiments it is evident that both, a protonophor and an ionophor, are responsible for the enhanced activity. We conclude that the proteoliposomes were relatively tight in terms of their permeability for protons and potassium ions. Horse heart cytochrome c was neither oxidized by blank liposomes nor by proteoliposomes pre-incubated with KCN (not shown). Thus, we conclude that the cbb<sub>3</sub>-type oxidase embedded into liposomes can couple electron flow to the generation of an electrochemical proton gradient.
To show whether protons are translocated across the vesicular membranes, we performed a proton pump experiment with a pH electrode. The observations are documented in Fig. 4. The experiment was performed in the presence of valinomycin to avoid a membrane potential that counteracts the proton extrusion. Upon a pulse of reduced horse heart cytochrome c to the proteoliposomes, a relatively rapid and pronounced acidification was observed. Following the immediate decrease of pH in the surrounding medium, an alkalization back to the original pH level occurred. This alkalization progressed further because of proton consumption inside the vesicles for the formation of H₂O.

![Fig. 4. Proton pump experiment with 2 nmol reconstituted B. japonicum cbb₃-type oxidase. In the presence of 5 μM valinomycin (Val), immediately upon a pulse of 16 nmol of horse heart cytochrome c (arrow), an acidification and subsequent alkalization of the surrounding buffer occurred. Addition of 10μM CCCP to the probe and again pulsing with the same amount of horse heart cytochrome c caused only an alkalization.](image)

Addition of CCCP disrupted ΔpH, and under this condition (with the same pulse of horse heart cytochrome c) only water formation was observed (Fig. 4). As a control, the same experiment was performed with the aa₃-type oxidase of P. denitrificans, which is a well-known proton pump (Solioz et al., 1982). The stoichiometries of proton pumping and proton consumption for water formation were calculated from these experiments and are listed in Table 1. With similar RCR values and ~1 H⁺/e⁻ for water formation, the reconstituted cbb₃-type oxidase had a substantially lower proton pumping activity (0.2H⁺/e⁻ to 0.4H⁺/e⁻) than the reconstituted aa₃-type oxidase. Moreover, water formation in the case of the cbb₃-type oxidase was always slower (Fig. 4) than that
observed with the \textit{aa}_3\text{-type oxidase (not shown). We do not have a reasonable explanation for this behavior.}

The lower level of proton translocation observed with the \textit{cbb}_3\text{-oxidase as compared with cytochrome \textit{aa}_3 may be due to the slow turnover of the enzyme (Table 1). Perhaps, horse heart cytochrome \textit{c} is not an ideal substrate for this oxidase, which, \textit{in vivo}, may obtain electrons from cytochrome \textit{c}_1 as the natural substrate. A low proton pumping efficiency was also shown recently for the \textit{cbb}_3\text{-type oxidase of \textit{Helicobacter pylori} (Tsukita et al., 1999), although precise values were not reported. Likewise, the \textit{in vivo} proton pumping of the \textit{cbb}_3\text{-type oxidase of \textit{P. denitrificans} was two times lower than that of the \textit{aa}_3\text{-type of the same organism (de Gier et al., 1996). In \textit{Rhodobacter sphaeroides}, an oxygen pulse experiment with a strain suffering multiple deletions of genes encoding alternative terminal oxidases, leaving the \textit{cbb}_3\text{-type oxidase as the solely remaining functional oxidase, a proton pumping activity with a value of 1H^+/e^- has been reported (Toledo-Cuevas et al., 1998).}

As mentioned, the \textit{cbb}_3\text{-type oxidase of \textit{B. japonicum} has a very high affinity for oxygen. In symbiotic conditions, the proton pump must support energy conservation under extremely low O_2 tensions and thus might be slow. When compared with the classical proton-pumping heme-copper oxidase such as cytochrome \textit{aa}_3, it is obvious that several of the conserved amino acid side chains of subunit I believed to form the proton translocating channels (Iwata et al., 1995) are missing in FixN (de Gier et al., 1996; Zufferey et al., 1998). In the \textit{P. denitrificans aa}_3\text{-type oxidase the so-called K-channel consists of Ser^{291}, Lys^{354}, Thr^{351} and Tyr^{280} and directs protons from the cytoplasm to the binuclear centre. The second, so-called D-channel consists of Asp^{124}, Asn^{113}, Asn^{131}, Asn^{199}, Tyr^{35}, Ser^{134}, Ser^{193} and Glu^{278} is believed to be for proton extrusion (Iwata et al., 1995; Schäfer et al., 1999). The residues corresponding to Asp^{124} and Glu^{278} are crucial for the proton pump activity of the \textit{bo}_3\text{oxidase of \textit{Escherichia coli} (Thomas et al., 1993; Verkhovskaya et al., 1997). Therefore, despite the similar setup of cofactors for redox chemistry, the \textit{cbb}_3\text{-type oxidase possibly uses a different route for proton translocation than the classical heme-copper oxidases. A similar suggestion has been made for some archaeal terminal oxidases (Gleissner et al., 1994; Schäfer et al., 1999).}
Chapter II

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CHAPTER III

Redox potentials of the $cbb_3$-type oxidase heme cofactors
III. 1. Introduction

Some respiratory complexes are associated with different cofactors like 2Fe-2S clusters or FMN (flavine mononucleotide) or cations in their subunits. Other respiratory complexes have cofactors like heme A, B, D, C and O. The latter proteins, also called cytochromes, are usually localized in biological membranes as subunits of larger complexes (oxidoreductases). Hence, cytochromes are hemoproteins that are often constituents of the electron transfer chain (Thöny-Meyer, 1997). The heme molecule carries a Fe-cation in its centre. If the Fe-cation is reduced by one electron, it is said to be the ferrous state (Fe$^{2+}$), whereas the oxidized form is called the ferric state (Fe$^{3+}$). A commonly used diagnostic characteristic of cytochromes is their absorption in the UV/VIS region (190nm–900nm). Each heme type in the peptide absorbs at specific wavelengths (for a review see Thöny-Meyer, [1997]).

The transfer of electrons from NADH$_2$ to O$_2$ is the main task of respiratory complexes (see chapter I). The electrons are passed from one redox centre to the next, where cations in hemoproteins become reduced and oxidized. Therefore, a redox potential for each cofactor in a protein or protein complex can be determined by electrochemistry. The redox potential quantitatively describes the affinity of peptide cofactors for electrons. Profound knowledge about the redox potential in a protein complex may help suggest the electron pathway through the enzyme complex. Large enzyme complexes, like terminal oxidases, are suitable for investigations by electrochemical methods (Hellwig, 1998; Moss et al., 1990). Since the oxidases have heme cofactors (see above) that facilitate their investigation with a spectrophotometer, they have been analyzed frequently. The reduction or oxidation of the cofactor can easily be followed by alteration of the redox potential in the bulk medium surrounding the oxidase, and by simultaneous detection of the absorption differences at a defined wavelength. The correlation between the absorption and the potential $A(p)$ of the bulk medium is described in the formula (next page), where $A_{\text{max}}$ is the absorption, $p$ is the potential, $E_m$ is the midpoint potential, $n$ is the amount of transferred electrons, $F$ is the Faraday constant, $R$ is the universal gas constant and $T$ is the absolute temperature. The formula (next page) is another type of the Nernst equation.
The equation mathematically describes the corresponding absorption (Hellwig, 1998) to each potential, which can be adjusted by the researcher. Redox titration of oxidases starts at −400mV (completely reduced conditions, and no O₂) and is performed until +400mV (completely oxidized conditions) in 50-mV steps. During each 50-mV step, equilibrium is achieved after 5 to 10min, and again an absorption spectrum is recorded.

If the absorption point is drawn to the corresponding potential, a mono-, bi- or oligophasic series of points is obtained (depending on the number of cofactors present). The fitting is achieved with a special software package, called MSPEK (Moss et al., 1990) for evaluation of data points from redox titrations. In the interactive modulation of the Nernst curve to the measured points, the midpoint potentials \( E_m \), the amplitude and the \( n \)-value (0.5-1) are varied as long as the best fit to the line connecting the points is achieved. The criterion for the best fit of the curve to the points depends upon the professional judgement of the researcher (Hellwig, [1998], and W. Mäntele, personal communication).

The \( cbb_3 \)-type oxidase of \( B. \) japonicum is a cytochrome c oxidase, which consists of three redox-active subunits (Preisig, 1994; Zufferey et al., 1996a; Zufferey et al., 1996b). The largest subunit, FixN (subunit I, \( M_r = 62300 \)), consists of 12 transmembrane helices. There are 6 strictly conserved histidines, like in all subunit I homologues of heme copper oxidases. FixN harbors the classical unique bimetallic centre of oxidases (\( b_3 \)-CuB), at which the reduction of O₂ to H₂O occurs (Garcia-Horsman et al., 1994a; Zufferey et al., 1996b). FixO (\( M_r =27300 \)) and FixP (\( M_r =31000 \)) are membrane bound mono- and diheme c-type cytochromes, respectively (Zufferey et al., 1997). FixQ (\( M_r =6000 \)) is the smallest subunit of the \( cbb_3 \) oxidase and is also membrane bound. It was demonstrated that the \( cbb_3 \) oxidase of \( B. \) japonicum, which is symbiotically essential, is a proton pump (Arslan et al., 2000). As of present, there is only little knowledge about the redox potentials of the cofactors of \( cbb_3 \)-type oxidases (Gray et al., 1994; Pereira, 2000). The main goal of this project was to identify the redox-active cofactors in the three subunits (FixN, FixO and FixP) by a suitable electrochemical method (OTTLE, optically transparent thin-layer electrochemistry),
and to propose an electron transport route through this terminal oxidase, based on the redox potentials measured. The results were to be discussed and embedded into the current knowledge of cbb₃ oxidases from other bacteria (Gray et al., 1994; Pereira et al., 2000a).

III. 2. Materials and Methods

Sample preparation. The cbb₃-type oxidase of B. japonicum was purified as described by Arslan et al. (2000). The buffer was exchanged with Centricon™, 100 kDa cut-off membrane, to 100 mM KP₄-buffer, pH 7, with 100 mM KCl and 0.01% (w/v) n-dodecyl-β-D-maltoside. During the exchange of the buffer, the oxidase was concentrated to approximately 0.2 mM (based on heme C absorption at 551nm). Six to eight μl of this concentrated probe was used for electrochemistry and redox titration.

Electrochemistry. The ultra-thin-layer spectrophotometric cell for UV/VIS was used as previously described (Hellwig, 1998). The gold grid working electrode was chemically modified by a 2 mM cysteamine solution as reported (Hellwig et al., 1998). To accelerate the redox reaction, 16 different mediators were added, as stated in Hellwig et al. (1998), to a final concentration of 45 μM each. At this concentration, no spectral contributions from the mediators in the VIS range were detected in control experiments lacking the protein (Hellwig et al., 1998). Potentials quoted in the Results section refer to the Ag/AgCl/3M KCl reference electrode; therefore, one must add +210 mV (pH7) for SHE' (standard hydrogen potential at pH7) to the potentials.

VIS-Spectroscopy. Difference spectra, as a function of the applied potential, were obtained from the sample. Firstly, the protein was equilibrated with an initial potential (-400mV) at the electrode, and single-beam spectra in the VIS range were recorded. The reduced oxidase consumes, at this stage, all available O₂ to form H₂O in the sample. Therefore, anaerobic conditions are generated. A potential step (+25mV) toward the final potential (+400mV) was then applied, and again a single-beam spectrum of this state was recorded after equilibration (4-6 min). Difference spectra were then calculated from the two single-beam spectra with the initial spectrum taken as reference. After each potential-step equilibration, a further single-beam spectrum was
recorded and subtracted from the initial step as reference. The protein was cooled down to 5°C during VIS-spectroscopy for redox titration.

*Redox-titration.* The redox-dependent absorbance changes of the *cbb*₃-type oxidase of *B. japonicum* were studied by performing electrochemical redox titration in the VIS range. Stepwise setting of the potential was achieved as described above. The midpoint potentials $E_m$, and the number $n$ of transferred electrons were obtained by adjusting a calculated Nernst curve to the measured absorbance change at single wavelengths (560 nm for heme B, 549 nm for heme C) by an interactive fit (MSPEK). All parameters had to be adjusted manually until the theoretical Nernst curve and the measured data matched well (*fit by eye*) (Hellwig, 1998; Hellwig *et al.* 1998; Hellwig *et al.*, 1999a; Hellwig *et al.*, 1999b).
III. 3. Results and discussion

*Electrochemically Induced VIS Difference Spectra.* Figure 1A (next page) shows the completely reduced and oxidized absolute spectrum of the cbb\(^3\)-type oxidase of *B. japonicum*. Figure 1B demonstrates the reduced minus oxidized VIS-difference spectrum of the cbb\(^3\)-type oxidase of *B. japonicum* obtained for the large potential step from \(-400\text{mV}\) to \(+400\text{mV}\) (vs Ag/AgCl/3M KCl). In the reduced minus oxidized spectrum, the negative values correspond to the oxidized spectra while the positive values correlate to the reduced form of the enzyme as it is shown in Figure 1A. The difference spectra are exact mirror images of the oxidized minus reduced spectra (data not shown), indicating the full reversibility of the electrochemical reaction. Similar results were obtained with dithionite-reduced minus air-oxidized difference spectroscopy of the cbb\(_3\) oxidase (Arslan et al., 2000; Preisig, 1994; Zufferey et al., 1998). The difference in values observed, in the range of 500 nm to 600 nm, can be assigned to absorbance contributions from cytochromes *b* and *c* (Thöny-Meyer, 1997). The absorbance maximum for heme *B*, including neglectable influences of heme *C* in the \(\alpha\) region, was determined to be 560 nm; whereas for heme *C* it was determined 549 nm. It was impossible to find a corresponding wavelength pair in the \(\gamma\) region of the VIS spectrum.

*VIS Redox Titration.* Initial observations indicated the disappearance of the heme *B* centres’ absorption during oxidative titration. Further increase of the potential led to the disappearance of heme *C* moiety absorbancy. The same result, but *vice-versa*, was observed for reductive titration, i. e., beginning with the appearance of heme *C* and then heme *B* (data not shown). This result suggests a flow of electrons from the heme *C* centres to the heme *B* centres, which seems evident, because the terminal reduction of \(O_2\) occurs at high-spin heme *B*-Cu\(_B\) binuclear centre (Garcia-Horsman *et al.*, 1994a; Garcia-Horsman *et al.*, 1994b).
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Fig. 1A. Electrochemically induced absolute spectra of the reduced (solid-line) and the oxidized (dotted-line) cbb₃-type oxidase of *B. japonicum*. Reduction of the oxidase was achieved by setting the potential to \(-400\) mV, whereas the oxidation was achieved with \(+400\) mV.

Fig. 1B. Electrochemically induced VIS-difference (red minus ox) spectrum of the *cbb₃*-type oxidase of *B. japonicum*. 
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Fig. 2A. Potentiometric, oxidative titration of the cbb₃-type oxidase of B. japonicum. The difference spectrum was followed at 549 nm, as described in Material and Methods. The potentials (3 inflection points of the graph, see arrows) are against Ag/AgCl/KCl (X-axes) electrode. As explained in Material and Methods, +210 mV must be added for obtaining the midpoint-potential of the centres.

Figure 2A shows the points for the oxidative titration of the cbb₃ oxidase of B. japonicum in the −400 mV to +400 mV range, obtained after ‘step-by-step’ oxidation of the heme C moieties followed at 549 nm. A triphasic Nernst curve fit was achieved, unambiguously indicating the presence of three heme C moieties in the cbb₃ oxidase. The evaluated $E_m^1$, $E_m^2$ and $E_m^3$ values (midpoint-potentials, inflection points) were $-300 \text{ mV (±15 mV, } n_1 = 0.8)$, $-120 \text{ mV (±15 mV, } n_2 = 0.6)$ and $+120 \text{ mV (±10 mV, } n_3 = 0.7)$, respectively. These are the same midpoint potentials, which were revealed in reductive redox titration (data not shown). It is not possible to assign definitely to which of the subunits (FixO or FixP) these potentials belong; however, one inflection point must belong to FixO as a monoheme c-type cytochrome and the other two must belong to FixP, the diheme c-type cytochrome.

In the work of Gray et al. (1994) midpoint-potentials of the cbb₃-type oxidase of R. capsulatus were reported. Anaerobic potentiometric titration was performed, as described by Dutton (1978) and cannot be compared with the method used in our project (Hellwig, 1998). The results were +265 mV and +320 mV (adjusted to SHE’). The sheer presence of two heme C binding sites in CcoP and one heme C binding site in
CcoO would lead to the expectation of resolving three different redox transitions. However, this was not the case in the \( cbb_3 \) enzyme of \( R. \) capsulatus. The authors discuss this discrepancy, but they could only conclude that at least two redox-active c-type centres are present (Gray et al., 1994). There is no information regarding the expected third centre in that work. The assignment of the two midpoint potentials was that +265mV belongs to CcoP and +320mV to CcoO (Gray et al., 1994). These assignments were based on the potentiometric titration of mutant membranes lacking all c-type cytochromes except CcoO (M7G-CBC1, Gray et al., 1994). Titration of membranes isolated from this strain demonstrated the presence of one c-type redox-active centre with a midpoint-potential of +320mV. Hence, it was assigned to CcoO. This assignment demands an electron flow from CcoP to CcoO, with the first electron acceptor CcoP obtaining the electrons from a yet unknown cytochrome c, and then downwards to the reduction of the heme B centres of the \( cbb_3 \) oxidase.

Also in the case of \( Rhodothermus \) marinus, only two redox-active c-type hemes (-50mV and +195mV) were identified for the purified \( cbb_3 \) oxidase. The authors conclude that the mono-heme c-type cytochrome (FixO homologue) was lost during purification, or it is not present in this organism (Pereira et al., 2000a). However, the measured cytochrome c oxidation activity with reduced horse heart cytochrome c argues against the possibility of having lost a subunit. One of the identified potentials is close to a proposed potential in FixP in \( B. \) japonicum (see above, -90mV), whereas the other is not (see above, +195mV). Either, they lost FixO during purification, or this organism has no FixO (not present on a SDS-PAGE). The authors comment that perhaps FixO is not needed for an electron flow at high temperatures (60°C), where this organism has its habitat.

In the \( cbb_3 \) enzyme of \( B. \) japonicum three redox-active c-type heme centres were unambiguously identified. One potential (+330mV, adjusted to SHE') is close to the one of CcoO (+320mV) of \( R. \) capsulatus. Therefore this potential might result from FixO in the \( cbb_3 \) oxidase of \( B. \) japonicum, suggesting that the remaining two would belong to FixP (-90mV and +90mV, adjusted to SHE'). This energetically descending but, redox-potential-wise ascending order from FixP to FixO makes biologically sense and fits with previous results which came out of the assembly studies done by Zufferey et al. (1996). It was demonstrated that FixNO could build a stable precomplex in the membranes without the presence of FixP. Furthermore, first attempts to purify the \( cbb_3 \) oxidase of \( B. \) japonicum resulted in loosing FixP from the rest of the complex (FixNO).
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(R. Zufferey, personal communication). This result indicates that FixP is not tightly interacting with the precomplex, and may underline its putative function as a natural electron donor for the FixNO precomplex. In the work of Keefe and Maier (1993) it was demonstrated that the cbb\textsubscript{3}-type oxidase of \textit{B. japonicum} was copurified together with the \textit{bc}_{1}\textit{c}-complex from bacteroids. This result may underline the possibility that the \textit{c}_{1} cytochrome of the \textit{bc}_{1}\textit{c}-complex may serve as a natural electron donor for FixP (see Fig. 3).

The potential-dependent development of the \textalpha region absorption at 560nm (Figure 2B) is a ‘step-by-step’ oxidation of the heme B moieties.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2b.png}
\caption{Midpoint potential (Ag/AgCl/KCl) (V) \textit{Fig. 2B.} Potentiometric, oxidative titration of the \textit{cbb}_{3}-type oxidase of \textit{B. japonicum}. The difference spectrum was followed at 560 nm, as described in Material and Methods. The indicated potentials (2 inflection points of the graph, see arrows) are against Ag/AgCl/KCl (X-axes) electrode. As explained in Material and Methods, +210mV must be added to arrive at the midpoint-potential of the centres.}
\end{figure}

In Figure 2B, a biphasic Nernst curve fit was obtained, indicating the presence of two redox-active heme B centres in the \textit{cbb}_{3} oxidase of \textit{B. japonicum}. The evaluated \textit{E}_{m1} is about \textminus270mV (±20mV, \textit{n}_{1} =0.8) whereas \textit{E}_{m2} is about +170mV (±15mV, \textit{n}_{2} =1). The same midpoint potentials were achieved in the reductive redox titration (data not shown).

The work of Gray \textit{et al.} (1994) demonstrated only one redox-active heme B centre (+380mV). This potential was assigned to the low-spin heme B in the \textit{cbb}_{3} oxidase of \textit{R.}
capsulatus. The discussion of a high-spin heme B is completely missing. In the work of Pereira et al. (2000a), two heme B centres were identified (−50mV and +120mV, adjusted to SHE'). They assigned the −50mV centre to the high-spin heme B and the +120mV centre to the low-spin heme B. These assignments were deduced from the work of Verkhovsky et al. (1995) with the aa3-type heme copper oxidase. In that work, it is described that the low-spin heme has a potential, which is by 200 mV higher (i.e., more positive) than that of the a3 high-spin centre. After the transfer of the electron to heme A, the electron is in a ‘thermodynamic hole’. It is not clear, how the electron then is transferred to heme A3. Perhaps the reductive or oxidative state of the hemes, or proton translocation through the oxidase, influence these potentials (Verkhovsky et al., 1995) in vivo.

Taking the work of Verkhovsky et al. (1995) into consideration, we suggest that the higher potential might be assigned also in the case of FixN (Fig. 2B) to the low-spin heme B moiety, whereas the lower potential might be assigned to the high-spin heme B. One potential (+380mV, adjusted to SHE') might be assigned to the low-spin heme B, and the other potential (−60mV, adjusted to SHE') to the high-spin heme B in the binuclear centre, if one follows the rationale in the work of Verkhovsky et al. (1995).

The redox-properties of the three cbb3-type oxidases, which have been purified and redox-titrated up to now, are summarized in Table 1.

<table>
<thead>
<tr>
<th>Redox centres in cytochromes</th>
<th>R. marinus</th>
<th>R. capsulatus</th>
<th>B. japonicum</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-type</td>
<td>−50mV</td>
<td>+265mV</td>
<td>−90mV</td>
<td>FixP, CcoP</td>
</tr>
<tr>
<td></td>
<td>+195mV</td>
<td></td>
<td>+90mV</td>
<td>FixP, CcoP</td>
</tr>
<tr>
<td></td>
<td>not present</td>
<td>+320mV</td>
<td>+330mV</td>
<td>FixO, CcoO</td>
</tr>
<tr>
<td>b-type (low-spin)</td>
<td>+120mV</td>
<td>+380mV</td>
<td>+380mV</td>
<td>FixN, CcoN</td>
</tr>
<tr>
<td></td>
<td>−50mV</td>
<td></td>
<td>−60mV</td>
<td>FixN, CcoN</td>
</tr>
<tr>
<td>b-type (high-spin)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Summary of the known redox-potentials (adjusted to SHE') in cbb3-type oxidases taken from Gray et al. (1994), Pereira et al. (2000a), and from the results of this chapter.
In the following, a hypothesis is presented that might serve as a model of how the electrons flow through the \( cbb_3 \) oxidases.

![Diagram of electron flow through oxidases](image)

**Fig. 3.** Putative model of the electrons transferred through the \( cbb_3 \) oxidases. All knowledge about the redox potentials is included. The electrons come from an unknown cytochrome \( c \), possibly \( c_1 \), and are first transferred to FixP (CcoP, or its homologue in \( R. m. \)). Then the electrons are passed to FixO (CcoO) in \( R. c. \) (B. japonicum) or \( R. c. \) (R. capsulatus). In \( R. m. \) (R. marinus) no mono-heme \( c \)-type cytochrome was detected (Pereira, 2000a), therefore it is assumed that the electrons pass directly from the FixP homologue to the FixN homologue. Black, diagonal bars represent the hemes, and the circle ( ● ) stands for the \( Cu\) atom.

In summary, the model is based on known potentials of other \( cbb_3 \)-type oxidase subunits as well as on the potentials measured in this chapter. Further experiments would be needed to present a conclusive result concerning the order with which these oxidases transfer their electrons. Furthermore, even though the exact midpoint potentials of the cofactors in the \( aao_3 \)-type oxidase are known, details regarding the electron flow through this oxidase are only roughly known. Models of the flow (see above) of electrons, based on comparable available data, have been suggested (Verkhovsky et al., 1999; Verkhovsky et al., 1995).
CHAPTER IV

Overproduction of the *Bradyrhizobium japonicum* c-type cytochrome subunits of the *cbb*$_3$ oxidase in *Escherichia coli*

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Overproduction of the *Bradyrhizobium japonicum* c-type cytochrome subunits of the *cbb*$_3$ oxidase in *Escherichia coli*

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IV. 1. Abstract

We report on a system to improve expression of mature c-type cytochromes in *Escherichia coli*. It is based on the use of plasmid pEC86 that expresses the *E. coli* cytochrome c maturation genes *ccmABCDEFGH* constitutively, whereby the production of both endogenous and foreign c-type cytochromes was increased substantially. The periplasmic soluble domains of the c-type cytochrome subunits FixO and FixP of the *B. japonicum cbb_3* oxidase could be expressed in *E. coli* only when pEC86 was provided in a *degP*-deficient strain. This shows that a stimulation of heme attachment by the Ccm maturase system combined with the diminished proteolytic activity in the periplasm can increase c-type cytochrome yields.

*Abbreviations:* PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; TMAO, trimethylamine-N-oxide
**Chapter IV**

**IV. 2. Introduction**

*Escherichia coli* produces c-type cytochromes under anaerobic conditions when grown in the presence of nitrite, nitrate or TMAO as the terminal electron acceptors. The covalent attachment of heme to the apocytochromes occurs in the periplasm and requires the function of eight cytochrome c maturation genes, *ccmABCDEFGH*, which are expressed in the so-called aeg46.5 operon under anaerobic conditions (Grove *et al.*, 1996; Page *et al.*, 1998; Thöny-Meyer *et al.*, 1995). These genes encode membrane proteins that comprise an ABC transporter (Kranz *et al.*, 1998; Thöny-Meyer, 1997), a periplasmically oriented thioredoxin system (Fabianek *et al.*, 1998), a heme chaperone (Schulz *et al.*, 1998), and a putative heme lyase (Kranz *et al.*, 1998; Page *et al.*, 1998; Thöny-Meyer, 1997). It was shown previously that a chromosomal deletion of the *ccm* genes led to a complete loss of holocytochrome c formation (Grove *et al.*, 1996; Thöny-Meyer *et al.*, 1995). When the *ccm* genes were provided on a plasmid together with the structural genes for the NapB and NapC c-type cytochromes of the periplasmic nitrate reductase, cytochrome c maturation was restored and even seemed to be increased (Thöny-Meyer and Künzler, 1997).

Cytochromes of the c-type are interesting molecules for structural and spectroscopic studies. Therefore, a system in which they can be overproduced is highly desirable. Overproduction of various heterologous c-type cytochromes has been attempted in *E. coli*, however, with variable success (Thöny-Meyer *et al.*, 1994b). For example, we were able to produce the soluble holocytochrome *c*₅₅₀ of *Bradyrhizobium japonicum* (Bott *et al.*, 1995) in *E. coli*; yet, this was achieved only under anaerobic, nitrate-dependent growth conditions, and the yield was rather low (Thöny-Meyer *et al.*, 1996). While studying the *B. japonicum cbb₃* type heme copper oxidase (Preisig *et al.*, 1996b) it became of particular interest to overproduce the mono- and diheme cytochrome c subunits II (FixO) and III (FixP) in *E. coli* in order to analyze their biochemical properties. Here we present an expression system for c-type cytochromes that makes use of a plasmid from which the *ccm* genes are expressed constitutively and independently of oxygen control. Co-expression of the *ccm* genes together with a structural gene for apocytochrome c can lead to high levels of holocytochrome c. To produce significant amounts of mature FixO and FixP in *E. coli*, it was necessary to use a strain in which the gene encoding the periplasmic protease DegP was defective (Strauch *et al.*, 1989).
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IV. 3. Materials and methods

Bacterial strains and growth conditions- E. coli MC1061 (hsdR araD139 Δ(araABC-leu)7679 galU galK Δ(lac) ΦX74 rpsl. thi) (Meissner et al., 1987) and HM125 (F ΔlacX74 galE galK thi rpsL(strA) ΔphoA degP41::ΩKan' eda51::Tn10(Tet') rpoH15 (Strauch et al., 1989) were used as hosts for cytochrome c expression. Aerobic growth was in LB medium (Miller, 1992), anaerobic growth in MS medium with either 20 mM NO₃⁻, 2.5 mM NO₂⁻ or 20 mM TMAO (Iobbi-Nivol et al., 1994). Antibiotics were added at the following final concentrations: ampicillin 200 μg ml⁻¹, chloramphenicol 10 μg ml⁻¹. Cells were grown at 37°C for expression of the B. japonicum cytochrome c₅₅₀ and the B. subtilis cytochrome c-550. Cells were grown at 30°C for expression of FixO and FixP. Cytochrome c-specific gene expression was induced at a cell density of A₆₀₀ = 0.6 with 0.8% arabinose for 4h.

Construction of plasmids. For the construction of pEC86, a PstI linker was fused in a first step to the EcoRV site of the pACYC184 vector in pEC66 (Thöny-Meyer et al., 1996), resulting in pEC83. This plasmid contained the napBCccmABCDEFGH sequence in the tet gene of the vector in the same orientation, thus allowing outreading transcription from the tet promoter. It was opened with PstI/AflIII to delete the napBCccmAB' segment, and the 1.2-kb NsiI/AflII fragment containing ccmAB' was inserted to restore an intact ccmABCDEFGH sequence.

To express a soluble FixO protein (FixOₙₕ), a Stul site was introduced at codons 33-35 of the fixO sequence by PCR and ligated with a Stul site at the end of the coding sequence for the OmpA signal peptide (21 codons). This resulted in an additional serine codon between the OmpA coding sequence and L₃₆ codon of fixO. The ATG start codon of the ompA gene was engineered into a Ndel site by PCR. The ompA'-fixO fusion was then cloned as an Ndel-XbaI fragment into the expression vector pISC-2 downstream of the arabinose-inducible p ara promoter to give pRJ4646 (Thöny-Meyer et al., 1996). A ompA'-fixP fusion was constructed on pRJ4591 using the same strategy. In this case the OmpA signal sequence was fused to FixP₅₄-2₉₀ at a Stul site introduced at codon 53 of FixP, again creating an additional serine codon. All PCR products and fusion sites were confirmed by sequencing.
Preparation of membranes and periplasmic fraction - Membranes were prepared from 1 l of stationary-phase grown cells as described previously (Thöny-Meyer and Künzler, 1997). Periplasmic fractions were prepared from 250 ml of aerobically grown cells induced for cytochrome c expression using lysozyme (Thöny-Meyer et al., 1996). For small-scale preparations, the treatment was applied to cells of 10 ml cultures of an $A_{600} = 0.6$, and the entire periplasmic fraction was precipitated with TCA.

Characterization of c-type cytochromes - Protein determination, SDS-PAGE, heme stains using 3,3'-dimethoxybenzidine, and Western blot analyses with antibodies against FixO and FixP were performed as described (Zufferey et al., 1996a), except that detection was done with 3-(4-methoxyspiro{1,2-dioxetan-3,2'- (5’chloro)tricyclo[3.3.1.13,7]decan}-4yl)phenylphosphate (CSPD; Boehringer, Mannheim, Germany) and goat anti-rabbit IgG alkaline phosphatase conjugate (BioRad, Glattpbrugg, Switzerland) as the secondary label. Optical difference spectra were recorded on a Hitachi model U-3300 spectrophotometer (Preisig et al., 1996b).
IV. 4. Results

*Overproduction of c-type cytochromes with pEC86 encoding the ccm genes*

We have previously established an expression system for the soluble monoheme cytochrome $c_{550}$ of *B. japonicum* in *E. coli* (Thöny-Meyer et al., 1996). The structural gene *cycA* was expressed from an arabinose-inducible promoter $p_{ara}$ in an *E. coli* MC1061 background under anaerobic growth conditions in the presence of nitrate. Under aerobic growth conditions, no holocytochrome $c_{550}$ was obtained. We reasoned that under such growth conditions a limitation of *ccm* gene products, which are expressed from the Fnr-dependent *aeg* promoter (Choe and Reznikoff, 1993), might have caused the absence of cytochrome $c$ maturation. Therefore, we constructed a plasmid that expressed the *ccm* genes from a Fnr-independent promoter. Plasmid pEC86 is derived from the vector pACYC184 and contains the *ccm* genes downstream of the *tet* promoter. Unlike the previously described plasmid pEC66 (Thöny-Meyer and Künzler, 1997), it did not contain the *napBC* genes encoding two of the endogenous *E. coli* c-type cytochromes and was therefore more suitable to enhance the formation of heterologous c-type cytochromes. Fig. 1A shows that pEC86 can in fact induce maturation of the *B. japonicum* cytochrome $c_{550}$ even under aerobic conditions. Stimulation of cytochrome $c$ maturation was also obtained under anaerobic conditions in the presence of nitrate (Fig. 1B). In this case, the chromosomally expressed *napB* gene product was also produced at detectable levels in the periplasm (Fig. 1B, lane 2).

```
<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
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<tbody>
<tr>
<td></td>
<td>aerobic</td>
<td>anaerobic</td>
</tr>
<tr>
<td>pRJ3268</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pEC86</td>
<td>-</td>
<td>+</td>
</tr>
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</table>
```

**Fig.1.** Overproduction of *B. japonicum* cytochrome $c_{550}$ in aerobically (A) or anaerobically (B) grown *E. coli*. Periplasmic protein obtained by small-scale preparations was TCA precipitated and separated by 15% SDS PAGE. The $c$-type cytochromes were visualized by heme staining.
Expression of a membrane-bound c-type cytochrome

It was shown previously that expression of the membrane-bound Bacillus subtilis cytochrome c-550 (encoded by cccA) in E. coli was possible under aerobic conditions (Jones and Poole, 1985), but required the presence of the ccm gene cluster (Thöny-Meyer et al., 1995). Although expression of the nap-ccm operon is induced anaerobically, sufficient amounts of Ccm polypeptides appear to be made under aerobic conditions to support maturation of the CccA polypeptide. We tried to stimulate the production of B. subtilis cytochrome c-550 by co-expressing the ccm genes on pEC86. Fig. 2 shows that similar levels of this cytochrome were produced independently of the presence of pEC86. When the ccm genes were co-expressed, the membranes accumulated the heme-binding form of the heme chaperone CcmE (Schulz et al., 1998), as visualized by heme staining. This result indicates that expression of the cccA gene from its own Bacillus promoter may be the limiting step for overproduction.

![pLUW1984 + + pEC86 - + CcmE Bs c-550](image)

Fig. 2. Overproduction of B. subtilis cytochrome c-550 in E. coli. Membrane proteins (40 µg per lane) were separated by 15% SDS-PAGE and proteins with covalently bound heme were visualized by heme staining.
**Chapter IV**

*Overproduction of the subunits FixO and FixP of the B. japonicum cbb\textsubscript{3}-type oxidase*

We were particularly interested to overproduce the *B. japonicum* c-type cytochrome subunits of the cbb\textsubscript{3} oxidase as individual proteins. To facilitate overproduction of these cytochromes that are normally membrane-anchored by an N-terminal, hydrophobic transmembrane helix, we constructed soluble, periplasmic versions by replacing the transmembrane helices with a cleavable OmpA signal sequence. It was shown previously, that FixO and FixP are extremely unstable in *B. japonicum* if their assembly with cofactors and other subunits of the FixNOP complex was disturbed (Zufferey et al., 1996a). A soluble version of FixO was constructed by fusing the cleavable OmpA signal sequence to Leu\textsubscript{36} of FixO. Expression of the fused gene was again from the para promoter. No mature FixO protein was obtained in the periplasmic fraction of induced, anaerobically grown MC1061 cells. However, when whole cell extracts of induced cells were loaded directly on an SDS polyacrylamide gel, a weak signal was obtained in a Western blot (not shown), which indicated that apocytochrome was formed and rapidly degraded. To improve holocytochrome c formation, fixO was co-expressed with the ccm genes on pEC86; however, maturation of FixO\textsubscript{sol} was still not observed (not shown). Next we combined this approach with the use of a strain that was deficient in the periplasmic protease DegP (Strauch et al., 1989). Mature FixO protein was detected as a 25-kDa heme-staining protein in periplasmic fractions of induced cells (Fig. 3, top panel, lane 2), which was in agreement with the theoretical molecular mass of 23'548 Da of FixO\textsubscript{sol} protein.

Maximal expression was obtained after 4h of induction with 0.8% arabinose (not shown). Western blot analysis (Fig 3, middle panel, lane 2) confirmed the identity of overexpressed FixO\textsubscript{sol}; As sometimes observed with cytochromes c, the protein migrated as a double band.

Can this system also be used for overproduction of the diheme cytochrome FixP? A similar construct with the cleavable OmpA signal sequence replacing the N-terminal membrane anchor amino acids (1-53) of Fix P was introduced into the *degP* strain, and cytochrome c expression was induced with arabinose. Again, FixP holoprotein was detected in the periplasm. The protein detected by heme stain (Fig. 3, top panel, lane 4) and Western blot analysis (Fig. 3, bottom panel, lane 4) migrated as a double band at about 25 kDa, again corresponding to the predicted molecular mass of 24’868 Da of the FixP\textsubscript{sol} polypeptide.
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<table>
<thead>
<tr>
<th>arabinose</th>
<th>pRJ4646</th>
<th>pRJ4591</th>
<th>cbb3</th>
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<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
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heme

stain

FixP

FixO

Anti FixO

FixO

FixOsol

Anti FixP

FixP

FixPsol

Fig. 3. Overproduction of soluble versions of the B. japonicum FixO and FixP proteins. Top panel: heme stain of periplasmic fractions (20 µg per lane) with overproduced FixOsol from pRJ4646 and FixPsol from pRJ4591, respectively. Purified cbb3-type cytochrome oxidase (2 µg) was loaded on the right as control (lane 5). Below are Western blots of identical gels probed with anti-FixO (middle panel) and anti-FixP (bottom panel) immunoglobulins (bottom panel).

The soluble FixO and FixP proteins were characterized spectrophotometrically. Reduced minus oxidized difference spectra of periplasmic fraction containing either FixO or FixP are shown in Fig. 4. The \( \alpha \)-absorption maxima of the difference spectrum of both proteins were at 551 nm, which is in good agreement with the symmetric peak at the same wavelength of the purified cbb3-type cytochrome oxidase that contains all three hemes C (Preisig et al., 1996b). We obtained only for the FixPsol, but not for the FixOsol protein, a typical CO-difference spectrum that has also been described for the entire cbb3-type oxidase. We conclude that the FixP protein is the CO-reactive c-type cytochrome of this oxidase. The yield of FixOsol and FixPsol was calculated to be 0.34 mg and 0.18 mg, respectively, per ml periplasmic fraction, using a molar extinction coefficient of 19.1 mM\(^{-1}\) cm\(^{-1}\) (Jones and Poole, 1985). These values corresponded to 3.5% and 2.4%, respectively, of the total periplasmic protein.
Fig. 4. Spectrophotometric characterization of FixO_{sol} and FixP_{sol}. A and B are the dithionite-reduced minus air-oxidized absorption difference spectra of periplasmic fractions with overproduced FixO_{sol} (0.49 mg ml^{-1} periplasmic protein) and FixP_{sol} (0.38 mg ml^{-1} periplasmic protein), respectively. C, dithionite + CO minus dithionite-reduced difference spectrum of the same fraction as in B.
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IV. 5. Discussion

We have established a system that allows overproduction and maturation of various c-type cytochromes in *E. coli*. It is based on two characteristics known to play a role in cytochrome c biogenesis. First, some apocytochromes appear to be extremely unstable polypeptides that are rapidly degraded in the periplasm if they do not bind heme. The use of a *degP*<sup>−</sup> strain reduces periplasmic proteolytic activity and thus may enhance the chances of heme binding. Second, high amounts of *ccm* gene products have been observed to stimulate heme attachment. The recently identified function of CcmE as a periplasmic heme chaperone that binds heme temporarily and releases it when apocytochrome c substrate is available (Schulz *et al.*, 1998) illustrates that restricted *ccm* gene expression might be limiting for the production of large amounts of holocytochrome c. Plasmid pEC86 provides a tool for constitutive *ccm* gene expression and in particular facilitates aerobic cytochrome c maturation. It can also be used to increase the amounts of endogenous c-type cytochromes.

In this work, we have used the *degP*<sup>−</sup>/pEC86 system to produce mature FixO and FixP proteins. These proteins are the subunits of the *B. japonicum cbb<sub>3</sub>*-type oxidase and are thought to be the electron donors for the reduction of O<sub>2</sub> at the binuclear heme-copper centre in FixN. It has not been possible to obtain mature FixO or FixP protein in the absence of the FixN subunit, which by itself is a b-type cytochrome. The reason for this may be lack of stability of the isolated subunit (Zufferey *et al.*, 1996a). When these subunits were overexpressed individually in *E. coli* we observed again a great instability of the apocytochromes. Mature holocytochromes could not be detected unless a *degP*<sup>−</sup> mutant strain was used to protect the polypeptides from periplasmic proteolysis. We succeeded at producing 1.36 mg FixO and 0.37 mg FixP per liter of cultured cells, which was sufficient to determine the spectroscopic characteristics of these proteins. In the reduced minus oxidized difference spectrum they showed α-bands with a maximum at 551 nm, respectively. FixP was CO-reactive, thus confirming the trough at 552 nm that had been detected previously for the entire *cbb<sub>3</sub>*-type oxidase (Preisig *et al.*, 1996b).

The *B. subtilis* membrane-bound cytochrome c-550 is expressed and matured in aerobically grown *E. coli* without pEC86. Although maturation of this cytochrome c depends on the *ccm* genes (Thöny-Meyer *et al.*, 1995), no stimulation of cytochrome c-550 production in the presence of pEC86 was observed. This indicates that low levels of *ccm* gene products must be synthesized already under aerobic conditions, and these are
sufficient for maturation of the entire pool of *B. subtilis* CccA polypeptide. The CccA polypeptide appears to be relatively stable even when heme is not bound to it, perhaps due to its localization to the membrane. By contrast, the *B. japonicum* CycA apocytochrome was shown to be extremely sensitive towards periplasmic degradation (Thöny-Meyer *et al.*, 1996). The latter cytochrome may be protected from proteolysis by interaction with, and heme incorporation by, the Ccm machinery, whereas the former may use the Ccm-maturase system only for heme attachment and not for additional stabilization.

In summary, plasmid pEC86 has been proven to be a valuable tool to enhance maturation and, therefore, production of soluble cytochromes of the c-type produced in *E. coli*. This will be of particular interest for applications such as structural and spectroscopic characterizations that require large amounts of mature protein to be produced from minimal culture volumes.

**Acknowledgments**

We thank G. Georgiou for providing the *degP*− strain, A. Hungerbühler for excellent technical assistance. H. Hennecke is greatly acknowledged for advice and generous support. This work was supported by the Swiss National Foundation for Scientific Research.
CHAPTER V

Cytochrome $bd$, a fifth terminal oxidase in the branched respiratory chain of *Bradyrhizobium japonicum*

*Draft of a manuscript for the* Journal of Bacteriology (2001)
Chapter V

Cytochrome bd, a fifth terminal oxidase in the branched respiratory chain of Bradyrhizobium japonicum

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Chapter V

V. 1. Abstract

*Bradyrhizobium japonicum* possesses a branched respiratory chain that is terminated by disparate oxidases belonging to the family of heme-copper oxidases. Here, we report on the discovery of the genes *cydAB* coding for the subunits of a *bd*-type oxidase belonging to a different oxidase family. These genes are closely linked with an upstream gene, *mqo*, that encodes a malate:quinone oxidoreductase. A *cydA*-‘*lacZ* fusion was expressed weakly under aerobic conditions in the presence of malate and/or lactate, suggesting that the *bd*-type oxidase is used as a terminal electron acceptor during malate oxidation. A *cydA::kan* insertion mutant was constructed and found to be required neither for aerobic growth under free-living conditions, nor for symbiotic nitrogen fixation in root nodule bacteroids. A *bd*-type oxidase was not detectable in membranes of *B. japonicum* grown under different conditions, most likely due to the low expression. Over-expression of the *cydAB* genes in an *Escherichia coli* strain deficient in its own *bd*-type oxidases revealed spectroscopic features of the *B. japonicum* enzyme characteristic for a *bd*-type oxidase. We conclude that the *B. japonicum* respiratory chain has a fifth branch that is terminated by this newly identified enzyme.
Chapter V

V. 2. Introduction

*Bradyrhizobium japonicum* is a diazotrophic, Gram-negative soil bacterium that can exist either free-living or in symbiosis with soybean *Glycine max* (Appleby, 1984). Inside the plant root nodules, the so-called bacteroids are able to fix atmospheric nitrogen to ammonia (Werner, 1992; Young, 1992). The key enzyme for this process, nitrogenase, is extremely sensitive to oxygen. In symbiosis, N$_2$-fixation occurs under near-anaerobic conditions (Hennecke, 1998), which is achieved by two means: (i) the plant synthesizes the hemoprotein leghemoglobin that binds oxygen with a high affinity (Appleby, 1984). (ii) The bacterium expresses a special, high-affinity oxidase of the cbb$_3$-type that is able to consume leghemoglobin-bound oxygen (Preisig, 1994; Preisig *et al.*, 1993; Preisig *et al.*, 1996b). This heme-copper-type cytochrome c oxidase (Garcia-Horsman *et al.*, 1994a; Garcia-Horsman *et al.*, 1994b) is used for energy production by acting as a proton pump (Arslan *et al.*, 2000).

The adaptation of the bacterial energy metabolism to optimal conditions for symbiosis is believed to be quite complex, because in *B. japonicum* a large set of respiratory cytochromes have been described, of which many are present predominantly in bacteroids (Appleby, 1984). Recently, the roles of individual cytochromes have become clearer due to the identification of the corresponding genes and the analysis of mutants. Figure 1 summarizes the present knowledge of the *B. japonicum* branched respiratory chain where the individual branches terminate with the reduction of molecular oxygen.

![Fig. 1. The branched respiratory chain of *B. japonicum*. The figure is adapted from (Hennecke, 1998), including the newly discovered bd-type quinol oxidase and the gene product of *mqo*.](image-url)
Electrons are channeled to the quinone pool by various dehydrogenases and are then transferred either via the $bc_1$ complex to cytochrome $c$ oxidases, or directly to quinol oxidases. The $bc_1$ complex and the $cbb_3$ oxidase are essential for respiration in symbiosis (Meyer, 1988; Preisig et al., 1993; Preisig et al., 1996b; Thöny-Meyer et al., 1989), whereas the main pathway for aerobic growth appears to involve the $bc_1$ complex, the membrane-bound cytochrome $c$ CycM (Bott et al., 1991) and the $aa_3$-type cytochrome $c$ oxidase (Bott et al., 1990) that is closely related to mitochondrial cytochrome $c$ oxidase. A second set of genes for this type of terminal oxidase, $coxMNOP$, is present in $B. japonicum$, but its function has not been revealed (Bott et al., 1992). In the absence of a functional $bc_1$ complex at least one alternative quinol oxidase of the $bb_3$ type encoded by $coxWXYZ$ has been reported to support aerobic growth (Surpin et al., 1994). However, this oxidase does not appear to be important for respiration under symbiotic conditions. The demands of energy during the plant infection process and the migration of the bacteria within the infection thread are not known. In particular, it has not been addressed which of the various oxidases are used during the initial stages of infection.

We have discovered a new gene cluster in $B. japonicum$ that appears to contain genes encoding the subunits I and II of a $bd$-type quinol oxidase. Here we describe the genetic analysis of the cyd locus and show that the derived enzyme is a $bd$-type oxidase that possibly receives electrons from a malate-quinone electron transport pathway.
V. 3. Materials and methods

Bacterial strains and plasmids. The bacterial strains and plasmids used in this work are listed in Table 1.

Media and growth conditions. *E. coli* was grown aerobically in Luria-Bertani (LB) medium (Miller, 1972) at 37°C. Where appropriate, antibiotics were added at the following concentrations (μg/ml): ampicillin, 200; chloramphenicol, 10; kanamycin, 30; tetracycline, 10. Microaerobic (0.5% O₂/99.5% N₂) cultures were prepared in minimal salts (MS) medium (Jobbib Nivol et al., 1994) supplemented with 40 mM fumarate, 0.4% glycerol and 5 mM nitrate. Expression of *cydB* was induced by the addition 0.1% (w/v) arabinose.

*B. japonicum* was grown aerobically in peptone-salts-yeast extract (PSY) medium (Regensburger and Hennecke, 1983) and anaerobically in yeast-extract-mannitol (YEM) medium that was supplemented with 10 mM KNO₃ (Daniel and Appleby, 1972) at 30°C. Microaerobic cultures (20 ml) in PSY medium were kept under a gas mixture consisting of 0.02% or 0.1% O₂ and 99.98% or 99.9% N₂, respectively, in sealed serum bottles (500 ml). The gas phase was periodically (approx. every 12 h) exchanged by flushing the bottles with the same gas mixture. Anaerobic cultures were kept under argon in serum bottles. HEPES-MES (HM) medium (Meyer, 1988) was used for growth in defined media. This medium was supplemented either with 1% (w/v) malate, 1% (w/v) lactate or malate/lactate mixture (1%, w/v/0.5%, w/v). When appropriate, antibiotics were present at the following concentrations (μg/ml): spectinomycin, 100; kanamycin, 100; tetracycline, 50 (plates) or 25 (liquid cultures).

DNA work and sequence analysis. Isolation of DNA, cloning and Southern blot hybridization were performed according to standard protocols (Sambrook et al., 1989). Probes for Southern blot hybridizations were generated by PCR and labeled with digoxigenin (supplier of kit). Double-stranded plasmid DNA was cycle-sequenced by the chain termination method of Sanger et al. (Sanger et al., 1992) with DNA sequencers (model 310; Applied Biosystems, Foster City, California, USA; and by Microsynth, Balgach, CH). For computer-assisted analysis of DNA and protein sequences, we used the software package (version 10.0) of the UWGCG (Genetics
Chapter V

Computer Group of the University of Wisconsin, Madison, WI). Homology searches were performed by using the National Center for Biotechnology Information BLAST network server (http://www.ncbi.nlm.nih.gov/BLAST/).

Cloning of the *cyd*-locus of *B. japonicum*. During an unrelated sequencing project the *cyd* locus was found in a partial genomic library of *B. japonicum* on the cosmid 12/1 (F. Narberhaus, personal communication). A 9-kb *SmaI* fragment from the cosmid was ligated into *EcoRV*-digested pUC18, resulting in pRJ5152 (Table 1). For sequencing, this 9-kb region was subcloned as 0.5 kb-1.5 kb fragments, into the pUC18/pUC19 and pACYC184 vectors (Table 1).

Construction of the *cydA* mutant. In pRJ4633 (Table 1), a 0.49-kb *SmaI/EcoRV* *cydA*-internal fragment was substituted by the kanamycin-cassette, with *kan* pointing into the direction opposite to *cydA* resulting in pRJ4640 (Table 1). The 3.8 kb-fragment of pRJ4640 (resulting by *EcoRI* digestion of pRJ4640) containing the *kan*-cassette and encompassing *B. japonicum* DNA was cloned into the vector pSUB202pol6K, resulting in pRJ4644. This vector was used for marker exchange mutagenesis of *B. japonicum* as described previously (Hahn and Hennecke, 1984). The correct genomic structure of the mutant strain Bj4644 was confirmed by Southern blot hybridization of genomic DNA.

Construction of a chromosomally integrated *cydA*'-*lacZ* fusion. For the construction of a translational *cydA*'-*lacZ* fusion, pRJ4628 (Table 1) was digested with *BamHI* and *XbaI* (*BamHI* overlaps with the start codon of *cydA* [ATGGATCC], whereas *XbaI* is in the polylinker site of pUC19). The resulting 0.85-kb fragment of *cydA* was substituted by the *lacZ*-gene of *E. coli* (5.1-kb-fragment, *lacZ* part from pNM480, pSUB480). This cloning resulted in a construct with 1.4-kb upstream of *cydA* ('mqo) and the *lacZ*-gene (10th codon) fused in frame to the start codon (ATG) of *cydA*. This fusion was cloned into pSUP202pol6K by digestion with *KpnI/XbaI* (6.5-kb) resulting in pRJ4641 (Table 1). This plasmid was integrated into the *B. japonicum* chromosome by conjugation and selection for a single crossover event by homologous recombination, using the *tet* marker of the vector for selection. The resulting strain, with a cointegration of the vector, was Bj4641, whose correct genomic structure was confirmed by Southern blot hybridization of genomic DNA.
Chapter V

Construction of the cydAB and cydAB\textsubscript{his6} plasmid for heterologous expression in E. coli.
pRJ4633 was partially digested with SphI, and the vector was isolated. pRJ4657 was digested with the same enzyme and the insert (cydB and parts of yoaN) was isolated. These two fragments resulted after ligation in pRJ4672. A PCR reaction was performed with primers FB101 (5'-CCAAGCTTGGCATATGGATCCGACGGCACTCCTCTCG-3' and FB102 (5'-CCAAGCTTGGGAATTCCCGCAGCTGTTGTTGACCATGATCC-3') resulting in a 480 bp fragment. This fragment (NdeI-site at the ATG of cydA) was cloned into a pUC18 (pRJ4680). For construction reasons the fragment was again subcloned after digestion of pRJ4680 with HindIII into pACYC177, resulting in pRJ4681. This plasmid was digested with PvuII and EcoRI, resulting in linearised vector plus NdeI-cydA\textsubscript{PvuII}. pRJ4672 was digested with PvuII and EcoRI and the insert (cydA\textsubscript{PvuII} until yoaN\textsubscript{EcoRI}) was ligated with the linearised pRJ4681 resulting in pRJ4682. This construct consists of cydAB and yoaN'. The pISC2 derivative for heterologous expression was opened with NdeI and EcoRI. pRJ4682 was digested with NdeI and EcoRI, and the insert was subcloned into the prepared pISC2 derivative, resulting in pRJ4683 (Table 1).

With the primers (831s) 5'-CAGCATCCTGATCTATCTT-3' and (cydABhis) 5'-CCGGAAATTCCTCAGTGGTGTTGAGTGGTGGTAGTGGTCCGCCGCAGC-3' a PCR reaction was performed using pRJ4672 (Table 2) as template. A 0.98-kb fragment was obtained and digested with EcoRI/HindIII. This fragment was cloned into EcoRI/HindIII- digested pRJ4683 (substitution of wt 3'-part of cydB by the 3'-his part of cydB), resulting in pRJ4687 (Table 1).

\textbf{β-Galactosidase assays.} β-Galactosidase activity assays were done as described previously (Grob et al., 1993).

\textbf{Plant infection test.} Symbiotic nitrogen fixation was determined in plant infection tests as described elsewhere, using the acetylene reduction assay of soybean root nodules after 21 days of infection (Göttfert et al., 1990).

\textbf{Heterologous expression of cydAB in E. coli.} Heterologous expression of cydAB or cydAB\textsubscript{his6} was performed in a strain deficient in its three terminal oxidases (Table 1). E. coli was grown in 2 ml LB medium containing 0.5% (w/v) glucose for 8 h and then diluted into 20 ml LB medium with 5 mM KNO\textsubscript{3} for microaerobic growth over night.

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The bacteria of this culture were harvested, washed twice in MS medium and then used to inoculate two 500 ml sealed serum bottles with MS medium as described previously. After induction with arabinose, cells were grown until the OD$_{600}$ was 0.4 (7-8 h) and then harvested by centrifugation at 8000xg for 15 min at 4°C. They were washed once with ice-cold 20 mM Tris-HCl, pH 7.5 and 50 mM NaCl, and then frozen for membrane preparation at −20 °C.

**Cell fractionation, membrane preparation, difference spectroscopy, and immunoblotting.** Cell fractionation and membrane preparation were performed as described in (Schulz et al., 2000). Antibodies specifically reacting with His-tagged proteins were purchased from Qiagen™. Visual difference spectroscopy was performed as described (Thöny-Meyer et al., 1995). Immunoblotting was performed as described by Fabianek et al. (1999). Concentrations of membrane proteins were determined by using the Bio-Rad assay (Bio-Rad Laboratories, Richmond, CA) with bovine serum albumin as the standard.

**Microaerobic growth complementation.** Microaerobic growth complementation was performed with the same cells that were used for membrane preparation. The cells were washed upon expression of cydAB (after 8h) two times with MS-medium. A microaerobic culture was started with these cells using MS-medium with 0.05% arabinose as inducer of the arabinose promoter. KNO$_3$ was substituted by 0.5% oxygen as the terminal electron acceptor. The gas atmosphere was exchanged every 7 to 9 hours (0.5% O$_2$, rest N$_2$). The optical density was measured at each gas exchange and a growth curve was developed.
### Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype or phenotype</th>
<th>Source or reference</th>
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<tr>
<td><strong>E. coli</strong></td>
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<tr>
<td>DH5α</td>
<td><em>supE44 ΔlacU169</em> (Φ80lacZΔM15) <em>hsdR17</em></td>
<td>Bethesda Research Laboratories, Inc., Gaithersburg, Md</td>
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<tr>
<td></td>
<td><em>recA1 gyrA96 thi-l relA1</em></td>
<td></td>
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<tr>
<td>S 17-1</td>
<td>*Sm&lt;sup&gt;+&lt;/sup&gt; *Sp&lt;sup&gt;+&lt;/sup&gt; <em>hsdR</em> (RP4-2 <em>kan::Tn7 tet::Mu</em>, integrated in the chromosome)</td>
<td>(Simon <em>et al.</em>, 1983)</td>
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<td>SB2115</td>
<td><em>HfrC relA pir-10 Δcyo::kan Δcyd::cat Δ(appB-appA)::kan zcc::Tn10</em></td>
<td>(Dassa <em>et al.</em>, 1991)</td>
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<td>(Sturr <em>et al.</em>, 1996)</td>
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<tr>
<td><strong>B. japonicum</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>110spc4</td>
<td>*Sp&lt;sup&gt;+&lt;/sup&gt;; wild type</td>
<td>(Regensburger and Hennecke, 1983)</td>
</tr>
<tr>
<td>4503</td>
<td>*ΔfixNOQP::kan, Sp&lt;sup&gt;+&lt;/sup&gt; <em>Km&lt;sup&gt;+&lt;/sup&gt;</em></td>
<td>(Zufferey <em>et al.</em>, 1996a)</td>
</tr>
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<td>4641</td>
<td>*Sp&lt;sup&gt;+&lt;/sup&gt; *Tc&lt;sup&gt;-&lt;/sup&gt; <em>cydA&lt;sup&gt;-&lt;/sup&gt;-'lacZ chromosomally integrated</em></td>
<td>This work</td>
</tr>
<tr>
<td>4644</td>
<td>*ΔcydA::kan, Sp&lt;sup&gt;+&lt;/sup&gt; <em>Km&lt;sup&gt;+&lt;/sup&gt;</em></td>
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<td><strong>Plasmids</strong></td>
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<td>pSUP480</td>
<td><em>Tc&lt;sup&gt;-&lt;/sup&gt;</em>; part of pNM480 in pSUP202pol4</td>
<td>H. M. Fischer, unpublished</td>
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<td>pISC2</td>
<td><em>Ap&lt;sup&gt;+&lt;/sup&gt;</em>; expression vector with <em>P&lt;sub&gt;ara&lt;/sub&gt;</em></td>
<td>Thöny-Meyer <em>et al.</em>, 1996</td>
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<td>(Norrander <em>et al.</em>, 1983)</td>
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<td>(Norrander <em>et al.</em>, 1983)</td>
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<td>pACYC177</td>
<td><em>Ap&lt;sup&gt;+&lt;/sup&gt;</em>; <em>Km&lt;sup&gt;+&lt;/sup&gt;</em>; cloning vector</td>
<td>(Chang and Cohen, 1978)</td>
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<td>pACYC184</td>
<td><em>Cm&lt;sup&gt;+&lt;/sup&gt;</em>; <em>Tc&lt;sup&gt;-&lt;/sup&gt;</em>; cloning vector</td>
<td>(Chang and Cohen, 1978)</td>
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<td>pSUP202pol6K</td>
<td><em>Tc&lt;sup&gt;-&lt;/sup&gt;</em>; pSUP202pol4 (<em>KpnI</em> linker in <em>Smal</em> site)</td>
<td>(Zufferey <em>et al.</em>, 1996a)</td>
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<tr>
<td>pRJ4628</td>
<td><em>Ap&lt;sup&gt;+&lt;/sup&gt;</em>; 2.3-kb fragment of <em>cydA</em> and <em>mqo</em> in <em>pUC18</em></td>
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<tr>
<td>pRJ4633</td>
<td><em>Ap&lt;sup&gt;+&lt;/sup&gt;</em>; 2.8-kb fragment of <em>mqo</em> and <em>cydA</em> in <em>pUC19</em></td>
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*continued*
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<th>Description</th>
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<td>0.49-kb fragment of cydA in This work</td>
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<td></td>
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<td>pRJ4633 substituted by kan cassette resulting in a 4.3-kb fragment, in pUC19</td>
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<tr>
<td>pRJ4641</td>
<td>Tc&lt;sup&gt;+&lt;/sup&gt;</td>
<td>6.5-kb fragment of pRJ4634, in This work</td>
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<tr>
<td>pRJ4657</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;, Km&lt;sup&gt;+&lt;/sup&gt;, Cm&lt;sup&gt;+&lt;/sup&gt;, Km&lt;sup&gt;+&lt;/sup&gt;</td>
<td>7-kb fragment of cydA::kan cydB This work</td>
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<td>pRJ4672</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>3.3-kb fragment of 'mqo-cydAB, in This work</td>
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<td>Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>560-bp fragment, in pUC18 This work</td>
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<td>pRJ4681</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>HindIII fragment from pRJ4680, in pACYC177</td>
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<td>pRJ4682</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>2.8-kb fragment (PvuII/EcoRI) from pRJ4682, in pRJ4681</td>
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<td>pRJ4683</td>
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<td>2.4-kb fragment of cydAB behind P&lt;sub&gt;ara&lt;/sub&gt; in pISC2</td>
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<tr>
<td>pRJ4687</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>2.4-kb fragment of cydAB&lt;sub&gt;his&lt;/sub&gt; behind P&lt;sub&gt;ara&lt;/sub&gt; in pISC2</td>
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<tr>
<td>pRJ5152</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>9-kb fragment of mqo-cydA, in pUC19 F. Narberhaus (ETH-Zürich)</td>
</tr>
</tbody>
</table>
V. 4. Results

The cyd-locus. The cyd locus was found fortuitously by identifying a cloned B. japonicum fragment encoding a peptide with high sequence similarity to the N terminus of subunit I of bd-type oxidases. The DNA encompassing this fragment was cloned and sequenced (Fig. 2A) and the sequence was deposited in GenBank, Accession No. xxx. It contained the genes cydA and cydB coding for subunit I and subunit II, respectively, of a putative B. japonicum bd-type oxidase (Fig. 2A).

Fig. 2 A. Physical map of the B. japonicum (Bj) cyd locus and mqo. The potential translation start sites (ATG) are indicated with 0, 1558 and 2968, for mqo, cydA and cydB, respectively, corresponding to the position relative to the upstream end of the sequenced DNA. The following restriction sites are indicated: B, BamHI; E, EcoRI; H, HindIII; K, KpnI; P, PstI; R, EcoRV; Sm, SmaI; Sp, SphI. Important subclones are indicated pRJ5152, pRJ4672, pRJ4633, pRJ4680, pRJ4681, pRJ4682 and pRJ4628.

The first gene, cydA, encodes a 468-amino-acid polypeptide showing significant sequence identity with CydA from Rickettsia prowazekii (40%), E. coli (30%) and Bacillus subtilis (29%). The second gene, cydB, codes for a protein of 328 amino acids that shares 39% sequence identity with R. prowazekii, 25% with E. coli and 26% with B. subtilis subunit II. The two genes are separated by two bp and each is preceded by a Shine-Dalgarno sequence 5'-GGAGAA-3' (Shine and Dalgarno, 1974) which in the case of cydB overlaps with the 3'-end of cydA.
The 20 strictly conserved amino acid residues of SUI (Jünemann, 1997) are also present in CydA of *B. japonicum*. Three of them have been proposed to be axial heme ligands: H186 and M393 for cytochrome *b*$_{558}$ (Fang *et al*., 1989; Kaysser *et al*., 1995), which was proposed to be the first acceptor for electrons (Jünemann, 1997), and H19 for cytochrome *b*$_{595}$ (Fang *et al*., 1989). Three of the remaining 17 conserved residues (K251, A253 and E257) comprise the so-called Q-loop (Jünemann, 1997), the putative quinol-binding site. The hemes of cytochromes *b*$_{595}$ and *d* seem to form a binuclear centre, where the reduction of oxygen to water occurs (Hill *et al*., 1993). SUII harbors two strictly conserved amino acid residues, G31 and P76, whose function is not clear (Kiyoshi *et al*., 1984; Newton *et al*., 1991; Oden and Gennis, 1991). These residues are also present in CydB of *B. japonicum*.

Upstream of *cydA*, in a distance of 19 bp, an open reading frame of 1539 bp was found (Fig. 2A) that codes for a 512-amino-acid polypeptide with 53% and 48% sequence identity to malate:quinone oxidoreductases (MQO) of *E. coli* and *Corynebacterium glutamicum*, respectively. It is preceded by a Shine-Dalgarno sequence 5'-GAAGGA-3'. The N-terminal domain of Mqo contains an FAD binding site (DhhhhGA/GG with h for the amino acids I, V or L) that is strictly conserved in MQOs. This motif is also found in glycerol-3-phosphate dehydrogenases, where it represents the FAD binding site of these enzymes (Austin and Larson, 1991; Molenaar *et al*., 1998). Due to the close vicinity of *mqo* and *cydAB*, we assume that they form an operon.

The *mqo-cyd* locus is flanked by two incompletely sequenced open reading frames, *fumX* and *yoaN*, that are predicted by sequence similarity to code for a fumarase C (Ching-Ping *et al*., 2001) and a legumin-like protein (Horstmann, 1983), respectively. The intergenic region between *fumX* and *mqo* does not contain any well-conserved promoter sequences typically found in *B. japonicum*.

**Expression of cydA’-’lacZ.** A translational *cydA’-’lacZ* fusion was constructed and co-integrated into the *B. japonicum* chromosome as described in Materials and Methods.
Fig. 2 B. The strain Bj4641 with the cointegrated cydA'-'lacZ fusion. The fusion site is at the ATG start codon of cydA.

β-Galactosidase activity was measured on X-gal plates or in liquid culture under various growth conditions. On plates, blue colonies were detected only on minimal medium supplemented with either malate and/or lactate at high cell density, but not in single colonies (not shown). The quantification of cydA expression in minimal medium liquid cultures showed that the expression level was very low, as it could be detected only with the sensitive fluorescent substrate 4-methylumbelliferyl-β-D-galactopyranoside (Grob et al., 1993).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>β-Galactosidase activity (Units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bj4641</td>
<td>cydA'-'lacZ</td>
<td>aerobic 0.1% O₂ 0.02% O₂</td>
</tr>
<tr>
<td></td>
<td></td>
<td>110 ± 20 50 ± 8 20 ± 5</td>
</tr>
</tbody>
</table>

*a Unit corresponds to 10 nmol of 4-methylumbelliferyl-β-D-galactopyranoside formed x ml⁻¹ x h⁻¹ x OD₆₀₀⁻¹. Background activity with 110scp4 was 5 ± 2 units. Numbers are mean values ± standard errors of at least three cultures that were assayed in duplicate.

Cells that were grown under aerobic conditions showed a five times higher expression than cells grown under microaerobic (0.02% O₂) conditions (Table 2). In comparison to the cells grown under 0.1% O₂, the expression level was two times higher. No expression was detected under the same conditions in rich medium (not shown). We conclude that the cydA'-'lacZ fusion is expressed to some extent in minimal medium supplemented with malate and/or lactate, and preferentially under aerobic conditions.

**Construction and characterization of a cydA mutant.** To investigate the functional importance of the putative bd-type oxidase for growth and symbiosis, a cydA deletion
mutant was constructed by insertion of a kanamycin cassette, and the construct was used for marker exchange mutagenesis (Fig. 2C).

![Fig. 2 C. The strain Bj4644 is the deletion-insertion mutation in cydA (SUI) of the bd oxidase.]

First, we tested whether the presence of the CydAB oxidase is needed for aerobic growth. However, an altered growth phenotype was observed neither in rich medium nor in minimal medium plus malate and/or lactate.

The most intriguing question was whether the cydA mutant had a deficiency in establishing an efficient symbiosis. Soybean plants were infected with wild type, the cydA mutant and a cbb\textsubscript{2}-oxidase-deficient strain. All three strains formed normal root nodules. When nitrogen fixation activities were compared, the cydA mutant had an even higher nitrogen fixation activity (140\%) than the wild type (100\%), whereas the cbb\textsubscript{2} - oxidase-negative mutant (BJ4503) showed only about 5\% residual nitrogenase activity, consistent with previous results (Preisig et al., 1993). The results are summarized in Table 3.

### Table 3. Symbiotic properties of the *B. japonicum* cydA mutant

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Number of nodules (mean ± SD)</th>
<th>Dry weight/nodule (mg)</th>
<th>Fix activity (% of wild type)</th>
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<tr>
<td>110spc4</td>
<td>fixNOQP\textsuperscript{+}, cydA\textsuperscript{+}</td>
<td>30 ± 6</td>
<td>1.1 ± 0.2</td>
<td>100 ± 33</td>
</tr>
<tr>
<td>Bj4644</td>
<td>cydA\textsuperscript{-}</td>
<td>40 ± 14</td>
<td>0.8 ± 0.2</td>
<td>139 ± 48</td>
</tr>
<tr>
<td>Bj4503</td>
<td>fixNOQP\textsuperscript{-}</td>
<td>42 ± 8</td>
<td>1.2 ± 0.1</td>
<td>5 ± 1</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Values are the means ± standard deviation of at least 11 individual plants. 100\% of nitrogenase activity (Fix-activity) corresponds to 146 \(\mu\)mol of C\textsubscript{2}H\textsubscript{2} x h\textsuperscript{-1} x mg of nodules\textsuperscript{-1} (dry weight).
We conclude that the *B. japonicum* *bd*-type oxidase is not involved in energy conservation during plant infection and for symbiotic nitrogen fixation.

**Expression of cydAB<sub>his6</sub> in *E. coli***. To show that cydAB actually encode a *bd*-type oxidase, we purified membranes from *B. japonicum* cells grown under the conditions where the highest levels of *cyd* gene expression was found and looked for the typical spectral characteristics of *bd*-type oxidases. However, no peak at 589 and 628 nm for one of the cytochromes *b* and *d*, respectively, was found (data not shown). This was not surprising, because the level of cydAB expression is very low. Therefore, and since there is no established system for overexpression of proteins in *B. japonicum* available, we decided to express the *B. japonicum* *bd*-type oxidase in *E. coli*. The genes cydAB were cloned into an arabinose-inducible expression vector in two versions (Fig. 2D), wild-type cydAB (pRJ4683) and cydAB<sub>his6</sub> (pRJ4687), in which codons for six C-terminal histidines are fused to the second gene.

![Diagram of expression vectors](image)

**Fig. 2.** pRJ4683 and pRJ4687, the constructs used for heterologous cydAB expression in *E. coli*.

Expression was done in a host strain deleted in the genes *cyo, cydAB* and *appBC*, which encode the three endogenous *E. coli* terminal oxidases *bo*, *bdI* and *bdII*, respectively (Dassa *et al.*, 1991). The use of this host background ensured that no spectral signals from endogenous *bd*-type cytochromes were obtained. As a control, we also tested the vector alone in the same host. In the following, only the results from experiments using the His-tagged version are presented. It was shown by immunoblot using anti His-tag immunoglobulins that membranes of cells carrying pRJ4687 were able to express the His-tagged *B. japonicum* CydB protein (Fig.3, pRJ4647). The membranes of the strain transformed with the vector control did not show any detectable signal (Fig.3, vector...
As a control for detection of a His-tag protein we loaded the purified $cbb_3$ type oxidase of *B. japonicum*, which also carries a C-terminal His tag in its FixN subunit (Zufferey *et al.*, 1998; Fig. 3, FixNHis).

These results demonstrate that the second subunit CydB is transcribed upon induction, translated and assembled into the membranes of *E. coli*. In a next experiment we tested whether the complete *bd* oxidase was assembled in *E. coli*. Absorption difference spectra were recorded for membranes of induced *E. coli* cells expressing CydAB$_{His6}$ (Fig. 4, pRJ4687). The characteristic peaks for a *bd*-type oxidase were found at 598 nm and 560 nm for the cytochromes $b$ and at 628 nm for cytochrome $d$. Membranes from cells containing only the vector showed no $d$ peak and a small $b$ peak in the region of 560 nm that most likely is due to the $b$-type cytochrome subunit SdhC of the succinate dehydrogenase (SdhABCD) of *E. coli* (Gennis, 1987). In the *E. coli bd* oxidase, the heme binding ligands have been mapped to SUI (CydA), but certain residues in the N-terminal domain of SUII were also shown to be involved in the correct assembly of the cofactors (Jüenemann, 1997). The appearance of all expected spectral bands indicates

---

**Fig. 3.** Immunoblot of the CydB$_{His6}$ (SUII) of the *bd* oxidase of *B. japonicum*. Monoclonal antibodies against tetra-His-tags were used for detection. The first two lanes (vector control and pRJ4687) contain 100 μg of membranes. The third lane (FixN$_{His}$) served as a positive control and contains 15 μg of purified $cbb_3$-type oxidase (Arslan *et al.*, 2000).
that CydAB proteins of *B. japonicum* assemble with the heme molecules to a functional oxidase when expressed in *E. coli*.

Fig. 4. Na-dithionite reduced minus air oxidized absorption difference spectra. Membrane proteins (1.5 mg/ml) from cells containing either the vector alone (vector control) or pRJ4687 expressing cydAB were analyzed. The insert (upper right) enlarges the bands characteristic for *b*- and *d*-type cytochromes.

To investigate the functionality of the *B. japonicum* CydAB proteins in the *E. coli* respiratory chain, we tested whether the overexpressed oxidase can support microaerobic growth of an *E. coli* strain deleted in the *cyo*, *cydAB* and *appBC* loci encoding the three endogenous quinol oxidases (Fig. 5, next page).
 Indeed, the oxidase-negative strain carrying plasmids pRJ4683 or pRJ4687 showed stimulation of microaerobic growth when expression of the B. japonicum genes cydAB was induced with arabinose (Fig. 5). This growth was comparable to that of a strain expressing the appBC genes (alternative bd type oxidase). By contrast, no growth stimulation was observed in the presence of the vector alone (Fig. 5). This result supports our view that the B. japonicum genes cydAB encode a bd-type quinol oxidase similar to those of E. coli.
The identification of a \textit{bd}-type quinol oxidase in \textit{B. japonicum} adds another branch to the respiratory chain of this organism (Fig. 1). Hence, this bacterium possesses at least five terminal oxidases for \textit{O}_2 reduction. The \textit{cbb}_3-type oxidase has so far been the only oxidase involved in energy production for symbiotic nitrogen fixation (Arslan et al., 2000; Preisig, 1994; Preisig et al., 1993). We have shown recently, that it functions as a proton pump, thereby directly producing a proton motive force for ATP synthesis. Since \textit{bd}-type oxidases are known to have either high affinities for oxygen (D'Mello et al., 1996; Rice and Hempfling, 1978) or high turnover rates to scavenge oxygen (Kolonay and Maier, 1997), we wondered whether the \textit{B. japonicum} \textit{bd}-type oxidase was involved in an oxygen scavenging process during symbiosis, as it is the case in the asymbiotic diazotroph \textit{A. vinelandii} (Kelly et al., 1990). If the \textit{bd}-type oxidase of \textit{B. japonicum} is important for nitrogen fixation, a \textit{cyd}^- mutant should show some defect in bacteroid formation or symbiotic nitrogen fixation. However, the results of our plant infection tests with this mutant revealed a wild-type-like phenotype, thus excluding a role of this oxidase in the establishment of an effective root nodule symbiosis. This is in contrast to what has been reported for \textit{Azorhizobium caulinodans}, another diazotroph, where the \textit{bd}-type oxidase is needed in addition to the \textit{cbb}_3-type oxidase for symbiotic nitrogen fixation (Kaminski et al., 1996).

An indication for the possible role of the \textit{bd} oxidase in \textit{B. japonicum} was the location of the \textit{cydAB} genes directly downstream of the gene \textit{mqo} that encodes a malate:quinone oxidoreductase (MQO). The distance of only 19 bp between the \textit{B. japonicum} genes \textit{mqo} and \textit{cydA} suggested that \textit{mqo} and \textit{cydAB} are co-transcribed. Moreover, a \textit{cydA}'\textquotesingle-\textit{lacZ} fusion was expressed only weakly under various growth conditions, and best in the presence of lactate and/or malate. In \textit{E. coli}, the oxidation of malate by MQO (\(\Delta G^{\circ} = -19\) kJ/mol) is energetically favorable compared with the endergonic reaction catalyzed by the TCA cycle enzyme malate dehydrogenase (MDH; \(\Delta G^{\circ} = +28\) kJ/mol; (Michel et al., 2000; Molenaar et al., 2000; Molenaar et al., 1998; Narindrasorasak et al., 1979). In contrast to MDH, MQO catalyzes oxidation of malate to oxaloacetate in a NAD$^+$-independent reaction, using quinone as an electron acceptor (Narindrasorasak et al., 1979). When lactate is metabolized by lactate dehydrogenase, the amount of NADH also increases. Thus, the cells may chose a pathway by which they can dump reduction
equivalents to electron transport components of the membrane, for example by inducing MQO that is linked to a respiratory enzyme such as the bd oxidase (Fig. 6).

\[ \text{NAD}^+ \rightarrow \text{NADH} + \text{H}^+ \]

\[ \text{malate} \rightarrow \text{oxaloacetate} \]

\[ \text{Q} \rightarrow \text{QH}_2 \]

\[ \text{H}_2\text{O} \rightarrow \text{CydAB} \rightarrow \text{O}_2 \]

---

**Fig. 6.** Model for the potential cooperation between MQO and the CydAB oxidase in *B. japonicum*.

In fact, membrane-associated MQOs have been described for *Corynebacterium glutamicum* and *Helicobacter pylori* (Kather et al., 2000; Molenaar et al., 1998). It is possible that co-expression of *mqo* and *cydAB* ensures this kind of pathway under certain growth conditions in *B. japonicum*.

As we could not find proof of the bd-type oxidase function in *B. japonicum*, we expressed the enzyme in *E. coli*. It is known that the additional genes *cydCD* are needed for assembly of a mature, heme containing oxidase of the bd type (Poole et al., 1994). In *B. subtilis*, these genes are located downstream of and co-transcribed with the *cydAB* genes (Winstedt et al., 1998), whereas in *E. coli*, the *cydDC* locus is not linked to the structural genes *cydAB* or *appBC* encoding the subunits of the two bd-type oxidases (Dassa et al., 1991). As in *B. japonicum* *cydCD*-like genes were not present downstream of *cydAB* (Fig. 2), we assume that they are located elsewhere on the chromosome. The *cydCD* genes encode the subunits for an ABC transporter that most likely transports a factor required for maturation of the oxidase. Therefore, we assumed that the presence of these genes in *E. coli* could allow assembly of the *B. japonicum* bd oxidase as well.

This assumption is supported by the successful production of a bd-type oxidase in an *E. coli* strain that is deleted for its endogenous bd-type oxidases in the presence of a plasmid carrying the *B. japonicum* *cydAB* genes. The spectroscopic characterization of this oxidase resulted in typical peaks for cytochromes b_{558}, b_{598} and d_{628}, indicating that the composition of cofactors is the same as in previously characterized bd-type oxidases. The oxidase was shown to support micro-aerobic growth of *E. coli* and therefore is a functional enzyme.
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Our work has revealed genetic and biochemical proof for the existence of a fifth terminal oxidase in the respiratory chain of *B. japonicum*. However, its benefit for the organism remains unclear, and further genetic analyses such as the construction of double and triple mutants affecting respiratory components will be required to better understand the branched electron transport pathway of this organism.

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CHAPTER VI

Future perspectives
Chapter VI

VI. 1. The \textit{cbb}_3-type oxidase

Chapter II dealt with the proton pumping activity of the \textit{B. japonicum} \textit{cbb}_3-type oxidase. Proton pumping activity had also been demonstrated for the \textit{cbb}_3-type oxidase of \textit{H. pylori} in a pre-steady-state pulse experiment (Tsukita \textit{et al.}, 1999). The proton-pump efficiency was in the same range as it was demonstrated in this work (0.2-0.5 \text{H}^+/\text{e}^-). Studies with other terminal oxidases of archaea have also demonstrated that their proton-pump efficiencies are lower then 1\text{H}^+/\text{e}^- (Kannt \textit{et al.}, 1998; Schäfer \textit{et al.}, 1999). One future direction in the investigation of \textit{cbb}_3-type oxidases should be in finding out how this oxidase pumps protons. Before doing so, a number of technical and biochemical aspects need to be considered.

\textit{Technical aspects:}

The reconstitution method established in this thesis is time-consuming (3 days) (Arslan \textit{et al.}, 2000). To reduce this time period, an improved reconstitution method would be desirable. As a positive control for the reconstitution, the established method in this thesis could be used. One promising method towards a better reconstitution is the systematic use of biobeads\textsuperscript{TM}. The detergent molecules adsorb to the beads and will no longer be released from there. This method is fast (one night) and could be done without an additional detergent for co-dialysis, as used by others (Ludwig and Schatz, 1980; Solioz \textit{et al.}, 1982) and in this work. In fact, the addition of a second detergent can lead to a decreased activity of the oxidase. There are two main products available on the market. One is from BioRad (Amberlite\textsuperscript{TM}) and the other is from Calbiochem (Calbioadsorb\textsuperscript{TM}). Systematic tests with each product and different protein-to-lipid ratios could lead to a more pronounced acidification signal produced by the reconstituted oxidase. The destabilization of the lipid with detergent and sonification is a further critical step in the reconstitution of a membrane protein. The longer it is sonified the more opalescent the solution looks and the more destabilized the mixture becomes. It was demonstrated for bacteriorhodopsin and the mitochondrial \textit{aa}_3-type oxidase that the reconstitution success varied in relation to the destabilized state of the mixture of lipid and detergent (three stage model, Lambert \textit{et al.}, 1998; Rigaud \textit{et al.}, 1995). A systematic investigation into the destabilizing procedure could increase the efficiency of the reconstitution method in such a way that the proton-pump efficiency
Chapter VI

results will be higher and more pronounced in a diagram where the pH changes related to the elapsed time of the experiment are recorded as shown in chapter II.

Biochemical aspects:
As outlined and extensively discussed in chapters I+II, the key amino acids with polar residues of the proton channel are completely missing in the subunit I (FixN) of the cbb\(_3\)-type oxidase (Arslan et al., 2000). In spite of this fact, we and others have demonstrated proton-pumping activity for this type of oxidase. Further, it turned out that the lack of certain key amino acids still allows proton pumping in the caa\(_3\)-type oxidase (Pereira et al., 2000b). The main question that should be considered now is which amino acid residue instead is important for proton pumping. The classical approach for finding such a crucial amino acid is to perform a site directed mutagenesis of the putative amino acid that is believed to be important. At the same time the cytochrome c oxidation activity of the purified cbb\(_3\)-type oxidase must be unaffected by such an amino acid substitution. It is not easy to obtain such a candidate. In the case of the aa\(_3\)-type and bo\(_3\)-type oxidases the key amino acids have already been identified (Garcia-Horsman et al., 1995; Ludwig, 1980). Several amino acids (Y, R, E, D) are strictly conserved between subunit I homologues of the cbb\(_3\)-type oxidases. Some of them could now be considered as potential targets for amino acid substitutions by site-directed mutagenesis.

Fig. 1. Putative topological model of subunit I (FixN) of the cbb\(_3\)-type oxidase. This model is based on lacZ and phoA fusions (Zufferey et al., 1996b). The potential transmembrane helices are indicated in gray and with roman numbers.

Potential amino acids for substitution could be the tyrosines (pK of 11), (Y80, Y185, Y194, Y212, Y235, Y278, Y296, Y297, Y308, Y310, Y324, Y334, Y390, Y436, Y468
and Y507). They are located either in cytoplasmic or in periplasmic domains or in helices. They are strictly conserved through FixN homologues. It was discussed that Y256 (R. marinus numbering) could be a functional substitute of the E278 (P. denitrificans numbering) (Pereira et al., 2000b). This tyrosine corresponds to Y278 in the middle of the transmembrane domain VI of subunit I of the cbb3-type oxidase in B. japonicum. Strictly conserved arginines (R126, R128, R159, R223, R375, R484 and R510) that seem to be exposed either to the periplasm or to the cytoplasm, have an ionizable residue with a pK-value of 12.5. These amino acids (Y (pK 10.1) and R) have high pK values determined in solution. However, the pK value of specific amino acids in the protein depends strongly on the surrounding amino acids (micro environment). This means that the pK value could be decreased to a more physiological value (pK 6-7), which would make these amino acids (Y and R) good candidates for site directed mutagenesis. In the first subunit of the P. denitrificans aa3-type and E. coli bo3-type oxidase aspartic and glutamic acids are important for proton translocation. Based on these findings, good candidates also in the first subunit of the cbb3-type oxidase of B. japonicum would be glutamic acids (E193, E196, E225 and E396). E225 and E396 are lying between helices IV and V, facing the cytoplasm (entrance into a putative proton channel?) or in the helix IX (proton channel?), respectively. E193 and E196 are localized in the periplasmic loop between helices III and IV (proton uptake for water formation?). These glutamic acids are strictly conserved among all FixN homologues, which make them suitable candidates for site-directed mutagenesis. The pK-value of glutamic acid is ideal to serve as proton acceptor (pK=4.3).

In the subunit I of the aa3-type oxidase and the bo3-type oxidase a so-called D-channel (D124, N113, N131, N199, Y35, S134 and E278 P. denitrificans numbering; D135, E. coli numbering) and K-channel (S135, K354, T351 and Y280) have been identified. Aspartic and glutamic acids D124 and E278 (E286, E. coli numbering) lying at the beginning and at the end of the D-channel, respectively, are crucial for proton translocation. It is believed that D124 is important for gating the protons into the D-channel, whereas E278 might be important for proton extrusion. Representatives of the so-called K-channel, like in the subunit I of aa3 and bo3 oxidases, have not been found among FixN homologues. Nevertheless, in multiple alignments of FixN homologues, the D202 and D377 lying in the transmembrane helix IV and towards the cytoplasm between helices XIII and IX, respectively, are strictly conserved. These amino acids should be considered as further potential candidates for site-directed mutagenesis.
In the future, only a systematic analysis of site-directed mutations in the cbb^-type oxidase would lead to a successful identification of crucial amino acid residues for proton pumping. It is evident that an X-ray crystal structure of this oxidase would accelerate its investigation tremendously. However, the bottleneck for such a project is the amount of cbb^-type oxidase available by purification from a B. japonicum strain. There are no appropriate over-expression systems known for B. japonicum. Heterologous expression in E. coli should be considered as an alternative and carefully developed in the future.

VI. 2. Biochemical characterization of the c-type subunits of the cbb^-type oxidase

The heterologous expression of the c-type subunits is a powerful tool, which was developed in this thesis (see chapter IV). It allows the maturation of holo c-type cytochromes aerobically in E. coli. The first result of this project was that FixPsoi interacts with CO gas, whereas FixOsoi does not. Because the maturation of FixOsoi,his and FixPsoi,his failed (not shown), a classical purification protocol without a Ni-NTA column should be considered. The best way to establish such a protocol would be to start with ammoniumsulfate precipitation. It is known that c-type cytochromes precipitate at 60% (w/v) ammoniumsulfate (Appleby et al., 1991). After dialysis, a MonoQ column could help for separating the cytochromes from the rest. Cytochrome c binds to a MonoQ (anion-exchanger) column, and a lot of other proteins do not. This step would certainly enrich for the specific target protein in the protein solution. It is to consider also using a SP-sepharose (cation-exchanger) column in order to bind contaminant proteins, whereas the target protein would flow through. The last step could be a gel filtration. Analytical gel filtration would also deliver directly the relative molecular mass of the protein.

As it was demonstrated in chapter III, the redox potentials of the c-type centres were identified. However, only speculative assignments are possible if the entire complex is investigated. The heterologous expression and purification of FixPsoi and FixOsoi could lead to an unambiguous assignment of the three heme C redox centres to their corresponding subunit. Only a definitive assignment of the redox potentials to the subunits would lead to a better prediction of the route of electrons through the cbb^-type oxidase.
The X-ray structure or NMR-structure of the purified c-type cytochromes might help to understand the mechanism of electron acceptance and transfer within the c-type cytochrome subunits, which is used by the \( cbb_3 \)-type of heme-copper oxidases. The X-ray structure either of FixP\textsubscript{sol} or FixO\textsubscript{sol} would show the heme C locations regarding to the surface of their proteins. Other heme-copper oxidases have a Cu\textsubscript{A} centre as the first electron acceptor in subunit II, whereas the \( cbb_3 \)-type oxidases lack this Cu\textsubscript{A} centre (Garcia-Horsman \textit{et al.}, 1994b; Gray \textit{et al.}, 1994). Based on these findings one could for example speculate which of the two heme C centres in FixP\textsubscript{sol} is the immediate electron acceptor from an electron-donating cytochrome c (probably \( c_1 \)).

\section*{VI. 3. Exact assignment of terminal oxidases for different growth conditions}

\begin{center}
\begin{tikzpicture}
  \node [align=center] (CI) at (0,0) {CI and CII};
  \node [align=center] (Cl) at (1,0) {Dehydrogenases};
  \node [align=center] (Quin) at (1.5,-1) {Quinone/quinol-pool};
  \node [align=center] (CIII) at (3,0) {CIII};
  \node [align=center] (CIV) at (4,0) {CIV};
  \node [align=center] (bc1) at (3,-1) {\( bc_1 \)};
  \node [align=center] (CycM) at (3.5,-2) {CycM};
  \node [align=center] (aa3) at (4,-2) {aa\textsubscript{3}};
  \node [align=center] (CoxMNOP) at (4.5,-2) {CoxMNOP};
  \node [align=center] (bb3) at (4.5,-3) {bb\textsubscript{3}};
  \node [align=center] (bd) at (4.5,-4) {bd};

  \draw [->] (CI) -- (Cl);
  \draw [->] (Cl) -- (Quin);
  \draw [->] (Quin) -- (bc1);
  \draw [->] (bc1) -- (CycM);
  \draw [->] (CycM) -- (aa3);
  \draw [->] (aa3) -- (CoxMNOP);
  \draw [->] (CoxMNOP) -- (bb3);
  \draw [->] (bb3) -- (bd);

  \draw [->] (CIII) -- (bc1);
  \draw [->] (CIII) -- (CycM);
  \draw [->] (CIII) -- (aa3);
  \draw [->] (CIV) -- (bb3);
  \draw [->] (CIV) -- (bd);
\end{tikzpicture}
\end{center}

\textbf{Fig. 2.} The actual branched respiratory chain of \textit{B. japonicum}. The cydAB genes code for a \textit{bd}-type oxidase (Chapter IV). Although the benefit for the cell from this terminal oxidase is not known, the respiratory chain is extended by one quinol branch. CI, CII, CIII and CIV stand for complexes I, II, III, and IV homologues.

In chapter V it was demonstrated that the cydAB genes of \textit{B. japonicum} show the features of a \textit{bd}-type oxidase. However, the benefit from the oxidase for \textit{B. japonicum} was not found. A project should now be the systematic investigation of several aerobic growth conditions. As it is demonstrated, this organism has the genes for at least five terminal oxidases. Currently, no information is known about the expression conditions of the oxidases encoded by cydAB and coxMNOP.
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Aerobically, under free-living conditions, the aa₃-type oxidase is the primary terminal oxidase (CoxBA). The a-type cytochrome (605nm) is visible in membranes of aerobically grown cells (Bott et al., 1990). The role of the CoxMNOP oxidase for *B. japonicum* remained enigmatic (Bott et al., 1992). A further oxidase shows the features of a bb₃-type quinol oxidase (Surpin et al., 1996; Surpin et al., 1994). The exact role for this oxidase is also not known. It was demonstrated that the symbiotic nitrogen fixation ability of the bb₃⁻ mutant decreased only marginally (Surpin and Maier, 1999). Under microaerobic conditions the cbb₃-type oxidase is expressed. It was demonstrated that this oxidase is needed for symbiotic nitrogen fixation (Preisig et al., 1993). The most powerful approach to demonstrate that several oxidases are functional simultaneously in *B. japonicum* would be the investigation of the aerobic and microaerobic growth of double and triple mutants. This analysis could reveal the functionality for those gene products, which so far have not been demonstrated to encode functional oxidases (cydAB, coxMNOP) in *B. japonicum*. This approach, unfortunately, can only reveal the oxidase that is strictly needed to support growth in a given double or triple mutant. One would then still have to demonstrate whether these oxidases (CydAB and CoxMNOP) are expressed in a wild type strain under the same conditions. Only such a result could deliver information about the benefit from CydAB or CoxMNOP for the organism.

**Demonstration of the functionality of the cydAB genes in *B. japonicum***:

A bc₁-minus mutant still grows aerobically (Thöny-Meyer et al., 1989). This observation led to the assumption that a quinol oxidase is present in this organism to support growth. Current knowledge underlines further this expectation with the finding of the genes for a second quinol oxidase (cydAB). The construction of a double mutant impaired in the bc₁ complex and the bb₃-type quinol oxidase might be promising. This mutant, if it is viable, could finally demonstrate the functionality of a bd-type oxidase in *B. japonicum*; however, the benefit of cytochrome bd for the wild type cells would still remain enigmatic. In general, it is not possible without finding the appropriate physiological conditions to postulate the benefits for the cells resulting from an oxidase. It is a clear growth or survival phenotype that has yet to be detected for the cydA-minus mutant.
Demonstration of the functionality of the coxMNOP genes in B. japonicum:
A triple mutant impaired in the cbb\textsubscript{3}, aa\textsubscript{3}- and bb\textsubscript{3}-type oxidases might uncover a functionality for the putative cytochrome c oxidase encoded by CoxMNOP. When growing cells posses a bc\textsubscript{1}-complex, they generate more ATP. This is because the bc\textsubscript{1}-complex is itself a proton pump. A strain lacking the three oxidases (cbb\textsubscript{3}, aa\textsubscript{3} and bb\textsubscript{3}) could benefit from the fact that growing with CoxMNOP delivers more ATP, whereas growing with the bd-type quinol oxidase delivers less ATP. Preparation of membranes would deliver the corresponding difference spectra, which would be indicative of which type of oxidase the coxMNOP genes code for. However, a direct relation between the gene and gene product can be only delivered with a quadruple oxidase mutant strain (cbb\textsubscript{3}, aa\textsubscript{3}, bb\textsubscript{3} and bd-minus). The number of antibiotic markers available for replacement mutagenesis limits this approach for B. japonicum to maximally a triple mutant.

Problem linked to the investigation of expression conditions for oxidases:
As the respiratory chain in B. japonicum is branched, always other terminal oxidases could take over the reduction of O\textsubscript{2} to H\textsubscript{2}O in certain mutant backgrounds. Therefore, it is difficult to assign the role of one particular oxidase for one particular growth condition.
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