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Respiratory terminal oxidases in the facultative chemoheterotrophic and dinitrogen fixing cyanobacterium *Anabaena variabilis* strain ATCC 29413: characterization of the *cox2* locus☆

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Abstract

Upon nitrogen step-down, some filamentous cyanobacteria differentiate heterocysts, cells specialized for dinitrogen fixation, a highly oxygen sensitive process. Aerobic respiration is one of the mechanisms responsible for a microaerobic environment in heterocysts and respiratory terminal oxidases are the key enzymes of the respiratory chains. We used *Anabaena variabilis* strain ATCC 29413, because it is one of the few heterocyst-forming facultatively chemoheterotrophic cyanobacteria amenable to genetic manipulation. Using PCR with degenerate primers, we found four gene loci for respiratory terminal oxidases, three of which code for putative cytochrome *c* oxidases and one whose genes are homologous to cytochrome *bd*-type quinol oxidases. One cytochrome *c* oxidase, Cox2, was the only enzyme whose expression, tested by RT-PCR, was evidently up-regulated in diazotrophy, and therefore cloned, sequenced, and characterized. Up-regulation of Cox2 was corroborated by Northern and primer extension analyses. Strains were constructed lacking Cox1 (a previously characterized cytochrome *c* oxidase), Cox2, or both, which all grew diazotrophically. In vitro cytochrome *c* oxidase and respiratory activities were determined in all strains, allowing for the first time to estimate the relative contributions to total respiration of the different respiratory electron transport branches under different external conditions. Especially adding fructose to the growth medium led to a dramatic enhancement of in vitro cytochrome *c* oxidation and in vivo respiratory activity without significantly influencing gene expression.

Keywords: Respiration; Nitrogen fixation; Cytochrome *c* oxidase; Quinol oxidase; Symbiosis

1. Introduction

Cyanobacteria are prokaryotes capable of oxygenic photosynthesis. In almost all cyanobacteria the photosynthetic electron transport chain is localized in intracytoplasmic membranes or thylakoids, which are also the site of a respiratory electron transport chain. The two processes share several components, making cyanobacteria the only cells in which these two most important bioenergetic processes occur in the same cellular compartment. The cytoplasmic membrane, in contrast, does not contain a functional photosynthetic electron transport chain, but probably contains a second respiratory chain [1] (for a review see Ref. [2]). In addition, some cyanobacteria are

Abbreviations: Synechocystis PCC 6803, *Synechocystis* sp. strain PCC 6803; *A. variabilis* ATCC 29413, *Anabaena variabilis* strain ATCC 29413 FD; *Anabaena* PCC 7120, *Anabaena* sp. strain PCC 7120; Cox, cytochrome *c* oxidase; Qox, cytochrome *bd*-type quinol oxidase; ARTO, alternate respiratory terminal oxidase; ORF, open reading frame; RT-PCR, reverse transcription-polymerase chain reaction; bp, base pair(s); kb, kilobase(s); kbp, kilobase pair(s); PCP, pentachlorophenol; HQNO, 2-heptyl-4-hydroxyquinoline N-oxide

☆ The nucleotide sequence for the *cox2* locus reported in this paper has been submitted to the EMBL database with accession number AJ296086.

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able to fix dinitrogen. In the absence of combined nitrogen, certain filamentous strains, belonging, e.g., to genera *Anabaena* or *Nostoc*, differentiate some of their cells into so-called heterocysts that are specialized for nitrogen fixation. Heterocysts are uniquely effective in protecting the highly O_2 sensitive key enzyme(s) of dinitrogen fixation, the nitrogenase(s), from oxygen produced in oxygenic photosynthesis in the neighboring vegetative cells. Recently, we demonstrated in the related but obligately photoautotrophic strain *Anabaena* sp. strain PCC 7120 that respiration plays an essential role in nitrogen fixation by heterocysts [3]. The aim of this work was to identify respiratory terminal oxidase(s) in *Anabaena variabilis* strain ATCC 29413, one of the very few heterocyst-forming cyanobacteria that are able to grow chemoheterotrophically in darkness, with combined nitrogen or with dinitrogen as the nitrogen source, and can be genetically manipulated using the conjugation system developed by Elhai and Wolk [4] and Wolk et al. [5].

All known respiratory terminal oxidases in cyanobacteria are members of only two protein families, the relatives of proton-pumping heme-copper cytochrome *c* oxidases (Cox, with three subunits, CoxB, CoxA, and CoxC) [6], and the cytochrome *bd*-type quinol oxidases (with two subunits, CydA and CydB) [7]. The former enzymes belong to two subgroups, the genuine cytochrome *c* oxidases that are present in all cyanobacteria [8] or the related enzymes with uncertain electron donors that have been described earlier [34] and under the name ARTO in *Synechocystis* PCC 6803 [9,1] and *Anabaena* PCC 7120 [3].

We previously identified in *A. variabilis* ATCC 29413 a cytochrome *c* oxidase (now renamed Cox1) that was shown to be essential for chemoheterotrophic growth using the Cox1 minus mutant strain CSW1 [10]. Strain CSW1 was able to differentiate functional heterocysts and grow on dinitrogen under phototrophic conditions. In vitro cytochrome *c* oxidase activities of membranes from CSW1 clearly showed that at least one other Cox must be present in *A. variabilis* ATCC 29413. We therefore searched for other respiratory terminal oxidases with PCR using degenerate primers in this strain and found a total of four: three putative cytochrome *c* oxidases, Cox1, Cox2, and Cox3 (ARTO-type), and one cytochrome *bd*-type quinol oxidase homolog. We cloned, mutated, and characterized in detail Cox2, since it was the only respiratory terminal oxidase significantly up-regulated under nitrogen-fixing conditions.

2. Materials and methods

2.1. Strains and growth conditions

The cyanobacterium *A. variabilis* strain ATCC 29413 was the source of a gene library in vector λEMBL3A. The variant *A. variabilis* strain ATCC 29413 FD [11] was used for construction of mutants and all other experiments, since it has a higher efficiency for gene transfer from *Escherichia coli*. *A. variabilis* strains were grown photoautotrophically, photoheterotrophically (+15 mM fructose and 10 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU)), mixotrophically (+15 mM fructose), or chemoheterotrophically (+15 mM fructose in the dark) at 32 °C in medium BG-11 or BG-11_0 [12] in 100-ml Erlenmeyer flasks in a shaker with 0.25% (vol/vol) CO_2 in air and 60 μmol quanta m^{-2} s^{-1} fluorescent white light. The media were supplemented with 10 μg ml^{-1} erythromycin, 20 μg ml^{-1} neomycin, and/or 50 μg ml^{-1} spectinomycin as needed.

Growth rate constants were estimated from the increase of protein concentration in shaken liquid cultures, grown at 30 °C in the light (75 μmol quanta m^{-2} s^{-1}) with air levels of CO_2. Protein concentrations were determined by a modified Lowry method [13] from 200-μl aliquots. The growth rate constant (μ) corresponds to $\ln 2 \times t_{d-1}$ where t_d represents the doubling time. *E. coli* strains DH5α, LE392, and HB101 were used for cloning, phage propagation, and conjugation, respectively. *E. coli* strains were grown in LB medium for cloning purposes and conjugation, and in NZY medium for phage propagation and plaque lifting.

2.2. Cloning, sequencing, and mutant construction

A list of plasmids and oligonucleotides used is presented in Table 1. Standard molecular biology procedures (like cloning or plaque lifting and hybridization) were performed according to Sambrook et al. [18]. For cloning of *cox* genes PCR was performed with primers DgCox-5′ and DgCox-3′ using about 1-μg total DNA from CSW1 (and *A. variabilis* ATCC 29413 as control), AccuTaq polymerase (Sigma-Aldrich) and the following cycle conditions: 20 s at 94 °C, 20 s at 46 °C, and 20 s at 72 °C (35 cycles). The *cyd* genes were identified by PCR with the degenerate Cyd primers (see Table 1) using about 1-μg total DNA from *A. variabilis* ATCC 29413 (and *Synechocystis* PCC 6803 as control), AccuTaq polymerase and the following cycle conditions: 30 s at 94 °C, 30 s at 42 °C, and 2–3 min (+2 s per cycle) for primer pairs CydA-5′/CydA-3′ and CydB-5′/CydB-3′ or 4–6 min (+4 s per cycle) for the primer pair CydA-5′/CydB-3′ at 72 °C (30 cycles).

For the partial removal of the genes *coxB2* and *coxA2*, plasmid pDPUV47 (Table 1) was constructed (using plasmids pDPUV42 to pDPUV46, pRL25V, and pRL425, see Table 1). This plasmid essentially contains the *cox2* locus of *A. variabilis* ATCC 29413 from the *Eco*RI restriction site at position 102 to the *Hin*dIII restriction site at position 4300 (positions according to EMBL acc. no. AJ296086), in which most of the *coxB2* and *coxA2* genes from the *Bsr*BI restriction site at position 983 to the *Hin*dIII restriction site at position 3164 was removed and replaced

Table 1
List of plasmids and oligonucleotides used in this work

Name	Relevant characteristics and use	Reference/source
Plasmids		
pUC19	Cloning and subcloning	[14]
pRL425	Source of erythromycin resistance cassette (~1 kbp *Ecl*136II fragment)	[15]
pRL25V	Vector for construction of the *cox2* minus mutant strains PDCn and PDC-Cn: pRL25 without the pDU1 part (removed with *Eco*RV), Neomycin resistance	[16,4]
pRL443	Conjugative plasmid	[17]
pRL528	Helper plasmid	[17]
pRL591-W45	Helper plasmid	[17]
pDPUV35	280 bp PCR fragment (primer: DgCox-5′, DgCox-3′) cloned in the *Sma*I site of pUC19, insert not cut by *Rca*I but cut by *Xmn*I and *Hae*II	Figs. 1B and C
pDPUV36	280 bp PCR fragment (primer: DgCox-5′, DgCox-3′) cloned in the *Sma*I site of pUC19, insert cut by *Rca*I and *Mfe*I	Figs. 1B and C
pDPUV42	3.2 kbp *Hin*dIII fragment of λDP35 cloned in the *Hin*dIII site of pUC19	Fig. 4A
pDPUV43	1.1 kbp *Hin*dIII fragment of λDP35 cloned in the *Hin*dIII site of pUC19	Fig. 4A
pDPUV44	1.6 kbp *Hin*dIII fragment of λDP35 cloned in the *Hin*dIII site of pUC19	Fig. 4A
pDPUV45	*Eco*RI–*Bsr*BI fragment (882 bp) from pDPUV42 cloned in the *Eco*RI and *Sma*I restriction sites of pDPUV43	Materials and methods
pDPUV46	*Ecl*136II fragment (erythromycin resistance cassette) from pRL425 cloned in the filled up *Bam*HI restriction site of pDPUV45	Materials and methods
pDPUV47	*Pvu*II fragment from pDPUV46 cloned in the *Eco*RV restriction site of pRL25V	Materials and methods
Oligonucleotides[a]		
Primers for degenerate PCR and relative quantitative RT-PCR		
DgCox-5′	5′-TGGGYNCAYCAYATGTT-3′	Fig. 1A
DgCox-3′	5′-ACRTARTGRAARTGNSCNACNAC-3′	Fig. 1A
CydA-5′	5′-TTTCAATTYGGTACGAACTGG-3′	Fig. 2A
CydA-3′	5′-CCCCCAATSACAAACAWAGARGTTTC-3′	Fig. 2A
CydB-5′	5′-GGCTCCACCTATCTCATYTT-3′	Fig. 2A
CydB-3′	5′-GTAATTGTAGATGTTGTAGAACAAC-3′	Fig. 2A
Primers for homozygocity check		
Cox2-5′	5′-ATAATACCGATGGCGTACCAGTGG-3′	Fig. 4A
Cox2-3′	5′-TAGTCATAAGGCCCATGAGTCACC-3′	Fig. 4A
Primers for relative quantitative RT-PCR		
Sd-Cox	5′-ATCAAAAGGCACGGCGTCCAAC-3′	Fig. 1C
RT-Cox	5′-TACCAGTGGTATTCCCGGCTGG-3′	Fig. 1C
Sd-35	5′-TCAAAAGGTGCTGTACCCATTG-3′	Fig. 1C
RT-35	5′-TACCAGTGGTACACCCGGTTGG-3′	Fig. 1C
Sd-36	5′-ATCAATTGGTACTGCGGAAAGC-3′	Fig. 1C
RT-36	5′-GCGGATAGTGTTCATGATTTCC-3′	Fig. 1C
SigA-5′	5′-GGGCNGANGAAGAAATTGAAC-3′	Materials and methods
SigA-3′	5′-CGGGAGATGGTTTCGTAGAGGTGGAC-3′	Materials and methods
Primers for probe used in Northern blot analysis		
CoxB1	5′-CAGCAAATTCCTGTTTCACT-3′	Fig. 4A
CoxB3	5′-CGGACATTCCCTGCAACGTA-3′	Fig. 4A
Primers for primer extension analysis		
CoxB-tsp1	5′-CGCCATCGCCCAAGTAG-3′	Fig. 4A
CoxB-tsp2	5′-CAATCCAAAGACTGATTACTCC-3′	Fig. 4A
CoxB-tsp3	5′-GGTTGAAGACATCTACAC-3′	Fig. 4A
CoxB-tsp4	5′-CACATTGACAACTAAGTCTGCG-3′	Fig. 4A

[a] Common IUPAC ambiguity codes: N=A, G, C, T; R=A, G; S=G, C; W=A, T; Y=C, T.

by an erythromycin resistance cassette. Plasmid pDPUV47 was introduced into *A. variabilis* ATCC 29413 FD and its derivative CSW1 [10] by triparental mating using conjugal plasmid pRL443 and helper plasmids pRL528 and pRL591-W45 [15,17]. Exconjugants were selected on BG-11 plates with erythromycin and further grown in liquid BG-11 medium containing erythromycin. Cells were propagated with successive dilutions until no *E. coli* cells were detectable and then plated on BG-11 agar with erythromycin. Thereafter three colonies that were neomycin-sensitive were tested for homozygocity at the mutated locus by PCR as described [9].

2.3. Southern blot analysis

Total DNA from *A. variabilis* strains ATCC 29413 FD and CSW1 was prepared according to Cai and Wolk [19], and hybridized with ^{32}P-labeled probes *coxA1*, *coxA2*, or *coxA3* (Fig. 1C).

2.4. Gene expression analysis

For relative quantitative RT-PCR, total RNA from *A. variabilis* strains was prepared with acid-equilibrated phenol essentially as described [20] except that freshly harvested cells were vortexed with glass beads (212-300

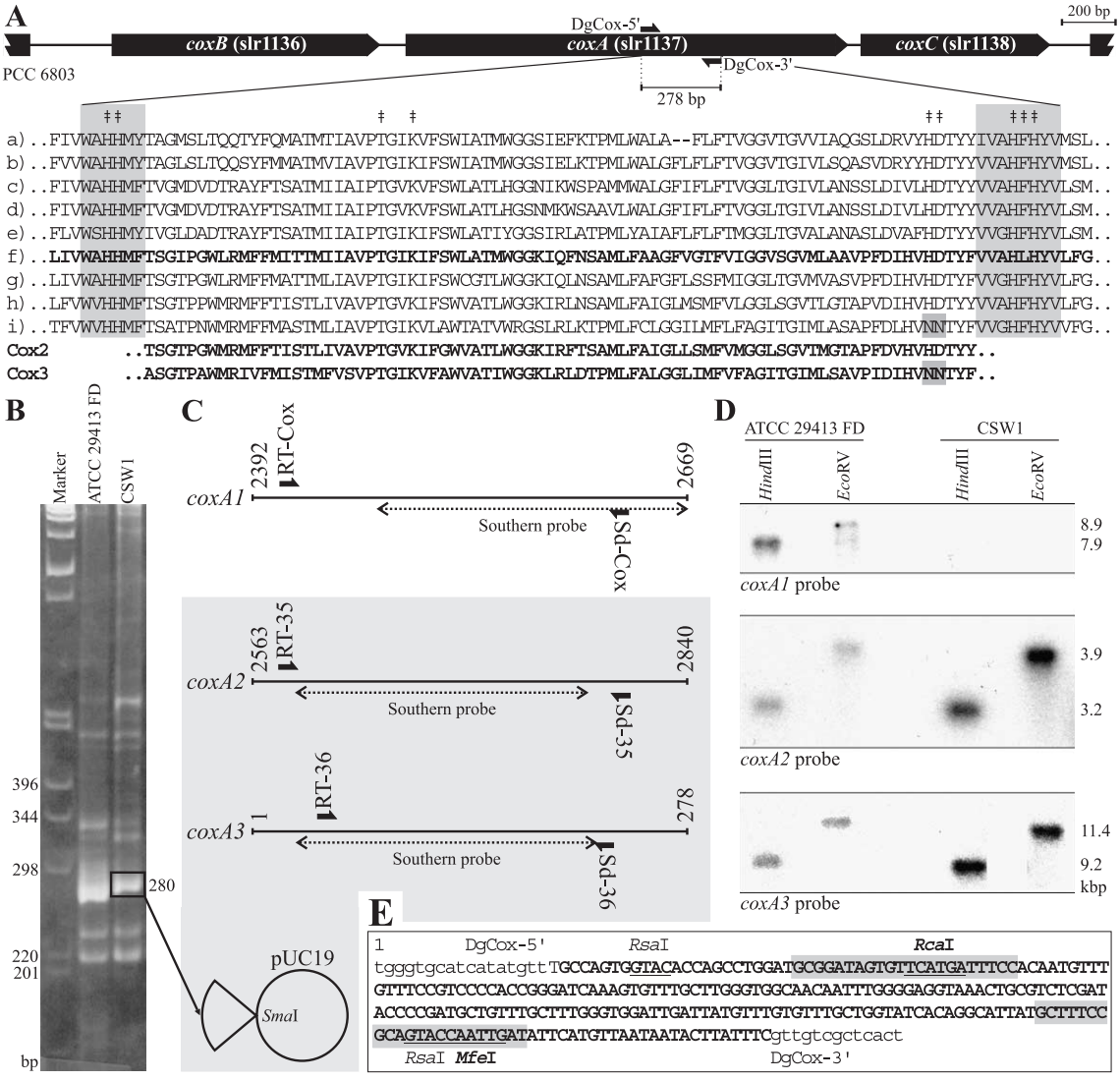

Fig. 1. The *cox* loci of *A. variabilis* strain ATCC 29413. (A) Scheme of the *cox* locus of *Synechocystis* PCC 6803 showing the location of the two highly conserved amino acid sequence regions (shaded boxes) selected for the design of the degenerate primers DgCox-5′ and DgCox-3′ (Table 1). The amino acid sequences used were from (a) *Paracoccus denitrificans* [28], (b) *Rhodobacter sphaeroides* [29], (c) *Bos taurus* [30], (d) *Homo sapiens* [31], (e) *Saccharomyces cerevisiae* [32], (f) *Anabaena variabilis* ATCC 29413, Cox1 [10], (g) *Synechocystis* PCC 6803, Cox [8], (h) *Synechococcus vulcanus* [33], (i) *Synechocystis* PCC 6803, ARTO [34]. Important amino acid residues identified with the help of the X-ray structure of cytochrome *c* oxidases [35–37] are marked by ‡: His^{325} and His^{326} Cu_B ligands, Thr^{351} and Lys^{354} proton K-pathway, His^{393} and Asp^{394} Mn^{2+}/Mg^{2+} ligands, His^{411} heme a_3 ligand, Phe^{412} electron transfer between hemes, and His^{413} heme *a* ligand (numbers are from *P. denitrificans*). The amino acid sequences of the corresponding part of the three *cox* loci of *A. variabilis* ATCC 29413 (including the two newly identified loci *cox2* and *cox3*) are shown in bold letters. The highlighted amino acid motif ‘NN’ is characteristic for a subtype of putative cytochrome *c* oxidases found exclusively in cyanobacteria, which lacks the typical Mg^{2+} binding motif ‘HD’ in subunit I (CoxA) and lacks the typical Cu_A binding motif in subunit II (CoxB). (B) PCR products obtained with primers DgCox-5′ and DgCox-3′ using total DNA of *A. variabilis* strains ATCC 29413 FD and CSW1 as template, separated on an 8% polyacrylamide gel. (C) The DNA fragments obtained by cloning the 280-bp PCR products (B) in pUC19, showing the *Rsa*I fragments used for the Southern blots (see D) and the location of the primers used for RT-PCR experiments (see Figs. 3A and B). Numbers are according to the corresponding EMBL sequences, acc. no. Z98264 for *coxA1* and acc. no. AJ296086 for *coxA2*, respectively. (D) Southern blot analysis with total DNA from *A. variabilis* strains ATCC 29413 FD and CSW1, digested with *Hin*dIII and *Eco*RV. Probes, see C. (E) DNA sequence of the PCR fragment containing a part of the *coxA3* gene of *A. variabilis* ATCC 29413. The amino acid sequence deduced from the part printed in bold letters is shown in A. The shaded boxes indicate the primers RT-36 and Sd-36 used for relative quantitative RT-PCR (see Fig. 3B).

microns, Sigma-Aldrich) for 4 min. Two-step RT-PCR was performed using the enhanced avian RT-PCR kit (Sigma-Aldrich). Total RNA was denatured together with the specific primer for 10 min at 85 °C and the reverse transcriptase reaction was performed for 40 min at 48 °C followed by 20 min at 60 °C. PCR cycle conditions were 45 s at 94 °C, 30 s at 45 °C, and 1–2 min (+2 s per cycle) at 72 °C (30 cycles). The PCR primers and conditions were evaluated with the plasmids carrying the corresponding genes as template and yielded comparably PCR products for all three *coxA* genes. No RT-PCR-standard to control equal loading of the lanes is available for *A. variabilis* ATCC 29413, when the strain is grown under different conditions. Therefore, we used for this purpose an RT-PCR reaction with primers SigA-5′ and SigA-3′ (Table 1) that were derived from highly conserved regions of the *sigA* genes of *Synechocystis* PCC 6803 (*slr0653*) and *Anabaena* PCC 7120 (*all5263*) [21]. The *sigA* gene, coding for a sigma factor, has been shown to be constitutively expressed in the closely related strain *Anabaena* PCC 7120 [22].

For analysis of RNA, cells were grown exponentionally in BG-11_0C medium (BG-11_0 plus 10 mM $NaHCO_3$) or BG-11_0C medium supplemented with 17.6 mM $NaNO_3$ or BG-11_0C medium supplemented with 5 to 8 mM NH_4Cl plus double concentration of 2-[(2-hydroxy-1,1-bis[hydroxymethyl]ethyl)amino]ethanesulfonic acid (TES)-NaOH buffer (pH 7.5) with or without 20 mM fructose. Cultures were bubbled with a mixture of 1% (vol/vol) CO_2 in air. For Northern blot and primer extension analysis, total RNA was isolated as described earlier [23] (based on Golden et al. [24]). About 30-μg total RNA per lane was electrophoresed on an 1% (mass/vol) denaturing agarose gel and transferred to a Hybond-N^+ membrane using 0.1 M NaOH. Hybridization and washing was performed as recommended by the manufacturer at 65 °C. Probes were ^{32}P-labeled with a Ready to Go™ DNA labeling kit (Amersham Biosciences) using [α-^{32}P]-dCTP. A control for the relative amounts of RNA loaded and transferred per lane was obtained by hybridization with the gene for the RNA subunit of ribonuclease P (*rnpB*) from *Anabaena* PCC 7120 [25]. For primer extension analysis, 25 μg of total RNA was used with primers Cox-tsp1, Cox-tsp2, Cox-tsp3, and Cox-tsp4 and the reverse transcriptase Superscript (Invitrogen). Oligonucleotide primers were radioactively end-labeled as described [26]. The accompanying sequencing ladder was obtained by the dideoxy chain termination method, using the T7 Sequencing Kit (Amersham Biosciences) and plasmid pDPUV42 as template.

2.5. Respiratory activities

Measurement of respiratory O_2 uptake activity in the dark with a Clark-type electrode at 32 °C in growth medium was described earlier [9]. In vitro horse heart cytochrome *c* oxidase activity was determined as described [10], except that the incubation on ice for 1 h was omitted. Chlorophyll *a* (chl) was determined as described [27].

3. Results

3.1. Cloning and identification of genes coding for respiratory terminal oxidases in A. variabilis ATCC 29413

The search for additional *cox* loci was performed by PCR in *A. variabilis* mutant strain CSW1 that lacks the genes for Cox1 [10]. Two degenerate primers, DgCox-5′ and DgCox-3′ (Table 1), were designed using the consensus amino acid sequences W(V/A)HHMF and VV(A/G)HFHYV, two highly conserved regions of subunit I of cytochrome *c* oxidases from cyanobacteria and other taxonomic groups (Fig. 1A). The 280-bp PCR products expected for *cox* loci were separated on an 8% polyacrylamide gel (Fig. 1B) and cloned into the *Sma*I site of pUC19. Eight such clones were screened with the restriction enzyme *Rca*I, which has one recognition site in the previously known sequence of *coxA1* (EMBL acc. no. Z98264). Three clones were cut by *Rca*I and five were not cut (data not shown), and one clone of each type (pDPUV35 and pDPUV36) was sequenced (cf. Figs. 1A and E). The translated amino acid sequences showed high similarity to CoxA proteins and the genes were therefore called *coxA2* and *coxA3*, respectively (Fig. 1). The partial amino acid sequence of the *cox3* locus showed two amino acid differences in the Mg^{2+} binding motif (highlighted in Fig. 1A) characteristic of this subtype of oxidases in cyanobacteria (ARTO) [9,34,3]. To find possible further *coxA* genes, 50 additional clones containing a 280-bp PCR product were screened with the following restriction enzymes (cf. Table 1): *Rca*I cutting *coxA1* and *coxA3*, *Pvu*II cutting *coxA1*, *Xmn*I and *Hae*II cutting *coxA2*, and *Mfe*I cutting *coxA3*. No further restriction patterns besides the ones for *coxA2* and *coxA3* were found (data not shown). Southern blot analysis with probes specific for *coxA1*, *coxA2*, and *coxA3* (*Rsa*I fragments shown in Fig. 1C) confirmed the existence of three different genes for *coxA* in *A. variabilis* ATCC 29413 and two in strain CSW1 (Fig. 1D).

A similar approach was chosen to find possible *cyd* genes in *A. variabilis* ATCC 29413 coding for cytochrome *bd*-type quinol oxidases. Four degenerate primers were designed using four highly conserved regions in the genes *cydA* and *cydB* of *Synechocystis* PCC 6803 and *Anabaena* PCC 7120 and used for three PCR reactions (Table 1 and Fig. 2A). Indeed, three PCR products with the same length as with total DNA from *Synechocystis* PCC 6803, namely ~380 bp, ~440 bp, and ~2.2 kbp, were found with total DNA from *A. variabilis* ATCC 29413 (Fig. 2B), indicating that both genes for a cytochrome *bd*-type quinol oxidase are present in *A. variabilis* ATCC 29413 in an operon-like locus similar to other cyanobacteria. Preliminary sequence data from the 2.2-kbp PCR product confirmed the identification

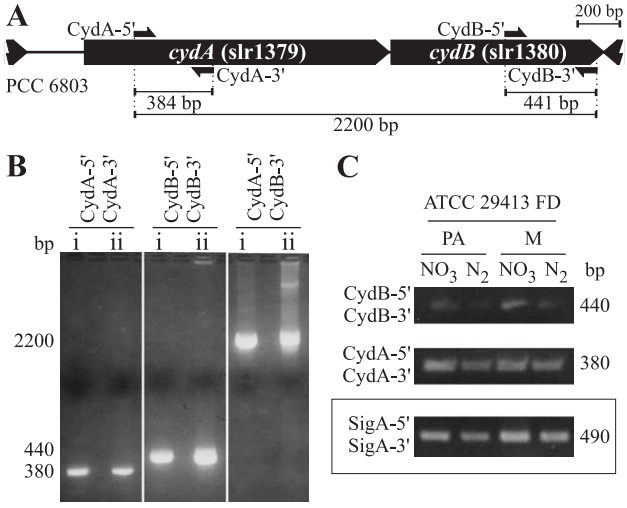

Fig. 2. The *cydAB* locus of *A. variabilis* strain ATCC 29413 FD. (A) Scheme of the *cydAB* locus of *Synechocystis* PCC 6803 showing the location of the degenerate primers used for identification and relative quantitative RT-PCR of the *A. variabilis* ATCC 29413 *cydAB* locus. (B) PCR products obtained with the indicated degenerate primer pairs using total DNA from *Synechocystis* PCC 6803 (i) or *A. variabilis* ATCC 29413 (ii) as template. (C) Expression analysis of the *A. variabilis* ATCC 29413 *cydA* and *cydB* genes using relative quantitative RT-PCR with the indicated primer pairs and 1-μg total RNA from cells grown photoautotrophically (PA) or mixotrophically (M) with N_2 or NO_3^- as the nitrogen source. RT-PCR with primers SigA-5′ and SigA-3′ was used as a control for loading equal amounts of RNA per lane.

as *cydAB* genes (A. Ludwig, D. Pils, and G. Schmetterer, unpublished results).

3.2. Expression studies of the three coxA genes and the cydAB locus using RT-PCR

For relative quantitative expression analysis, new primer pairs highly specific for *coxA1* (Sd-Cox, RT-Cox), *coxA2* (Sd-35, RT-35), and *coxA3* (Sd-36, RT-36) were designed (Table 1 and Fig. 1C) and evaluated with plasmids carrying the corresponding genes, while for the *cyd* genes the same primer pairs (see above) could be used. RT-PCR with these primers and 0.9- to 1.2-μg DNA-free total RNA from different strains (ATCC 29413 FD, CSW1 (Cox1 minus), and PDC-Cn (Cox1/Cox2 minus, see below)) grown on different nitrogen sources (nitrate or dinitrogen) and bioenergetic regimes (photoautotrophy, mixotrophy, or chemoheterotrophy) was performed (see Materials and methods). RT-PCR did not provide any evidence for a regulation of expression of *cydA* and *cydB* genes dependent on nitrogen source or bioenergetic regime (Fig. 2C). The relative quantitative RT-PCR experiments with the *coxA1* gene showed the same expression pattern as previously reported [10], namely up-regulation in cells grown with fructose both in the dark (chemoheterotrophy) and in the light (mixotrophy) (Figs. 3A and B).

The *coxA2* gene is expressed in both strains tested (ATCC 29413 FD and CSW1) under all growth conditions used. However, the RT-PCR experiments indicate that expression of *coxA2* might be higher in diazotrophically grown cells than in cells grown on nitrate irrespective of

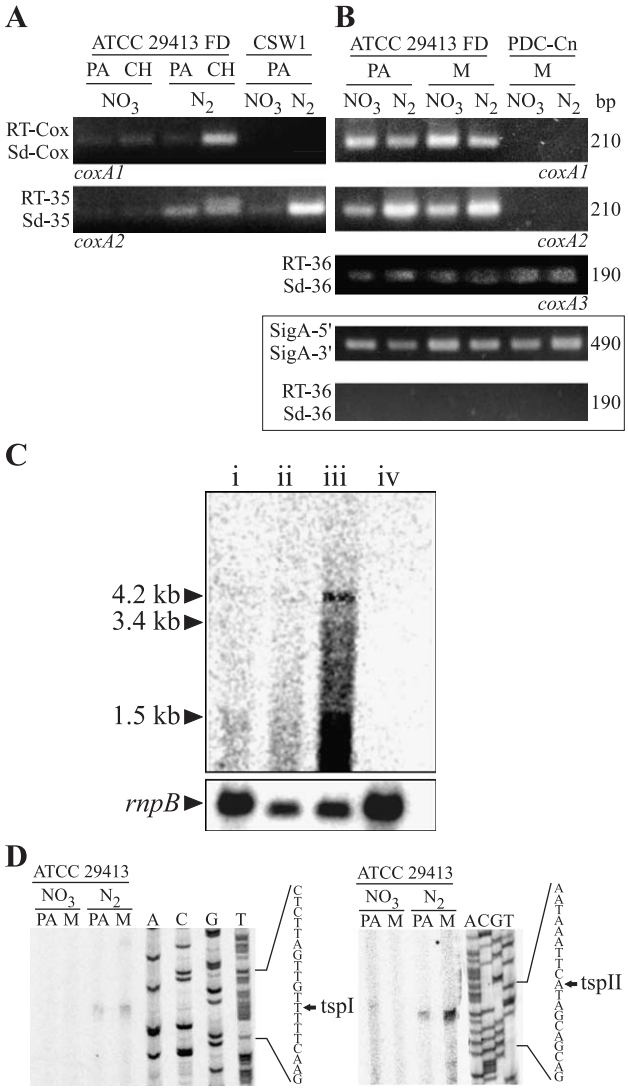

Fig. 3. Expression analysis of *A. variabilis* strain ATCC 29413 FD cytochrome *c* oxidase genes. (A and B) Relative quantitative RT-PCR with primer pairs specific for *coxA1*, *coxA2*, and *coxA3* used on total RNA from *A. variabilis* strains grown under different conditions: photoautotrophy (PA), mixotrophy (M), or chemoheterotrophy (CH). The contrast of the photo with *coxA3* was enhanced to show the very faint bands obtained. The loading control with the SigA primers was similar as in Fig. 2C. In view of the very low abundance of the *coxA3* transcript, a negative control confirming the absence of DNA in this sample was performed omitting the RT reaction from the RT-PCR protocol (B, bottom panel). The amounts of total RNA used were 0.9 μg for the experiments shown in A and 1.2 μg for B. (C) Northern blot analysis of total RNA isolated from *A. variabilis* ATCC 29413 grown on NH_4^+ (i), NO_3^- (ii), N_2 (iii), or NH_4^+ with 20 mM fructose added (iv). The probe consisted of a PCR product containing almost the entire *coxB2* gene (see Fig. 4A). The same filter was also hybridized with an *rnpB* probe that corresponds to the RNA subunit of RNase P of *Anabaena* PCC 7120 as a loading and transfer control. (D) Determination of the two putative tsps of the *cox2* locus by primer extension analysis. Total RNA was prepared from wild-type *A. variabilis* ATCC 29413 grown either on NO_3^- or on N_2, both under photoautotrophic (PA) or mixotrophic (M) conditions.

the bioenergetic regime employed (Figs. 3A and B). The expression of *coxA3* is very weak and not measurably regulated (Fig. 3B). As expected, no signal was obtained in strain CSW1 with *coxA1* primers (Fig. 3A) and in strain PDC-Cn with both *coxA1* and *coxA2* primers (Fig. 3B), confirming that the primers are specific for the corresponding *coxA* genes and that the strains are homozygous for the absence of *coxA1* and *coxA1* plus *coxA2*, respectively.

3.3. Cloning and sequencing of the cox2 locus of A. variabilis ATCC 29413

Of the respiratory terminal oxidases identified above, Cox2 was of particular interest, because it was the only one whose expression, analyzed by RT-PCR, was appreciably influenced by the nitrogen source. Therefore, this gene locus was investigated in more detail. To this end, a gene library consisting of partially *Sau*3AI digested total DNA from *A. variabilis* ATCC 29413 cloned into the *Bam*HI site of λEMBL3A was hybridized with the *coxA2* probe (Fig. 1C). One clone called λPD35 was obtained that covered the entire *cox2* locus (Fig. 4A).

Parts of λPD35 were subcloned (pDPUV42, pDPUV43, and pDPUV44) and sequenced until the complete *cox2* gene set was covered including the ends of the adjoining genes, *fdxB* (coding for a ferredoxin homologous to *asr2513* in *Anabaena* PCC 7120, probably involved in electron transport for nitrogen fixation), at the 5′ end and a gene of unknown function homologous to *Anabaena* PCC 7120 gene, *alr2517*, at the 3′ end (Fig. 4A). Three open reading frames were found whose translated amino acid sequences were highly homologous to *coxB*, *coxA*, and *coxC* gene products from other organisms, especially other cyanobacteria, and therefore called *coxB2*, *coxA2*, and *coxC2*. All important amino acid residues of CoxA and CoxB characteristic for genuine cytochrome *c* oxidases as derived from the available X-ray structures of the enzymes

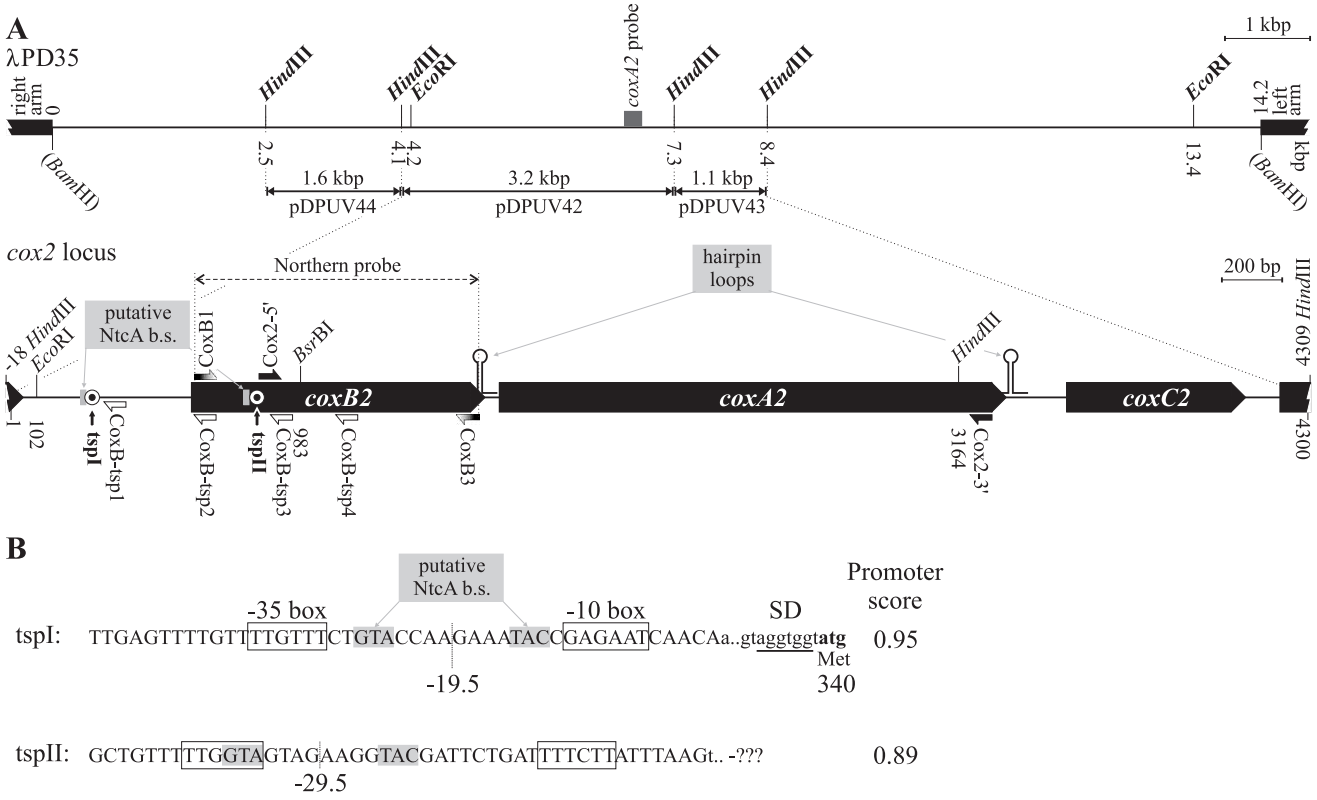

Fig. 4. The *cox2* locus of *A. variabilis* strain ATCC 29413. (A) In the upper scheme, a physical map of λPD35 and its subclones used for sequencing that contain the *cox2* locus is shown. The numbers in the *cox2* locus scheme below are nucleotide numbers according to the sequence in the EMBL database (acc. no. AJ296086). The locations of the adjoining genes are indicated as a black triangle on the left (*fdxB*) and as a black box on the right end (homolog of *Anabaena* PCC 7120 *alr2517*) of the *cox2* locus scheme. The two encircled dots show the location of the two putative transcriptional start points (tspI and tspII, see Figs. 3D and B). In front of each tsp there is a putative NtcA binding site (BS) indicated by a shadowed box (see B for details). Arrows denote the location of primers used for primer extension analysis (open), for the preparation of a probe for Northern hybridization (hatched), or for confirming the homozygocity of the *cox2* mutants by PCR (filled). The positions of hairpin loops (possible transcriptional stop signals) downstream of the *coxB2* gene and downstream of the *coxA2* gene are indicated (cf. Section 3.4). (B) Annotated sequences of the two putative promoter regions of the *cox2* locus upstream of tspI and tspII. The first bases of the RNA-products are marked by lowercase letters. The Shine–Dalgarno box is underlined (SD). Possible translational products of RNA molecules starting at tspII are unclear and therefore—in contrast to tspI—no distance from the tsp to the first translational start is indicated. Analysis of these sequences by the Neural Network Promoter Prediction software (http://www.fruitfly.org/seq_tools/promoter.html) yielded high promoter probability scores and predicted correctly the experimentally determined tsps.

from *P. denitrificans* and beef heart mitochondria [35–37] are present in Cox2 of *A. variabilis* ATCC 29413, except for CoxA Ser^{134} (CoxA2: Ala) and Glu^{278} (CoxA2: Ala) that are both part of the proton D-pathway (numbers for *P. denitrificans* [35]). It is assumed that amino acid composition may vary in the proton D- and K-pathways and Gomes et al. [39] showed that several amino acids of these pathways are not essential for efficient proton pumping in bacterial cytochrome *c* oxidases.

3.4. Analysis of the cox2 locus of A. variabilis ATCC 29413

A scheme of the *cox2* locus of *A. variabilis* ATCC 29413 and some of its features is depicted in Fig. 4. Since the *coxA2* gene was found to be regulated by the nitrogen regime, the *cox2* locus was searched for the occurrence of putative NtcA binding sites. The NtcA protein is a DNA binding protein acting as a central regulator of genes involved in nitrogen metabolism and heterocyst differentiation in cyanobacteria [47]. The recognition sequence is essentially GTA-N_8-TAC [40]. Two such sequences were detected in the *A. variabils* ATCC 29413 *cox2* locus, one 359.5 bp upstream of the putative *coxB2* translational start and the second 840.5 bp upstream of the putative *coxA2* translational start (within the ORF of *coxB2*) (Figs. 4A and B).

With primer extension analysis using primers CoxB-tsp1, CoxB-tsp2, CoxB-tsp3, or CoxB-tsp4 (see Table 1 and Fig. 4A), covering both putative NtcA binding sites and the complete *fdxB-coxB2* intergenic region, two putative transcriptional start points were found (tspI, 340 bp upstream of the putative *coxB2* translational start, and tspII, 811 bp upstream of the putative *coxA2* translational start but within the *coxB2* ORF) (Figs. 4 and 3D). Signals were obtained only with total RNA from cultures grown with dinitrogen as nitrogen source and were stronger from mixotrophically grown cultures than from those grown photoautotrophically (Fig. 3D). Both tsps are directly (19.5 and 29.5 bp, respectively) downstream of the two putative NtcA binding sites (Fig. 4B). For the sequence of the two putative promoters, possible −35 and −10 boxes, and the promoter scores estimated by the Neural Network Promoter Prediction software (http://www.fruitfly.org/seq_tools/promoter.html), see Fig. 4B.

In order to determine the lengths of the transcript(s) of the *cox2* locus, Northern blot analysis using a probe that consisted of almost the entire *coxB*2 gene (cf. Fig. 4A) was performed. When total RNA from cells grown on dinitrogen was used, three bands with lengths of 4.2, 3.4, and 1.5 kb were obtained (Fig. 3C). The largest transcript found for the *cox2* locus (4.2 kb) is sufficient to cover all three *cox2* genes showing that they constitute an operon. The second one (3.4 kb) corresponds either to a transcript starting at tspI and ending at the hairpin loop downstream of *coxA2* (~3050 b) or to a mRNA starting at tspII and ending downstream of *coxC2* (~3600 b). The third one (1.5 kb) corresponds to a transcript of *coxB2*, possibly ending at the hairpin loop downstream of *coxB2*. In view of the same regulatory pattern obtained for tspI and tspII (Fig. 3D), and the unusual location of tspII within an ORF (*coxB2*), it cannot be excluded that the mRNA starting at tspII is actually a processing product of a primary message starting at tspI. With regard to nitrogen control of *cox2* expression, the mechanism for this regulation remains unknown and warrants further investigation, since both putative NtcA binding sites overlap the region of the −35 and −10 boxes of the putative promoters (Fig. 4B), which is suggestive of NtcA repressor sites. With total RNA from cells grown on ammonium, nitrate, or ammonium plus 20 mM fructose, no transcripts were found (Fig. 3C).

Two palindromes representing putative RNA hairpin loops were detected immediately downstream of *coxB2* (AAG*UAG*AGACGUUCCAUACAACGUUUCUACAA; *stop codon; ΔG°=−59.5 kJ mol^{-1} at 37 °C, calculated with the RNA mfold server at http://www.bioinfo.rpi.edu/applications/mfold/old/rna/ [38] using the default settings) and *coxA2* (CC*UAA*CCCCCUAGCCCCCUUCCCUCGUAGGGAAGGGGGAA; *stop codon; ΔG°=−94.6 kJ mol^{-1} at 37 °C). Each ORF of the *cox2* locus is preceded by a putative Shine–Dalgarno box, *coxB2* (AGGUGG), *coxA2* (GGUGGU), and *coxC3* (GGAGAA).

3.5. Inactivation of the cox2 locus in A. variabilis strains ATCC 29413 FD and CSW1 and growth characteristics of the mutant strains

For the inactivation of the *cox2* locus, plasmid pDPUV47 (Table 1) was conjugated from *E. coli* into *A. variabilis* strains ATCC 29413 FD and CSW1 with selection for erythromycin resistance. After obtaining cultures free of *E. coli*, all single-cell colonies tested were found to be neomycin-sensitive confirming that gene displacement by double recombination had occurred. Since cyanobacteria contain several copies of the chromosome, complete segregation of the mutated *cox2* allele was tested by PCR in several clones with primers Cox2-5′ and Cox2-3′ (data not shown). One homozygous clone each derived from wild-type *A. variabilis* ATCC 29413 and strain CSW1 was isolated and called PDCn and PDC-Cn, respectively. Both strains, as well as *A. variabilis* strains ATCC 29413 FD and CSW1, were able to grow photoautotrophically (both in continuous light and in 8-h light–16-h dark cycles), photoheterotrophically, and mixotrophically, using nitrate or dinitrogen as the nitrogen source. However, chemoheterotrophic dark growth—with or without combined nitrogen—was observed only with strains ATCC 29413 FD and PDCn, but not with the Cox1 minus strains CSW1 and PDC-Cn. Independent of the nitrogen source, strains lacking Cox2 (PDCn and PDC-Cn) showed growth rate constants for photoautotrophic growth of 75% to 80% of the corresponding parent strain (Table 2).

Table 2
Growth rate constants of *A. variabilis* strains grown photoautotrophically with (NO_3^-) or without (N_2) combined nitrogen

Strain	Genotype	Growth rate constant, μ (day^{-1})	
		N-Source[a]	
		NO_3^-	N_2
29413	wild type	1.56±0.11	0.98±0.09
CSW1	*cox1*::Sp^r	1.65±0.07	0.82±0.06
PDCn	*cox2*::Em^r	1.26±0.13	0.78±0.21
PDC-Cn	*cox1*::Sp^r; *cox2*::Em^r	1.25±0.03	0.61±0.03

[a] NO_3^- (BG-11), N_2 (BG-11_0). Numbers are the mean and standard deviation of the results of four independent experiments. Sp, spectinomycin. Em, erythromycin.

3.6. In vitro cytochrome c oxidase activities

Isolated total membranes of all strains used in this study were tested for in vitro oxidation of prereduced horse heart cytochrome *c* (Table 3). Membranes isolated from the double mutant strain PDC-Cn that lacks both Cox1 and Cox2 had no detectable in vitro cytochrome *c* oxidase activity under all tested growth conditions, showing that in wild-type *A. variabilis* ATCC 29413 Cox1 and Cox2 are the only cytochrome *c* oxidases active in the assay used. Therefore, mutant strains CSW1 and PDCn allow the determination of the activity of exclusively one cytochrome *c* oxidase. Cytochrome *c* oxidase activity in strain CSW1 (due to Cox2) is much higher in cells grown with fructose than in its absence (+1929%) and further enhanced when combined nitrogen is replaced by dinitrogen (+143%). The activity of Cox1 in strain PDCn is also enhanced by fructose (+813%) but this activity is decreased by the removal of combined nitrogen (−35%). The wild type showed a higher activity in cells grown with nitrate without fructose than either of the two single mutant strains, the effects of fructose (+424%) and removal of combined nitrogen (a further +12%) being intermediate between those in strains CSW1 and PDCn. Adding glucose instead of fructose to the growth medium did not change the cytochrome *c* oxidase activity, correlating with the fact that glucose—in contrast to fructose—cannot be used as the sole carbon source in heterotrophic growth of *A. variabilis* ATCC 29413 [41]. Activity of wild-type cells grown chemoheterotrophically in the dark also showed an enhancement upon the transition from combined nitrogen to dinitrogen (+110%) that was lost in strain PDCn (−42%). Cells grown with dinitrogen as nitrogen source and CO_2 as the sole carbon source do not yield membranes with the protocol commonly used for all other growth conditions so that a different preparation method had to be employed [10]. Thus, the cytochrome *c* oxidase activity rates determined from these membranes are not comparable to others and therefore presented separately at the bottom of Table 3. Under these conditions, removal of Cox2 (in PDCn) led to a significant loss of cytochrome *c* oxidase activity (−98%), while removal of Cox1 (in CSW1) led to a small enhancement (+34%) compared to the wild-type strain.

3.7. Respiratory oxygen uptake by intact cells

Respiratory oxygen uptake rates by intact cells are presented in Table 4 for all strains and growth conditions. The majority of in vivo respiratory oxygen uptake measurements described in this paper was performed in the presence of added fructose, since this ensured reaction kinetics of zero-order with respect to O_2 for at least half an hour. In *A. variabilis* ATCC 29413 the addition of fructose to the assay medium during measurement increases respiratory activity only a little (within the range of +3.3% to +22.0%). Furthermore, cells grown on fructose could not be assayed in the absence of fructose, since this would have entailed a thorough washing of the cells, a step which had to be avoided to ensure reproducibility. A very striking result of the oxygen uptake measurements was that the removal of respiratory terminal oxidase(s) from the genome of *A. variabilis* ATCC 29413 did not diminish respiratory activity, irrespective of the conditions used for growth of the cells. Indeed, in mutants lacking Cox2 (PDCn and PDC-Cn), the total respiratory activity was generally even higher than in the wild type. With respect to strain PDC-Cn, this clearly

Table 3
In vitro cytochrome *c* oxidation of prereduced horse heart cytochrome *c* (cyt. *c*) by isolated total membranes of *A. variabilis* strains

Strain	Growth conditions			Horse heart cyt. *c* oxidase activity[a]
	N-Source[b]	L/D[c]	Frc[d]	nmol h^{-1} (mg chl.)$^{-1}$
29413	NO_3^-	L	−	810 (4)
	NO_3^-	L	+	4,244 (2)
	NO_3^-	L	Glc[d]	961 (1)
	N_2	L	+	4,741 (2)
CSW1	NO_3^-	L	−	199 (3)
	NO_3^-	L	+	3,937 (3)
	N_2	L	+	9,818 (3)
PDCn	NO_3^-	L	−	584 (2)
	NO_3^-	L	+	5,334 (2)
	N_2	L	+	3,475 (2)
PDC-Cn	NO_3^-	L	−	0 (1)
	N_2	L	+	0 (1)
29413	NO_3^-	D	+	7,241 (2)
	N_2	D	+	15,225 (2)
PDCn	NO_3^-	D	+	6,120 (2)
	N_2	D	+	3,539 (2)
29413	N_2	L	−	4,166 (1)
CSW1	N_2	L	−	5,591 (1)
PDCn	N_2	L	−	91 (1)
PDC-Cn	N_2	L	−	0 (1)

[a] Inhibition with 5 μM KCN was always >88%.
[b] NO_3^- (BG-11), N_2 (BG-11_0).
[c] (L)ight or (D)ark.
[d] 15 mM fructose, Frc (or 15 mM glucose, Glc). Numbers in parentheses denote the number of independent determinations. Variation was always <±11%.

Table 4
In vivo respiratory O_2 uptake in darkness by intact cells of *A. variabilis* strains

Strain	Growth conditions			μmol O_2 h^{-1} (mg chlorophyll)$^{-1}$	%Inhibition of frc respiration
	N-Source[a]	L/D[b]	Frc[c]	+10 mM Fructose[d]	(50 μM HQNO[e])
29413	NO_3^-	L	–	7.1	13.0
	NO_3^-	L	+	32.2	75.7
	NO_3^-	L	Glc[c]	15.1	45.9
	N_2	L	–	10.4	4.3
	N_2	L	+	58.2	56.7
CSW1	NO_3^-	L	–	6.1	41.7
	NO_3^-	L	+	30.9	87.7
	N_2	L	–	9.5	25.7
	N_2	L	+	69.5	58.5
PDCn	NO_3^-	L	–	9.7	21.4
	NO_3^-	L	+	54.9	78.0
	N_2	L	–	21.2	32.5
	N_2	L	+	53.6	59.8
PDC-Cn	NO_3^-	L	–	11.8	26.2
	NO_3^-	L	+	57.1	94.5
	N_2	L	–	22.4	24.2
	N_2	L	+	91.5	65.1
29413	NO_3^-	D	+	47.4	15.7
PDCn	NO_3^-	D	+	65.2	25.0

[a] NO_3^- (BG-11), N_2 (BG-11_0).
[b] (L)ight or (D)ark.
[c] 15 mM fructose, Frc (or 15 mM glucose, Glc).
[d] Endogenous respiratory rates (from strains grown without fructose and measured without fructose in the assay medium) were from 5.9 to 20.8 μmol O_2 h^{-1} (mg chlorophyll)$^{-1}$ (two independent experiments with variations < ±43.2%). Numbers of fructose respiration are the mean of two independent experiments with variations <±23.2%. Inhibition with 1 mM KCN was always ~100%.
[e] 2-Heptyl-4-hydroxyquinoline N-oxide. Numbers are the mean of two independent experiments with variations <±45.2%.

demonstrates that, in addition to Cox1 and Cox2, at least one of the other respiratory terminal oxidases must have a significant activity. In all strains, respiratory rates from cells grown mixotrophically with fructose were considerably higher (between +153% and +397%) than from cells grown photoautotrophically on the same nitrogen source. The transition from growth in combined nitrogen to dinitrogen generally also led to an increase (up to +125%) of respiratory activity.

In *Synechocystis* PCC 6803 three inhibitors of respiratory activity have been characterized, KCN, 2-heptyl-4-hydroxyquinoline N-oxide (HQNO), and pentachlorophenol (PCP) [1,9]. As in *Synechocystis* PCC 6803, oxygen uptake in *A. variabilis* ATCC 29413 was completely inhibited by 1 mM KCN, demonstrating the absence of a cyanide-resistant terminal oxidase known from plant and fungal mitochondria [42]. HQNO is a quinone analog inhibiting both cytochrome *bd* and cytochrome *bo* respiratory terminal oxidases in *E. coli* [43] and the cytochrome *bd*-type quinol oxidase in *Synechocystis* PCC 6803 [1,9]. PCP inhibits isolated cytochrome *bd* from *E. coli* [44] and in *Synechocystis* PCC 6803 the cytochrome *bd*-type quinol oxidase in vivo [1,9]. In *A. variabilis* ATCC 29413 respiration was completely inhibited by PCP in strains CSW1 and PDC-Cn, while in strains FD and PDCn inhibition by PCP was higher in cells grown with N_2 (96.5% and 89.8%, respectively) than in cells grown with nitrate (56.5% and 63.5%, respectively). Table 4 shows that the inhibition of respiratory rates by HQNO depended highly on the respiratory terminal oxidases present in the different mutants and the growth conditions. HQNO did not inhibit respiration completely in any strain under all growth conditions used. The addition of fructose to the growth medium always led to a significantly higher HQNO inhibition of respiratory activity. In strains FD and CSW1 that contain Cox2, HQNO inhibition is lower in cells grown with N_2 than in cells grown with nitrate, both in the absence and in the presence of fructose in the growth medium.

4. Discussion

4.1. Respiratory terminal oxidases and their relative contribution to total respiration

Our data show that in wild-type *A. variabilis* ATCC 29413, at least four respiratory terminal oxidases are expressed and active: Cox1, described earlier [10]; Cox2, main topic of this work; Cox3, a new subtype of putative cytochrome *c* oxidases found only in cyanobacteria; and a cytochrome *bd*-type quinol oxidase. Recently, a draft version of the sequence of the complete genome of *A. variabilis* ATCC 29413 was made open to the public from the Joint Genome Institute (http://genome.ornl.gov/microbial/avar/). A preliminary analysis of the subunit II of the *cox3* locus (CoxB3) revealed that as in the corresponding proteins from *Synechocystis* PCC 6803 [34] and *Anabaena* PCC 7120 [3], the characteristic Cu_A binding motif [34] is missing. As shown above (Fig. 1A), the typical Mg^{2+} binding motif 'HD' is missing in subunit I of Cox3 (CoxA3) as well. Both facts indicate that Cox3 of *A. variabilis* ATCC 29413 is a paralogue to this subtype of putative cytochrome *c* oxidases. The missing Cu_A binding motif and the fact that this enzyme has no measurable in vitro horse heart cytochrome *c* oxidase activity (Table 3) [1,9] would suggest that this oxidase is no genuine cytochrome *c* oxidase. However, in a previous work [1] we got some evidence that this enzyme can use cytochrome *c* in vivo to generate a proton gradient across the cytoplasmic membrane. Further investigations are necessary to characterize this oxidase-subtype.

There is no direct method to assay the contributions of the different respiratory branches to total in vivo respiratory electron transport. However, the data presented in this work allow for the first time in a nitrogen fixing cyanobacterium to estimate these contributions (Fig. 5) using deletion

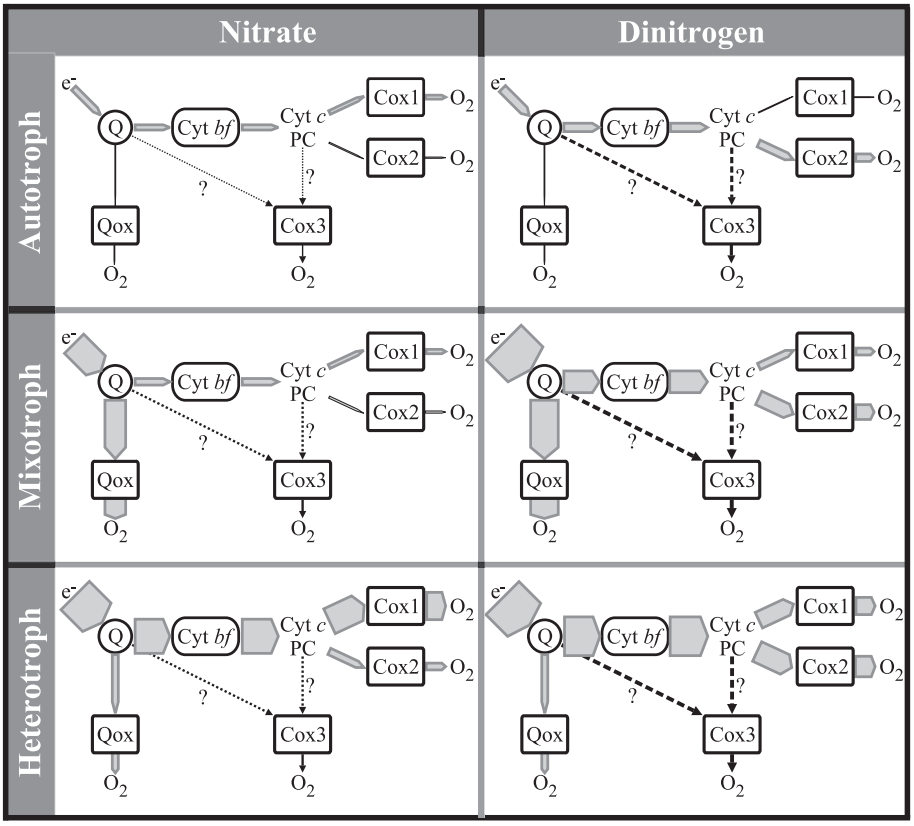

Fig. 5. A model showing the relative distribution of electron flows through the different respiratory branches under different external conditions. All available data presented in this work and earlier [10] were used. Only the oxidative ends of the branches are shown. The thickness of the e^- input arrows corresponds to the total respiratory rates shown in Table 4. The other arrows are the estimated relative contributions of the respiratory branches (see Discussion). The question marks at the arrows leading to Cox3 indicate that the direct electron donor of Cox3 is possibly cytochrome c_6 [1] but not known with certainty. Due to the lack of unequivocal information about the cellular location (cell membrane or intracellular membranes, vegetative cells or heterocysts) of the respiratory components in *A. variabilis*, no attempt has been made to show this in these schemes. Quinone pool (Q), cytochrome b_6f complex (Cyt *bf*), cytochrome c_6 (Cyt *c*), plastocyanin (PC), cytochrome *bd*-type quinol oxidase (Qox), cytochrome *c* oxidases (Cox1, Cox2, and Cox3).

mutants and specific inhibitors of respiratory terminal oxidases and the in vitro cytochrome *c* oxidase assay. The main result is that these contributions depend highly on the growth conditions used. HQNO is a known specific inhibitor of the cytochrome *bd*-type quinol oxidase in the unicellular cyanobacterium *Synechocystis* PCC 6803 [1]. Therefore, the HQNO inhibitable part of respiratory activity was considered to be due to the electron transport branch ending in the cytochrome *bd*-type quinol oxidase. HQNO inhibits all investigated strains only partly (Table 4), which is of special interest in the Cox1–Cox2 double mutant PDC-Cn since it shows (assuming complete HQNO inhibition of the cytochrome *bd*-type quinol oxidase as in *Synechocystis* PCC 6803 [1]) that, besides the HQNO sensitive cytochrome *bd*-type quinol oxidase, this strain contains another active respiratory terminal oxidase, namely the HQNO-resistant Cox3. The respiratory activity of strain CSW1 that lacks Cox1 but contains all other respiratory terminal oxidases was 100% inhibited by PCP. This implies that all respiratory branches except the one ending in Cox1 contain at least one PCP-sensitive component. Therefore, the PCP-resistant respiratory activity was used to estimate the contribution of Cox1 to the total respiratory activity. As PCP can function as an uncoupling reagent as well, PCP inhibition data should be interpreted carefully. However, previous data from *Synechocystis* PCC 6803 showed no measurable uncoupling activity of PCP [1]. Cox1 is the cytochrome *c* oxidase essential for chemoheterotrophy [10]. Accordingly, cells grown chemoheterotrophically in the dark showed rather high respiratory rates that were fairly resistant to HQNO (Table 4), indicating a high contribution of the Cox branches. Under photoautotrophic conditions, PCP inhibition in both strain ATCC 29413 FD and strain PDCn was considerably higher in cells grown in N_2 than in nitrate, showing that Cox1 has a lower contribution under diazotrophy. Cox2 is the dominating cytochrome *c* oxidase under diazotrophy, concordant with its enhanced expression under this condition. This is especially shown by the in vitro cytochrome *c* oxidation (Table 3) of membranes from strains containing (ATCC 29413 FD and CSW1) or lacking (PDCn) Cox2, the latter having only a very small activity in diazotrophy in the absence of fructose. A similar effect is not observed in NO_3^- grown cells. However, both Cox2 minus strains, PDCn and PDC-Cn, are able to grow on dinitrogen (PDCn even chemoheterotrophically), albeit at a

slower growth rate (Table 2). We have recently obtained evidence in *Anabaena* PCC 7120 that both Cox2 and Cox3 are involved in aerobic nitrogen fixation [3]. Indeed, our data may imply that the activity of Cox3 is higher in *A. variabilis* ATCC 29413 grown with dinitrogen than with combined nitrogen. In strain PDC-Cn that has only Cox3 and the cytochrome *bd*-type quinol oxidase, HQNO-resistant respiratory activity (due to Cox3) (Table 4) amounts to 73.8% of 11.8=8.7 μmol O_2 h^{-1} (mg chl)$^{-1}$ in combined nitrogen and 75.8% of 22.4=17.0 μmol O_2 h^{-1} (mg chl)$^{-1}$ in dinitrogen, a doubling of the activity. Similarly, in the presence of fructose, a change from combined nitrogen to dinitrogen enhances HQNO-resistant respiratory activity in strain PDC-Cn from 3.1 to 31.9 μmol O_2 h^{-1} (mg chl)$^{-1}$.

4.2. Effect of fructose on respiration in A. variabilis ATCC 29413

Growth with fructose (but not with glucose) has a profound influence on the properties of *A. variabilis* ATCC 29413, as has been noted earlier [45]. Previously [10] and in Figs. 3A and B, *cox1* expression was shown to be up-regulated when *A. variabilis* ATCC 29413 was grown in the presence of fructose. Concomitantly, in vitro Cox1 activity in strain PDCn (in which Cox1 is the only cytochrome *c* oxidase) rose about 10-fold (Table 3). Surprisingly, when strain CSW1 (that lacks Cox1) was grown with fructose and combined nitrogen, in vitro cytochrome *c* oxidase activity (which must be due entirely to Cox2 in this strain) rose even more, about 20-fold (Table 3), compared to cells grown in the absence of fructose. However, in contrast to *cox1*, RT-PCR detected only a low expression of *cox2* in nitrate grown cells both in the presence and in the absence of fructose, and neither primer extension (Fig. 3D) nor Northern blot analysis (Fig. 3C) detected any signal at all under these conditions. Thus, the expression level of *cox2* could not explain the large rise in activity of Cox2 in fructose grown CSW1 cells. However, the Cox1–Cox2 double mutant PDC-Cn showed no in vitro cytochrome *c* oxidase activity under all growth conditions (Table 3) ruling out the contribution of Cox3 or any other oxidase to the rise of cytochrome *c* oxidase activity in the Cox1 minus mutant CSW1. The possibility that fructose enhances translation and/or protein activity of Cox2 should therefore be considered.

Fructose in the growth medium enhanced total respiratory activity under all growth conditions and in all strains investigated (Table 4). These higher rates were invariably linked to a higher HQNO sensitivity (Table 4), suggesting a high contribution of the cytochrome *bd*-type quinol oxidase (up to 94.5%) to the total respiratory rate of fructose-grown cells. However, the expression studies (Fig. 2C) did not reflect a corresponding rise in *cydAB* (cytochrome *bd*-type quinol oxidase) transcription. As for Cox2, fructose may influence translation and/or activity of the cytochrome *bd*-type quinol oxidase, but we propose an alternative explanation that takes into account the postulated function of the cytochrome *bd*-type quinol oxidase in *Synechocystis* PCC 6803. Several experiments have produced evidence [46,1] that in *Synechocystis* the cytochrome *bd*-type quinol oxidase may act as a valve for removal of electrons from an overreduced quinone pool. Thus, an essentially constant amount of cytochrome *bd*-type quinol oxidase protein could be present, but the electron transfer rate of the branch ending in the cytochrome *bd*-type quinol oxidase would be only partially saturated to a different degree under different growth conditions.

The highest respiratory rates were observed in cells grown diazotrophically in the presence of fructose (Table 4). This is reminiscent of the effect of exogenous carbohydrates on nitrogen fixation and cell differentiation in the symbiotic cyanobacterium *Nostoc* sp. strain PCC 9229 [47]. In *Nostoc* exogenous fructose induces heterocyst development in darkness, even in a growth medium containing combined nitrogen. This process may be decisive for the symbiotic relationship of the cyanobacterium with its host, in that carbohydrates supplied by the host plant induce heterocyst differentiation and nitrogen fixation [47]. In *A. variabilis* ATCC 29413, growth with exogenous fructose in the light (mixotrophic growth) also induces heterocyst development in growth medium containing combined nitrogen, with heterocysts appearing underdeveloped (Pils and Schmetterer, unpublished observations).

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