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Fusing two cytochromes *b* of *Rhodobacter capsulatus* cytochrome bc_1 using various linkers defines a set of protein templates for asymmetric mutagenesis

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Cytochrome bc_1 (mitochondrial complex III), one of the key enzymes of biological energy conversion, is a functional homodimer in which each monomer contains three catalytic subunits: cytochrome c_1, the iron–sulfur subunit and cytochrome *b*. The latter is composed of eight transmembrane α-helices which, in duplicate, form a hydrophobic core of a dimer. We show that two cytochromes *b* can be fused into one 16-helical subunit using a number of different peptide linkers that vary in length but all connect the C-terminus of one cytochrome with the N-terminus of the other. The fusion proteins replace two cytochromes *b* in the dimer defining a set of available protein templates for introducing mutations that allow breaking symmetry of a dimer. A more detailed comparison of the form with the shortest, 3 amino acid, linker to the form with 12 amino acid linker established that both forms display similar level of structural plasticity to accommodate several, but not all, asymmetric patterns of mutations that knock out individual segments of cofactor chains. While the system based on a fused gene does not allow for the assessments of the functionality of electron-transfer paths *in vivo*, the family of proteins with fused cytochrome *b* offers attractive model for detailed investigations of molecular mechanism of catalysis at *in vitro*/reconstitution level.

Keywords: cytochrome bc_1/fusion membrane protein/homodimer/linker/mutagenesis

Introduction

Purple photosynthetic bacteria, such as *Rhodobacter (Rb.) sphaeroides* or *Rb.capsulatus*, have traditionally been used in studies aiming at elucidating the mechanism of energy conversion supported by components of electron transport chains. Of those components, the photosynthetic reaction center and cytochrome bc_1 (Gennis *et al.*, 1993; Cooley *et al.*, 2004; Berry *et al.*, 2009) have drawn a particular attention, as they form a simple cyclic electron-transfer system suitable for experimental investigations on several levels. It is amenable for structural alterations through genetic manipulations (Atta-Asafo-Adjei and Daldal, 1991) and the availability of light-activatable chromatophore vesicles makes it convenient for kinetic studies of function (Dutton and Prince, 1978; Crofts *et al.*, 1983; Ding *et al.*, 1995). In addition, the membranous components can be extracted from the membranes and obtained in the isolated forms (Robertson *et al.*, 1993; Valkova-Valchanova *et al.*, 1998). This makes them convenient for several enzymological and spectroscopic studies. The architecture of the catalytic core and the mechanism of its action are highly conserved through the evolution (Berry *et al.*, 2004; Kramer *et al.*, 2009); therefore, the results obtained with the bacterial system provide important insights into the functioning of all cytochromes bc_1, including mitochondrial complex III—a counterpart of bacterial cytochrome bc_1.

In *Rb.capsulatus*, cytochrome bc_1 has its simplest composition and consists of just the three subunits: cytochrome *b*, cytochrome c_1 and the iron–sulfur (FeS) subunit (Darrouzet *et al.*, 2004). They form the catalytic core that embeds all redox cofactors necessary for the operation of the two catalytic quinone oxidation/reduction sites. Cytochrome c_1 and the FeS subunit have water-soluble domains anchored into the membrane with transmembrane α-helix. The domain of cytochrome c_1 embeds heme c_1, while that the FeS subunit 2-iron–2-sulfur cluster. Cytochrome *b* is composed of eight transmembrane α-helices connected by loop regions. First four helices form attachment site for two hemes b (b_L and b_H).

Cytochrome bc_1 is a homodimer in which each monomer contains all three catalytic subunits just described. Two cytochromes *b* face each other and form a hydrophobic core of a dimer. In recent study, we have shown that two cytochromes *b* of *Rb.capsulatus* cytochrome bc_1 can be fused into one 16-helical subunit that assembled with other subunits of the complex (Świerczek *et al.*, 2010). The fusion was achieved by introducing a linker made of 12 amino acids that connected the C-terminus of one cytochrome *b* with the N-terminus of the other. With such system we were able to break the symmetry of the dimer by introducing strategically positioned point mutations that selectively eliminated individual segments of the dimer in various combinations. Even though not all possible combinations of mutations were tolerated, with those that were, we were able to test all major electron-transfer paths within the dimer. This revealed fundamental principles of its operation demonstrating that electrons move freely within and between monomers, crossing an electron-transfer bridge between two hemes in the core of

dimer (Świerczek *et al.*, 2010). The so formed H-shaped electron-transfer system distributes electrons between four quinone catalytic sites at the corners of the dimer within the millisecond timescale of catalytic turnover.

Other bacterial systems that allow breaking symmetry of homodimeric cytochrome bc_1 have also been recently described (Castellani *et al.*, 2010; Lanciano *et al.*, 2011). They are based on parallel expression of two plasmids and isolations of heterodimers with a use of two different tags. With the help of those systems, one study has shown that cytochrome bc_1 with only one quinone oxidation site is as active as the native enzyme with two active sites (Castellani *et al.*, 2010), while the other study has implied that the inter-monomer electron transfer is able to support the growth of bacterial cells (Lanciano *et al.*, 2011).

Clearly, the experimental accessibility to the asymmetric forms of cytochrome bc_1 now becomes highly desirable in studies on mechanism of its operation. From this perspective, in this work we explore possibilities to use other linker sequences for fusing two cytochromes *b* (see Fig. 1) in attempt to increase a number of available protein templates suitable for asymmetric mutagenesis within the complex.

Materials and methods

Bacterial strains and plasmids

Rb.capsulatus and *Escherichia (E.) coli* (HB101, DH5α) were grown in liquid or solid mineral–peptone–yeast extract (MPYE) at 30°C and Luria-Bertani at 37°C media, respectively, supplemented with appropriate antibiotics as needed. Respiratory growth of *Rb.capsulatus* strains was achieved at 30°C in the dark under semiaerobic conditions. The cells were grown on plates for 3 days and in liquid medium no >3 days in three steps of 2 ml, 25 ml and 1 l cultures. Photosynthetic growth abilities of *Rb.capsulatus* strains carrying the fusion B-B form (engineered cytochrome bc_1-like complex with two cytochrome *b* subunits fused together) was tested on MPYE plates using anaerobic jars (GasPak™ EZ Anaerobe Container System, BD) at 30°C under continuous light. The *Rb.capsulatus* strains (Atta-Asafo-Adjei and Daldal, 1991) used were pMTS1/MT-RBC1 which overproduces wild-type cytochrome bc_1 from the expression vector pMTS1 (contains a copy of *pet*ABC operon coding for all three subunits of cytochrome bc_1), and MT-RBC1 which is a *pet*ABC—operon deletion background. The mutagenized pMTS1 derivatives were introduced to *Rb.capsulatus* MT-RBC1 via triparental crosses as described (Atta-Asafo-Adjei and Daldal, 1991). Plasmid pPET1 (a derivative of pBR322 containing a wild-type copy of *pet*ABC) was used as a template for polymerase chain reaction (PCR) and in some of the subcloning procedures.

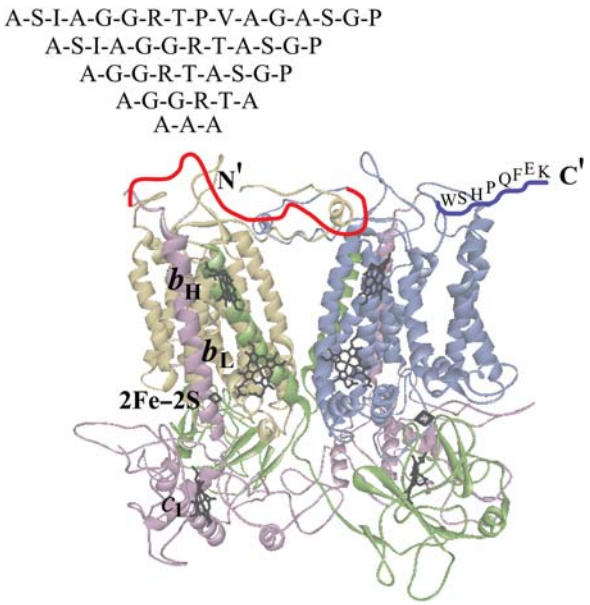

Fig. 1. Schematic structural model of fusion protein B-B. Cytochrome *bb*, a fusion of two cytochromes *b* (yellow and blue), forms a hydrophobic core of the complex. The remaining subunits, cytochrome c_1 and the FeS subunit, are shown in violet and green, respectively. Cofactors are overlaid in black. The red line indicates a linker connecting the C-terminus of one cytochrome *b* with the N-terminus of the other. Above the line, possible amino acid compositions of the linker are shown. Blue line and the letters above it indicate Strep-tag and its amino acid composition. Note that the red and blue lines are just schematic visualizations and do not correspond to a real conformation of the linker and Strep-tag peptides. The figure was generated using the crystal structure of *Rb.capsulatus* cytochrome bc_1 (Berry *et al.*, 2004) (protein Data Bank ID:1ZRT).

Construction of plasmids for expression of B-B with different linkers

The strategy of constructing plasmids for expression of B-B follows the general approach described in Świerczek *et al.* (2010). The plasmid pPET1-BL containing NotI restriction site at the 3′ end of *pet*B was created by PCR using QuikChange Site-Directed Mutagenesis Kit (Stratagene) and the mutagenic oligonucleotides, EndNotI-F: 5′-CAC TAC GGC AAT CCG GCG GCC GCC GGA AAG GAA CCG AC-3′ and EndNotI-R: 5′-CGG TTC CTT TCC GGC GGC CGC CGG ATT GCC GTA GTG GC-3′ (Fig. 2A). The plasmid pUC-BLST containing NotI restriction site at the 5′ end of *pet*B and a sequence encoding Strep-tag added to its 3′ end was constructed in several steps summarized in Fig. 2B. First, ∼1.4 kb DNA fragment (*pet*B and 111bp of *pet*C) from pPET1 was PCR-amplified using primers StartNotI-F: 5′-GCG GCC GCC TCC GGA ATT CCG CAC GAC CAT TAC G-3′ and AsuII-R: 5′-GCC GAA GAT CCC TTC GAA GCT GAA GGC GTG-3′ and then inserted into pCR-Blunt II-TOPO (from Invitrogen). This created plasmid TOPO1-StartNot. Next, the XbaI/KpnI fragment of TOPO1-StartNot was cloned into pUC19 to create pUC-BL. Finally, XmaI/SfuI fragment of pUC-BL was exchanged with its counterpart in pPET1-ST (derivative of pPET1 in which *pet*B was extended with a sequence encoding Strep-tag II inserted between GAG coding for C-terminal E^{437} and TGA stop codon) which created pUC-BLST (Fig. 2B).

The plasmids pPET1-BL and pUC-BLST and their derivatives were used to construct a family of plasmids pMTS1-BLBST (where L denotes sequences encoding linkers shown in Table I) as summarized in Fig. 2C. Generally, the NotI/SfuI fragment of pPET1-BL was exchanged with NotI/SfuI fragment of pUC-BLST to create pPET1-BLBST plasmid containing *pet*BLBST gene flanked by original *pet*A and *pet*C genes. Then, the BstXI/SfuI fragment of pPET1-BLBST was exchanged with its counterpart in pMTS1 to create the expression vector pMTS1-BLBST. Without additional steps this created pMTS1-BLBST with the sequence coding for the linker composed of three amino acids (Table I).

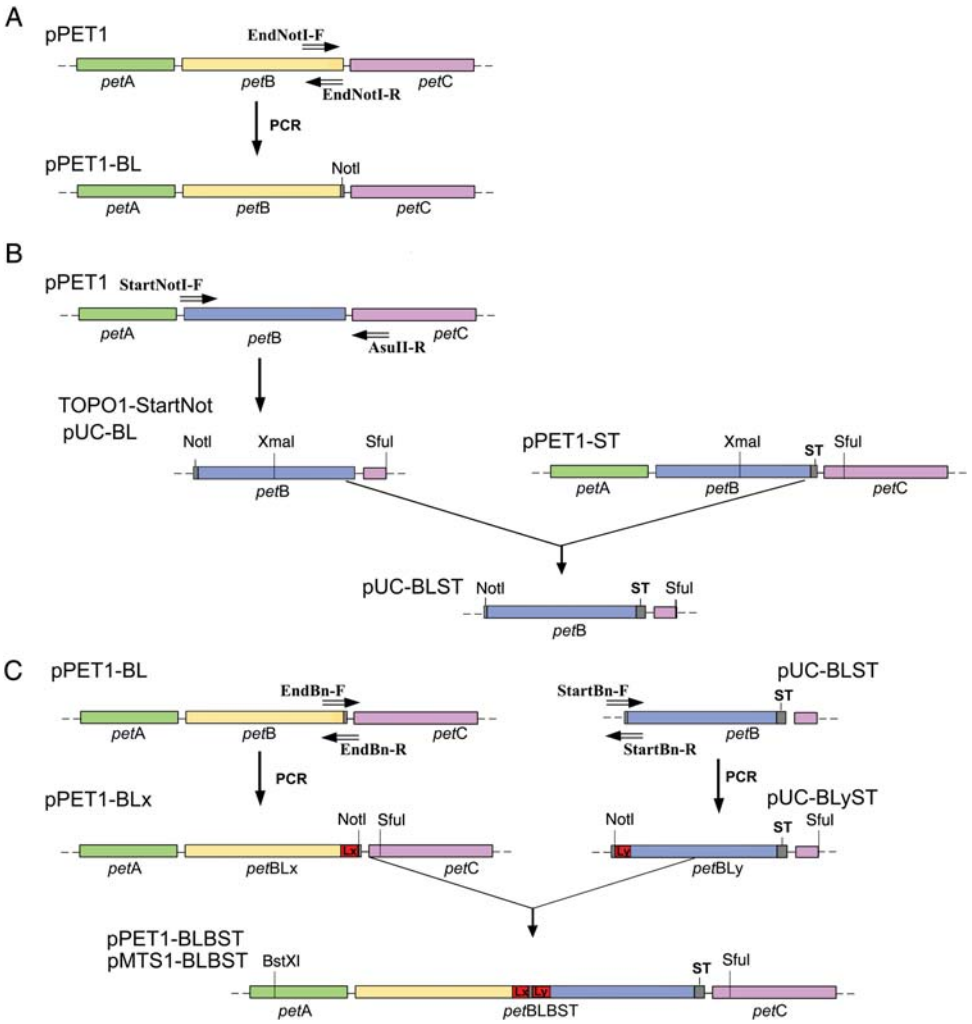

Fig. 2. Scheme of plasmid family pMTS1-BLBST construction. (**A**) Introducing NotI restriction site into 3′ end of *pet*B gene by PCR. The pPET1 plasmid contains operon encoding cytochrome bc_1 subunits: *pet*A encoding FeS protein (green), *pet*B encoding cytochrome *b* (yellow) and *pet*C encoding cytochrome c_1 (violet). Positions of mutagenic primers are indicated with horizontal arrows. (**B**) Steps of construction of pUC-BLST plasmid with NotI restriction site to the 5′ end site of *pet*B and sequence encoding Strep-tag (ST) attached to the 3′ end of *pet*B. Positions of mutagenic primers and restriction sites used for plasmid construction are indicated with horizontal arrows and vertical lines, respectively. (**C**) Final steps of construction of pMTS1-BLBST: introducing parts of linker by PCR performed on pPET1-BL using primers EndBn-F, EndBn-R and on pUC-BLST using primers StartBn-F, StartBn-R (n in the primer names marks varying number of nucleotides); inserting NotI/SfuI fragment of pUC-BLyST into pPET1-BLx. Red boxes denote parts of added sequences (Lx, Ly) that together form sequences of the linkers.

Table I. Assembly of B-B constructed using various linkers

Name of fusion protein	Length of linker[a]	Amino acid composition of linkers	Assembly of fusion protein
B-B[3]	3	A-A-A	+
B-B[6]	6	A-G-G-R-T-A	+
B-B[9]	9	A-G-G-R-T-A-S-G-P	+
B-B[12]	12	A-S-I-A-G-G-R-T-A-S-G-P	+
B-B[16]	16	A-S-I-A-G-G-R-T-P-V-A-G-A-S-G-P	+
B-B[20]	20	A-S-V-G-S-G-I-A-G-G-R-T-P-V-A-G-A-S-G-P	−

[a]The length of the linker corresponds to all amino acid residues that were changed and added between the fused C and N termini before the NotI ligation step and as a result are flanked by the following amino acid sequences: G-N-P^{435} from the C terminus and ^{1}S-G-I from the N terminus of cytochrome *b*.

To create pMTS1-BLBST with sequences encoding longer linkers (Table I) additional PCR steps were performed. In those steps sets of primers EndBn-F, EndBn-R for pPET1-BL and StartBn-F, StartBn-R for pUC-BLST were used to create pPET1-BLx and pUC-BLyST, respectively (Fig. 2C). Those plasmids were then used in NotI/SfuI and BstXI/SfuI insertion steps, which created a family of pPET1-BLBST and pMTS1-BLBST containing Lx and Ly sequences connected together to form sequences encoding for desired linkers (in the scheme of Fig. 2C, Lx and Ly denote sequences that were progressively elongated in consecutive steps of PCRs; x and y mark varying number of added/replaced nucleotides).

Introducing point mutations to the petBLBST template

Plasmids pMTS1-BLBST containing various combinations of point mutations corresponding to G158W and H212N in cytochrome *b* were generated in the following way. First, mutations were introduced to pPET1-BL or pUC-BLST using a PCR-based mutagenesis (QuickChange from Stratagene) and/or appropriate restriction cuts and ligations (as described in Świerczek *et al.*, 2010) and then pPET1-BL (mutated or non-mutated) was ligated with NotI/SfuI fragment of pUC-BLST (mutated or non-mutated) to create pPET1-BLBST variant containing desired combinations of mutations (as depicted in Table II). Finally, the BstXI/SfuI fragment of pPET1-BLBST was exchanged with its counterpart in pMTS1 to create the expression vector pMTS1-BLBST. With this strategy we obtained plasmids used to express a family of B-B[3] (B-B with linker made of three amino acids) forms containing G158W and/or H212N mutations in various combinations. Similar strategy was adopted to obtain a respective family of B-B[12] (B-B with linker made of 12 amino acids) described in Świerczek *et al.* (2010) in which case mutations were introduced to the derivatives of pPET1-BL or pUC-BLST (pPET1-BLx and pUC-BLyST containing appropriate Lx and Ly extensions).

DNA sequence and correct size of all constructs were verified both at the stage of preparing the constructs and after the re-isolation of plasmids from *Rb.capsulatus* strains and appropriate cloning procedures (DNA sequence of *pet*BLBST for B-B and all mutant forms listed in Table II was verified from two parts of pMTS1-BLBST that were separated by its digestion with XmaI followed by cloning of the XmaI-XmaI insert into pUC19 and self-ligation of the remaining part of pMTS1-BLBST). Re-isolation of plasmids from *Rb.capsulatus* strains was followed by the amplification step in the *E.coli* HB101 strain.

Isolation, electrophoresis and western blot of various B-B forms

The chromatophore membranes of WT (native form of cytochrome bc_1) and the B-B derivatives were prepared from semiaerobically grown cultures of *Rb.capsulatus* as described (Valkova-Valchanova *et al.*, 1998; Świerczek *et al.*, 2010). During isolations, special care was taken to use a mixture of inhibitors phenylmethylsulfonyl fluoride, benzamidine and 6-aminocaproic acid (Lee *et al.*, 2008). Membranes were diluted to a final protein concentration 10 mg/ml and solubilized with *n*-dodecyl-β-D-maltoside (DDM) (1 mg protein:1.3 mg detergent) by 30 min at 4°C. The mixture was ultracentrifuged (45 min 45 000 g) and the supernatant was loaded onto a DEAE-Biogel (Bio-rad) or Strep-tag column (IBA-Biotechnology). All purification steps were performed at 4°C.

The native form of cytochrome bc_1 was purified using DEAE-Biogel column according to the protocol described (Valkova-Valchanova *et al.*, 1998). To purify B-B forms, special care was taken to limit the whole isolation procedure (starting from membrane solubilization) to 1 day and the following modifications were introduced. Appropriate amounts of DDM-solubilized membranes were loaded onto 5–7 ml DEAE-Biogel column pre-equilibrated with a Tris buffer (50 mM Tris pH 8, 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 20% glycerol, 0.01% DDM). Then the column was washed with two to three column volumes of the equilibration buffer. In the next step, the column was washed with two to three column volumes of Tris buffer containing 150 mM NaCl. B-B proteins were eluted in Tris buffer containing 400 mM NaCl.

To purify B-B forms by affinity chromatography, we followed the protocol for Strep-tag purification supplied by the manufacturer (IBA), with the following modifications. Typically, 3–5 ml of DDM-solubilized membranes were loaded onto the 1 ml Strep-tag sepharose column (IBA) pre-equilibrated with a Washing Buffer (100 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1 mM EDTA) containing additionally 20% glycerol and 0.01% DDM (Buffer WG). In order to remove unbound proteins and photosynthetic pigments the column was washed with two to three column volumes of Buffer WG. The absorbed proteins were eluted with three column volumes of Buffer WG containing 2,5 mM desthiobiotin.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) was performed as described (Osyczka *et al.*, 2001). Samples of protein were incubated under reducing conditions at 60°C for 5 min prior to the loading on gels (4% stacking gel and 15% linear separating gel was used). The gels were stained with Coomassie blue.

The western blot against Strep-tag was performed according to the protocol supplied by IBA with HRP-streptactin used for detection of Strep-tag.

For each protein sample, the cells growth, and all steps of membrane and protein preparation and analysis were repeated several times.

Table II. Assembly of fusion protein B-B[3] and B-B[12] containing additional mutations W and/or N in various combinations

Mutation pattern[a]	Assembly of fusion protein	
	B-B[3]	B-B[12]
$_{W}$B-B	+	+
B-B$_{W}$	+	+
$_{W}$B-B$_{W}$	−	−
^{N}B-B	+	+
B-B^{N}	+	−
^{N}B-B^{N}	−	−
$_{W}$B-B^{N}	+	+
^{N}B-B$_{W}$	−	−
$^{N}_{W}$B-B	−	+
B-B$^{N}_{W}$	+	+

[a]W and N refer to G158W and H212N point mutations in cytochrome *b*.

Optical and electron paramagnetic resonance (EPR) spectroscopy and enzymatic activity measurements

Optical spectra for b- and c-type cytochromes were recorded using a Shimadzu UV-2450 spectrophotometer. The optical spectra of purified protein were obtained with samples that were first oxidized by an addition of a potassium ferricyanide, and then reduced by using either sodium ascorbate or a minimal amount of solid sodium dithionite. EPR and enzymatic activities measurements of membranes were performed as described in Sarewicz *et al.* (2009) and Świerczek *et al.* (2010).

Guarding against reversions

Because of the risk of reversions (see Results and discussion), special care was taken each time when preparing a new batch of membranes and/or proteins to ensure that it is homogenous and suitable for kinetic analysis. This included the following steps: (i) the colonies only from freshly prepared plates originated directly from −80°C stock were taken to inoculate liquid cultures for preparative purposes, (ii) the cultivation of the cells in liquid media was kept to minimum and did not exceeded 3 days (1 day for each of the 2 ml, 25 ml and 1 l cultures), (iii) the integrity of the fused gene was verified at the final cultivation stage (1 l culture), (iv) the content of membranes was analyzed electrophoretically by western blots (using antibodies against Strep-tag) and by SDS–PAGE of complexes isolated from membranes with two parallelly applied methods, ion-exchange DEAE-Biogel or affinity Strep-tag chromatography, which comprehensively identified all bc-type complexes present in membranes, (v) spectroscopic properties of complexes in membranes were analyzed by optical and EPR spectroscopy, to verify structural asymmetry of all forms with asymmetrically introduced mutations. Only the samples that met all the purity criteria related to points (iii)–(v) (see Results and discussion) were considered as suitable for further kinetic and functional analysis (for the forms denoted as '+' in Tables I and II, occasional preparations that contained elevated levels of reverted forms of proteins were discarded).

Results

Fusion proteins with linkers of different length

The N- and C-termini of the eight-transmembrane α-helical cytochrome *b* subunit of cytochrome bc_1 protrude at the cytoplasmic side of the membrane. As shown previously the C-terminus of one cytochrome can be joined with the N-terminus of the other if a linker made of 12 amino acids is used (Świerczek *et al.*, 2010). This creates a fusion protein (cytochrome *bb*) which in a dimer replaces two separate cytochromes *b* (creating an engineered cytochrome bc_1-like complex, named B-B). Adopting similar strategy, we tested several linkers of different length for successful fusion. Figure 2 summarizes steps of construction of plasmids containing the operon coding for cytochrome bc_1 in which the gene encoding cytochrome *b* was extended with the second copy of the same gene containing Strep-tag at its C-terminus. The extension procedures described in Fig. 2 yielded constructs containing sequences coding for the gene with the linkers of length between 3 and 20 amino acids. In all cases, the other two genes of the operon, encoding the FeS subunit and cytochrome c_1, were left unchanged.

In designing the sequences of the linkers the following points were considered. We aimed for the sequence that maintained similarity with motifs already present in the loop regions of native cytochrome *b*. Bulky and hydrophobic residues were avoided to secure polarity and flexibility of the linker. The proline residues were intentionally incorporated at the end and/or in the middle of longer linkers to minimize the probability of creation of the ordered secondary structure. One constraint in the amino acid composition came from the presence of the NotI restriction site in the nucleotide sequence necessary for creation of the fused gene.

Table I shows the sequences of the tested linkers and summarizes the results of expression of the respective plasmids in *Rb.capsulatus* cells. The assembly of B-B was examined at the level of membranes and isolated complexes. The size of cytochrome *bb* was verified by western blots on membranes and the subunit composition of B-B was analyzed by SDS–PAGE of complexes isolated by ion-exchange (DEAE) and affinity (Strep-tag) chromatography. Figure 3A compares blots for membranes containing B-B complexes containing cytochrome *bb* made with 3-, 6-, 9-, 12- or 16-amino acid liker (B-B[3], B-B[6], B-B[9], B-B[12] and B-B[16], respectively). Figure 3B and C compares electrophoretic profiles of those forms.

It is clear that in all cases cytochrome *bb* replaces cytochrome *b* subunits present in the native cytochrome bc_1 dimer. This cytochrome is two times larger than cytochrome *b*, as expected for the size of fusion protein, and is accompanied by the two remaining catalytic subunits of cytochrome bc_1: cytochrome c_1 and the FeS subunit. While in the case of native cytochrome bc_1, affinity and ion exchange chromatographies yield samples of similar purities (Fig. 3B and C, line 1), in the case of B-B, ion exchange allows only for partial purification (Fig. 3C, lines 2–6) and pure complexes can be obtained using affinity chromatography (Fig. 3B, lines 2–6). The spectroscopic properties of B-B forms (optical spectra of hemes b and c and EPR spectra of the FeS cluster) in membranes were similar to those of native cytochrome bc_1 and B-B[12] reported in Świerczek *et al.* (2010). The B-B forms isolated by either ion exchange or affinity chromatography retained the native-like optical spectra as exemplified by a comparison for the wild-type bc_1 and B-B[12] as shown in Fig. 4. We note a slight increase in the heme b to heme c absorption ratio in the spectrum of complex isolated by Strep-tag which reflects an increased probability of dissociation of subunits not-containing Strep-tag during the purification using the affinity chromatography (this holds also for native cytochrome bc_1). Enzymatic activities of the B-B forms measured at the level of membranes were similar to the wild type (not shown).

It can be concluded that the connection provided by the 3-, 6-, 9- and 16-amino acid linker in cytochrome *bb* allows for successful fusion and assembly of B-B (Fig. 1), as reported earlier for B-B[12] (Świerczek *et al.*, 2010). On the other hand, our attempts to obtain B-B containing two cytochromes *b* fused with longer (20 amino acids) linker failed. In this case, during the crossing procedure, only few single colonies appeared on selective plates within the characteristic 3 days time. The remaining colonies (that corresponded in number to the typical yield of the crossing procedure) appeared with 1-day delay. Further analysis of the plasmid DNA isolated from these cells showed the presence of the short version of the gene (corresponding in size to a single copy of *pet*B) in addition to a fusion gene (Fig. 5). Although the reason of this phenomenon is unknown, we speculate that B-B[20] might not have been inert to the cells (perhaps improperly folded fusion protein affected integrity of the membrane), which by impeding the growth of the cells advanced efficient selection toward the non-fused form from the early stages of the plasmid acquisition (see further discussion on this issue in the following paragraphs).

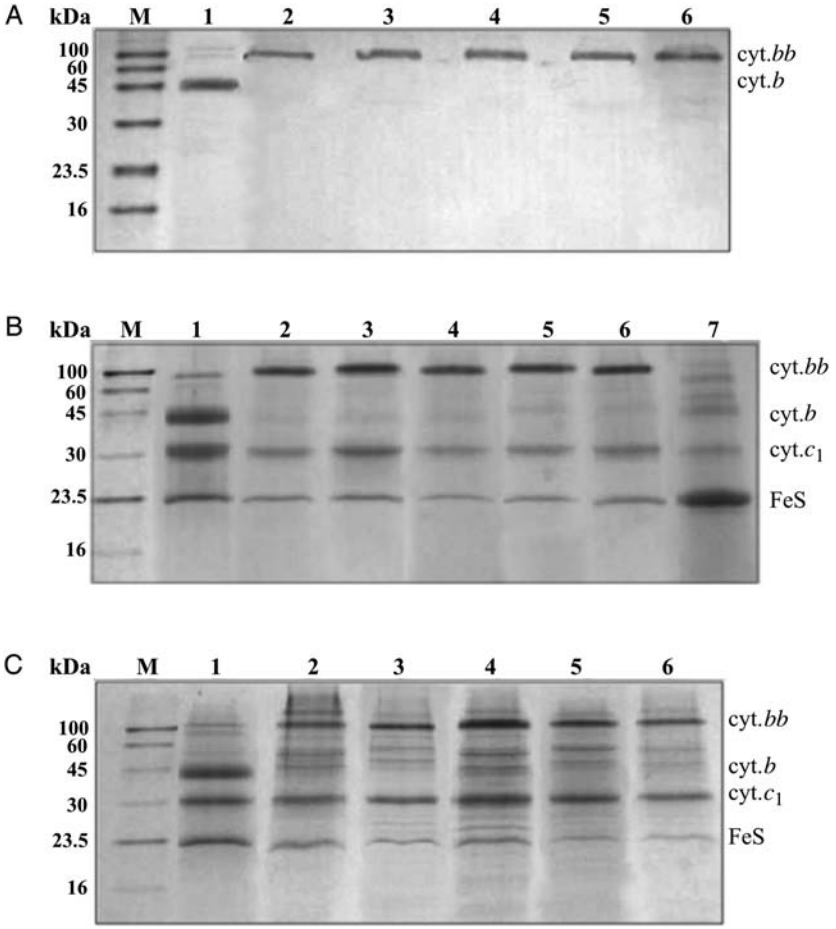

Fig. 3. Western blot and SDS–PAGE analyses of B-B containing cytochrome *bb* fused with various linkers. (**A**) Western blot against Strep-tag II of chromatophore membranes. (**B**) SDS–PAGE analysis of B-B complexes isolated using affinity chromatography (Strep-tag). (**C**) SDS–PAGE analysis of B-B complexes isolated using ion-exchange chromatography (DEAE Bio-gel). Lines: M marker (IBA); 1, native form of cytochrome bc_1; 2, B-B[3]; 3, B-B[6]; 4, B-B[9]; 5, B-B[12]; 6, B-B[16]. For comparison, line 7 in B shows electrophoretic profile of an engineered cytochrome bc_1-like complex that misses cytochrome *b* subunit (this is purified by Strep-tag b_6c_1 complex where *pet*B is split into two parts, reminiscent of cytochrome b_6 and subunit *IV* present in cytochrome b_6f as described in Saribas *et al.*, 1999).

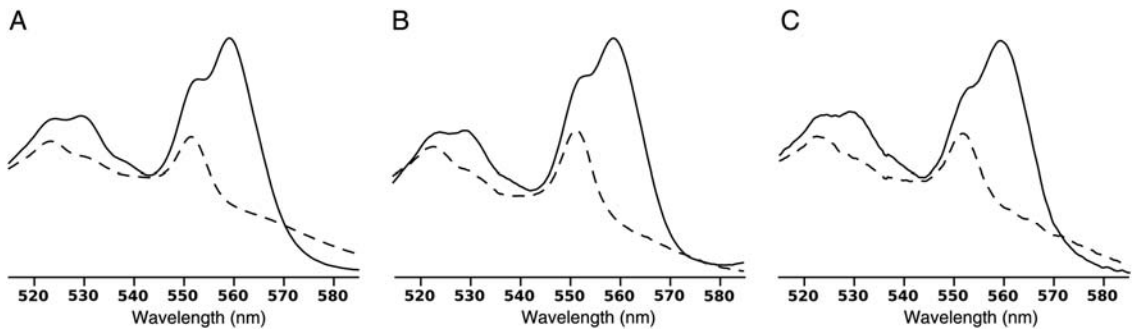

Fig. 4. Optical absorption spectra of hemes in purified complexes. (**A**) Native form of cytochrome bc_1 isolated by ion-exchange chromatography. (**B**) B-B[12] purified by ion-exchange chromatography. (**C**) B-B[12] purified by affinity chromatography. Solid and dashed lines indicate dithionite-reduced and ascorbate-reduced spectra, respectively.

Incompetence of fusion protein to support photosynthetic growth

Photosynthetic growth of *Rb.capsulatus* depends on functional cytochrome bc_1, thus testing photosynthetic phenotype of the cells carrying mutated genes coding for cytochrome bc_1 provides a simple way to verify functionality of the mutated complexes *in vivo*. Such tests, done for B-B[3] and B-B[12] indicated lack of photosynthetic competence of cells carrying genes coding for the fusion protein. Furthermore, when cells grown semiaerobically were transferred on plates for photosynthetic growth, reversions to Ps + (photosynthetic growth) occured. Further restriction analysis of the plasmid DNA isolated from these cells revealed that only the short version of the gene was present (its size corresponded to a single copy of *pet*B).

The typical serial dilutions experiment, in which the number of colonies that gain Ps + phenotype were compared to the number of all cells that grow under aerobic conditions at a given concentration of cells (see example in Fig. 6), provided an estimation of 10^{-3}–10^{-4} for the frequency of reversion. We note that additional 2–3 days of cultivation under Ps + conditions were necessary before any of the Ps + colonies appeared visible on the photosynthetic plates (compared with the characteristic 3-day time required for growth of single colonies on those plates). We also note that this frequency level was maintained at all stages of cultivation (i.e. 2 ml, 25 ml and 1 l cultures).

The reason of the inability of the cells expressing B-B to grow photosynthetically is currently unknown. One possibility is that the energetic cost of incorporation of cytochrome *bb* into membrane and its assembly with the remaining parts of the complex is so high that it results in the overall failure of B-B to support growth when cells absolutely depend on it (photosynthetic conditions). This could, for example, be associated with an altered stoichiometry of gene product vs. subunit composition. Because an assembly of B-B requires two cytochromes c_1 and two FeS proteins per one cytochrome *bb*, the expression of the whole operon containing *pet*BLBST leads to an excess of cytochrome *bb* which in the cells cannot be maintained as individual subunit and represents a possible case of energetic extravagance that the cells cannot afford when their growth absolutely depends on cytochrome bc_1 function.

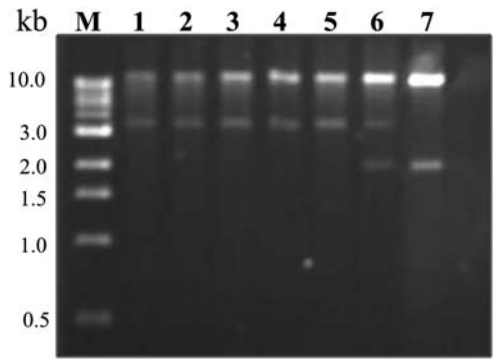

Fig. 5. Restriction analysis of pMTS1-BLBST plasmids isolated from various *Rb.capsulatus* strains. Plasmids were digested with BstXI and SfuI restriction enzymes. The presence of ~3.3 kb DNA fragment indicates that plasmid bears the fusion gene *pet*BLBST, while the ~2 kb DNA fragment corresponds to the native form of *pet*B gene. Plasmids are visualized on ethidiumbromide-stained 1% agarose gel. Lines: M—marker, plasmids isolated from the strains transformed with pMTS1-BLBST plasmids that encoded for the following forms: 1, B-B[3]; 2, B-B[6]; 3, B-B[9]; 4, B-B[12]; 5, B-B[16]; 6, B-B[20]; 7, control plasmid (pMTS1 containing native operon).

The reversions observed under photosynthetic conditions raise an issue of a genetic stability of the system. This issue requires particular attention in all cases when two copies of the same gene are parallelly introduced to the cells. In our case, careful examination of the samples originating from the semiaerobically grown cells (when the growth does not depend on cytochrome bc_1 function) performed at the level of DNA, membranes and isolated proteins confirmed that B-B can stably be maintained in the cells during the period of cultivation under those conditions at such level that the reversions, if occur, are below a threshold of detection for the biochemical and spectroscopic methods used.

We emphasize, however, that because of this risk of the reversions, a special care must be taken to carefully examine the samples every time a new batch of membranes and/or proteins is prepared so that for further functional/kinetic analysis are taken only the samples that meet the criteria applied in Świerczek *et al.* (2010): the integrity of *pet*BLBST isolated from the cells, the positive results from western blots on membranes and from SDS electrophoresis of complexes isolated using ion exchange and affinity chromatography showing the presence of only the fusion protein, and the proper spectroscopic properties of the complexes (optical and EPR). As certain point mutations are known to change specifically the spectral properties of cytochrome bc_1, the spectral analysis of the asymmetrically mutated forms (see below) offers additional level of verification whether both parts of cytochrome *bb* are properly incorporated and integrated with the remaining subunits. In such case, the

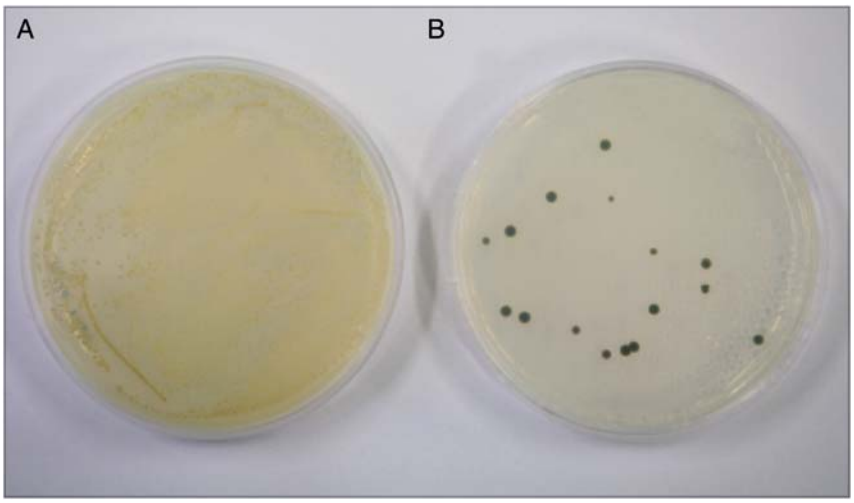

Fig. 6. The comparison of heterotrophic and photosynthetic growth of *Rb.capsulatus* strain transformed with genes coding for B-B[12]. The same amount of bacterial cells deriving from the semiaerobic liquid culture were transferred onto the agar plates and placed under aerobic conditions in dark (**A**) or anaerobic conditions in light (**B**). Few colonies in B represent the revertants that gain PS + phenotype by losing part of *pet*BLBST gene.

spectrum of asymmetric form is a combination of that of the native and the mutant form, as described in Świerczek *et al.* (2010).

We also emphasize that cultivation of cells for periods longer than a 3-day propagation involving three stages of 2 ml, 25 ml and 1 l cultures (described under 'Materials and methods') should be avoided and samples of membranes/proteins should always be prepared from freshly grown cultures originating directly from −80°C stocks.

Tolerance to asymmetric and symmetric mutations patterns

Since the native operon coding does not differentiate between the monomers of cytochrome bc_1, the system based on a fusion of two cytochromes *b* was specifically designed to enable introducing individual point mutations at the level of dimer that would break its symmetry. Permutations of just two strategically positioned point mutations knocked-out individual segments of the dimer in such a way that all major electron-transfer paths were exposed for kinetic examination. Those mutations included H212N (denoted as N) which caused a loss of one heme b rendering the Q_i site (quinone reduction site) non-functional (Osyczka *et al.*, 2004), and G158W (denoted as W) which distorted the quinone binding pocket rendering the Q_o site (quinol oxidation site) non-functional (Ding *et al.*, 1995).

As individual mutation can be introduced either to the first or the second copy of the fused gene, each variant can, in principle, have its mirror form (for example, for single W it can be either $_W$B-B or B-B$_W$). However, studies with B-B[12] show that not all possible variants can be obtained (Table II), as reported in Świerczek *et al.* (2010). Figure 7 summarizes western blots of membranes and SDS–PAGE profiles of complexes purified from the cells carrying *pet*BLBST genes with various combinations of asymmetric mutations. Within a group of single mutants, it was possible to obtain both variants containing W ($_W$B-B, B-B$_W$) (Fig. 7, lines 4 and 5), but only one variant with N (^{N}B-B) assembled as fusion protein (line 2). In the case of B-B^N, the fusion protein was absent in the cells and the complexes containing fragments shorter than cytochrome *bb* could have been isolated only using ion-exchange chromatography (line 3) (clearly, the second portion of the protein ending with the Strep-tag was missing). Within the group of double mutants, both combinations containing W and N on the same side (^N_WB-B, B-B^N_W) were tolerated (lines 8 and 9), while only one combination containing mutations across was expressed as a fusion protein ($_W$B-B^N) (line 6). In the case of ^{N}B-B$_W$, the cells expressed significant amount of complexes containing non-fused cytochome *b*, which co-purified with B-B (line 7). Thus, even though ^{N}B-B$_W$ is seen in the form of the complex with fused cytochrome *bb*, the presence of non-fused cytochrome *b* precludes the usage of ^{N}B-B$_W$ for further kinetic/functional analysis (this is an example not meeting criteria described in proceeding paragraph). We note that a comparison of electrophoretic profiles of complexes purified independently by the ion

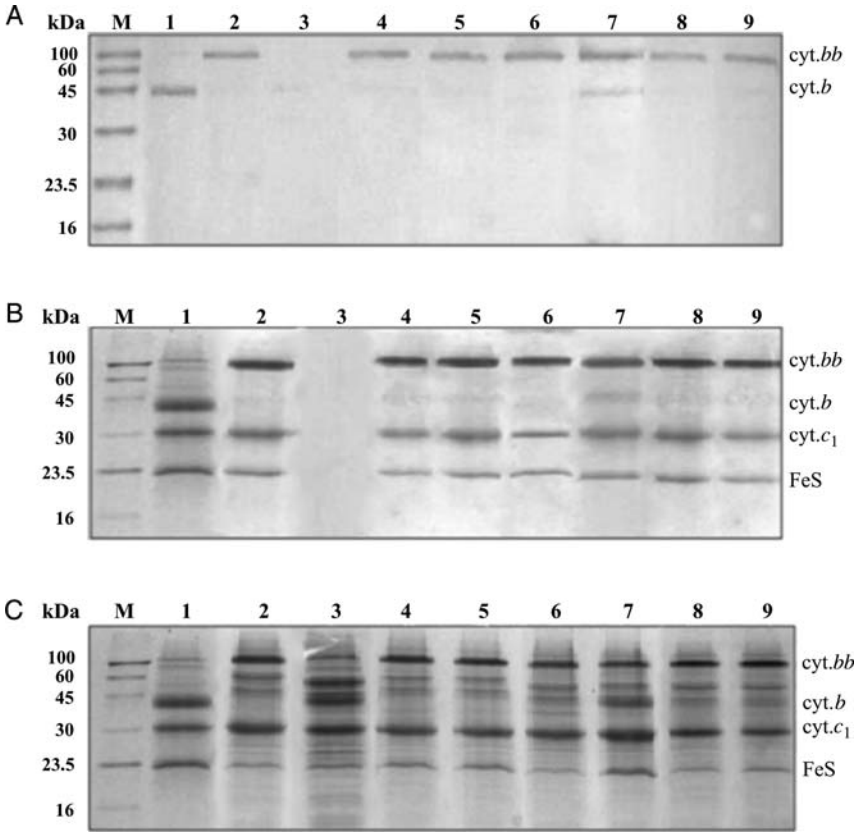

Fig. 7. Western blot and SDS–PAGE analyses of B-B[12] containing additional mutations W and/or N in various combinations. (**A**) Western blot against Strep-tag II of chromatophore membranes. (**B**) and (**C**) SDS–PAGE analysis of B-B[12] complexes isolated using affinity chromatography (Strep-tag) and ion-exchange chromatography (DEAE Bio-gel), respectively. Lines: M marker (IBA); 1, native form of cytochrome bc_1; 2, ^{N}B-B; 3, B-B^N; 4, $_W$B-B; 5, B-B$_W$; 6, $_W$B-B^N; 7, ^{N}B-B$_W$; 8, ^N_WB-B; 9, B-B^N_W.

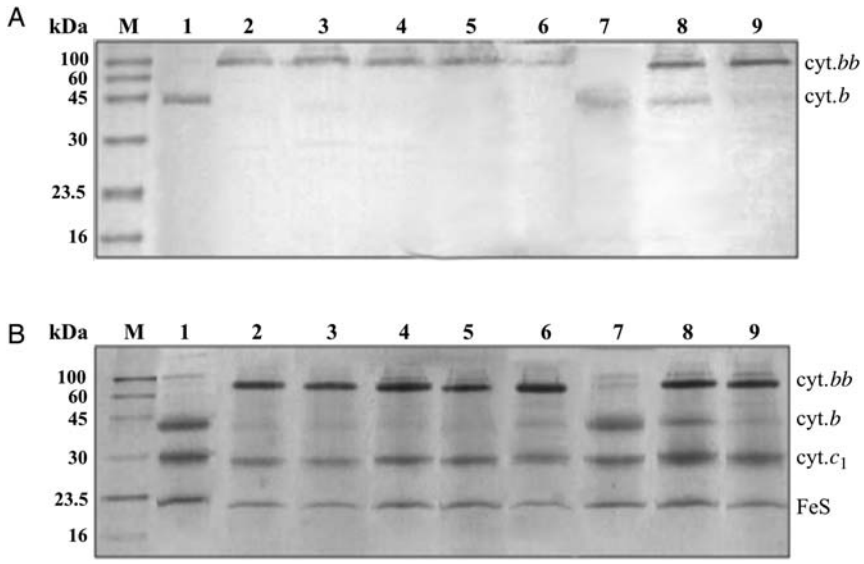

Fig. 8. Western blot and SDS–PAGE analyses of B-B[3] containing additional mutations W and/or N in various combinations. (**A**) Western blot against Strep-tag II of chromatophore membranes. (**B**) SDS–PAGE analysis of B-B[3] complexes isolated using affinity chromatography (Strep-tag). Lines: M marker (IBA); numbering as in Fig. 7.

exchange (isolates all complexes) or affinity chromatography (isolates Strep-tagged complexes) shown in Fig. 7 identifies comprehensively all the forms of bc-type complexes present in membranes. This is best visualized by the examples of lines 3 and 7.

For asymmetric variants that assembled as fusion protein, the reversion frequency was checked adopting similar strategy as described above for B-B[3] and B-B[12]. The reversion frequency was estimated to be at 10^{-3}–10^{-4} at all stages of cell cultivation, which was within a similar range as found for B-B[3] or B-B[12].

To explain the inability to obtain some forms of the fusion protein, we suggest that there is less of structural flexibility in the overall assembly of one 16-helical subunit in B-B than it is in the assembly of two 8-helical cytochromes *b* in the native dimer. Consequently, B-B might experience more difficulty in accommodating structural changes enforced by additional mutations even to the point that if the fusion protein does assemble, it bears significant structural alterations. If such alterations are not inert to the cell and impede the growth (for example, by affecting the integrity of the membrane), a selection toward the non-fused form advances more or less effectively during the cultivation period depending on how much the presence of a given type of fused protein affects physiological functions.

To test whether and how this sensitivity to mutational change depends on the linker itself, we examined the effect of introducing point mutations to B-B[3] repeating the permutation pattern of W and N used for B-B[12]. The results are summarized in Table II and Fig. 8. Both variants containing single W ($_{W}$B-B, B-B$_{W}$), or single N (^{N}B-B, B-B^{N}) assembled as fusion protein. From the combinations of W and N across, only $_{W}$B-B^{N} assembled as fusion protein. From the combinations of W and N on the same side, only B-B$^{N}_{W}$ was tolerated, while in the case of $^{N}_{W}$B-B, cells also expressed a significant amount of non-fused cytochrome *b*. These results demonstrate that in the case of B-B[3], like in B-B[12], there are some asymmetric mutation patterns that end up effectively advancing selection toward the non-fused form during the cultivation period. However, specific patterns causing these effects do not necessarily have to be the same.

A separate case of mutation patterns that cannot be accommodated by any of the fusion protein tested in this work (this regards all templates described in Table II) are symmetric ^{N}B-B^{N} and $_{W}$B-B$_{W}$. For those mutants, introducing the corresponding genes to the cells resulted in the similar type of phenomenon as described earlier for the B-B[20]. During the crossing procedure, few single colonies appeared on selective plates with 1 day of delay to the characteristic 3 days time. The remaining colonies (that corresponded in the number to the typical yield of the crossing procedure) appeared with a 2- to 3-day delay. Further restriction analysis of the plasmid DNA isolated from these cells revealed that only the short version of the gene was present (its size corresponded to a single copy of *pet*B). We follow the same reasoning as discussed for the B-B[20] and consider these results as an indication that the presence of ^{N}B-B^{N} or $_{W}$B-B$_{W}$ might somehow be damaging to the cells (improperly folded protein affects integrity of the membrane), and the resulting growth impediment advances efficient selection toward the non-fused form from the early stages of the plasmid acquisition.

Discussion

Our study shows that successful fusion of two cytochromes *b* and an integration of the fused protein with other subunits of the cytochrome bc_1 of *Rb.capsulatus* can be achieved with variety of linkers that connect the C-terminus of one cytochrome *b* with the N-terminus of the other (Fig. 1). This indicates that the termini have significant conformational flexibility in accommodating specific structural constrains imposed by the presence of an individual linker. Spectral properties and subunit composition of modified complexes indicate that under these circumstances the overall assembly

of the 16-helical construct resembles an assembly of two 8-helical cytochromes *b* in the dimer. Such resemblance requires a preservation of the attachment sites for four hemes B, a formation of four quinone binding sites, and retention of specificity in interactions with other subunits, cytochrome c_1 and the FeS subunit. The competent versions of B-B listed in Table I come thus as remarkable examples of the overall structural plasticity of cytochrome bc_1 complex and its hydrophobic core built by cytochromes *b*.

The B-B complex, however, should not be treated as exact replica of the native cytochrome bc_1. The two major differences should be emphasized. First, cells expressing B-B, despite high enzymatic and electron-transfer activity of B-B (as reported by flash-induced and steady-state kinetic measurements (Świerczek *et al.*, 2010)), are not able to grow photosynthetically. Second, B-B exhibits less engineering tolerance for further mutagenic changes, which manifested itself in the apparent difficulty of B-B to accommodate certain mutation patterns (understood as specific combinations of point mutations introduced to the first, or the second, or both halves of the fused protein). These two sets of conditions (i.e. the photosynthetic growth conditions or the presence of certain mutations) effectively advance selection toward the pseudo-native form containing non-fused cytochrome *b*. As proposed in the Results, in the first case, this may be because of the too costly energetically biosynthesis and incorporation of B-B to the membrane, while in the second case, this may be due to the non-inert for the cells presence of somewhat 'distorted' B-B. Keeping with this proposal, one may expect that a degree of negative impact of the non-inert forms will depend on the level of structural distortion experienced by individual type of B-B. This could very well be at roots of the observed differences in the time needed for the selection of revertants: in more severe cases (symmetric ^{N}B-B^{N} and $_{W}$B-B$_{W}$) the effective selection manifests itself at initial stages of cell cultivation, while in less severe cases (for example asymmetric ^{N}B-B$_{W}$ on B-B[12] template, or $^{N}_{W}$B-B on B-B[3] template), it takes place at later stages.

Table II indicates that in some cases a success in an assembly of B-B with asymmetric mutations depended on whether a particular mutation was placed in the first or the second halve of the fusion protein. For example, with B-B[12], $_{W}$B-B^{N} assembled, but ^{N}B-B$_{W}$ failed to do so. This points to the fact that the linker itself and/or C-terminal somewhat lowers the degree of symmetry intrinsic to the homodimeric structure of native cytochrome bc_1. Consequently, B-B might experience difficulty in accommodating certain changes associated with additional structural modifications, which could be one of the reasons of the observed increase in sensitivity of B-B to point mutations in comparison with native cytochrome bc_1.

While this increased sensitivity holds with all linkers tested, irrespective of their length, a set of specific mutation patterns not tolerated may differ depending on the type of linker used (Table II). For example, $^{N}_{W}$B-B is tolerated in B-B[12] but not in B-B[3]. Clearly, the forms with various linkers may, in certain cases, differently respond to the necessity to accommodate mutational changes. Considering all cases of failure listed in Table II it is also clear that there is no distinction between the first or second halve of the fused protein in terms of the origin of failure, i.e. mutations located either in the first or the second halve can result in a failure.

Nevertheless, our results indicate that B-B does accommodate several asymmetric mutational patterns that can be maintained in the cells during the cultivation period under semiaerobic conditions when the growth of cells does not depend on cytochrome bc_1. Furthermore, the presence of the fusion protein is easily verifiable, providing us with effective means to control the status of the membrane and protein samples at every experimental step. This also includes means to confirm the presence of desired mutation(s) (like N or W used here) at appropriate position(s) within cytochrome *bb* verified at the level of DNA sequence of plasmids isolated from the *Rb.capsulatus* cells. We believe that this type of control is important for any genetic system based on two copies of the same gene which face recombination possibilities.

This system thus provides an attractive way to break structural symmetry of dimer to expose individual electron-transfer paths for kinetic testing. Those types of experiments can be performed at the level of membranes isolated from the semiaerobically grown cells (flash-induced electron-transfer kinetics, enzymatic activities) as described in Świerczek *et al.* (2010), or purified complexes (manuscript in preparation). They have already proven valuable in discerning mechanistic principles of the operation of dimer of cytochrome bc_1. It is noteworthy that till now, this system appears to be the only one to provide the experimental access to certain electron-transfer paths (such as those exposed by B-B with single asymmetric mutation N or W, or double-mutations N and W placed on the same side) for flash-induced electron-transfer kinetic analysis at the level of membranes.

This system also has its limitations which relate to the discussed earlier observation that a duplication of a gene implemented to construct a fusion protein, as might have been expected, is prone to reversions. This is an undesirable feature that must be taken into account when using the system and preparing the samples for kinetic analysis. This means experimental rigor at all stages of sample preparation and analysis implemented to obtain membranous fractions that contain almost exclusively the assembled B-B complexes containing the fusion protein. In such cases, a comprehensive chromatographic analysis (using independently ion exchange and affinity chromatography) must show just the presence of the B-B complexes, while spectroscopic analysis (optical and EPR) of the forms containing asymmetric mutations must confirm structural asymmetry. Applying those analyses among others, described in detail in Materials and methods and Results, provides criteria for clear recognition and separation of the cases where the level of undesired background of reverted forms of proteins would compromise further kinetic analysis. We documented several of such cases here.

Because of the photosynthetic incompetence of B-B and associated with it reversions, the other limitation of this system is that it cannot be used to asses functionality of individual electron-transfer paths *in vivo*. It can only be used as a model for *in vitro*/reconstitution measurements. In this context, the recently described system based on parallel expression of two plasmids and isolations of heterodimers with a use of two different tags offered an attractive way to test one of the paths *in vivo* (Lanciano *et al.*, 2011). This path has always been of particular interest from the mechanistic

point of view as it involves the electron transfer across the dimer engaging the centrally located two-heme bridge (see discussions in Osyczka *et al.*, 2004; Shinkarev and Wraight, 2007; Crofts *et al.*, 2008; Castellani *et al.*, 2010; Świerczek *et al.*, 2010; Lanciano *et al.*, 2011). Clearly, the availability of those two alternative systems for asymmetric mutagenesis opens now new avenues of investigations and it can be anticipated that they will complement each other in providing new insights into the mechanisms of cytochrome bc_1 action.

In literature, several cases of successful fusion of proteins have been described, including the closely related to our study fusion of two membrane-anchored cytochromes *c* in *Rb.capsulatus* (Lee *et al.*, 2008); for other examples see Hanada *et al.* (1987), Sahin-Tóth *et al.* (1994) and Arai *et al.* (2001). From the protein engineering point of view, however, our cytochrome bc_1-like complexes represent an interesting and rare example of multisubunit protein complex where two transmembranous cytochromes (containing attachment sites for hemes and binding sites for quinones) can be fused to one subunit that assembles with other subunits (membrane-anchored proteins) of the catalytic core of the enzyme. It is thus interesting to contemplate the fusion protein from the perspective of biogenesis of cytochrome bc_1. This process, despite several studies with bacterial and eukaryotic systems (Thony-Meyer, 1997; Sanders *et al.*, 2010; Conte and Zara, 2011), remains largely unknown. In particular, it is not certain, at which stage and how the dimeric structure is assembled. The assembly of the complex containing cytochrome *bb* makes it tempting to speculate that it is an assembly of dimeric cytochrome *b* core (reminiscent of cytochrome *bb*) that acts to nucleate formation of the entire complex by attracting the remaining subunits, cytochrome c_1 and the FeS subunit, and enforcing the specific ternary binding alignment.

The fused protein system developed to break the symmetry of cytochrome bc_1 dimer originally utilized 12 amino acid linker for fusion. From the present study it is clear that the employment of other linkers offers additional possibilities to expand the family of asymmetric variants for further kinetic and structural investigations.

While this paper was under review, Daldal and collaborators raised concerns about the risk of heterogeneity that might limit the fusion protein system for studies of heterodimeric cytochromes bc_1 (Khalfaoui-Hassani *et al.*, 2011). The present work complements our earlier work (Świerczek *et al.*, 2010) to remove these concerns by demonstrating that the fusion system enables us to obtain biochemically and spectrosopically homogenous samples of asymmetric forms of B-B suitable for kinetic analysis.

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