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Genetic and functional characterisation of *Rhodobacter capsulatus* reaction centres carrying triple mutations near to or far from quinone sites

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Abstract

In *Rhodobacter capsulatus*, two triple mutants were constructed. In these non-photosynthetic mutants, two amino acids near the quinone Q_B have been mutated to two alanines: in the Q_A site have been mutated to alanine-aspartic acid and glutamic acid-alanine. Several spontaneous mutants derived from original constructs were selected. DNA sequencing experiments on originally designed mutant strains and their spontaneous mutants were performed to identify possible genetic reversions at quinone site-specific locations. Constructed mutants carry double alanines in the Q_B site and single alanine in the Q_A site. Spontaneous mutants carry additional compensating mutations, aspartic acid (L225), cysteine (M231), and serine (M231) far from Q_A and Q_B sites, which may be involved in quinone binding by the photosynthetic reaction centres.

Additional key words: DNA sequencing; L and M subunits; spontaneous mutants; sub-cloning.

Introduction

Rhodobacter (R.) capsulatus is a Gram-negative, non-sulphur photosynthetic purple bacterium. It is capable of living photo- or chemo-heterotrophically depending on culture conditions (Fonstein *et al.* 1995). The bacterium is a model organism for studying the photosynthetic reaction centre, RC (Youvan *et al.* 1984, Deisenhofer and Michel 1991, Allen and Williams 1998). *R. capsulatus* has a photosynthetic unit consisting of pigment-protein complexes held together *via* non-covalent bonds. These complexes are photosynthetic RCs and light-harvesting complexes (Taguchi *et al.* 1993). The overall structure of photosynthetic RC consists of three integral membrane proteins, L, M, and H. The L and M protein subunits bind bacteriochlorophylls (P; B_A, and B_B), bacteriopheophytins, quinones, a non-heme ferrous iron ion, and a carotenoid molecule. The function of RC in the cell membrane is to convert radiant energy to chemical energy in successive reductions by help of P, B_A, H_A, Q_A, and Q_B. The core protein subunits and cofactors show twofold symmetry in the RC structure but not in the complex's function (Allen and Williams 1998). The M protein site of the RC has a role in the primary electron transfer process although the L protein site, especially Q_B, plays a role in secondary electron transfer (Allen and Williams 1998).

The specific aim was to construct new site-specific RC mutants in the important amino acids of the L and M subunits, and then to isolate spontaneous revertants of these mutants so as to enlarge the collection of RC mutants.

Materials and methods

Bacterial strains: Phagemid pBS –/+, *E. coli* DH5α competent cells (from *Gibco Laboratories-Bethesda Research Laboratories*), *E. coli* S17-1 competent cells (Simon *et al.* 1983), and *R. capsulatus* strains U43 (Youvan *et al.* 1984, Bylina *et al.* 1986, Hanson *et al.* 1993, Taguchi *et al.* 1993, 1996). *R. capsulatus* U43[pU29] and U43[pU2922], and strains containing plasmids with various mutants in RC genes including U43[pRLAAM86BglII], U43[pQArev1L], and U43[pRQrev4A] (Hanson *et al.* 1993, Hanson and Schiffer 1998) were supplied by Dr. D.K. Hanson. *R. capsulatus* plasmids constructed in these studies were pRAA+1L/KS and pRAA+4A/KS. *E. coli* transformants, sub-clones, *Rb. capsulatus* transconjugants, and photosynthetic phenotypic revertants were isolated in this work (Table 1).

Chemicals: EcoRI, SacI, AscI, HindIII, BamHI, BglII,

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Table 1. A list of strains subjected to this research. This table contains only strains used for further analysis.

	Strains
Background strains	U43[pU2922], U43[pRAABglII], U43[pQArev1L], U43[pRQrev4A]
E. coli DH5α transformants	DH5α[pRAA+1L/KS], DH5α[pRAA+4A/KS]
E. coli S17-1 transformants	S17-1[pRAA+1L/KS], S17-1[pRAA+4A/KS]
R. capsulatus transconjugants	U43[pRAA+1L/KS], U43[pRAA+4A/KS]
Re-constructed *E. coli* DH5α transformants	DH5α[RCAA+1LR9], DH5α[RCAA+1LR18], DH5α[RCAA+1LR23], DH5 α[RCAA+4AR7], DH5α[RCAA+4AR19]
Re-constructed *E. coli* S17-1 transformants	S17-1[RCAA+1LR9], S17-1[RCAA+1LR18], S17-1[RCAA+1LR23], S17-1[RCAA+4AR7], S17-1[RCAA+4AR19]
Photosynthetic spontaneous mutants	U43[pRAA+1L]R18, U43[pRAA+1L]R23, U43[pRAA+4A]R7, U43[pRAA+4A]R19
Re-constructed spontaneous mutants	U43[pRAA+1LR18], U43[pRAA+1LR23], U43[pRAA+4AR7], U43[pRAA+4AR19]
E. coli DH5α subclones	DH5α[RCAA+1L/ES], DH5α [RCAA+4A/ES], DH5α[RCAA+1LR9/ES], DH5α[RCAA+1LR18/ES], DH5α[RCAA+1LR23/ES], DH5α[RCAA+4AR7/ES], DH5α[RCAA+4AR19/ES]

and T_4 DNA ligase were obtained from *Bethesda Research Laboratories*, *New England BioLabs*, and *Sigma*. Lambda DNA cleaved with HindIII was from *Promega*. *Bacto*-agar, *Bacto*-tryptone, *Difco*-yeast extract, *Bacto*-peptone, ampicillin, carbenicillin, kanamycin, and tetracycline were purchased from *Sigma*. Kits: *Qiagen Tip-20/100* from *Qiagen*; *Geneclean II Kit* from *Bio 101*; *Sequenase* version *2.0* sequencing kit from *United States Biochemical Corporation*; *Polymerized Acryl-a-mix® 6* from *Promega*; *Bio-Bag Environmental Chamber Type A* from *Becton Dickinson Microbiology Systems*.

Media: RB, LB, and 2XTY medium (Sambrook *et al.* 1989) (with antibiotics or without them) were used to maintain the *E. coli* cells. MPYE (Davidson *et al.* 1989), RPYE (Hanson *et al.* 1992, Xiao *et al.* 1994, Taguchi *et al.* 1996), RCV (Yen and Marrs 1977, Bauer *et al.* 1988, Biel and Marrs 1983, Taguchi *et al.* 1993) were prepared for culturing of *R. capsulatus* mutant strains.

Isolation of plasmid DNA: *Tens alkaline miniprep* isolation protocol (Birnboim 1983) and *Qiagen miniprep* kits were used to isolate plasmid DNA. Plasmid DNA was quantified by absorbance and gel electrophoresis (Sambrook *et al.* 1989). The quantification standard was lambda DNA cleaved with HindIII.

Restriction enzyme digestion of plasmid DNA: To identify loss of the BglII marker from KpnI-SacI digested plasmids, miniprep DNA was digested with BglII. To identify correct sub-clones made from EcoRI-SacI digested DNA, miniprep DNA was digested with EcoRI and BamHI. After the gel run of the digestion was completed, the gel was photographed, the smallest fragment was cut out from the gel and purified using the *Geneclean* kit using instructions supplied by the manufacturer. To confirm DNA bands, fragments purified using *Geneclean* were re-run on gels.

Ligation of digested DNA of mutant plasmids: Each of the KpnI-SacI and EcoRI-SacI digests was ligated by using T4 DNA ligase (*New England Biolabs*) in the recommended buffer for 4 h at room temperature. Individual ligation reactions were run to reconstruct RC plasmids. EcoRI and SacI digested pBS+/– was ligated with each of EcoRI and SacI digested DNAs from the original plasmids.

Transformation: The RbCl method utilised for transformation was adapted from the procedure of the John Innes Institute (Norwich, England) *via* Joseph Utermohlen from the University of Arizona. Competent cells were prepared and stored in a freezer at –70 °C, and used as needed.

Conjugation: Mutant plasmids were returned from *E. coli* to *R. capsulatus* by conjugation (modified from Youvan *et al.* 1984).

Spotting test for photosynthetic phenotypic characterisation: Photosynthetic phenotypic expression was observed by using the spotting test (modified from Bylina *et al.* 1986).

Sub-cloning of plasmids into *E. coli* strains: The RC plasmid carrying mutant amino acid is ~32 kilobase (kb), and this size of DNA cannot be used for sequencing. Therefore, EcoRI-SacI subclones of each mutant *puf* operon were required. They were prepared as follows: (*1*) The 3.5 kb EcoRI and SacI fragment was isolated on a preparative gel after digestion of miniprep DNA. (*2*) This fragment was ligated to phagemid pBS +/–, which has been also digested with EcoRI and SacI, gel isolated, and gene-cleaned. (*3*) A portion of the ligation reaction was used to transform *E. coli* DH5α. (*4*) Several white colonies were inoculated for miniprep DNA isolation to check for the right insert. (*5*) Miniprep DNA isolated, and di-

gested with EcoRI and BamHI to obtain a characteristic pattern of three bands.

Sequencing of plasmid DNA: Sanger's method was performed to sequence double-stranded DNA of mutant plasmids to prove the original constructs were carrying the right mutations, and find out new mutations occurring *via* spontaneous mutations. Samples from each plasmid DNA were loaded into polymerised acryl-A-mix® 6, and run in an electrical field at 2 000 V and 55 °C.

Photosynthetic spontaneous mutants from the original mutant constructs were isolated by the respective isolation technique (modified from Hanson *et al.* 1993 and Taguchi *et al.* 1996).

Re-cloning of photosynthetic spontaneous mutants was done to compare their phenotypic expression on plates. Two-days-old dark-semi-aerobically grown cell cultures from the original photosynthetic *R. capsulatus* mutant strains were prepared in 3 cm^3 RPYE+Kan medium in non-clear Falcon tubes, and incubated in a dark shaker at 34 °C. Miniprep DNA from these cultures was isolated and used to transform *E. coli* DH5α competent cells. Overnight cultures from those transformants were prepared. Freezer stock of each was prepared and miniprep DNA was isolated by using the *Tens* alkaline method. An aliquot of miniprep DNA was used to transform *E. coli* S17-1 competent cells. A single colony of *E. coli* S17-1 transformants was grown for preparation of freezer stocks and conjugation to *R. capsulatus*. *E. coli* S17-1 cells carrying re-cloned mutant plasmid were conjugated to *R. capsulatus*. Two single trans-conjugant colonies of *R. capsulatus* were purified and freezed stocks were prepared for future studies. The spotting test was performed to test the phenotypic expression of original photosynthetic mutants and re-cloned ones.

Results

Two different genetically engineered RC plasmids (pRAA+1L/KS and pRAA+4A/KS) were constructed, transformed into *E. coli* cells, and selected successfully.

Photosynthetic spontaneous mutant colonies appeared in the light-anaerobic conditioned bottles or culture tubes in 1-2 weeks. Purple coloured colonies on dark conditioned plates appeared in 2 d. The sum of the photosynthetic revertants isolated from each original construct was 24 phenotypic revertants of U43[pRAA+1L/KS] and 34 of U43[pRAA+4A/KS]. Results of spotting tests showed that U43[pRAA+1L/KS] and U43[pRAA+4A/KS] strains were non-photosynthetic (Table 2).

Table 2. Reaction centre mutants from *Rhodobacter capsulatus*. Alanine (A), aspartic acid (D), arginine (R), glutamic acid (E), cysteine (C), glycine (G), serine (S). PS: photosynthetic.

Strain		Q_B site			Q_A site			PS
		L212	L213	L225	M231	M246	M247	
Wild type	U43[pU2922]	E	D	G	R	A	A	Yes
Background strains	U43[pRLAAM86BglII]	A	A	G	R	A	A	No
	U43[pQArev1L]	A	D	G	R	A	A	No
	U43[pRQrev4A]	E	D	G	R	E	A	No
Constructed mutants	U43[pRAA+1L/KS]	A	A	G	R	A	D	No
	U43[pRAA+4A/KS]	A	A	G	R	E	A	No
Spontaneous mutants	U43[pRAA+1L]R9	A	D	G	R	A	D	Yes
	U43[pRAA+1L]R18	A	A	G	C	A	D	Yes
	U43[pRAA+1L]R23	A	A	G	S	A	D	Yes
	U43[pRAA+4A]R7	A	A	D	R	E	A	Yes
	U43[pRAA+4A]R19	A	A	G	S	E	A	Yes

A total of 60 spontaneous revertant strains were spotted on agar plates for phenotypic characterisation. Spotting tests showed that all the isolated spontaneous mutants had photosynthetic character with diverse colours on test plates such as brown colonies from U43[pRAA+4A/KS] revertants, yellow colonies from U43[pRAA+1L/KS] revertants, and wild type-like purple spots.

A total of 60 spontaneous revertant strains were initially re-cloned. The sums of the "re-constructed" transconjugants include 48 U43[pRAA+1L/KS] revertants and 68 U43[pRAA+4/AKS] revertants. The original spontaneous and re-constructed spontaneous revertant strains were all photosynthetic and there was no difference in phenotypic expression between any original spontaneous revertant and the corresponding reconstructed one. Thus, all these mutations resulting in reversion to photosynthesis were plasmid-borne and they occurred in the L and M segment DNA synthesising the helices of L and M subunits.

Table 3. DNA sequence results of spontaneous mutants. A: adenine, C: cytosine, G: guanine, T: thymine. Superscript [a] shows original constructed mutant plasmid. DNA sequences and corresponding amino acids: ACA and ACG: cysteine; CCC and CCG: glycine; CGC and CGG: alanine; CTC: glutamic acid; CTG: aspartic acid; GCA and GCG: arginine; TGC: serine.

Plasmid DNA	L segment			M segment		
	L212	L213	L225	M231	M246	M247
pU29 (WT)	CTC	CTG	CCG	GCG	CGG	CGG
RCAA+1L/ES[a]	CGC	CGG	CCG	GCG	CGC	CTG
RCAA+1LR9/ES	CGC	CTG	CCG	GCG	CGC	CTG
RCAA+1LR18/ES	CGC	CGG	CCG	ACG	CGC	CTG
RCAA+1LR23/ES	CGC	CGG	CCG	AGC	CGC	CTG
RCAA+4A/ES[a]	CGC	CGG	CCG	GCG	CGG	CTG
RCAA+4AR7/ES	CGC	CGG	CTG	GCG	CTC	CGG
RCAA+4AR19/ES	CGC	CGG	CCG	TGC	CTC	CGG

A total of seven plasmids were used for sub-cloning. The sub-cloning process was highly efficient; there were at least 50 colonies from each sub-cloned strain per plate. A total of 7 plasmid DNA were sequenced to discover new amino acid locations in the L and M segments that are involved in the Q_A and Q_B sites (Table 3).

Discussion

One of the unique approaches in this research was to construct triple mutants of *R. capsulatus*, which is different from previous works in which only single mutants in RC were studied (Paddock *et al.* 1989, Takahashi and Wraight 1990, Okamura and Feher 1995). "RC" mutants from *Rhodobacter* species, carrying glutamate(L212), aspartic acid(L212), asparagine(L213), serine(213), threonine(L213), leucine(L213), and glutamate(L217) were reported to affect proton transfer (Paddock *et al.* 1989, Takahashi and Wraight 1990, Deisenhofer and Michel 1991, Okamura and Feher 1995). I extended this work by converting the residues responsible for proton transfer to double alanines at positions L212-L213.

Another group of amino acids in the RCs from *Rhodobacter* species did not affect proton transfer (Okamura and Feher 1995). In this research, only one double mutated RC [in U43[pRAA+1L]R9 with mutations alanine(L212) and aspartic acid(M247)] was discovered. It is slightly photosynthetic because of the aspartic acid(L213) reversion in the L sub-unit.

When phenotypic expression of each strain carrying triple mutated RCs was examined, both strains were found to be non-photosynthetic. The additional mutations in these two RCs did not restore wild type because of the reversed amino acid properties of the Q_A and Q_B sites.

These constructions proved that double alanines are essential amino acids for the photosynthetic phenotype only at positions M246-M247, not at L212-L213, in the Q_A pocket.

U43[pRAA+4A]R7 had triple mutated RCs. It gained photosynthetic capacity by reverting at position L225. When phenotypic expression of each strain carrying triple mutated RCs was compared to wild type, this mutant had the strongest photosynthetic phenotype. Phenotypic expression of this mutant may be correlated with introduction of acidic aspartic acid at position L225.

U43[pRAA+1L]R18, U43[pRAA+1L]R23, and U43[pRAA+4A]R19 had quadruple mutated RCs. When phenotypic expression of each strain carrying quadruple mutated RCs is compared to wild type, only U43[pRAA+4A]R19 shows a wild type like photosynthetic phenotype.

Except for the as yet unexplained difference between U43[pRAA+4A]R19 and U43[pRAA+1L]R23 the strongest photosynthetic phenotype requires a polar residue at M231 and the acidic glutamic acid at M246.

Each photosynthetic strain had at least one new mutation at the original site or somewhere else in the L and M subunits, and hence that strain had a photosynthetic phenotype. U43[pRAA+4A]R7 gained a PS+ phenotype by adding an additional mutation at position L225 (which compensates L212-L213), in the Q_B pocket. Conversion of L225 from wild type glycine to aspartic acid or alanine compensates for alanine(L212)-alanine(L213) mutations in the Q_B pocket. The glycine(L225) → alanine(L225) change observed in this research is a new discovery in studies of RC structure.

Previously studied mutations enhancing quinone binding in the RC include glycine(L222), proline(L223), and methionine(L229) (Bylina *et al.* 1986). L225 neighbours L222, L223, and L229. Considering this, it may be that the L225 changes in this work may also affect quinone binding in a positive way, leading to regaining of photosynthetic phenotypes.

Photosynthetic mutants carrying M231 substitutions are U43[pRAA+1L]R18, U43[pRED+1L]R23, and U43[pRAA+4A]R19. Conversion of M231 from wild type arginine(M231) to cysteine compensates for alanine(M246)-aspartic acid(M247), conversion of M231 from wild type arginine M231 to serine compensates for

glutamic acid(M246)-alanine(M247). The M231 reversions observed in this research [arginine(M231) → serine and cysteine] is a new discovery.

Leucine(M231) forms salt bridges with glutamic acids of the H helix [glutamic acid(H125) and glutamic acid(H232)] (Sebban *et al.* 1995). If non-polar leucine makes conservative salt bridges with amino acids of the H helix, possibly basic arginine and polar serine have different interactions. The M231 mutation shows that M231 is essential for photosynthesis if alanine(L212)-alanine(L213)-alanine(M246) or glutamic acid(M246)-alanine(M247) or aspartic acid(M247) are present in the RC.

Results of sequencing studies pointed out that some of the sequences at those specific locations had to have reversions to give a photosynthetic phenotype. In addition to that, some other reversions in the RCs had to occur at different locations to suppress the non-photosynthetic phenotypes. In summary, Q_A site and Q_B site in each unique RC have their own mechanisms to become photosynthetic. There is no requirement to revert to wild type or keep the same amino acids in the same positions. I have performed extensive mutation analyses of RC mutations yielding a number of well-defined mutants that may block specific steps of electron and proton transfer pathways.

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