

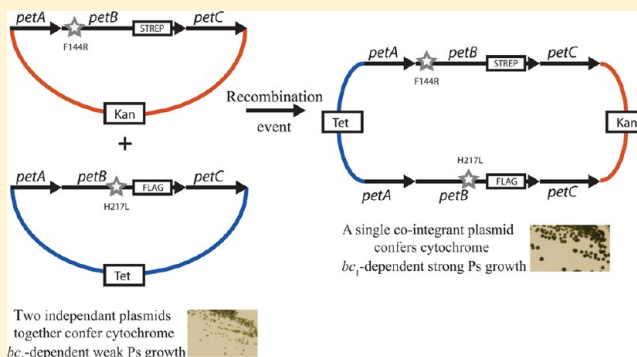
A Robust Genetic System for Producing Heterodimeric Native and Mutant Cytochrome bc_1

Bahia Khalfaoui-Hassani, Pascal Lanciano, and Fevzi Daldal*

Department of Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19104, United States

S Supporting Information

ABSTRACT: The ubiquinone:cytochrome c oxidoreductase, or cytochrome bc_1 , is central to the production of ATP by oxidative phosphorylation and photophosphorylation in many organisms. Its three-dimensional structure depicts it as a homodimer with each monomer composed of the Fe–S protein, cytochrome b , and cytochrome c_1 subunits. Recent genetic approaches successfully produced heterodimeric variants of this enzyme, providing insights into its mechanism of function. However, these experimental setups are inherently prone to genetic rearrangements as they carry repeated copies of cytochrome bc_1 structural genes. Duplications present on a single replicon (one-plasmid system) or a double replicon (two-plasmid system) could yield heterogeneous populations via homologous recombination or other genetic events at different frequencies, especially under selective growth conditions. In this work, we assessed the origins and frequencies of genetic variations encountered in these systems and describe an improved variant of the two-plasmid system. We found that use of a recombination-deficient background (*recA*) minimizes spontaneous formation of co-integrant plasmids and renders the homologous recombination within the cytochrome b gene copies inconsequential. On the basis of the data, we conclude that both the newly improved *RecA*-deficient and the previously used *RecA*-proficient two-plasmid systems reliably produce native and mutant heterodimeric cytochrome bc_1 variants. The two-plasmid system developed here might contribute to the study of “mitochondrial heteroplasmy”-like heterogeneous states in model bacteria (e.g., *Rhodobacter* species) suitable for bioenergetics studies. In the following paper (DOI 10.1021/bi400561e), we describe the use of the two-plasmid system to produce and characterize, in membranes and in purified states, an active heterodimeric cytochrome bc_1 variant with unusual intermonomer electron transfer properties.



The ubiquinone:cytochrome c oxidoreductase (cytochrome bc_1 or mitochondrial complex III) is a multi-subunit membrane integral enzyme required for both photosynthetic (Ps) and respiratory (Res) electron transfer in many organisms. It oxidizes hydroquinones (QH₂) to produce quinones (Q) and reduces metalloprotein electron carriers, which are often c -type cytochromes. This reaction is coupled with the translocation of protons across the membrane for ATP production. The three-dimensional structures of various cytochrome bc_1 forms are symmetrical homodimers.^{1–4} In most bacteria, each monomer consists of three conserved subunits: cytochrome b , the Fe–S (also called Rieske) protein, and cytochrome c_1 . Cytochrome b has two b -type hemes (a low-potential b_L heme and a high-potential b_H heme) located on the p and n sides of the membrane. The Fe–S protein and cytochrome c_1 carry a high-potential [2F-2S] cluster and a c -type heme, respectively.⁴

The modified Q cycle describes the mechanism of electron transfer and proton translocation within cytochrome bc_1 .^{5–7} Accordingly, two QH₂ molecules are oxidized, one Q molecule is reduced, and the resulting electrons are conveyed to the terminal acceptors (e.g., cytochrome c oxidase or mitochondrial complex IV in respiration) via a complex series of reactions.^{8,9}

As generally described, the Q cycle mechanism is confined to a single monomer of the dimeric cytochrome bc_1 and does not consider any possible interaction between the monomers.¹⁰ A mechanism with pathways of electrons through a dimeric cytochrome bc_1 was first formulated by De Vries et al.,¹¹ and a dimeric organization of this enzyme was observed by electron microscopy.¹² Similar ideas were rejuvenated after its dimeric architecture was established firmly via its three-dimensional structures.^{1–4} Various interesting features of the dimeric enzyme raised intriguing questions, including electron transfer possibilities between the monomers through the b_L - b_L hemes because of their proximity to each other, or conformational changes during the catalytic steps of a dimeric cytochrome bc_1 . These observations, together with experimental data^{13–18} and theoretical calculations,¹⁹ led to models that invoked allosteric interactions and electron transfer reactions between the monomers of cytochrome bc_1 .^{13–15}

Received: May 2, 2013

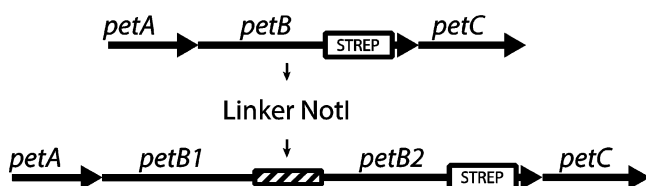
Revised: September 10, 2013

Published: September 12, 2013



Recently, the intermonomer electron transfer issue was experimentally probed by various groups,^{20,21} including ours,²² using genetic approaches aimed at breaking the symmetry of the dimeric cytochrome *bc*₁. Using *Rhodobacter capsulatus*, Osyczka's group produced a cytochrome *bc*₁ variant (*b-bc*₁) in which two cytochromes *b* were genetically fused together with an amino acid linker. A single plasmid (termed the one-plasmid system) carrying this engineered *petAB1-B2C* operon yielded a heterodimeric cytochrome *b-bc*₁, which was purified using an epitope tag added to the cyt *b-b* fusion subunit²¹ (Figure 1A).

A. One-Plasmid System



B. Two-Plasmids System

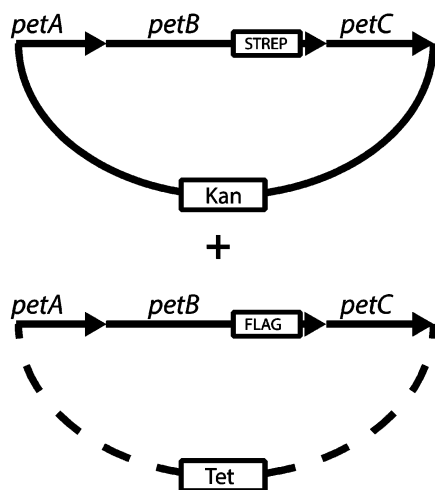


Figure 1. Genetic systems producing cytochrome *bc*₁ heterodimers. (A) In the one-plasmid system, the *petB* gene was duplicated (*petB1* and *petB2*) and linked together with the 12-amino acid NotI linker (ASIAGGRTASGP). The *petB2* gene is C-terminally tagged with the Strep epitope (boxed). For the sake of simplicity, the remaining part of plasmid pMTS1 (Kan^R derivative of pRK415) carrying these constructs is not shown. (B) In the two-plasmid system, two *petABC* operons are carried by two plasmids (solid and dashed lines) marked with different antibiotic resistance cassettes, as indicated by boxes labeled Kan (kanamycin) and Tet (tetracycline). The *petB* genes of the Kan and Tet plasmids are C-terminally tagged with the Strep and Flag tags, respectively.

Independently, we developed a different genetic setup that duplicated the entire *petABC* operon (encoding the three catalytic subunits of cytochrome *bc*₁) on two separate plasmids (termed the two-plasmid system), in which each *petB* gene is marked with a different epitope tag²² (Figure 1B). This system produced two-homodimeric (i.e., same tag and same mutation on both monomers) and one-heterodimeric (i.e., different tags and different mutations in each monomer) cytochrome *bc*₁ variants in the same cell. Using judiciously chosen mutations

inactivating the Q_o site of one monomer and the Q_i site of the other monomer, both one-plasmid and two-plasmid systems produced heterodimeric variants to expose the occurrence of the *b*_L-*b*_L heme intermolecular electron transfer.^{21,22} Salient properties of these genetic systems were reviewed recently.²³

A more recent study by Hong et al.²⁴ attempted to reproduce Osyczka's work²⁵ using *Rhodobacter sphaeroides* instead of *R. capsulatus*. These authors²⁴ reported the occurrence of genetically rearranged forms of cytochrome *b* fusions that were thought to arise from homologous recombination. On the basis of these data, they questioned the validity of the one-plasmid system²¹ and the occurrence of intermonomer electron transfer in cytochrome *bc*₁.^{21,22} Furthermore, they extended their skepticism to the two-plasmid system^{20,22} for which they had no experimental data. Unfortunately, in this work, the authors²⁴ used inappropriate experimental conditions (i.e., Ps growth), which is strongly selective for better growing cells, invalidating their findings. The basis of the strong growth disadvantage seen with the one-plasmid system is not yet understood²³ and may not necessarily originate from the intermonomer electron transfer in cytochrome *bc*₁. Thus, no conclusion about the occurrence or rate of intermonomer electron transfer can be reached on the basis of the work by Hong et al.²⁴

Following the course of our work, we report here a detailed assessment of the genetic rearrangements exhibited by *R. capsulatus* strains harboring the one- and two-plasmid systems. In each case, we monitored the growth phenotype that they confer, the frequency of the "revertants" that they form under selective (Ps) and nonselective (Res) growth conditions, and the molecular nature of the DNA rearrangements and sequence changes that they undergo. Furthermore, we isolated a *R. capsulatus* strain lacking the RecA-dependent homologous recombination pathway to assess its role in the observed genetic rearrangements. We found that a RecA⁻ background minimized to different extents the frequency of DNA rearrangements observed with both the one- and two-plasmid systems. We conclude that the earlier RecA⁺ and the newly developed RecA⁻ versions of the two-plasmid system reliably produce native and mutant forms of heterodimeric cytochrome *bc*₁. In the following paper (DOI 10.1021/bi400561e), we describe the production, purification, and characterization of an active heterodimeric cytochrome *bc*₁ with interesting electron transfer properties. Our findings are discussed with respect to the capabilities of the two-plasmid system to produce reliably heterodimeric multisubunit protein complexes and to generate heterogeneous populations of cytochrome *bc*₁ in a bacterial model.

MATERIALS AND METHODS

Growth Conditions and Genetic Crosses. *Escherichia coli* strains were grown at 37 °C on LB medium supplemented with antibiotics [100 µg/mL ampicillin (Amp), 50 µg/mL kanamycin (Kan), and 12.5 µg/mL tetracycline (Tet)]. *R. capsulatus* wild-type and mutant strains were grown at 35 °C under respiratory (Res, aerobic dark) or photosynthetic (Ps, anaerobic light) conditions in liquid or solid enriched MPYE medium, supplemented with antibiotics as needed [10 µg/mL Kan, 2.5 µg/mL Tet, 10 µg/mL spectinomycin (Spe), and 1 µg/mL gentamicin (Gen)] as described previously.²⁶ For Ps growth, completely filled culture vessels or solid medium-containing plates were placed in anaerobic jars with gas packs

generating H₂ and CO₂ (Becton, Dickinson Inc.) and incubated in temperature-controlled Percival light incubators.

R. capsulatus strains harboring a single plasmid, or coharboring two plasmids, were obtained by conjugation between appropriate *E. coli* HB101 derivatives used as donors and *R. capsulatus* RecA⁺ strain MT-RBC1 with a complete chromosomal deletion of the *petABC* operon (a *bc₁*[−] mutant)²⁷ or its RecA[−] derivative BK-RBC1 as recipients. For the two-plasmid system, plasmids marked with different antibiotic resistance (Kan^R and Tet^R) genes were introduced into appropriate recipients by consecutive conjugative transfer. Strains coharboring two plasmids were maintained in the presence of one-half of the normal concentration of the antibiotics used (i.e., 5 μg/mL Kan and 1.25 μg/mL Tet).

Plasmids and Strains Used. All strains and plasmids used are listed in Table S1 of the Supporting Information. Molecular genetic techniques were performed using standard procedures.²⁸ All constructs were confirmed using appropriate restriction enzyme digestions and DNA sequencing. For the isolation of a RecA[−] derivative of MT-RBC1, the *R. capsulatus* ORF RCC01751 encoding the *recA* gene was first amplified via polymerase chain reaction (PCR) using the *recA*For (5′-GTCGTCGCGGGTACCGAAGCGATA-3′ with the KpnI site underlined) and *recA*Rev (5′-CGTCATCGGTGTTCTAGACGGTGACCA-3′ with the XbaI site underlined) primers. The PCR product thus obtained was digested with KpnI and XbaI restriction enzymes and cloned into the similarly digested plasmid pBluescript to yield plasmid pWX1 (Table S1 of the Supporting Information). The 600 bp SmaI–HindIII portion within *recA* carried by pWX1 was deleted and replaced with a gentamycin (*gen*) resistance gene digested with the same restriction enzymes to yield pWX2. Plasmid pWX2 was digested with KpnI and XbaI, and the DNA fragment carrying the Δ(*recA::gen*) allele was cloned into a similarly digested pRK415, to yield plasmid pCWX3 (Table S1 of the Supporting Information). This plasmid was conjugated into gene transfer agent (GTA) overproducer strain Y262,²⁹ and a chromosomal RecA null derivative of MT-RBC1 carrying the Δ(*recA::gen*) and Δ(*petABC::spe*) alleles, named BK-RBC1 (Table S1 of the Supporting Information), was obtained by GTA crosses.²⁶ Strain BK-RBC1 subsequently received the one- or two-plasmid systems by conjugative transfer.

Construction of the One-Plasmid Genetic System. The one-plasmid system was generated as described in refs 21 and 25. The *petAB1-B2C* fusion plasmid (pBK6) contained a 12-amino acid (ASIAGGRTASGP) linker with a NotI site between *petB1* and *petB2* and a Strep tag at the C-terminus of *petB2* (Figure 1A). First, the *petA* and *petB1* genes were amplified via PCR using the *pet*-BamHI (5′-AAATATCTGTGCTGGATCCGCTGCGCTATG-3′) and *petL2* (5′-AACAGCCACTACGGCAATCCGGCGTCGATCGCCGGCGGCGCCGACCG-3′) forward and reverse primers, with the NotI site underlined. Primer *petL2* is located at the end of the *petB* gene where it overlapped Pro435 of cytochrome *b* and contained a NotI restriction site. The PCR product obtained was cloned into the pBluescript plasmid after digestion with the BamHI and NotI restriction enzymes, to yield plasmid pBK8 (Table S1 of the Supporting Information). Separately, a *petB2* gene with a Strep (−S) tag at its C-terminus and the adjacent *petC* gene was amplified via PCR using *petL1* (5′-GCCGCGCGGCCGACCGCATCGGGCCCCGTCCGGAATTCGCGACGACCAT-3′, with the NotI site underlined) and *pet*-HindIII (5′-CGCCACAGGAAGCTTTGATAGGCATCGA-3′) primers, respec-

tively. The *petL1* primer is located at the beginning of the *petB2* gene where Ser2 of cytochrome *b* was linked to the second part of the NotI linker. The PCR product obtained was also cloned into the pBluescript plasmid after digestion with NotI and HindIII restriction enzymes, to yield plasmid pBK7 (Table S1 of the Supporting Information). Plasmids pBK7 and pBK8 were digested with the NotI and HindIII enzymes and the NotI and BamHI enzymes, respectively, to yield the *petAB1*-(NotI linker 1) and (NotI linker 2)-*petB2C* DNA fragments. These fragments were ligated into pMTS1 (Kan^R derivative of plasmid pRK415) digested with the HindIII and BamHI enzymes to yield pBK6 (Table S1 of the Supporting Information). Plasmid pBK6 harbored the fused form of *petB1-B2* (in the *petAB1*-Linker 1 NotI Linker 2-*petB2C* operon) with a NotI linker between *petB1* and *petB2* (Figure 1A).

Similarly, plasmid pBK32 (*petAB1*^{F144R}-*B2*^{H217L}*C*) containing the Q_o site cyt *b* F144R mutation in *petB1*³⁰ and the Q_s site cyt *b* H217L mutation in *petB2*³¹ was constructed. Plasmid pBK8 (*petA-petB1*^{wt}) was used as a template for PCR-mediated site-directed mutagenesis to generate the *petB1*^{F144R} mutation using the F144For (5′-TGATGGGCACCGCCCGCATGGGCTACGTGC-3′) and F144Rev (5′-TGATGGGCACCGCCCGCATGGGCTACGTGC-3′) primers, yielding plasmid pBK22 (Table S1 of the Supporting Information). Plasmid pBK7 (*petB2*^{wt}-*petC*) was used as a template for PCR-mediated site-directed mutagenesis to generate the *petB2*^{H217L} mutation using the H217LFor2 (5′-ATCTGGGCCTTCCTACCACCGGCAAC-3′) and H217LRev2 (5′-ATCTGGGCCTTCCTACCACCGGCAAC-3′) primers, yielding plasmid pBK27 (Table S1 of the Supporting Information). As described above, plasmids pBK22 and pBK27 were digested with the NotI and HindIII enzymes and the NotI and BamHI enzymes, respectively. The *petAB1*^{F144R} and *petB2*^{H217L}*C* DNA fragments obtained were ligated into plasmid pMTS1 digested with HindIII and BamHI enzymes to yield pBK32 (Table S1 of the Supporting Information).

Construction of the Two-Plasmid Genetic System. The two-plasmid system was generated as described previously,²² except that the previously used pRK-pPET1 composite plasmid was replaced with the smaller pRK415 (Tet^R) derivative. Plasmid pMTS1-S or pMTS1-F carrying *petABC* with a Strep or a Flag tag at the carboxyl end of *petB* was digested with the HindIII and BamHI restriction enzymes, and the 3.5 kb DNA fragments containing the *petABC* operon were cloned into a similarly digested pRK415 (Tet^R), yielding plasmids pBK20 and pBK21, respectively. The previously described plasmids pPET1-F and pPET1-S²² were used as templates to introduce the cyt *b* H217L mutation into pPET1-F using primers H217LFor2 and H217LRev2 to yield pBK18 and the cyt *b* F144R mutation into pPET1-S using primers F144RFor and F144RRev to yield pBK19, respectively. Plasmid pBK19 was digested with EcoRI and HindIII enzymes to produce the *petAB*^{F144R}*C* fragment, which was used to replace its wild-type counterpart in pMTS1 (Kan^R) to yield plasmid pBK25-F. Similarly, plasmid pBK18 was digested with ApaLI and HindIII enzymes to produce the *petAB*^{H217L}*C* fragment, which was used to replace its wild-type counterpart in pBK20 to yield plasmid pBK28-S (Table S1 of the Supporting Information).

Biochemical Techniques. Cultures (1L) of freshly controlled cells were grown by Res to prepare intracytoplasmic (chromatophore) membranes. Frozen washed cell pellets resuspended in MOPS-KCl buffer [50 mM MOPS (pH 7.0), 1 mM KCl, and 1 mM EDTA] were broken via two passages

through a French press, as described in ref 32. Protein concentrations were determined using bicinchoninic acid with bovine serum albumin as a standard.³³ Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (12.5%) was conducted as described in ref 34, and prior to being loaded, samples were solubilized in a loading buffer to obtain final concentrations of 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 0.1 M dithiothreitol, 25% glycerol, and 0.01% bromophenol blue by incubation at room temperature for 10 min. Immunoblot analyses were conducted as described in ref 35 using polyclonal antibodies against *R. capsulatus* cytochrome *b* and commercially available monoclonal anti-Flag (Sigma-Aldrich, Inc.) and anti-Strep II (Novagen, Inc.) antibodies.

Steady-state cytochrome *bc*₁ activity was measured using decylbenzohydroquinone (DBH₂) as an electron donor and horse heart cytochrome *c* as an electron acceptor at 25 °C. The reaction was initiated by enzyme addition and monitored at 550 nm for 1 min, and the portion of the initial rate that is famoxadone sensitive was taken as the enzyme activity. Optical spectra were recorded on a Cary 60 spectrophotometer (Agilent Technologies Inc.). Absorption difference spectra for the *c*- and *b*-type cytochromes were obtained using chromatophore membranes (0.3 mg of total protein/mL), oxidized with potassium ferricyanide, and reduced with sodium ascorbate or sodium dithionite, as appropriate.²⁷

RESULTS

Isolation of a RecA[−] Mutant of *R. capsulatus*. During our previous work using the two-plasmid system, we observed that *R. capsulatus* strains that produce heterodimeric cytochrome *bc*₁ variants yield large Ps⁺ colonies at a frequency of ~10^{−4} among a predominantly small Ps^{+/−} population.²² We sought a RecA[−] mutant to evaluate the role of homologous recombination in the occurrence of the large Ps⁺ colonies. We thought that abolishing homologous recombination between the multiple copies of *petABC* might decrease the frequency of large Ps⁺ colonies produced by these strains. As a *R. capsulatus* RecA[−] mutant was unavailable, we scanned its genome and found ORF RCC01751 that is homologous to the *recA* gene encoding RecA in many organisms.^{36,37} We cloned and knocked out this chromosomal gene in a cytochrome *bc*₁-null mutant (MT-RBC1) to isolate strain BK-RBC1 [Δ (*petABC::spe*) Δ (*recA::gen*)] (Materials and Methods). *E. coli* RecA[−] strains exhibit slower growth than their wild-type counterparts and are hypersensitive to alkylating agents like methylmethanesulfonate (MMS) because of their DNA repair deficiency.³⁸ As expected, BK-RBC1 was Ps[−] due to the absence of cytochrome *bc*₁, grew slowly (~50% slower rate compared to that of its parent) under the Res growth conditions, and was sensitive to MMS unlike its parent MT-RBC1 [Δ (*petABC::spe*)]. The two isogenic MT-RBC1 (RecA⁺) and BK-RBC1 (RecA[−]) strains were used comparatively in all subsequent studies to assess the role of homologous recombination in the genetic rearrangements that occur in strains carrying the one- or two-plasmid systems.

One-Plasmid System. *R. capsulatus* strains pBK6/MT-RBC1 and pBK6/BK-RBC1 carrying plasmid pBK6 with the *petAB1-B2C* operon (two fused copies of wild-type *petB*) were taken to be “wild-type” analogues for the one-plasmid system (Figure 1A and Table S1 of the Supporting Information). They were compared with pMTS1/MT-RBC1 and pMTS1/BK-RBC1 (native *petABC* operon) with respect to their cytochrome *bc*₁-dependent Ps growth ability, frequency of

large Ps⁺ colonies that they yielded, and the molecular nature of the genetic rearrangements and amounts of heterodimeric enzymes that they produced, as described below.

Growth Properties of Strains Carrying the One-Plasmid System in RecA⁺ and RecA[−] Backgrounds. The Res growth phenotypes of pBK6/MT-RBC1 and pBK6/BK-RBC1 (*petAB1-B2C*) strains were similar to those of pMTS1/MT-RBC1 and pMTS1/BK-RBC1 (*petABC*). However, their Ps growth abilities were remarkably different (Figure 2A,B).

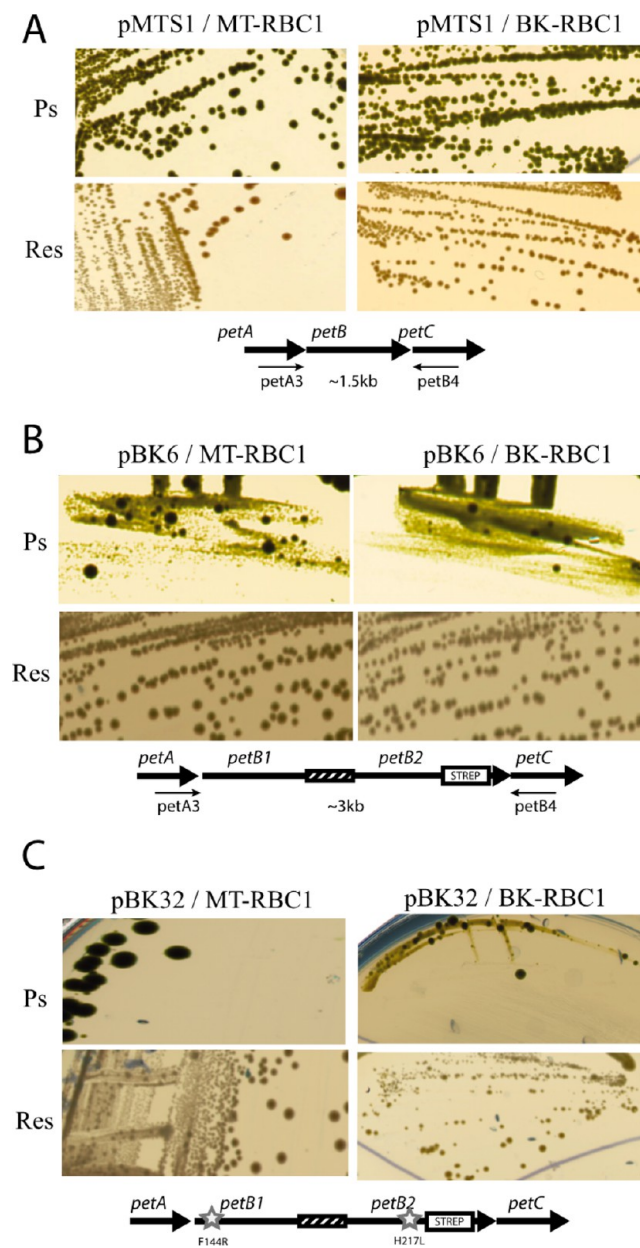


Figure 2. Growth phenotypes of strains carrying the one-plasmid system. Photosynthetic (Ps) and respiratory (Res) growth on enriched medium of *R. capsulatus* strains MT-RBC1 (RecA⁺) and BK-RBC1 (RecA[−]) harboring plasmid (A) pMTS1 carrying a wild-type *petABC* operon, (B) pBK6 carrying *petAB1-B2C*, and (C) pBK32 carrying the mutated *petAB1^{F144R}-B2^{H217L}C* fusion operons. In each case, the appropriate operon constructs are shown below the colony pictures, and mutations *cyt b* F144R and *cyt b* H217L are indicated with stars. Primers *petA3* and *petB4* (see the text) were used for PCR amplification.

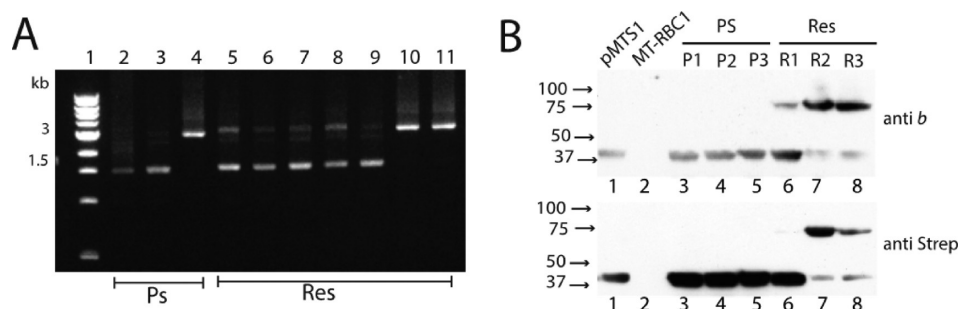


Figure 3. Genetic rearrangements in a strain carrying the one-plasmid system. (A) PCR-amplified DNA mixtures using the *petA3* and *petB4* primers were run on a 1% agarose gel, and each lane corresponds to plasmid DNA extracted from independent colonies of strain pBK6/MT-RBC1 grown under Ps and Res conditions (Materials and Methods): lane 1, DNA size markers; lane 2, control plasmid pMTS1 carrying the *petABC* operon; lane 3, plasmid extracted from a colony carrying the one-plasmid system and growing as a wild type under photosynthetic (Ps) conditions; lane 4, plasmid extracted from a colony carrying the one-plasmid system and growing poorly under Ps conditions; lanes 5–11, plasmids extracted from similar colonies growing under respiratory (Res) conditions. The 1.5 and 3 kb bands correspond to wild-type *petB* and the *petB1-petB2* fusion, respectively. (B) SDS–PAGE and immunoblot analyses of chromatophore membranes (40 μ g of total proteins) from cells harboring the one-plasmid system: lane 1, pMTS1/MT-RBC1; lane 2, MT-RBC1; lanes 3–8, Ps- and Res-grown colonies of pBK6/MT-RBC1 compared for their cytochrome *b* contents using anti-cytochrome *b* (anti *b*) and anti-Strep (anti Strep) antibodies.

The strains carrying pBK6 formed colonies under Ps growth conditions only upon prolonged incubation (~ 5 days for pBK6 vs ~ 2 days for pMTS1 derivatives, and longer incubation was needed for the *RecA*[−] derivatives). Thus, a fused *petB1-petB2* construct was deficient for Ps growth, although it contained two wild-type *petB* alleles. The basis of this growth defect was not studied, but a possibility is that these strains may contain inadequate amounts of cytochrome *bc*₁ to sustain Ps growth of *R. capsulatus*.³⁹ In addition, pBK6-containing strains exhibited larger Ps⁺ colonies at frequencies of $\sim 10^{-2}$ and $\sim 10^{-3}$ in the *RecA*⁺ and *RecA*[−] backgrounds, respectively (Table S2A of the Supporting Information). Moreover, when plasmid pBK32 carrying the fused form of the *petAB1*^{F144R}-*B2*^{H217L}-*C* operon with the Q_o site *cyt b* F144R³⁰ and Q_i site *cyt b* H217L³¹ mutations [for detailed properties of these mutations, see the following paper (DOI 10.1021/bi400561e)] was tested, both pBK32/MT-RBC1 (*RecA*⁺) and pBK32/BK-RBC1 (*RecA*[−]) showed no Ps growth but produced few large Ps⁺ colonies (Figure 2C). Thus, neither pBK6- nor pBK32-containing strains were able to support adequate Ps growth, and the contribution of the *RecA* pathway upon abolishing the formation of large Ps⁺ colonies was approximately an order of magnitude (Table S2A of the Supporting Information).

Genetic Rearrangements in Strains Carrying the One-Plasmid System in *RecA*⁺ and *RecA*[−] Backgrounds. In strains containing a plasmid-borne *petAB1-B2C* operon, genetic instability could originate from DNA events that occur either on the plasmid (e.g., pBK6 and pBK32) or on the host chromosome. Here, only the plasmid-borne events were examined. Plasmid DNA isolated from both large and small Ps⁺ colonies after subculture under Res conditions (i.e., one colony inoculated into 1 mL, and after overnight incubation, transferred to 10 mL for another overnight incubation, and cells harvested for DNA extraction) was subjected to PCR amplification together with a control plasmid carrying the native *petABC* operon (pMTS1) using appropriate primers located on *petA* and *petC*, respectively (Figure 3A). The data showed that plasmids extracted from the large Ps⁺ colonies (lane 3) yielded a DNA fragment of a size (~ 1.5 kb) identical to that seen with a control plasmid (lane 2) carrying a single *petB* gene (Figure 1A). On the other hand, plasmids extracted from the small Ps⁺ colonies yielded a larger DNA fragment

(~ 3.0 kb), indicating that they still carried the intact *petB1-petB2* fusion (lane 4). Under Res growth conditions, colonies harboring the one-plasmid system showed no readily discernible phenotype on solid media (Figure 2B). However, a similar analysis using randomly chosen Res grown colonies indicated that they also exhibited heterogeneity, producing both the ~ 1.5 and ~ 3.0 kb DNA PCR fragments in variable amounts (Figure 3A, lanes 5–11). DNA sequencing of the plasmids yielding the 1.5 kb fragment confirmed that they carried only one copy of *petB* with its 3' end still tagged with the DNA sequence corresponding to the Strep epitope used. Data similar to those described above were observed with the large Ps⁺ (Figure 2C) derivatives of pBK32/MT-RBC1 harboring the *petA-B1*^{F144R}*B2*^{H217L}-*C* operon (data not shown). DNA sequencing indicated that the plasmids extracted from these colonies contained only one copy of the wild-type *petB* gene without the *cyt b* F144R or *cyt b* H217L mutation but still contained the Strep tag. This finding implied that rare double-crossover events between the *petB1*^{F144R} and *petB2*^{H217L} copies occurred in these cells to yield a wild-type *petB* copy. Overall, the data indicated that complex intramolecular genetic events occur in strains carrying *petB1-petB2* fusions under both selective (i.e., cytochrome *bc*₁-dependent Ps) and nonselective (i.e., cytochrome *bc*₁-independent Res via an alternative quinol oxidase) growth conditions to yield heterogeneous populations of fused and nonfused forms of cytochrome *b* variants.

Production of Heterodimeric Cytochrome *b-bc*₁ with the One-Plasmid System. Cytochrome *b-bc*₁ production by strain pBK6/MT-RBC1 carrying the *petAB1-B2C* operon grown under Ps or Res growth conditions was examined. Chromatophore membranes of appropriate strains were prepared and analyzed by SDS–PAGE and immunoblots using *R. capsulatus* cytochrome *b* polyclonal and Strep epitope monoclonal antibodies (Figure 3B). Compared with a strain lacking cytochrome *bc*₁ (MT-RBC1, lane 2), membranes of pMTS1/MT-RBC1 carrying the native *petABC* operon produced an ~ 40 kDa band corresponding to a cytochrome *b* monomer detected by both antibodies (lane 1). A similar pattern was observed with membranes of all large Ps⁺ colonies of pBK6/MT-RBC1 (lanes 3–5). Importantly, membranes from randomly chosen Res-grown colonies of pBK6/MT-RBC1 produced variable amounts of the ~ 40 kDa band

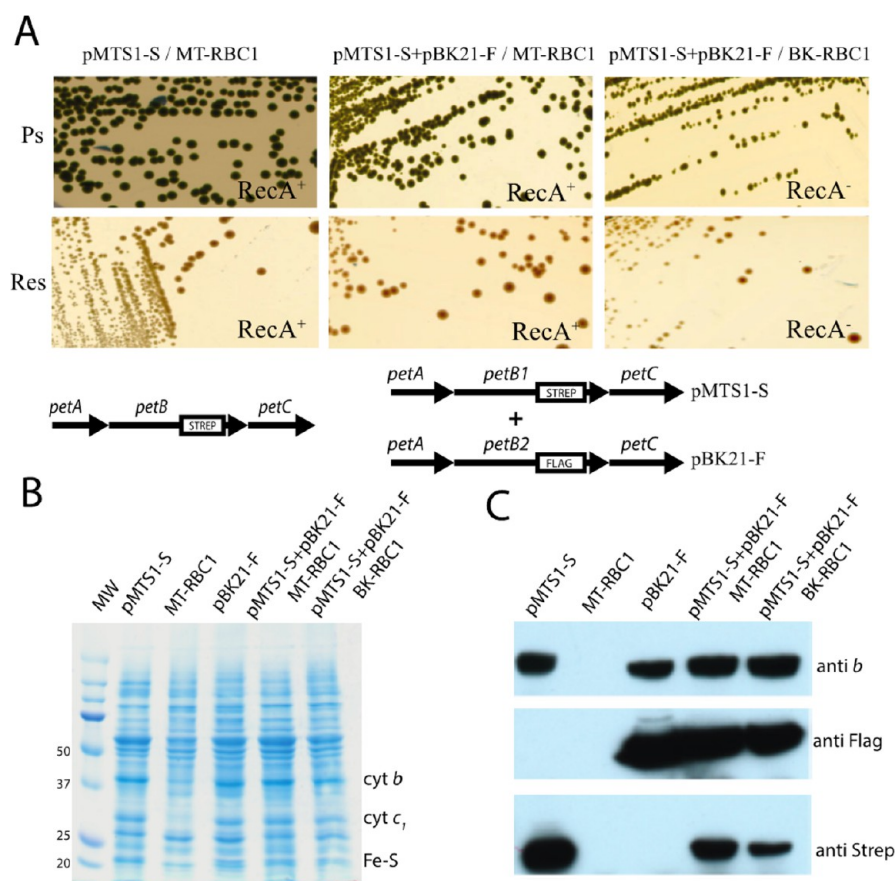


Figure 4. Growth and biochemical properties of strains carrying the two-plasmid system. (A) Photosynthetic (Ps) and respiration (Res) growth on enriched medium of wild-type *R. capsulatus* strain pMTS1/MT-RBC1 compared to that on MT-RBC1 (RecA⁺) and BK-RBC1 (RecA⁻) strains harboring the two-plasmid system (pMTS1-S + pBK21/MT-RBC1 and pMTS1-S + pBK21/BK-RBC1). In each case, the plasmids carrying the *petABC* operon are shown below the colony pictures. (B) SDS–PAGE and (C) immunoblot analyses of cytochrome *bc*₁ subunits in a strain carrying the two-plasmid system. Chromatophore membranes (40 μ g of total proteins per lane, from left to right) from pMTS1-S/MT-RBC1, MT-RBC1, pBK21-F/MT-RBC1, (pMTS1-S + pBK21)/MT-RBC1, and (pMTS1-S + pBK21)/BK-RBC1 were probed with polyclonal anti-cytochrome *b* and monoclonal anti-Flag and anti-Strep antibodies (see Materials and Methods).

corresponding to cytochrome *b* and of a larger band corresponding to a relative molecular mass of 75–100 kDa under our experimental conditions (lanes 6–8). The molecular nature(s) of the higher-molecular mass band, possibly corresponding to the fused forms of cytochrome *b* dimers, was not pursued further. In a manner independent of the higher-molecular mass band, all strains harboring the *petB1*-*B2* fusion also produced varying amounts of the ~40 kDa band corresponding to the size of the native cytochrome *b* monomer.

Two-Plasmid System. Strains carrying the two-plasmid system were also examined with respect to their cytochrome *bc*₁-dependent Ps growth ability, the frequency of large (Ps⁺) and small (Ps⁻) colonies that they yielded, and the molecular nature of the genetic rearrangements and the amounts of heterodimeric enzymes that they produced (Figure 1B). The two-plasmid system used here was similar to that reported previously,²² except that two identical plasmids (i.e., with the same pRK replicon) were chosen instead of two different replicons (e.g., pRK and “pRK-composite” plasmids).

Growth Properties of Strains Carrying the Two-Plasmid System in a RecA⁺ or RecA⁻ Background and Production of Heterodimeric Cytochrome *bc*₁. As a wild-type control, Kan^R pMTS1 (*petAB*-SC) and Tet^R pBK21 (*petAB*-FC) were introduced into the RecA⁺ and RecA⁻ backgrounds to yield the (pMTS1-S + pBK21-F/MT-RBC1)

and (pMTS1-S + pBK21-F/BK-RBC1) strains, respectively (Table S1 of the Supporting Information). Comparison of the Ps and Res growth phenotypes on solid media of these strains with those carrying only one of the plasmids (pMTS1-S) showed no discernible difference in terms of colony size or population homogeneity under either growth condition (Figure 4A). The Ps growth conferred by this “wild-type” two-plasmid system was robust, unlike that conferred by the one-plasmid system carrying a fused “wild-type” *petB1*-*petB2*, which had very poor Ps growth (Figure 2B). In addition, both RecA⁺ and RecA⁻ derivatives (pBK25-S + pBK28-F/MT-RBC1) and (pBK25-S + pBK28-F/BK-RBC1), respectively, harboring the two-plasmid system with the *cyt b*-S F144R and *cyt b*-F H217L mutations exhibited predominantly slightly smaller Ps⁺ colonies together with rare large Ps⁺ colonies [following paper (DOI 10.1021/bi400561e)]. The frequency of the large Ps⁺ colonies was around 10⁻⁴ for both RecA⁺ and RecA⁻ backgrounds (Table S2B of the Supporting Information). Furthermore, both Ps- and Res-grown liquid cultures of these strains formed approximately the same number of colonies in a manner independent of their sizes ($n = 7$ cultures, 1.5–1.9 $\times 10^8$ and 2.2–2.3 $\times 10^8$ colony forming units/mL under Ps and Res growth conditions, respectively). Thus, only the rare large Ps⁺ colonies probably underwent a genetic event(s) to exhibit a different (large vs small) growth phenotype (see below).

Table 1. Production of Native, Active, and Inactive Cytochrome *bc*₁ Heterodimers

two-plasmid system	$\Delta(petABC)$	Ps growth ^a	plasmid selection in <i>E. coli</i>	tag exchange	mutation exchange
Wild-Type Heterodimers					
(a) pMTS1-S (Kan ^R) + pBK21-F (Tet ^R)	RecA ⁺	Ps ⁺	165 of 210 <u>Kan^R</u> Tet ^R 80 of 148 <u>Tet^R</u> Kan ^R (<i>n</i> = 6) ^b	yes (3 of 8 Kan ^R -Flag; 0 of 3 Tet ^R -Strep)	NA ^c
(b) pMTS1-S (Kan ^R) + pBK21-F (Tet ^R)	RecA ⁻	Ps ⁺	0 of 224 <u>Kan^R</u> Tet ^R 0 of 341 <u>Tet^R</u> Kan ^R (<i>n</i> = 3)	no (0 of 3 <u>Kan^R</u> -Flag; 0 of 3 Tet ^R -Strep)	NA ^c
Mutant Heterodimers					
(c) cyt <i>b</i> -S F144R + cyt <i>b</i> -F H217L	RecA ⁺	Ps ^{+/-}	74 of 100 <u>Kan^R</u> Tet ^R 36 of 50 <u>Tet^R</u> Kan ^R (<i>n</i> = 3)	yes (2 of 10 Kan ^R -Flag; 3 of 13 Tet ^R -Strep)	tag-linked ^d
small Ps colonies	RecA ⁻	Ps ^{+/-}	0 of 1734 <u>Kan^R</u> Tet ^R	no (0 of 10 Kan ^R -Flag; 0 of 10 Tet ^R -Strep)	no ^e
(d) cyt <i>b</i> -S F144R + cyt <i>b</i> -F H217L	RecA ⁻	Ps ⁺	0 of 1366 <u>Tet^R</u> Kan ^R (<i>n</i> = 6)	yes (9 of 10 Kan ^R -Flag; 9 of 13 Tet ^R -Strep)	tag-linked ^d
large Ps colonies			999 of 1000 <u>Kan^R</u> Tet ^R		
(e) cyt <i>b</i> -S F144R + cyt <i>b</i> -F H217L			398 of 400 <u>Tet^R</u> Kan ^R (<i>n</i> = 3)		
no Ps colonies					
(f) cyt <i>b</i> -S F144R + cyt <i>b</i> -F T288S	RecA ⁺	Ps ⁻	NA ^c	NA ^c	

^aPs^{+/-} and Ps⁺, small and large colonies, respectively, formed under Ps growth conditions. ^b*n* is the number of cultures of *R. capsulatus* used to extract the plasmids; Kan^R and Tet^R indicate resistance to kanamycin and tetracycline, respectively, obtained after transformation of HB101 with plasmids from *R. capsulatus*. Clones were selected on Kan or Tet (underlined) and scored for the unselected marker (not underlined). ^cNot applicable. ^dTag-linked, cytochrome *b* mutations and their associated tags remained together. ^eIn the 10 Kan^R and 10 Tet^R plasmids sequenced, all mutations were associated with their tags. No tag or mutation exchange was seen among the 10 Kan^R and 10 Tet^R clones sequenced.

Strains (pMTS1-S + pBK21-F/MT-RBC1) and (pMTS1-S + pBK21-F/BK-RBC1) carried two wild-type *petABC* operons in which only *petB* is tagged with Strep or Flag epitopes. Thus, cells would produce wild-type but tagged homodimeric (Strep–Strep and Flag–Flag) and heterodimeric (Strep–Flag and Flag–Strep) cytochrome *bc*₁ variants. SDS–PAGE analyses (Figure 4B) indicated that, unlike a cytochrome *bc*₁-lacking strain (MT-RBC1), the strains harboring only pMTS1-S or pBK21-F and those harboring the two-plasmid system in RecA⁺ (pMTS1-S + pBK21-F/MT-RBC1) or RecA⁻ (pMTS1-S + pBK21-F/BK-RBC1) backgrounds produced all subunits of cytochrome *bc*₁. Immunoblot analyses with antibodies against cytochrome *b* detected this subunit in all cases except MT-RBC1, whereas the anti-Strep and anti-Flag epitope-specific antibodies detected it only in pMTS1-S and pBK21-F, respectively, and in both RecA⁺ and RecA⁻ strains with the two-plasmid system (Figure 4C).

Genetic Rearrangements Seen with Strains Carrying the Two-Plasmid System in a RecA⁺ or RecA⁻ Background. The genetic rearrangements that occur in the two-plasmid system were examined by extracting plasmid DNA from appropriate cells grown under Res conditions. Extracted DNA was transformed into *E. coli* RecA⁻ strain HB101, and Kan^R or Tet^R transformants were selected separately. The frequency of Tet^R clones found among the selected Kan^R clones and the frequency of Kan^R clones found among the selected Tet^R clones were scored after purification on the appropriate plates. The plasmids harbored by these clones were also analyzed by DNA sequencing (Table 1). When the DNA originated from the RecA⁺ strain (pMTS1-S + pBK21-F/MT-RBC1) grown under Res conditions, approximately one-half of the transformants were Kan^R Tec^S or Kan^S Tec^R (*n* = 6 independent experiments) and harbored the initial plasmids; 165 of 210 selected Kan^R transformants were also Tet^R, and 80 of 148 selected Tet^R transformants were also Kan^R (i.e., simultaneously Kan^R and Tet^R). The latter transformants

remained Kan^R Tet^R despite extensive purification, suggesting that they were not cotransformants with two coresiding plasmids in the same cell (Table 1, entry a). DNA sequencing of the Kan^R Tet^S and Kan^S Tet^R plasmids indicated that the antibiotic resistance markers with respect to the epitope tags were frequently (3 of 8 and 0 of 3) exchanged between the plasmids to generate pMTS1-F and pBK21-S instead of pMTS1-S and pBK21-F (Table 1, entry a). Given that in most bacteria the RecA-dependent recombination pathway is involved in the formation of the co-integrand between identical plasmids and their resolution,^{40,41} the RecA⁻ strain (pMTS1-S + pBK21-F/BK-RBC1) carrying the same plasmids was examined. Among the ~600 clones tested after Kan^R or Tet^R selection from three repeat cultures (*n* = 3), no Kan^R Tet^R colonies (0 of 224 and 0 of 341, respectively) were found (Table 1, entry b). DNA sequencing of the plasmids extracted from the Kan^R Tet^S and Tet^R Kan^S clones showed no antibiotic resistance marker or epitope tag exchange between the plasmids (0 of 3 in each case with *n* = 3). Thus, inactivation of the RecA pathway in a strain harboring the two-plasmid system drastically decreased the frequency of occurrence of Kan^R Tet^R co-integrand plasmid formation seen in the RecA⁺ background (Table 1, entry b).

Similar analyses were also performed using a two-plasmid system carrying two cytochrome *b* mutations to assess the degree of segregation between the mutations on the different plasmids or between a given mutation and its associated epitope tag on the same plasmid. RecA⁺ and RecA⁻ strains (pBK25-S + pBK28-F/MT-RBC1) and (pBK25-S + pBK28-F/BK-RBC1) (Table S1 of the Supporting Information) carrying a two-plasmid system with cyt *b*-S F144R³⁰ and cyt *b*-F H217L mutations³¹ were examined. The data indicated that the formation of Kan^R Tet^R co-integrand plasmids and epitope tag exchange were seen again only in RecA⁺ [74 of 100 and 36 of 50 (*n* = 3)], not in RecA⁻ [0 of 1734 and 0 of 1366 (*n* = 6)], backgrounds (Table 1, entries c and d). In the case of the

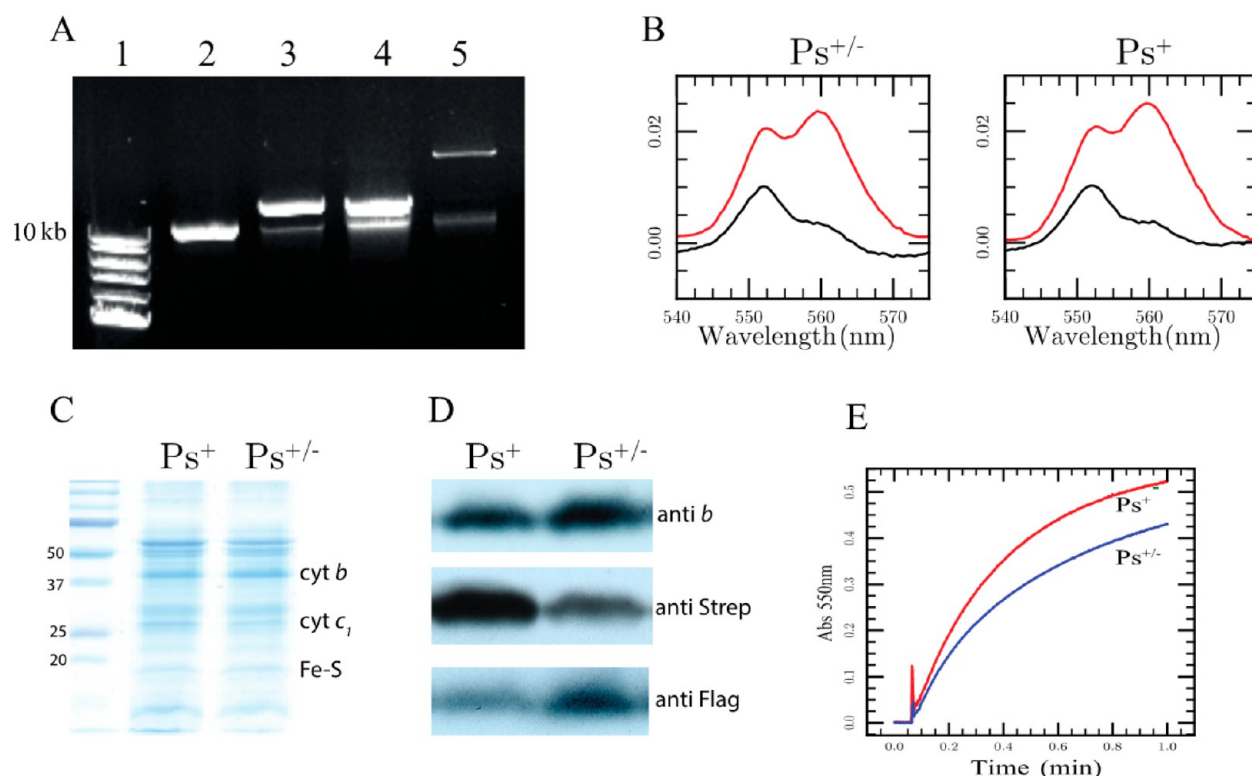


Figure 5. Large Ps⁺ colonies produced by a strain carrying the two-plasmid system contain large co-integrand plasmids. (A) Agarose gel showing the plasmid DNA extracted from RecA⁻ cells carrying the pRK415 vector (lane 2), pMTS1-S (lane 3), pBK21-F (lane 4), and a co-integrand plasmid formed between two resident plasmids found in the large Ps⁺ colonies carrying both plasmids from strain pBK25-S + pBK28-F/BK-RBC1 (lane 5). In each case, the upper band corresponds to the pRK415 derivatives and the identity of the lower band is not defined. DNA size markers are shown in lane 1. (B) Optical redox difference spectra of total b-type (dithionite minus ferricyanide colored red) and c-type (ascorbate minus ferricyanide colored black) cytochrome contents of chromatophore membranes obtained from a large Ps⁺ and a small Ps^{+/-} colony derivative of pBK25-S + pBK28-F/BK-RBC1. (C) SDS-PAGE and (D) immunoblot analyses of the chromatophore membranes obtained from the same large Ps⁺ and a small Ps^{+/-} as described for panel B using cytochrome b polyclonal antibodies and commercially available monoclonal antibodies against the Strep and Flag epitope tags. (E) Steady-state DBH₂:cytochrome c reduction activity (Materials and Methods) monitored at 550 nm exhibited by the same chromatophore membranes as in panel B, and the corresponding rates obtained using the same amounts of proteins (see the text).

RecA⁺ background, DNA sequencing of the plasmids extracted from Kan^R Tet^S [i.e., pBK25-S (cyt b-S F144R)] or Kan^S Tet^R [i.e., pBK28-F (cyt b-F H217L)] indicated that 2 of 10 of the Kan^R Tet^S clones contained the cyt b-F H217L mutation and 3 of 13 Tet^R Kan^S clones contained the cyt b-S F144R mutation ($n = 3$). Although the Kan^R and Tet^R markers were exchanged between the two plasmids, no segregation was seen between given mutations and their associated epitope on the same copy of *petB*. In other words, in all instances (~70 sequences), the cyt b F144R mutation remained linked to the Strep epitope tag and the cyt b H217L mutation to the Flag epitope tag, regardless of the exchange of antibiotic resistance markers between the plasmids (Table 1, entry c). With the RecA⁻ background, indeed no Kan^R Tet^R colony and no segregation between the cytochrome b mutations and their associated epitope tags were seen among the ~3000 clones ($n = 6$) tested (Table 1, entry d). This finding suggested that in the RecA⁺ background, co-integrants between the two resident plasmids (pMTS1-S and pBK21-F or pBK25 and pBK28/MT-RBC1) of quasi-identical DNA sequences (except the cytochrome b mutations and their associated epitope tags) could be continuously formed and resolved, leading to frequent antibiotic marker exchanges between the plasmids.^{40,41} Importantly, because of the short distances that separate the cytochrome b mutations and their associated tags, the genetic rearrangements did not segregate them at a detectable rate.

Large Ps⁺ Clones Observed Using the Two-Plasmid System in a RecA⁻ Background Are Co-Integrants between the Two Plasmids. We inquired about the correlation between the occurrence of the large Ps⁺ colonies and the formation of co-integrand Kan^R Tet^R plasmids. Although these colonies appear in both RecA⁺ and RecA⁻ backgrounds, we analyzed them in the latter case in which the two-plasmid system is more robust (Table 1). Using strain pBK25-S + pBK28-F/BK-RBC1, we deliberately sought large Ps⁺ colonies, which appeared only after multiple subcultures, in agreement with their low frequency (approximately $<10^{-4}$) (Table S2B of the Supporting Information). We purified these derivatives under Ps growth conditions and examined the genetic rearrangements that they underwent. Transformation of plasmid DNA extracted from these large Ps⁺ clones into an *E. coli* strain (HB101) almost exclusively yielded Kan^R Tet^R transformants independent of the initial Kan^R or Tet^R selection (only ~1 of 1000 clones was Kan^R Tet^S upon Kan^R selection, and 2 of 400 clones were Tet^R Kan^S upon Tet^R selection) (Table 1, entry e). Furthermore, DNA analyses revealed that the Kan^R Tet^R clones contained large co-integrand plasmids formed by the fusion of the two initial plasmids (Figure 5A, lane 5) but frequently not resolved back to the initial plasmids. Upon digestion with the appropriate restriction enzyme, religation, and transformation into *E. coli*, the large co-integrand plasmids regenerated the initial Kan^R Tet^S and Tet^R Kan^S

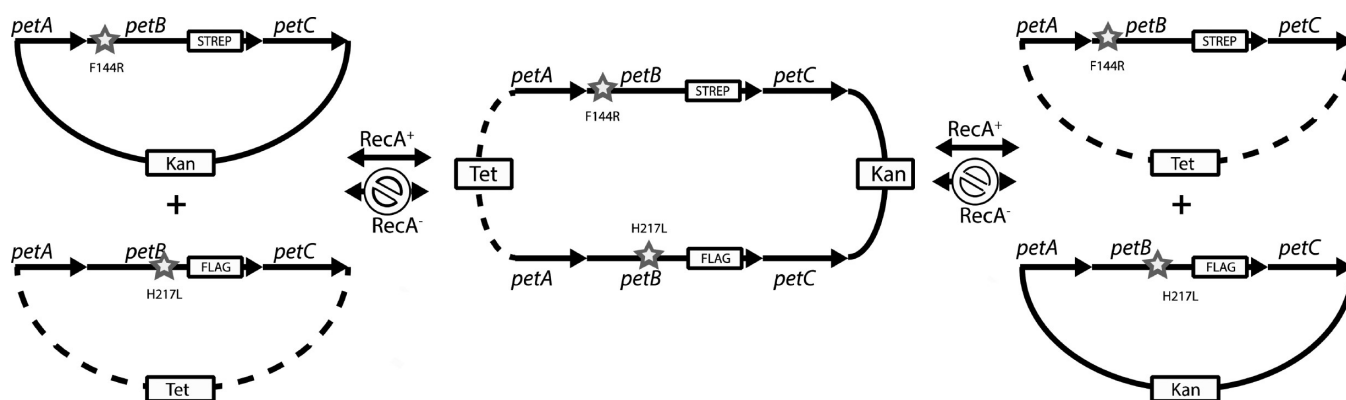


Figure 6. Formation of co-integrants between the resident plasmids in cells carrying the two-plasmid system. Continuous co-integrant plasmid formation and resolution via the homologous recombination (RecA) pathway between two plasmids carrying the same type of replicon coresiding in a cell are depicted. The frequency of occurrence of these single-recombination events is decreased drastically in the absence of the RecA protein (RecA⁻ background). Segregations of the genetic markers carried by the co-integrant plasmids formed are distance-dependent. In a RecA⁺ background, exchange between the antibiotic resistance markers is detected upon the formation and resolution of the co-integrants, but no recombination or segregation between the cytochrome *b* mutations or the mutations and their associated epitope tags is seen, because of the short distances that separate these markers.

plasmids. DNA sequence analyses of several ($n = 23$) such plasmids revealed that those carrying the *cyt b*-S F144R mutation remained associated with their Strep tag and those carrying the *cyt b*-F H217L mutation remained associated with their Flag tag. As observed with the RecA⁺ background, although exchange of antibiotic resistance markers (Tet^R and Kan^R) could be seen, in no instances did we detect any plasmid carrying a wild-type cytochrome *b* or an allele with both mutations (i.e., *cyt b* F144R and *cyt b* H217L carried by a single *petB*), or any segregation between a cytochrome *b* mutation and its associated epitope tag (Table 1, entry e). The data demonstrated that the large Ps⁺ colonies were not due to the loss of cytochrome *b* mutations by reversion or recombination and implied that they resulted from the formation of RecA-dependent co-integrants between the two resident plasmids. Importantly, the co-integrated plasmids also carried faithfully the initial cytochrome *b* mutations and their associated epitope tags and produced the expected heterodimeric cytochrome *bc*₁ variants (Figure 6).

Better Ps⁺ growth of the large colonies suggested that they might contain increased amounts of active heterodimeric cytochrome *bc*₁ variants. Optical redox difference spectra at 560 and 550 nm, visualizing the total amounts of cytochromes *b* and *c*, respectively, indicated that both the selected large Ps⁺ and the normal smaller Ps^{+/-} clones had comparable amounts of total cytochrome *bc*₁ (Figure 5B). Similar data were also obtained via SDS-PAGE analyses using chromatophore membranes of Ps⁺ and Ps^{+/-} derivatives of pBK25-S + pBK28-F/BK-RBC1 (Figure 5C). However, immunoblot analyses using antibodies against cytochrome *b* and Strep and Flag epitopes revealed that these clones showed different levels of Strep and Flag epitope tag contents (Figure 5D). The Strep-tagged subpopulation (homodimers and heterodimers) was larger in the Ps⁺ clones than in the Ps^{+/-} clones. Conversely, the amount of the Flag-tagged subpopulation (homodimers and heterodimers) was larger in the Ps^{+/-} clones than in the Ps⁺ clones. Moreover, the chromatophore membranes of the large Ps⁺ colonies exhibited a cytochrome *bc*₁ activity (0.33 and 0.24 μ mol of cytochrome *c* reduced per minute per milligram of total membrane proteins, respectively, which correspond to 36 and 26%, respectively, of the wild-type activity) higher than that

of the smaller Ps^{+/-} colonies (Figure 5E). The data suggested that the formation of co-integrants between the two resident plasmids (Figure 6) increased the level of production of the Strep-tagged cytochrome *b* subunits in comparison to that of their Flag-tagged counterparts and consequently yielded an increased level of active heterodimeric cytochrome *bc*₁ variants that supported better Ps⁺ growth.

DISCUSSION

In this work, we assessed the two genetic systems^{20–22} previously reported to successfully produce heterodimeric cytochrome *bc*₁ variants. Because of the dimeric structure of the enzyme, these systems carry DNA duplications that are naturally prone to genetic rearrangements, including homologous and nonhomologous recombination.^{40,41} We aimed to elucidate the genetic events that could occur using these genetic systems and to evaluate their impact(s) on the production of heterodimeric cytochrome *bc*₁ variants. A thorough assessment of the properties of these systems is of critical importance because the validity of the information gained about dimeric cytochrome *bc*₁ directly depends on their experimental reliability and proper use. Their misuse could lead to erroneous conclusions, as reported in ref 24.

One-Plasmid System. Using the one-plasmid system, with a fusion of two “wild-type” or two mutant alleles of cytochrome *b*, we observed extremely slow or no Ps growth, respectively. Frequent genetic rearrangements were seen under both selective (Ps) and nonselective (Res) growth conditions. Both “wild-type” and mutant *petB1-B2* fusions yielded better growing derivatives that carried a single copy of cytochrome *b* at a frequency of $\sim 10^{-2}$ under our conditions. A RecA⁻ background minimized the occurrence of these genetic rearrangements by approximately an order of magnitude ($\sim 10^{-3}$). Consequently, strains carrying the one-plasmid system seem to be under strong selective growth pressure and propagate as heterogeneous populations.²⁵ In an attempt to reproduce the one-plasmid system in *R. sphaeroides*, the Crofts group reported that they obtained only wild-type-like derivatives while growing their strains under Ps conditions.²⁴ This finding is not surprising, but rather expected, as the Ps growth conditions are strongly selective against even slightly

Ps-defective mutants, including those carrying the one-plasmid system. Failure to produce a heterodimeric cytochrome bc_1 under inappropriate experimental conditions using a fragile genetic system is expected. Therefore, the findings reported in ref 24 are inconclusive for the production of heterodimeric cytochrome $b-bc_1$ variants or the occurrence and rate of intermonomer electron transfer in cytochrome bc_1 . Genetic systems using mutants should not be exposed to selective conditions. Otherwise, mutant populations rapidly “evolve” better-adapted variants that supersede the initial populations. Heterogeneous microbial populations are not suitable for many studies, especially for biophysical and biochemical characterizations.

The molecular basis underlying the strong selective pressure seen with strains carrying the one-plasmid system is unknown. Although they were not studied here beyond documenting that this system leads to marked population heterogeneity, a possible explanation is that the selection is not caused solely by inadequate cytochrome bc_1 activity (e.g., slow intermonomer electron transfer) or exclusively by RecA-dependent recombination events, and its molecular basis may lie elsewhere. Indeed, even a wild-type *petB1-petB2* fusion is defective for Ps growth. A Ps growth defect could only indicate that heterodimeric cytochrome $b-bc_1$ variants are insufficiently functional (or not maintained in sufficient amounts) if this phenotype is directly correlated with the absence of an active cytochrome bc_1 . Nonetheless, meticulously controlled short culture periods seem to minimize the observed heterogeneity to allow purification of cytochrome $b-bc_1$ variants.³⁹ It has been reported that different cytochrome b fusions exhibit different “levels of engineering tolerance” because of the type of mutation(s) in cytochrome b or the size of the artificial linker sequence fusing the two copies of *petB*.²⁵ Moreover, a variant of the one-plasmid system, which uses a *R. sphaeroides* cytochrome b fused with that of *R. capsulatus* (*petBs-petBc* instead of *petB1-petB2*), was recently described to provide enhanced stability and Ps-proficient growth.⁴²

Two-Plasmid System. Our previously reported two-plasmid system based on two different replicons produced mutant heterodimeric cytochrome bc_1 that supported slow Ps growth via the intermonomer electron transfer pathway.²² In an attempt to produce comparable amounts of homodimeric and heterodimeric cytochrome bc_1 in a given cell, we tested a variant of this system in which the two plasmids used have the same type of replicon. However, biogenesis of cytochrome bc_1 is complex and not well-known.⁴³ The cytochrome b mutations and their associated epitope tags (e.g., Strep vs Flag) might interfere differently with the replication, expression, biogenesis, and degradation processes *in vivo*. Expecting that cells with a two-plasmid system would produce predetermined amounts of homodimers and heterodimers is unreasonable.²⁴ In cells carrying two plasmids with the same type of replicons, the copy number of each plasmid might vary, although the total copy number of plasmids per cell is constant. Consequently, the amount of homo- and heterodimers produced can vary. In contrast, when a co-integrant plasmid is formed, the copies of the two plasmids are “equalized” as they become parts of the same molecule maintained at a given copy number, and the amounts of homo- and heterodimers are more similar, as seen here with the large Ps^+ colonies.

The two-plasmid system yields a population of predominantly small $Ps^{+/-}$ and fewer (at a frequency of $\sim 10^{-4}$) large Ps^+ colonies.²² RecA is important for frequent co-integrant

formation and resolution; however, in the absence of RecA, co-integrants are still formed but apparently not resolved. More detailed studies are required to better define this process. In a population, the frequency of colonies with a different phenotype (e.g., small $Ps^{+/-}$ vs large Ps^+) reflects how rapidly such colonies arise. This phenotype might originate from various molecular events, and this “reversion” should not be equated with a “wild-type” genotype. Hong et al.²⁴ postulated that the small $Ps^{+/-}$ colonies might correspond to cells that have already reverted, rendering them partially Ps-proficient, and that the large Ps^+ colonies acquired a second mutation that further improved their Ps growth ability. The observed frequency of this event already indicates that this cannot be the case. Moreover, cultures carrying the two-plasmid system produce the same number of colonies (independent of their size) on solid media under both Ps and Res growth conditions. With each colony being the result of successive divisions of a single cell, the data demonstrate that all cells are capable of growth under both selective (Ps) and nonselective (Res) growth conditions. The small $Ps^{+/-}$ colonies are formed at a frequency of ~ 1 , demonstrating that they cannot be “revertants” even though their Ps phenotype (i.e., colony size) is different than a wild-type strain. Only the large Ps^+ colonies that appear at a low frequency reflect cells that underwent genetic rearrangement(s) (i.e., revertants). Monitoring the frequency of these events is the key to ensuring the proper production of heterodimeric cytochrome bc_1 variants using one- or two-plasmid systems.

Duplicated genetic materials usually undergo homologous recombination, which is a “distance-dependent” event. The frequency of homologous recombination segregating two genetic markers is lower when the markers are closer to each other. In the two-plasmid system carrying two Q_{ϕ} site defective cytochrome b mutations (cyt b -S F144R and cyt b -F T288S), no Ps^+ colony was observed. Also, this strain did not produce any $Ps^{+/-}$ colonies as both Q_{ϕ} sites are defective (Table 1, entry f) [see also the following paper (DOI 10.1021/bi400561e)]. Similarly, the large number (~ 70) of plasmids thus far sequenced consistently showed the initial cytochrome b mutations linked to their associated epitope tags, in a manner independent of their phenotype. Thus, no recombination between two mutations in *petB* or a mutation and its associated epitope tag carried by *petB* is readily detected, whereas similar RecA-dependent exchanges between the antibiotic resistance markers (Kan^R and Tet^R) are often observed between the two plasmids (Table 1 and Figure 6). This finding is not unexpected given that the distance separating the cytochrome b mutations or a mutation and its associated epitope tag (~ 1 kb) is ~ 10 times shorter than that separating the Kan^R and Tet^R markers (~ 10 kb) on co-integrant plasmids. However, an increased recombination frequency might be seen between genetic markers that are physically far apart (e.g., a mutation located at the N-terminus of the Fe-S protein together with one at the C-terminus of cytochrome c_1), although the use of a $RecA^-$ background might minimize such events (Figure 6).

A two-plasmid system that is very similar to that analyzed here was also used in *Paracoccus denitrificans*.²⁰ That study suggested that one Q_{ϕ} site is sufficient for the activity of cytochrome bc_1 and that the intermonomer electron transfer might occur via the b_L - b_L hemes. This genetic setup has not been analyzed further, and the results obtained with *R. capsulatus* may not be directly translatable to the *P. denitrificans* system. The recombination pathways might differ between

different organisms and their plasmids. The occurrence of co-integrant plasmids and the determination of the recombination frequency yielding a wild-type homodimeric cytochrome bc_1 would be useful for further evaluation of this system. We note that the two-plasmid system developed here could be useful not only for the study of electronic communication between the monomers of a dimeric cytochrome bc_1 but also for other homodimeric multisubunit enzymes. For example, it could be informative in the case of the dimeric cytochrome c nitrite reductase (ccNiR) or the dimeric photosynthetic reaction center type I (PSI) for which dimer interactions are not well-known. The three-dimensional structure of ccNiR is a homodimer in which the distance separating the hemes located at the edge of the dimer interface is short (~ 12 Å),^{44,45} whereas that of PSI is a pseudohomodimer containing similar but not identical (PsaA and PsaB) monomers.^{46,47}

Finally, we note that the two-plasmid system, which can generate heterogeneous populations, has the potential to contribute to the study of the mitochondrial "heteroplasmy state" seen in mammalian cells. In the case of cytochrome bc_1 , analyses of heteroplasmic cytochrome b often require the generation of "cybrid" lines.^{48,49} Using the *R. capsulatus* two-plasmid system, generation of heterogeneous populations might provide an alternative approach, and comparative studies using bacteria and cybrids carrying human disease-causing mutations might be very informative.^{49,50}

■ ASSOCIATED CONTENT

■ Supporting Information

Plasmids and strains used in this work (Table S1) and photosynthetic (Ps) and respiratory (Res) colony forming abilities of appropriate strains that produce mutant cytochrome bc_1 heterodimers (Table S2). This material is available free of charge via the Internet at <http://pubs.acs.org>.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: fdaldal@sas.upenn.edu. Telephone: (215) 898-4394. Fax: (215) 898-8780.

Funding

This work was supported by National Institutes of Health Grant GM 38237 and the Division of Chemical Sciences, Geosciences and Biosciences, Office of Basic Energy Sciences of the U.S. Department of Energy, via Grant DE-FG02-91ER20052 to F.D.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Xiaomin Wu for isolation of the *recA* gene and construction of a *RecA*[−] mutant.

■ ABBREVIATIONS

Q, quinone; SQ, semiquinone; QH₂, hydroquinone; Res, respiration; Ps, photosynthesis; Kan^R, kanamycin resistance; Tet^R, tetracycline resistance.

■ REFERENCES

(1) Xia, D., Yu, C. A., Kim, H., Xia, J. Z., Kachurin, A. M., Zhang, L., Yu, L., and Deisenhofer, J. (1997) Crystal structure of the cytochrome bc_1 complex from bovine heart mitochondria. *Science* 277, 60–66.

(2) Zhang, Z., Huang, L., Shulmeister, V. M., Chi, Y. I., Kim, K. K., Hung, L. W., Crofts, A. R., Berry, E. A., and Kim, S. H. (1998) Electron transfer by domain movement in cytochrome bc_1 . *Nature* 392, 677–684.

(3) Iwata, S., Lee, J. W., Okada, K., Lee, J. K., Iwata, M., Rasmussen, B., Link, T. A., Ramaswamy, S., and Jap, B. K. (1998) Complete structure of the 11-subunit bovine mitochondrial cytochrome bc_1 complex. *Science* 281, 64–71.

(4) Berry, E. A., Huang, L.-S., Saechao, L. K., Pon, N. G., Valkova-Valchanova, M., and Daldal, F. (2004) X-ray structure of *Rhodobacter capsulatus* cytochrome bc_1 : Comparison with its mitochondrial and chloroplast counterparts. *Photosynth. Res.*, 251–275.

(5) Mitchell, P. (1976) Possible molecular mechanisms of the protonmotive function of cytochrome systems. *J. Theor. Biol.* 62, 327–367.

(6) Crofts, A. R., Meinhardt, S. W., Jones, K. R., and Snozzi, M. (1983) The role of the quinone pool in the cyclic electron-transfer chain of *Rhodospseudomonas sphaeroides*: A modified Q-cycle mechanism. *Biochim. Biophys. Acta* 723, 202–218.

(7) Trumpower, B. L. (1990) The protonmotive Q cycle. Energy transduction by coupling of proton translocation to electron transfer by the cytochrome bc_1 complex. *J. Biol. Chem.* 265, 11409–11412.

(8) Darrouzet, E., Valkova-Valchanova, M., Moser, C. C., Dutton, P. L., and Daldal, F. (2000) Uncovering the [2Fe2S] domain movement in cytochrome bc_1 and its implications for energy conversion. *Proc. Natl. Acad. Sci. U.S.A.* 97, 4567–4572.

(9) Darrouzet, E., and Daldal, F. (2002) Movement of the iron-sulfur subunit beyond the *ef* loop of cytochrome b is required for multiple turnovers of the bc_1 complex but not for single turnover Q_o site catalysis. *J. Biol. Chem.* 277, 3471–3476.

(10) Crofts, A. R., Holland, J. T., Victoria, D., Kolling, D. R., Dikanov, S. A., Gilbreth, R., Lhee, S., Kuras, R., and Kuras, M. G. (2008) The Q-cycle reviewed: How well does a monomeric mechanism of the bc_1 complex account for the function of a dimeric complex? *Biochim. Biophys. Acta* 1777, 1001–1019.

(11) De Vries, S., Albracht, S. P., Berden, J. A., and Slater, E. C. (1982) The pathway of electrons through QH₂:cytochrome c oxidoreductase studied by pre-steady-state kinetics. *Biochim. Biophys. Acta* 681, 41–53.

(12) Weiss, H., Leonard, K., and Neupert, W. (1990) Puzzling subunits of mitochondrial cytochrome reductase. *Trends Biochem. Sci.* 15, 178–180.

(13) Gupta, O. A., Feniouk, B. A., Junge, W., and Mulkidjanian, A. Y. (1998) The cytochrome bc_1 complex of *Rhodobacter capsulatus*: Ubiquinol oxidation in a dimeric Q-cycle? *FEBS Lett.* 431, 291–296.

(14) Trumpower, B. L. (2002) A concerted, alternating sites mechanism of ubiquinol oxidation by the dimeric cytochrome bc_1 complex. *Biochim. Biophys. Acta* 1555, 166–173.

(15) Cooley, J. W., Lee, D. W., and Daldal, F. (2009) Across membrane communication between the Q_o and Q_i active sites of cytochrome bc_1 . *Biochemistry* 48, 1888–1899.

(16) Covian, R., Gutierrez-Cirlos, E. B., and Trumpower, B. L. (2004) Anti-cooperative oxidation of ubiquinol by the yeast cytochrome bc_1 complex. *J. Biol. Chem.* 279, 15040–15049.

(17) Cooley, J. W., Ohnishi, T., and Daldal, F. (2005) Binding dynamics at the quinone reduction Q_i site influence the equilibrium interactions of the iron sulfur protein and hydroquinone oxidation Q_o site of the cytochrome bc_1 complex. *Biochemistry* 44, 10520–10532.

(18) Covian, R., and Trumpower, B. L. (2005) Rapid electron transfer between monomers when the cytochrome bc_1 complex dimer is reduced through center N. *J. Biol. Chem.* 280, 22732–22740.

(19) Shinkarev, V. P., and Wraight, C. A. (2007) Intermonomer electron transfer in the bc_1 complex dimer is controlled by the energized state and by impaired electron transfer between low and high potential hemes. *FEBS Lett.* 581, 1535–1541.

(20) Castellani, M., Covian, R., Kleinschroth, T., Anderka, O., Ludwig, B., and Trumpower, B. L. (2010) Direct demonstration of half-of-the-sites reactivity in the dimeric cytochrome bc_1 complex: Enzyme with one inactive monomer is fully active but unable to

activate the second ubiquinol oxidation site in response to ligand binding at the ubiquinone reduction site. *J. Biol. Chem.* 285, 502–510.

(21) Swierczek, M., Cieluch, E., Sarewicz, M., Borek, A., Moser, C. C., Dutton, P. L., and Osyczka, A. (2010) An electronic bus bar lies in the core of cytochrome *bc*₁. *Science* 329, 451–454.

(22) Lanciano, P., Lee, D. W., Yang, H., Darrouzet, E., and Daldal, F. (2011) Intermonomer electron transfer between the low-potential *b* hemes of cytochrome *bc*₁. *Biochemistry* 50, 1651–1663.

(23) Khalfaoui-Hassani, B., Lanciano, P., Lee, D. W., Darrouzet, E., and Daldal, F. (2011) Recent advances in cytochrome *bc*₁: Inter monomer electronic communication? *FEBS Lett.* 586, 617–621.

(24) Hong, S., Victoria, D., and Crofts, A. R. (2012) Inter-monomer electron transfer is too slow to compete with monomeric turnover in *bc*₁ complex. *Biochim. Biophys. Acta* 1817, 1053–1062.

(25) Czapla, M., Borek, A., Sarewicz, M., and Osyczka, A. (2011) Fusing two cytochromes *b* of *Rhodobacter capsulatus* cytochrome *bc*₁ using various linkers defines a set of protein templates for asymmetric mutagenesis. *Protein Eng., Des. Sel.* 25, 15–25.

(26) Daldal, F., Cheng, S., Applebaum, J., Davidson, E., and Prince, R. C. (1986) Cytochrome *c*₂ is not essential for photosynthetic growth of *Rhodospseudomonas capsulata*. *Proc. Natl. Acad. Sci. U.S.A.* 83, 2012–2016.

(27) Atta-Asafo-Adjei, E., and Daldal, F. (1991) Size of the amino acid side chain at position 158 of cytochrome *b* is critical for an active cytochrome *bc*₁ complex and for photosynthetic growth of *Rhodobacter capsulatus*. *Proc. Natl. Acad. Sci. U.S.A.* 88, 492–496.

(28) Sambrook, J., and Russell, D. W. (2001) *Molecular Cloning: A laboratory manual*, 3rd ed., Cold Spring Harbor Laboratory Press, Plainview, NY.

(29) Yen, H. C., Hu, N. T., and Marrs, B. L. (1979) Characterization of the gene transfer agent made by an overproducer mutant of *Rhodospseudomonas capsulata*. *J. Mol. Biol.* 131, 157–168.

(30) Ding, H., Daldal, F., and Dutton, P. L. (1995) Ion pair formation between basic residues at 144 of the cyt *b* polypeptide and the ubiquinones at the Q_o site of the cyt *bc*₁ complex. *Biochemistry* 34, 15997–16003.

(31) Gray, K. A., Dutton, P. L., and Daldal, F. (1994) Requirement of histidine 217 for ubiquinone reductase activity (Q_o site) in the cytochrome *bc*₁ complex. *Biochemistry* 33, 723–733.

(32) Gray, K. A., Davidson, E., and Daldal, F. (1992) Mutagenesis of methionine-183 drastically affects the physicochemical properties of cytochrome *c*₁ of the *bc*₁ complex of *Rhodobacter capsulatus*. *Biochemistry* 31, 11864–11873.

(33) Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 150, 76–85.

(34) Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.

(35) Davidson, E., Ohnishi, T., Tokito, M., and Daldal, F. (1992) *Rhodobacter capsulatus* mutants lacking the Rieske FeS protein form a stable cytochrome *bc*₁ subcomplex with an intact quinone reduction site. *Biochemistry* 31, 3351–3358.

(36) Radding, C. M. (1981) Recombination activities of *E. coli* RecA protein. *Cell* 25, 3–4.

(37) Cox, M. M. (2003) The bacterial RecA protein as a motor protein. *Annu. Rev. Microbiol.* 57, 551–577.

(38) Todd, P. A., Brouwer, J., and Glickman, B. W. (1981) Influence of DNA-repair deficiencies on MMS- and EMS-induced mutagenesis in *Escherichia coli* K-12. *Mutat. Res.* 82, 239–250.

(39) Czapla, M., Borek, A., Sarewicz, M., and Osyczka, A. (2012) Enzymatic Activities of Isolated Cytochrome *bc*₁-like Complexes Containing Fused Cytochrome *b* Subunits with Asymmetrically Inactivated Segments of Electron Transfer Chains. *Biochemistry* 51, 829–835.

(40) Bedbrook, J. R., and Ausubel, F. M. (1976) Recombination between bacterial plasmids leading to the formation of plasmid multimers. *Cell* 9, 707–716.

(41) Potter, H., and Dressler, D. (1977) On the mechanism of genetic recombination: The maturation of recombination intermediates. *Proc. Natl. Acad. Sci. U.S.A.* 74, 4168–4172.

(42) Czapla, M., Cieluch, E., Borek, A., Sarewicz, M., and Osyczka, A. (2013) Catalytically-relevant electron transfer between two hemes *b*_L in the hybrid cytochrome *bc*₁-like complex containing a fusion of *Rhodobacter sphaeroides* and *capsulatus* cytochromes *b*. *Biochim. Biophys. Acta* 1827, 751–760.

(43) Smith, P. M., Fox, J. L., and Winge, D. R. (2012) Biogenesis of the cytochrome *bc*₁ complex and role of assembly factors. *Biochim. Biophys. Acta* 1817, 276–286.

(44) Einsle, O., Messerschmidt, A., Stach, P., Bourenkov, G. P., Bartunik, H. D., Huber, R., and Kroneck, P. M. (1999) Structure of cytochrome *c* nitrite reductase. *Nature* 400, 476–480.

(45) Youngblut, M., Judd, E. T., Srajer, V., Sayyed, B., Goelzer, T., Elliott, S. J., Schmidt, M., and Pacheco, A. A. (2012) Laue crystal structure of *Shewanella oneidensis* cytochrome *c* nitrite reductase from a high-yield expression system. *JBIC, J. Biol. Inorg. Chem.* 17, 647–662.

(46) Jordan, P., Fromme, P., Witt, H. T., Klukas, O., Saenger, W., and Krauss, N. (2001) Three-dimensional structure of cyanobacterial photosystem I at 2.5 Å resolution. *Nature* 411, 909–917.

(47) Jagannathan, B., and Golbeck, J. H. (2009) Breaking biological symmetry in membrane proteins: The asymmetrical orientation of PsaC on the pseudo-C2 symmetric Photosystem I core. *Cell. Mol. Life Sci.* 66, 1257–1270.

(48) King, M. P., and Attardi, G. (1989) Human cells lacking mtDNA: Repopulation with exogenous mitochondria by complementation. *Science* 246, 500–503.

(49) Ghelli, A., Tropeano, C. V., Calvaruso, M. A., Marchesini, A., Iommarini, L., Porcelli, A. M., Zanna, C., De Nardo, V., Martinuzzi, A., Wibbrand, F., Vissing, J., Kurelac, I., Gasparre, G., Selamoglu, N., Daldal, F., and Rugolo, M. (2013) The cytochrome *b* p.278Y > C mutation causative of a multisystem disorder enhances superoxide production and alters supramolecular interactions of respiratory chain complexes. *Hum. Mol. Genet.* 22, 2141–51.

(50) Lanciano, P., Khalfaoui-Hassani, B., Selamoglu, N., Ghelli, A., Rugolo, M., and Daldal, F. (2013) Molecular mechanisms of superoxide production by complex III: A bacterial versus human mitochondrial comparative case study. *Biochim. Biophys. Acta* 1827, 1332–1339.

(51) Ditta, G., Schmidhauser, T., Yakobson, E., Lu, P., Liang, X. W., Finlay, D. R., Guiney, D., and Helinski, D. R. (1985) Plasmids related to the broad host range vector, pRK290, useful for gene cloning and for monitoring gene expression. *Plasmid* 13, 149–153.