Norges teknisk-naturvitenskapelige universitet (NTNU)
Fakultet for naturvitenskap
Institutt for bioingeniørfag

NADH dehydrogenase sin rolle i aerobisk og anaerobisk vekst av *Cereibacter sphaeroides*

Complex I and its role in aerobic and anaerobic growth of *Cereibacter sphaeroides*

Av / by
Eline Fondevik
Columbus, OH, USA, 2022

Preface

The following report was written as a part of the bachelor's degree in biomedical laboratory science at the Norwegian University of Science and Technology (NTNU), Trondheim, Norway. The project was executed over three months (March – June 2022), and the experiments were performed in the laboratory of Dr. Birgit Alber in the Department of Microbiology at the Ohio State University (OSU), Columbus, Ohio, USA.

I would like to thank Dr. Alber for granting me the opportunity to conduct my experiments in her laboratory, and for practical and scientific guidance throughout my stay in the lab. I am grateful to Camilla Wolowczyk for guiding me through the writing process.
I would also like to thank Dr. Patrice Hamel and Kristin Solum Steinsbekk for putting the Trondbuss-program together and giving me the chance to do my project at OSU. Finally, I would like to thank the other members of the Alber laboratory for their help in the lab and for making my days in the lab more enjoyable: Ross Garrison, Asha Raghavan, Cassie Skrant, and Vig Lalgudi.

Abstract

Complex I is a big protein complex in the electron transport chain and works as the entry point of electrons from NADH into the electron transport chain. Complex I in *Cereibacter sphaeroides* differs from complex I in many other bacteria, as there are two gene clusters that code for the protein complex: complex I_A and complex I_E. Both isozymes consist of the subunits NuoA – NuoN.

C. sphaeroides was randomly mutagenized using transposon mutagenesis during a teaching class at Ohio State University, and two of the mutants obtained were Sp10-28-8 E2 and SP10-JAI. These are, however, believed to be the same mutant, with the transposon insertion at *nuoN* in the gene cluster coding for complex I_E. *C. sphaeroides* was also randomly mutagenized in the Alber laboratory. A mutant isolated from this screen was called ABC21CM32 and had the transposon insertion at a gene with an unknown function in the gene cluster coding for complex I_A. This mutant was phenotyped to not grow aerobically with D-lactate as the carbon source.

The aim of this experiment was to phenotype Sp10-28-8 E2, Sp10-JAI, and ABC21CM32 by performing growth experiments with different carbon sources. To further investigate the function of complex I, a clean in-frame deletion of *nuoE* in the gene cluster coding for complex I_E was performed. A third approach to study the carbon assimilation of *C. sphaeroides* was to perform a transposon mutagenesis experiment and map the transposon insertion sites on mutants which did not grow on certain carbon substrates.

The results of the growth experiments were surprising, as the phenotype of ABC21CM32 was different from the phenotype that was found last year. The phenotype of Sp10-28-8 E2/Sp10-JAI was also different from what was expected. Based on the experiment performed in this thesis, the preculture of the growth experiment influences the results.
Single crossovers of *nuoE* were successfully obtained, but due to lack of time, a double crossover was not obtained. Three transposon mutants that showed compromised growth on acetate were isolated, and the transposon insertion sites were mapped to be on genes which have an unknown function in *C. sphaeroides*' ability to use acetate as a carbon source.

Table of content

1 Introduction

1.1 *Cereibacter sphaeroides*

Cereibacter sphaeroides, formerly known as *Rhodobacter sphaeroides*, is a Gram-negative, purple non-sulfur bacterium, meaning it is a phototroph and can use light as an energy source. The organism can also respire aerobically in the dark or use dimethyl-sulfoxide (DMSO) as the terminal electron acceptor for anaerobic respiration. During respiration, the carbon substrate is used both as a carbon source and as an energy source. For energy conservation, some of the carbon is oxidized to CO_2 and the electrons are transferred to the terminal electron acceptor. During phototrophy, however, the carbon substrate is solely used as a carbon source, as the energy source is light [1] .

The organism is able to use a large variety of carbon sources in its metabolism, including CO_2 and various organic acids. This makes the bacteria an ideal model to study carbon assimilation.

C. sphaeroides has two chromosomes, one is 3 Mb (CI) and the other is 900 Kb (CII). The two chromosomes share many of the same genes, but they appear to be differently regulated. Several of the open reading frames of CII also appear to code for proteins with unknown functions [2]. The microbe also has five plasmids (A – E) [3].

1.2 The electron transport chain

In the electron transport chain, protein complexes transfer electrons from electron donors to electron acceptors via redox reactions. These reactions are coupled with proton translocations across an energy-transducing membrane.

This flow of electrons is an exergonic process, meaning energy is released and the change in the free energy is negative. This energy goes into creating an electrochemical proton gradient which drives ATP synthesis [4].

In aerobic respiration, the final electron acceptor is oxygen, whereas the final electron acceptor can, for example, be the organic solvent DMSO in anaerobic respiration [5].

1.2.1. Oxidative phosphorylation

Electrons that enter the electron transport chain come from NADH and quinol, which can be produced during the citric acid cycle, as well cytochrome c. For example, in the citric acid cycle, malate is oxidized to oxaloacetate by malate dehydrogenase, which also reduces NAD^+

to NADH. Succinate is oxidized to fumarate in the cycle, this reduces ubiquinone to ubiquinol. This reaction is catalyzed by succinate dehydrogenase (complex III). NADH transfers electrons directly to complex I and gets oxidized back to NAD^+, which releases energy. Complex I and II transfer their electrons to ubiquinone, which reduces it into quinol. In aerobic respiration, quinol travels through the membrane and delivers the electrons to complex III, and electrons are transferred from quinol to cytochrome c, which is another electron carrier. Cytochrome c carries the electrons to complex IV, where more protons get pumped across the membrane. Finally, complex IV transfers the electrons to O_2, which then splits into two oxygen atoms and accepts protons to form water. Two H_2O molecules are formed in the process, with four electrons transferred, as shown in figure 1.1 [6].
In anaerobic respiration, complex III and complex IV are not needed. Instead, DMSO reductase transfers the electrons from quinol to DMSO, which reduces DMSO into DMS, and H_2O is formed [7].

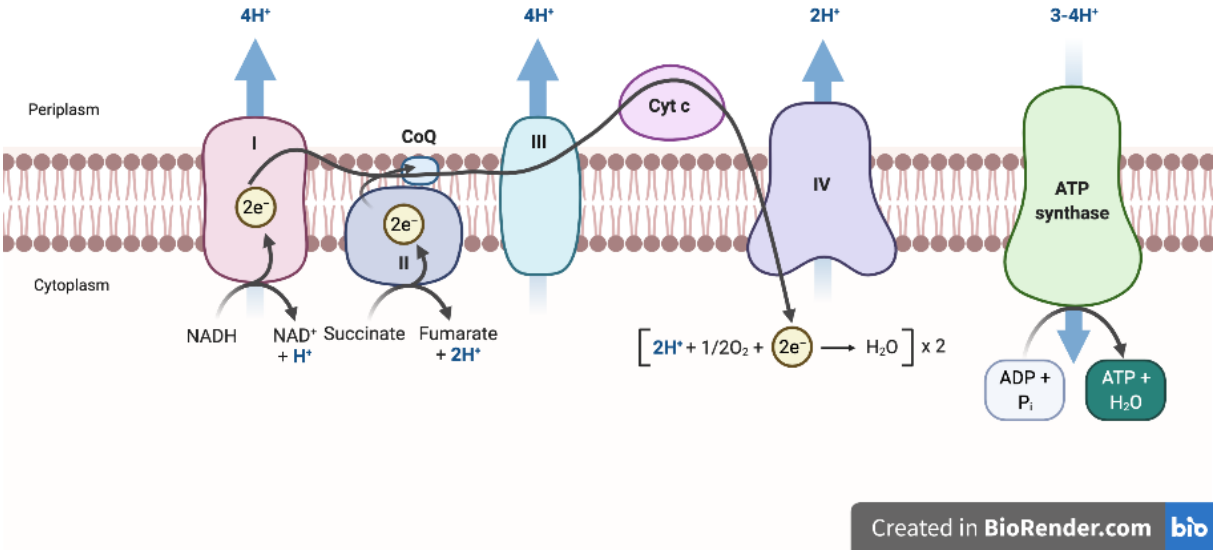


Figure 1.1: Oxidative phosphorylation in C. sphaeroides*, with the periplasm (the space between the inner and outer membrane in Gram-negative bacteria) at the top and the cytoplasm at the bottom. The energy created goes into making an electrochemical proton gradient which drives ATP synthase, shown to the right. The figure was made using BioRender.*

1.2.2 Photophosphorylation

The way to achieve proton translocation during photophosphorylation differs from the process in respiration. It is cyclic electron transport and involves a reaction center, light-harvesting complexes, the quinone pool, the bc_1-complex, and cytochrome c_2. The energy conserved from proton translocation is used for the synthesis of ATP from ADP + P_i.

When *C. sphaeroides* grows using phototrophy, its inner membrane turns into a network of vesicle-like intracellular membranes, shown in figure 1.2. At these energy transducing membranes, there are reaction centers (RCs) that catalyze light-driven photooxidation, after light gets absorbed by light-harvesting antenna complexes (LH-1 and LH-2). These light-harvesting complexes consist of protein-bound bacteriochlorophylls and the carotenoid sphaeroiden [8]. The reaction center consists of a bacteriochlorophyll dimer called "special pair", bacteriochlorophyll, bacteriochlorophaeophytin (bacteriochlorophyll without Mg^{2+}), and quinones (Q_A and Q_B) [9].

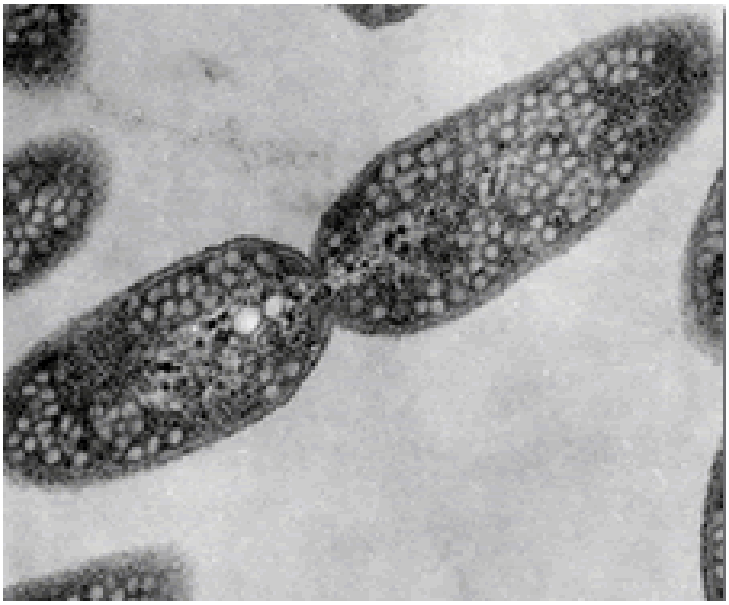


Figure 1.2: C. sphaeroides *with intracellular membranes that contain the reaction center, light harvesting complex, electron transport system and c-type chromosomes, which are formed when it is grown with light as the energy source. The picture is taken from Genome Portal [10].*

1.3 Complex I

Complex I, also known as NADH:ubiquinone oxidoreductase or NADH dehydrogenase type I, is an enzyme of the respiratory chain and is a membrane-bound protein complex that links two reactions. The enzyme catalyzes the transfer of electrons from NADH to quinone, making quinol, and translocates four protons across the inner membrane per molecule of oxidized NADH. This helps create the electrochemical potential difference which is used to produce ATP [11].

Complex I is present both in eukaryotic cells and bacterial cells, but the gene cluster encoding complex I in eukaryotic cells is larger than in prokaryotic cells. The complex consists of 45 subunits in human cells, whereas the core bacterial complex I only consists of 14 subunits, NuoA – NuoN. Another difference is that complex I is located in the inner membrane of the mitochondria in eukaryotic cells, whereas in prokaryotic cells it is located in the cytoplasmic membrane [12].

C. sphaeroides has two gene clusters in its genome that code for complex I enzymes: complex I_A and complex I_E. These gene clusters share many of the same subunits, but some of them are different. For example, the gene *nuoE* is longer on the gene cluster coding for complex I_A than the one for complex I_E, as shown in figure 1.3. The function of the extended C-terminal on *nuoE* in the cluster that codes for complex I_A is unknown. Complex I_A also has several subunits that have unknown functions. The isozyme I_A is a member of clade A and is similar to complex I enzymes found in many other alphaproteobacteria, whereas I_E is a member of clade E and is similar to complex I enzymes found in many gammaproteobacteria. The study "Different Functions of Phylogenetically Distinct Bacterial Complex I Isozymes" by Spero et al. claim that complex I_A is a necessity for aerobic growth, whereas complex I_E is not. Transcripts from complex I_E are low during aerobic growth, so in principle, complex I_E deficient cells should grow the same as the wild type during aerobic growth [13].

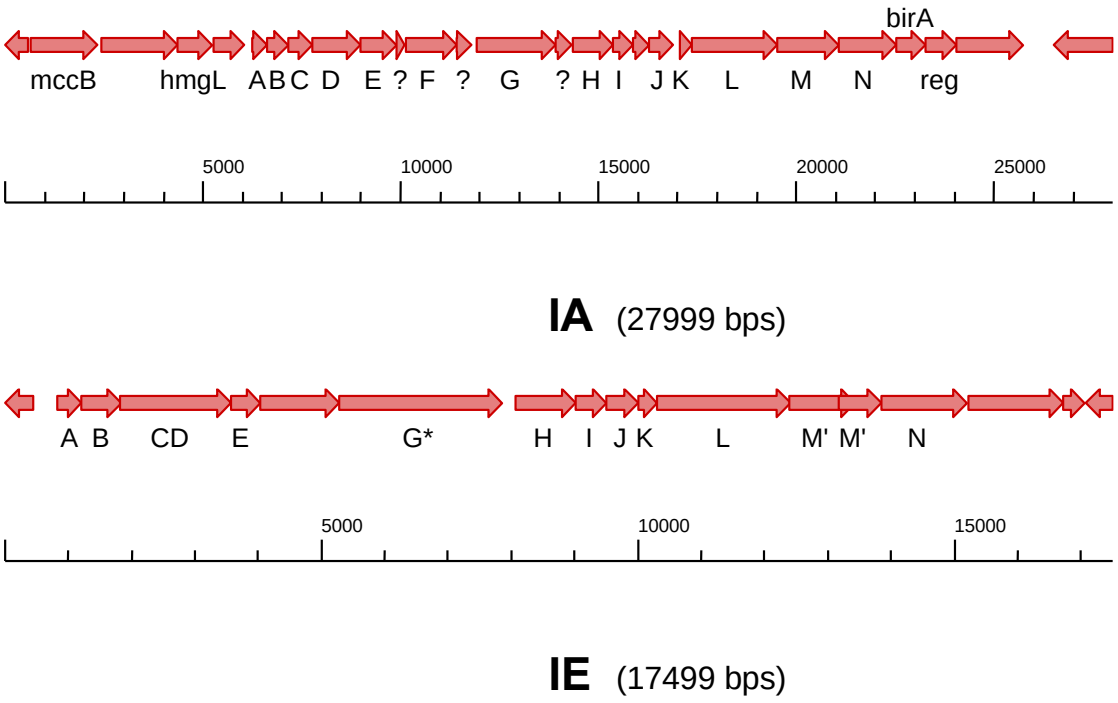


Figure 1.3: Gene clusters coding for complex I_A (upper) and complex I_E (lower), with the subunits NuoA – NuoN. The question marks on complex I_A represent genes that code for proteins with unknown functions. The scales under the gene clusters depict the size of each gene cluster. As shown, the gene cluster for complex I_A is larger than the one for complex I_E. The figure was made using Clone Manager.

1.4 Background for the project

There is no clear reason why *C. sphaeroides* has two complex I isozymes. It is assumed that complex I is a necessity in respiration, however, previous studies have shown that the microbe can grow using respiration, even with interrupted genes in the gene clusters coding for complex I [13]. Another striking discovery that was made in the paper "Genetic disruption of the respiratory NADH-ubiquinone reductase of *Rhodobacter capsulatus*" by Dupuis et al. is that *Rhodobacter capsulatus*, which can also use phototrophy, cannot grow with light as the energy source when a gene in the complex I gene clusters was interrupted [14]. This is an interesting discovery because it is believed that complex I is only needed in respiration, and not photophosphorylation. Therefore, many functions of complex I remain unsolved.

This project aimed to further investigate why *C. sphaeroides* has two gene clusters for complex I, and how the isozymes are involved in both respiration and phototrophy. To do this, in-frame deletion of the *nuoE*-gene in the gene cluster coding for complex I_E will be done, using the plasmid pKB77. This plasmid contains the in-frame deletion of *nuoE* along with the flanking regions and was available for use in the Alber laboratory.

In addition, mutants that have previously received a transposon insertion in genes coding for subunits in complex I_E (Sp10-28-8 E2 and Sp10-JAI) and complex I_A (ABC21CM32) will be tested in liquid medium with different carbon sources, to see if the absence of a functional complex I impacts growth. According to figure 2B in the article "Different Functions of Phylogenetically Distinct Bacterial Complex I Isozymes" by Spero et al., both complex I_A and complex I_E mutant strains did not grow with D/L-lactate, whereas only the complex I_A mutant strain did not grow with succinate as the carbon substrate. However, when DMSO was included, the cells were rescued and were able to grow with both carbon substrates. The paper also concludes from figure 7 that only complex I_A is needed for aerobic growth, while complex I_E is not [13]. The growth experiments will therefore include these carbon sources and will be inoculated both aerobically and anaerobically. The data will be compared with the data received in this thesis, to look for similarities and differences. ABC21CM32 was phenotyped to be D-lactate negative last year by another student in the Alber laboratory at Ohio State University. This is consistent with the data found in the paper by Spero et al., as they also found that their complex I_A mutant strain was D/L-lactate negative. This mutant should therefore be negative for D-lactate in the growth experiment. Another idea to test is whether the preculture of the growth experiment matters; therefore, some growth experiments will be done where DMSO is included in the preculture, and some where DMSO is not included.

A new transposon mutagenesis experiment will also be done, and the mutants that show no growth or compromised growth on different carbon sources will be isolated, and the transposon insertion location will be mapped.

2 Materials and methods

2.1 Bacterial strains, plasmids, primers, and enzymes

For this project, different strains for *C. sphaeroides* and *E. coli* were needed, as well as a plasmid. The bacterial strains and plasmid that were used in this thesis are shown in table 2.1.

Table 2.1 shows the names of the bacterial strains and plasmid that were used in this thesis, and why they were used.

Name	**Function**
Cereibacter sphaeroides 2.4.1	Wild type, DSMZ 158
Cereibacter sphaeroides Sp-10-JA1/Sp10-28-8 E2 (RSP_0112)	Mutant with a transposon insertion in *nuoN*-gene in gene cluster coding for complex I_E (accession number: EP_011337995.1)
Cereibacter sphaeroides ABC21CM32 (RSP_2520)	Mutant with a transposon insertion in a gene that has an unknown function in gene cluster coding for complex I_A (accession number: WP_011337548.1)
Escherichia coli S17-1	Competent cells, used for conjugation
Escherichia coli WM2672 (pRL27)	Donor with transposon-containing plasmid, used for transposon mutagenesis. Transposons are DNA sequences that can "jump" from one place to another and cause random mutations. The suicide vector pRL27 contains the transposon, as well as other components needed to insert the transposon on *C. sphaeroides'* genome. The plasmid also contains the gene *aph*, which gives kanamycin resistance
Escherichia coli DH5-αλpir-WM2826	Competent cells, used for transformation
pKB77	Plasmid used for in-frame deletion of *nuoE* on gene cluster coding for complex I_E. Contains a deletion of *nuoE* and flanking regions.

Enzymes are needed in experiments to catalyze different reactions. The enzymes used in this project and their functions are shown in table 2.2.

Table 2.2: Names of the enzymes that were used in this thesis, and why they were used.

Enzyme	**Function**
*Nco*I (New England Biolabs)	Restriction enzyme, cuts at C/CATGG
T4 DNA ligase (New England Biolabs)	Ligation enzyme, used to ligate ends at linear DNA to make it circular
DNA polymerase (GXL)	Enzyme used in PCR, to copy DNA

To start DNA synthesis, primers are needed as a starting point. The primers used in this project are shown in table 2.3.

Table 2.3: Names of the primers that were used for sequencing and PCR, and why they were used.

Primer number	**Primer name**	**Primer sequence**	**Function**
1275	tnpRL 17-1	AACAAGCCAGGGATGTAACG	Used in DNA sequencing to map transposon insertions site
1276	tnpRL 13-2	CAGCAACACCTTCTTCACGA	Used in DNA sequencing to map transposon insertion site
983	RSP_0103 rev1	GCCGTTGACGATCAGATAGCTC	Used in PCR, binds in the beginning of *nuoN* – binds to plasmid
984	RSP_0103 for1	TCGACCCATGGCGTGTTAC	Used in PCR, binds in the middle of *nuoCD* – does not bind to plasmid
985	Geno0103down_for	GCCTCACGCAATATGCCATC	Used in PCR, binds towards the end of *nuoCD* – binds to plasmid
986	Geno0103down_rev	GCGGTCATCTGCAACAGCTC	Used in PCR, binds towards the end of *nuoF* – does not bind to plasmid

2.2 Media

2.2.1 LB (Luria-Bertani) medium

LB medium is a complex/rich medium, where several microbes can grow. It was for example needed to grow wild-type *E. coli* in this project. The composition of the medium is shown below.

LB medium

Composition for volume: 1000 mL

- 10 g tryptone
- 5 g yeast extract
- 5 g NaCl

The pH was adjusted to 7.0 with NaOH.

For solid medium, 500 mL was transferred to a 1 L Erlenmeyer flask containing 7.5 g agar. Kanamycin was added when selection for mutants with the gene that gives kanamycin-resistance was done. Appropriate amounts of 20 mg/mL kanamycin were added when needed (final concentration: 50 µg/mL).

For liquid medium, aliquots of 25 mL media were added to 50 mL Erlenmeyer flasks. If needed, 62.5 µL 20 mg/mL kanamycin was added (final concentration: 50 µg/mL).

2.2.2 MM (minimal medium)

Minimal medium is a specific medium for *C. sphaeroides*. The composition of the medium is shown below.

Minimal medium

Composition for volume: 1000 mL

- 1.2 g NH_4Cl (final concentration 22 mM)
- 0.2 g $MgSO_4 \cdot 7H_2O$ (final concentration 0.8 mM)
- 0.07 g $CaCl_2 \cdot 2H_2O$ (final concentration 0.5 mM)
- 10 mL of a 100-fold trace element solution, which consists of (per liter):
    - 500 mg Disodium EDTA
    - 300 mg $FeSO_4 \cdot 7H_2O$
    - 3 mg $MnCl_2 \cdot 4H_2O$
    - 50 mg $CoCl_2 \cdot 6H_2O$
    - 1 mg $CuCl_2 \cdot 2H_2O$

    - 2 mg $NiCl_2 \cdot 6H_2O$
    - 3 mg $Na_2MoO_4 \cdot 2H_2O$
    - 5 mg $ZnSO_4 \cdot 7H_2O$
    - 2 mg H_3BO_3 (boric acid)

For solid medium, 500 mL was transferred to a 1 L Erlenmeyer flask containing 12.5 g agar. For liquid medium, aliquots of 50 mL media were transferred to bottles, and then made anaerobic.

The solution was autoclaved, and then the following components were added.

- 10 mL 1M carbon substrate (final concentration: 10 mM)
- 1.5 mL vitamin solution, which consists of:
    - 100 mg Cyanocobalamin (Vitamin B12)
    - 300 mg Pyridoxamine-2 HCl (Vitamin B6)
    - 100 mg Calcium-D(+)-Pantothenate
    - 200 mg Thiamine chloride (Vitamin B1)
    - 200 mg Nicotinic acid
    - 80 mg 4-Aminobenzoic acid (Vitamin H1)
    - 20 mg D(+)-Biotin
- 15 mL 1 M phosphate buffer, pH 6.7 (final concentration 15 mM)
- (500 μL 20 mg/mL kanamycin, (final concentration 20 μg/mL))
- (7.8 mL 12.8 M DMSO (used for anaerobic respiration) (final concentration 100 mM))

When sucrose was needed in the media, the volume was 800 mL instead of 1000 mL, with the same composition. Then, 400 mL was transferred to an Erlenmeyer flask with 12.5 g agar, the solution was autoclaved, and the same components were added. Finally, 100 mL 50 % sucrose was added to the solution.

2.3 Methods

2.3.1 Conjugation

2.3.1.1 Preparation of E. coli *S17-1 pre-culture*

The plasmid pKB77 was introduced into *E. coli* S17-1 using transformation explained in 2.3.3.1.

Six single colonies from the overnight incubation obtained in 2.3.3.1 were re-streaked in 6 different sections on two LB-plates, where one plate contained sucrose and the other contained kanamycin.

After incubation, cells that grew on the plate with kanamycin, and not on sucrose, were inoculated in 5 mL LB/kan at 37 °C in an O_2-shaker. The cells that grew in the presence of sucrose were avoided because the plasmid pKB77 contains the gene *sacB* which confers sucrose sensitivity.

2.3.1.2 Plasmid transfer via conjugation

A 0.9 mL overnight culture of wild-type *C. sphaeroides* 2.4.1., (grown in LB), was inoculated in 25 mL LB medium, the OD_{578} was measured, and the cells were incubated at 30 °C in the dark. For the transposon mutagenesis, a 0.5 mL overnight culture of *E. coli* WM2672 (pRL27) was inoculated in 25 mL LB with kanamycin, the OD_{578} was measured, and the cells were incubated at 37 °C in an O_2-shaker. For the in-frame deletion, the same procedure was done with *E. coli* S17-1(pKB77). The cells were centrifuged for 10 minutes at 8,900 x g at 4 °C, and the supernatant was removed completely. The cell pellets were resuspended in 1 mL LB and transferred to an Eppendorf tube. The tube with the cell suspension was centrifuged for 2 minutes at 8,900 x g at 4 °C, and the supernatant was again completely removed. *E. coli* had an extra washing step, meaning it was resuspended in 700 µL LB media, and then centrifuged for 2.5 minutes at 4 °C. This was to make sure that all the kanamycin in the original media was gone because wild-type *C. sphaeroides* cannot grow in the presence of kanamycin. Finally, the cell pellets were resuspended in the amount of µL that corresponds to the final OD_{578} that was measured x 1,000.

C. sphaeroides and *E. coli* were mixed in a 1:1 ratio in an Eppendorf tube. The ratio was determined by measuring the OD_{578} (990 µL media + 10 µL cells) of both concentrated cell cultures and adding more of the cells with the lowest OD_{578}. The conjugation mix was centrifuged for 2 minutes at 9,000 x g at 4 °C, and the supernatant was removed.

2.3.2 Obtaining transposon mutants

The suicide vector pRL27 was introduced into *C. sphaeroides* using conjugation, which is explained in 2.3.1.2. The conjugation mix pellet was resuspended in 100 µL LB media, and this suspension was separated into 6 spots on 2 LB plates, in which one spot contained 10-30 µL of the suspension. The plate was incubated aerobically at 30 °C in the dark for about 20 hours.

A dilution series was set up from the cells obtained using conjugation. Most of the cells in one spot were scraped off the plate and resuspended in 300 µL of MM in an Eppendorf tube. A series of 1:10 dilutions with minimal media were performed until a dilution of 1×10^{-6} was obtained.

Two transposon mutagenesis experiments were performed, these experiments were called Leo and Sp22. An aliquot of 30 µL of each of the dilutions was spread onto plates with and without kanamycin and with Na-acetate/$NaHCO_3^-$ (Leo) or Na-L-malate (Sp22) as the carbon sources. The undiluted solution and the 1:10 dilution were plated on plates with kanamycin, which selects for *C. sphaeroides* that have received the transposon. The more diluted solutions were spread on the media plates without kanamycin, where wild-type *C. sphaeroides* 2.4.1 will also grow. The purpose of this was to calculate the efficiency of the conjugation and transposition. The plates were incubated for about a week at 30 °C aerobically in the dark.

2.3.3 Patching and screening cells that received the transposon through conjugation

A screening was done to detect the phenotypes of the mutations obtained after the conjugation. To do this, single colonies were picked using a sterile flat toothpick from the plates containing acetate/HCO_3^- with kanamycin or L-malate with kanamycin and then patched on plates containing D-lactate, L-lactate, acetate, succinate, or L-malate, which all contained kanamycin. The new plates containing acetate/HCO_3^- and L-malate were used as positive controls for the colonies picked from the plates containing acetate/HCO_3^- and L-malate, respectively. The purpose of this was to find mutants that can no longer use one or more of the carbon substrates. The plates were incubated at 30 °C aerobically in the dark for about a week after patching.

After the cells were patched and incubated, they were screened. To do this, growth on the different carbon sources was evaluated, and the mutants that showed no growth or slow growth were noted down.

The mutants that showed little to no growth on the screening, were re-streaked on plates with the same media as their control, to ensure growth. These plates were incubated aerobically at 30 °C in the dark. After incubation, the mutants were re-patched on the different carbon sources, to make sure that the first patching had been done correctly. These mutants were again screened after incubation, and the mutants that showed the same behavior as the first screening were re-streaked on carbon substrates they grew well on.

2.3.4 Isolation of single crossover

The plasmid pKB77 was introduced into *C. sphaeroides* using conjugation, which is explained in 2.3.1.2. The conjugation mix pellet was resuspended in 50 µL LB media, and this suspension was applied to an LB-plate as one single drop, and then incubated aerobically at 30 °C. Most of the cells in the spot were scraped off the plate and resuspended in 300 µL of MM in an Eppendorf tube. A dilution series was then set up, with the dilutions 1:10 and 1:100. The dilutions were plated on plates containing MM L-malate/kan/DMSO and MM D/L-lactate/kan/DMSO and incubated aerobically at 30 °C. Two small colonies and two big colonies were picked from MM L-malate/kan/DMSO, and two small colonies and two big colonies were picked from MM D/L-lactate/kan/DMSO. A three-phase streak was performed for these colonies, on the same type of plates as the ones they were picked from.

2.3.5 Transformation of competent *E. coli* cells

2.3.5.1 Transformation of S17-1 cells using uncut vector plasmid pKB77

An aliquot of about 30 ng/µL 1 µL vector plasmid was added to 200 µL of competent *E. coli* cells. As a negative control, 10 µL of water was added to 200 µL of competent *E. coli* cells. The mix was incubated on ice for 20 minutes, to allow the plasmid DNA to mix with the cells. Then the mix was incubated at 42 °C for 2 minutes. The heat causes the lipids in the cell membranes to get more separated, so the plasmid can get into the cell. The mix was then again incubated on ice for 10 minutes, to allow the lipids to close again. An aliquot of 1 mL LB media was added, and the suspension was incubated at 37 °C for 1 hour, so the antibiotic resistance gene will be expressed. An aliquot of 50 µL cell suspension was then plated on LB media containing kanamycin overnight.

2.3.5.2 Transformation of DH5αλpir cells using a ligation mix

An aliquot of 30 µL ligation mix was added to 200 µL of competent DH5α λpir *E. coli* cells. The mix was incubated on ice for 20 minutes. The mix was incubated at 42°C for 2 minutes, and then it was again incubated on ice for 10 minutes. An aliquot of 865 µL LB media was added, and the suspension was incubated at 37 °C for 1 hour. The mixture was centrifuged at 8,000 x g for 2.5 minutes at 4 °C, to collect all the cells at the bottom of the tube. Then, 975 µL of the solution was removed, leaving 100 µL in the tube, with the cell pellet on at the bottom. Then the cells were resuspended, and all 100 µL of the cell suspension was then plated on LB media containing kanamycin overnight.

2.3.6 BLAST search

A BLAST alignment search was done between the two NuoE-subunits of complex I_A and complex I_E to look for similarities and differences between the gene clusters. The C-terminal of *nuoE* on complex I_A is more extended than the C-terminal on complex I_E (it extends beyond 175 amino acids). In order to see if there are any homologs (similar proteins) that have C-terminal extensions that match the one on the *nuoE*-gene on complex I_A, a BLAST search was done. To exclude the N-terminal that codes for NuoE, and other complex I-proteins, the BLAST search started from 189 amino acids. The search also excluded all alphaproteobacteria, since most of these have *nuoE*-genes in complex I_A, like the one in *C. sphaeroides*.

2.3.7 DNA isolation

2.3.7.1 Genome DNA isolation

There are two approaches to obtaining cells for genomic DNA isolation, both of which yield approximately the same number of cells. If the cells were taken from a media plate, some cells were scooped up and put in about 200 µL of LB media in an Eppendorf tube. The cells were then centrifuged at 9,000 x g for 2.5 minutes at 4 °C. If the cells were taken from a liquid cell culture, 900 µL of a fully grown cell culture was harvested, added into an Eppendorf tube, then centrifuged.

The QIAGEN QIAprep Spin MiniPrep genomic DNA kit was used to isolate the DNA, and the procedure is as follows:

An aliquot of 180 µL Buffer ATL was added, and the cells were resuspended and 20 µL Proteinase K was added and the solution was mixed by vortexing. Then the cells were

incubated at 56 °C for 30 minutes until the cells are completely lysed. The tube was mixed occasionally during incubation. 200 µL Buffer AL was added, and the solutions were mixed by vortexing for 15 seconds. The tube was incubated at 70 °C for 10 minutes. The tube was briefly centrifuged to remove drops from the lid.

An aliquot of 200 µL 100% ethanol was added, and the tube was vortexed for 15 seconds. The tube was briefly centrifuged to remove drops from the lid. The mixture was pipetted into a QIAamp Mini spin column (in a 2 mL collection tube). The tube was centrifuged at 6,000 x g for 1 minute. The flowthrough and collection tube were discarded. The QIAamp Mini spin column was placed in a new 2 mL collection tube and 500 µL Buffer AW1 was added. The tube was centrifuged at 6,000 x g for 1 minute. The flowthrough and collection tube were discarded.

The QIAamp Mini spin column was placed in a new 2 mL collection tube and 500 µL Buffer AW2 was added. The tube was centrifuged at 6,000 x g for 1 minute. The flowthrough was discarded. The QIAamp Mini spin column was placed in the same collection tube, and the tube was centrifuged on full spin for 1 minute, to eliminate the chance of possible Buffer AW2 carryover. The QIAamp Mini spin column was placed in a new 1.5 mL Eppendorf tube, and the lid was left open for 5 minutes, then 200 µL Buffer AE was added, and the solution was incubated at room temperature for 1 minute. The solution was centrifuged at 6,000 x g for 1 minute to elute the DNA.

2.3.7.2 Plasmid DNA isolation

The QIAGEN QIAprep Spin MiniPrep plasmid DNA kit was used to isolate plasmid DNA, and the procedure is as follows:

An aliquot of 3 mL bacterial overnight culture was centrifuged at 8,000 rpm for 3 minutes at about 4 °C to get a cell pellet. The cell pellet was resuspended in 250 µL Buffer P1 (resuspension buffer). 250 µL Buffer P2 (lysis buffer) was added, and the solution was mixed by inverting the tube 4-6 times until the solution became clear. 350 µL Buffer N3 (neutralization buffer) was added, and the solution was mixed by flicking the tube 4-6 times. The solution was centrifuged at 13,000 rpm at 4 °C. 800 µL of the supernatant was added to a spin column, then it was centrifuged for 60 seconds. The flowthrough was discarded. The spin column was washed by using 750 µL Buffer PB (binding buffer). The solution was centrifuged for 60 seconds at 13,000 rpm at 4 °C, and the flowthrough was discarded. The spin column was then washed by using 750 µL Buffer PE (wash buffer). The solution was centrifuged for 60 seconds, and the flowthrough was discarded. The spin column was once

again centrifuged for 1 minute, to avoid carry-over of the wash solution. To elute the DNA, 50 µL Buffer EB (elution buffer) was added to the spin column. The spin column was let stand for 1 minute and centrifuged for 1 minute at 13,000 rpm at room temperature.

2.3.8 *Nco*I Digest

2.3.8.1 Genomic DNA digest

An aliquot of 34 µL of genomic DNA was transferred to a labeled tube, 4 µL of #3.1 NEB buffer was added, and the tube was vortexed and placed in a centrifuge for a quick spin.
An aliquot of 2 µL of *Nco*I enzyme was transferred to the tube, and the solution was mixed by pipetting up and down. The solution was quickly spun, then incubated for 6 hours at 37 °C. Then the solution was placed in a -20 °C freezer, and later used in a ligation reaction

2.3.8.2 Plasmid DNA digest

After a plasmid had been isolated, a mix of 27 µL H_2O, 4 µL buffer, and 1 µL *Nco*I was made for three reactions. Then 8 µL of this mix was pipetted into an Eppendorf tube, along with 2 µL plasmid. This mix was incubated for 1 hour at 37 °C.

2.3.9 Ligation

A tube with *Nco*I-restricted genomic DNA was heated for 15 minutes in a thermomixer at 80 °C, then the mixture was cooled down to room temperature. This step is done to inactivate the restriction enzyme and to prevent cohesive-end interactions that may reduce ligation efficiency.
The mixture was placed in a centrifuge for a quick spin, then 148 µL of sterile dH_2O was added. 20 µL of ligase buffer was added to the tube and then vortexed and briefly centrifuged. An aliquot of 2 µL of T4 DNA ligase (NEB) was added and mixed by pipetting up and down. The mixture was set at room temperature overnight to decrease sticky ends coming apart.

2.3.10 Gel electrophoresis

Gel electrophoresis is a method used to separate mixtures of DNA, RNA, or proteins based on their molecular size. The molecules are separated by an electrical field pushing through a gel with small pores.
TAE buffer was used to make the gel. A 50x solution consists of 121 g TRIS, 28.55 mL Acetic acid, 50.0 mL NaEDTA pH = 8.0/HCl 10.5 M and H_2O to 500 mL.

Agarose powder (0.6 g) was dissolved in 75 mL TAE buffer (1x) at 190 °C until the solution was completely clear. The solution was then cooled down to about 50 °C, and then 11 µL 10 mg/mL ethidium bromide was added. The solution was then poured into a casting tray, which contained an electrophoresis comb to make the chambers. Once the solution was solidified, the comb was removed, and the samples were mixed with loading dye (1/6 of the sample's volume), which consists of 0.25% Orange G, 0.25% Xylene Cyanol and 30% glycerol (6-fold concentrated). The samples were then loaded into the wells, and the gel ran at a constant 80V, with a variable current (mA).

2.3.11 DNA sequencing

DNA sequencing was not done in the lab and therefore had to be sent to the laboratory at Azenta Life Sciences. Before sending the DNA, the concentration and size of the DNA fraction(s) had to be determined. The size was determined using gel electrophoresis, and the concentration was determined by measuring its absorbance at 260 nm.
To find the genotype of a mutant, it must be sequenced. Table 2.4 shows the general setup for a DNA sequence.

Table 2.4: General setup for DNA sequencing.

Material	Volume
< 100 ng/µL plasmid DNA 100-200 ng/µL plasmid DNA	10 µL 5 µL + 5 µL H_2O
5 µM Primer	5 µL

2.3.12 Growth experiments

To determine a phenotype of a mutant and see if it grew slower in certain conditions, it was grown in several different conditions. The preculture of the experiment contained MM L-malate/DMSO, and the cells had been transferred about once every week, nine times in total. For the first experiment, 100 µL of the preculture was used to inoculate 4 mL MM succinate, MM L-malate, and MM acetate, both aerobically in an O_2-shaker and anaerobically in a light chamber. The OD_{578} in each condition was measured every few hours, to determine an accurate doubling time for the mutant. The doubling time was determined by using the formula shown in (1).

$$\frac{(t_1 - t_2)\ x\ 0.301}{log_{10}(OD_1) - log_{10}(OD_2)} \quad (1)$$

For the next experiment, 100 µL of cells in a preculture with MM L-malate/DMSO was used to inoculate MM D-lactate and MM L-lactate and were inoculated either aerobically in an O_2-shaker or anaerobically in a light chamber. The cells were also inoculated in MM L-malate/DMSO and MM succinate/DMSO anaerobically in a light chamber, to see if the growth could be rescued with anaerobic respiration. Since the mutants seemed to behave the same as the wild type, fresh inoculums were made using cultures and the same substrate from the previous growth experiment. Cells were inoculated from D-lactate to D-lactate, L-lactate to L-lactate and L-malate/DMSO to L-malate, all anaerobically, and the OD_{578} was again measured every few hours. Finally, 100 µL of cells were used to inoculate from a preculture that was grown one time with L-malate without DMSO, to D-lactate, L-lactate, succinate, and L-malate, anaerobically and aerobically. This was done to prevent carryover of DMSO from the precultures to the experiments.

A final experiment was done, where the cell culture with MM D-lactate from the previous experiment was used as a preculture. This was done to get another transfer without DMSO, to prevent carryover. The preculture was used to inoculate MM D-lactate, anaerobically in a light chamber.

2.3.13 PCR

PCR is a method to synthesize new strands of DNA, complementary to a template strand, using DNA polymerase. The enzyme can perform this when there are primers present, which can flank the target region. In the PCR, there also must be free nucleotides (dNTP), which are used to make the new DNA strand. A buffer with optimal concentrations of salts, optimal pH etc. for the DNA polymerase also need to be in the reaction. PCR was performed in this project to detect possible single crossovers.

To do a PCR, a master mix was made. The total volume of a master mix for one PCR reaction is 50 µL. The contents of this PCR mix are shown in table 2.5.

Table 2.5: General setup for one PCR reaction.

Solution	Volume (µL)
ddH_2O	34.5
5 x GXL buffer	10
100 µM primer 1	0.25
100 µM primer 2	0.25
(GXL) 2.5 mM dNTP	4
(GXL) DNA polymerase	1

The total volume of 50 µL was added to a PCR tube. In the case of colony PCR, a toothpick was used to carefully touch the top of a colony grown on a Petri dish, to get as few cells as possible, then added to the PCR mix. In the case of genomic DNA, 1 µL DNA was added to the mix. The program chosen for the PCR reaction is shown in table 2.6.

Table 2.6: The program used for PCR.

Step	Temperature (°C)	Time
1	98	5 minutes
2	98	45 seconds
3	60	30 seconds
4	68	2 minutes
5	5	Hold

Step 2 through 4 in table 2.6 was repeated 37 times.

2.3.14 Making a double crossover

A single crossover mutant was inoculated in 100 µL L-malate/DMSO in a microcentrifuge tube, aerobically at 30 °C in O_2-shaker for 20 hours. After incubation, a dilution series was set up. 1:100 and 1:1000 dilutions were plated on plates containing MM L-malate/sucrose and MM L-malate/sucrose/DMSO. These plates were incubated aerobically in the dark at 30 °C for about a week.

After incubation, single colonies grown on MM L-malate/sucrose were patched on plates containing MM L-malate/kan, MM L-malate/sucrose, and MM L-malate. Single colonies

grown on MM L-malate/sucrose/DMSO were patched on plates containing MM L-malate/kan/DMSO, MM L-malate/sucrose/DMSO and MM L-malate/DMSO.

3 Results

3.1 Transposon mutagenesis

Wild-type *C. sphaeroides* was randomly mutagenized using a transposon in order to obtain mutants that had lost the ability to use a specific carbon substrate. The carbon substrates tested were succinate, acetate, D-lactate, and L-lactate. The transposon-containing plasmid pRL27 was already present in *E. coli* and was introduced into *C. sphaeroides* through conjugation.

The efficiency of the conjugation and transposition of wild-type *C. sphaeroides* and *E. coli* WM2672 (pRL27) was calculated to see how productive the transposon mutagenesis experiment was, by counting the cell amount on plates containing kanamycin, which are the now kanamycin-resistant mutants, and the cell amount on plates without kanamycin, which are wild-type cells. This was done when growth of colonies was obtained after plating the dilution series on plates containing MM L-malate (Sp22) and MM acetate/HCO_3^- (Leo), with and without kanamycin. The results are shown in table 3.1.

Table 3.1: Efficiency of conjugation and transposition of wild-type C. sphaeroides *and* E. coli *WM2672 (pRL27)*

Mutant strain name	**Media**	**Average number of mutants in 30 µL**	**Average number of total cells in 30 µL**	**Efficiency**
Leo	Acetate/HCO_3^-, kanamycin	20	11×10^6	1.8×10^{-6} → 1 cell in 1,800,000
Sp22	L-malate, kanamycin	1560	82×10^6	1.6×10^{-5} → 1 cell in 160,000

The transposon mutants Sp22EF-138, Sp22RG-53 and Sp22VRL-60 were obtained by the transposon mutagenesis experiment, as they all showed compromised growth with acetate. To determine the transposon insertion site, the genomic DNA of the mutants was isolated, digested, ligated, and the ligation mix was used to transform *E. coli* DH5αλpir cells. The plasmid was isolated using primers reading outwards from the transposon, to find the transposon insertion sites, which are given in table 3.2.

Table 3.2: The transposon mutants obtained by transposon mutagenesis, their genotype, and their phenotype.

Transposon mutant	Genotype	Annotation	Phenotype
Sp22EF-138	Insertion in position 1783 on plasmid C (CP000146).	Codes for a hypothetical protein with an unknown function	Showed compromised growth with acetate
Sp22RG-53	Insertion in position 240904 on chromosome 1 (CP000143).	Codes for a probable peptidase	Showed compromised growth with acetate
Sp22VRL-60	Insertion in position 194904 on chromosome 2 (CP000244).	Codes for a conserved hypothetical protein – most likely transcribed with a putative tartrate dehydratase alpha subunit	Showed compromised growth with acetate

3.2 NuoE in complex I_A and complex I_E

3.2.1 BLAST search

A BLAST alignment search was done between the two NuoE-subunits of complex I_A and complex I_E to compare the gene on the two different gene clusters, as explained in 2.3.6. No genes coding for proteins with known functions were found from the BLAST search that corresponded to the extended C-terminal of NuoE in complex I_A.

3.2.2 In-frame deletion of *nuoE* in complex I_E, using pKB77

The plasmid pKB77 was used to obtain a mutant with a deletion on *nuoE* in the gene cluster coding for complex I_E in *C. sphaeroides*. This is the same gene cluster as the transposon in Sp10-28-8 E2/Sp10-JAI was mapped to.

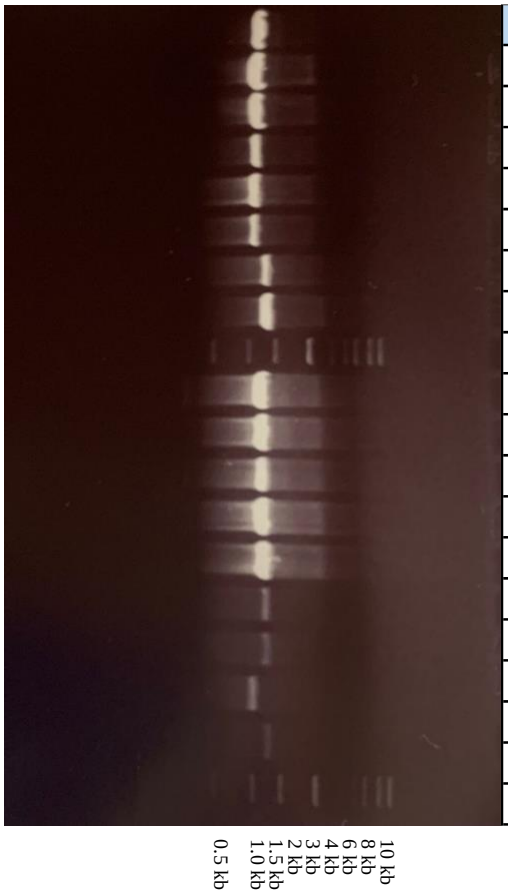
The plasmid was transferred to *E. coli* using transformation, and then transferred to *C. sphaeroides* using conjugation.

From the conjugation experiment, eight single colonies were chosen from the conjugation and these mutants were given names based on whether the original colony that was picked from was big or small, for example wt::pKB77-1b (big colony) or wt::pKB77-2s (small colony). To determine if the single crossover was an upstream- or downstream crossover, a colony PCR was performed, using the primer pairs 983x984 and 985x986. The expected size of the mutants using the different primer pairs is shown in table 3.3.

Table 3.3: The primers used in the colony PCR reaction and the expected sizes of the upstream- and downstream crossover mutants.

Primers	**Expected size for upstream crossover (kb)**	**Expected size for downstream crossover (kb)**	**Expected size for wild type (kb)**
983x984	1.62	1.85	1.85
984x985	1.77	1.54	1.77

The results of the PCR are shown in figure 3.1, and the frequency for up- and downstream crossover using the plasmid pKB77 is shown in table 3.4. The results show that there is a favor in upstream crossovers, even though there should be a 50 % chance of getting either one.



Lane	Sample
1	PCR #983x984, pKB77-1b
2	PCR #983x984, pKB77-2s
3	PCR #983x984, pKB77-3b
4	PCR #983x984, pKB77-4s
5	PCR #983x984, pKB77-5b
6	PCR #983x984, pKB77-6s
7	PCR #983x984, pKB77-7b
8	PCR #983x984, pKB77-8s
9	1 kB ladder
10	PCR #985x986, pKB77-1b
11	PCR #985x986, pKB77-2s
12	PCR #985x986, pKB77-3b
13	PCR #985x986, pKB77-4s
14	PCR #985x986, pKB77-5b
15	PCR #985x986, pKB77-6s
16	PCR #985x986, pKB77-7b
17	PCR #985x986, pKB77-8s
18	PCR #985x986, Sp10-28-8 E2 (control)
19	1 kB ladder

Figure 3.1: The gel electrophoresis on the left shows the PCR products of the single crossover, using the primer pairs 983x984 and 985x986. The numbers at the bottom represent the sizes of the fragments in the ladder. The table on the right shows the order of where the samples are loaded. Genomic DNA of Sp10-28-8 E2 is used as a "wild type" control, as this mutant does not have an interruption where the primers bind. A control is not included in the gel with the primer pai 983x984, as the control did not work in the PCR reaction.

Table 3.4: The rate of upstream- and downstream crossovers, based on colony PCR.

Mutant strain	Crossover
wt::pKB77-1b	Upstream
wt::pKB77-2s	Upstream
wt::pKB77-3b	Upstream
wt::pKB77-4s	Upstream
wt::pKB77-5b	Upstream
wt::pKB77-6s	Upstream
wt::pKB77-7b	Behaves like wild type, although kanamycin resistant
wt::pKB77-8s	Downstream

The single crossovers wt::pkB77-1b and wt::pkB77-8s were chosen to be included in a double crossover. Single colonies of the double crossover for wt::pKB77 were obtained, but due to lack of time, a PCR to identify the mutants was not performed.

3.3 Transposon mutants Sp10-JA1 (JAI B5), Sp10-28-8 E2 (JAI E2) and ABC21CM32 (PB5D3)

3.3.1 Mapping the transposon insertion site

For Sp-10-JAI, Sp10-28-8 E2, and ABC21CM32, the transposon insertion sites were mapped. In ABC21CM32, the transposon insertion was mapped to be on a gene that has an unknown function in the gene cluster coding for complex I_A. Sp10-28-8 E2 and Sp10-JA1 were found to be the same mutant, as they both had the transposon inserted on the same position on the *nuoN* gene in the gene cluster coding for complex I_E. Therefore, Sp10-28-8 E2 and SP10-JAI are two isolates of the same strain.

3.3.2 Growth experiments

Growth experiments were performed on the transposon mutants Sp10-JAI, Sp10-28-8 E2 and ABC21CM32, as explained in 2.3.10. The calculated doubling time for the graphs, along with the standard deviation, are used to compare the mutants to each other. When the doubling time for a mutant is significantly different from the wild type, the conclusion is that said mutant grows with a different growth rate than the wild type. When the standard deviation is too high (≥10 % of the doubling time), the doubling time is replaced with n.d. (not determined). In this scenario, the conclusion is solely based on a comparison of the graphs, instead of the doubling time. Sp10-JAI and Sp10-28-8 E2 should behave the same, as they are the same mutant, only revived from different stocks.

Based on the growth experiment, the mutants grew slower than the wild type when they were grown aerobically with L-malate as the carbon source, as shown in figure 3.2, both when the preculture was with and without DMSO.

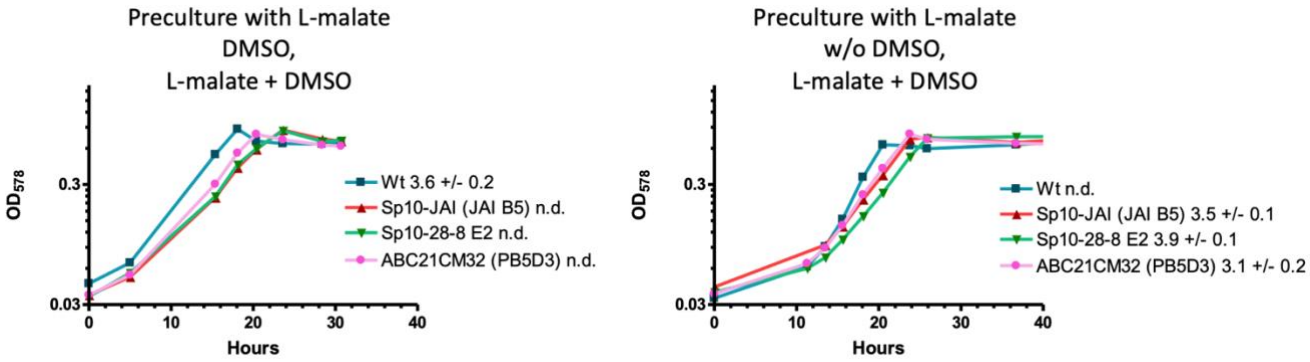


Figure 3.2: Aerobic growth of wild type and complex I mutant strains with L-malate as the carbon source. The graph to the left shows an experiment where DMSO is present in the preculture, but not in the experiment, and was performed 4/21. The graph to the right shows an experiment done where DMSO is neither present in the experiment nor the preculture and was performed 5/29. The numbers next to the strain names are the calculated growth rate +/- the standard deviation. N.d. stands for not determined and is given when the standard deviation is too high to give an accurate growth rate.

When the strains were grown with L-malate anaerobically in the light, none of the mutants grew slower than the wild type, as shown in figure 3.3.

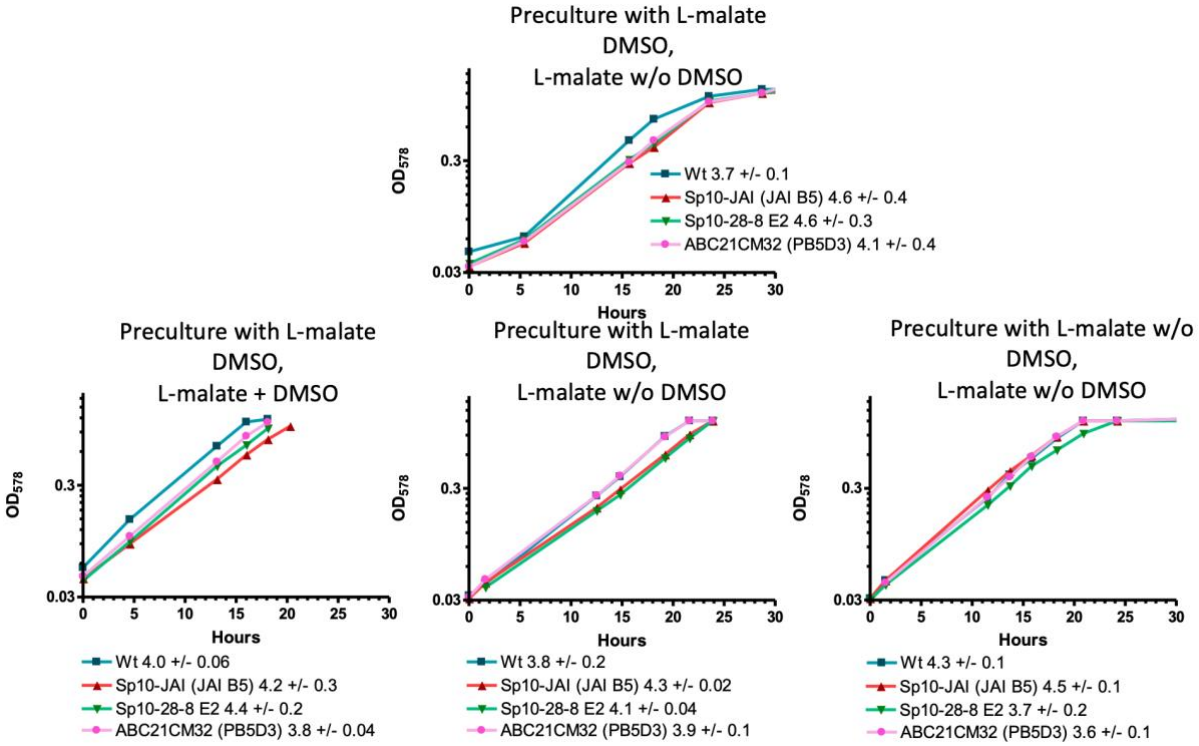


Figure 3.3: Phototrophic growth of wild type and complex I mutant strains with L-malate as the carbon source. The graph to the left shows an experiment where DMSO is included, and where the pre-culture also contains DMSO, and was performed 5/25. The two graphs in the middle show the same experiment, where the upper was performed 4/21 and the latter was performed 5/27. These graphs show experiments where DMSO is not included in the experiment, but it is present in the preculture. The graph to the right shows an experiment where DMSO is neither in the experiment nor the preculture and was performed 5/29. The numbers next to the strain names are the calculated growth rate +/- the standard deviation.

The complex I_E mutant strains showed slower growth than the wild type and complex I_A mutant strain with succinate as the carbon source when DMSO was not present in aerobic growth, as shown in figure 3.4. It therefore seems like DMSO carryover affects the growth in succinate.

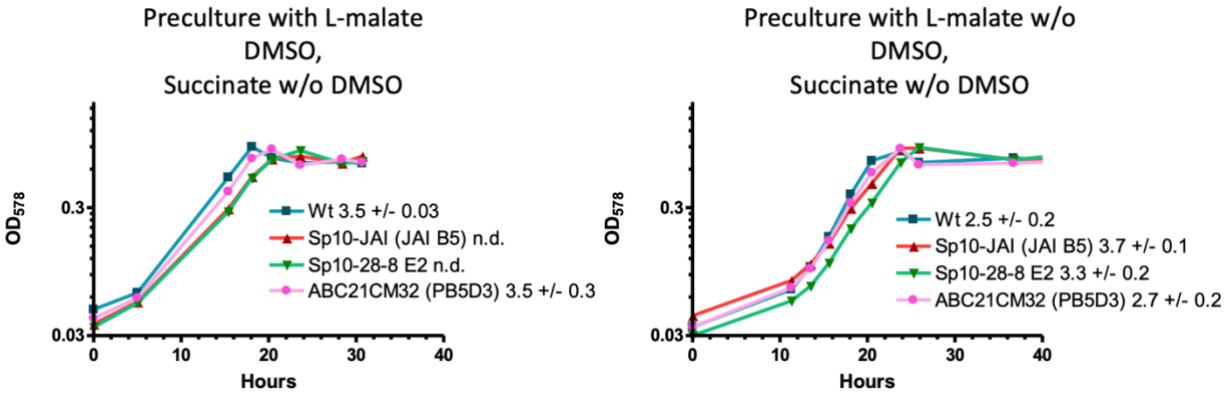


Figure 3.4: Aerobic growth of wild type and complex I mutant strains with succinate as the carbon source. The graph to the left shows an experiment where DMSO was included in the preculture but not in the experiment and was performed 4/21. The graph to the right shows an experiment where DMSO is neither in the experiment nor the preculture and was performed 5/29. The numbers next to the strain names are the calculated growth rate +/- the standard deviation. N.d. stands for not determined and is given when the standard deviation is too high to give an accurate growth rate.

The complex I_E-mutant also showed slower growth than the other strains with succinate as the carbon source when DMSO was not present in phototrophic growth, as shown in figure 3.5. It seems like there is a DMSO carryover when the preculture contains DMSO.

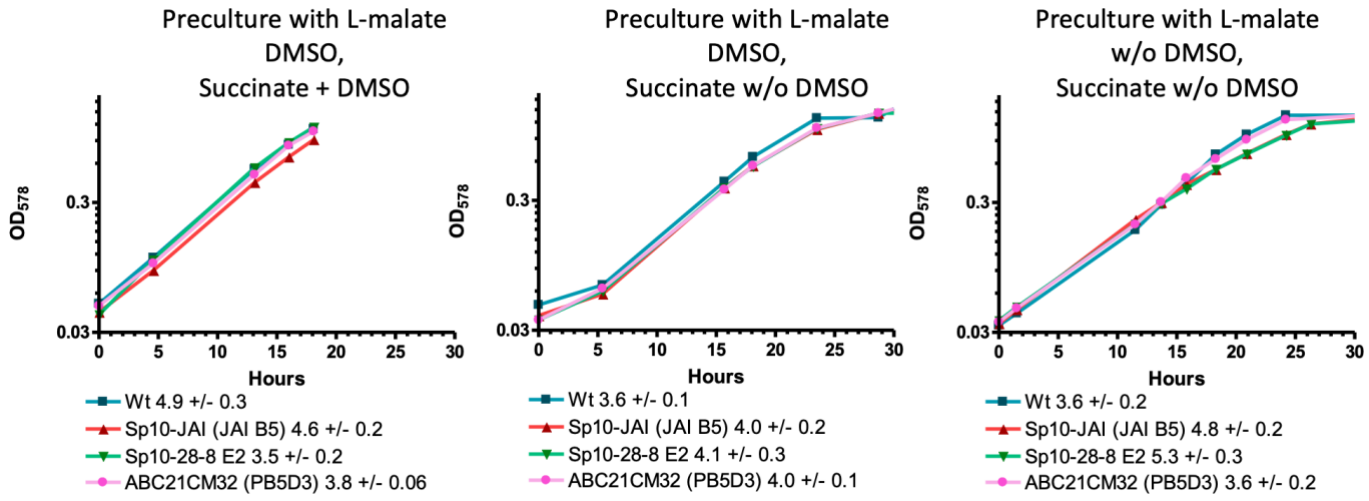


Figure 3.5: Phototrophic growth of wild type and complex I mutant strains with succinate as the carbon source. The graph to the left shows an experiment where DMSO is present in both the preculture and the experiment and was performed 5/25. The graph in the middle shows an experiment where DMSO is included in the preculture, but not the experiment and was performed 4/21. The graph to the right shows an experiment where DMSO is neither in the preculture nor the experiment and was performed 5/29t. The numbers next to the strain names are the calculated growth rate +/- the standard deviation.

None of the mutants grew slower than the wild type when they were grown aerobically with D-lactate as the carbon source, as shown in figure 3.6.

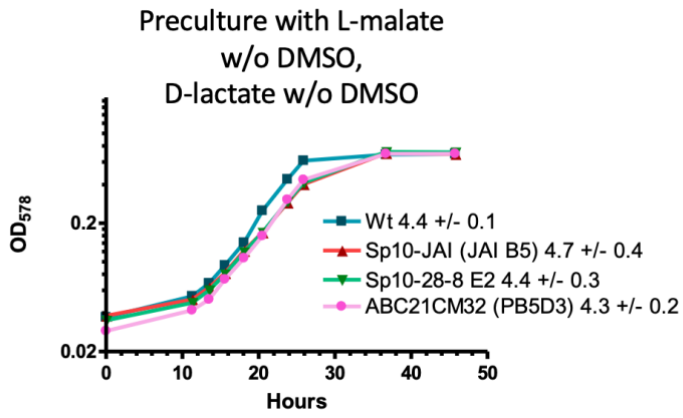


Figure 3.6: Aerobic growth of wild type and complex I mutant strains with D-lactate as the carbon source. The graph shows an experiment where DMSO is neither in the experiment nor the preculture and was performed 5/29. The numbers next to the strain names are the calculated growth rate +/- the standard deviation.

Both the complex I_E mutant strains and the complex I_A mutant strain grew slower than the wild type when they were grown anaerobically in the light, as shown in figure 3.7. The complex I_E mutant strains grew the slowest. The mutants grew slower when the preculture was with L-malate w/o DMSO than when it was with D-lactate w/o DMSO.

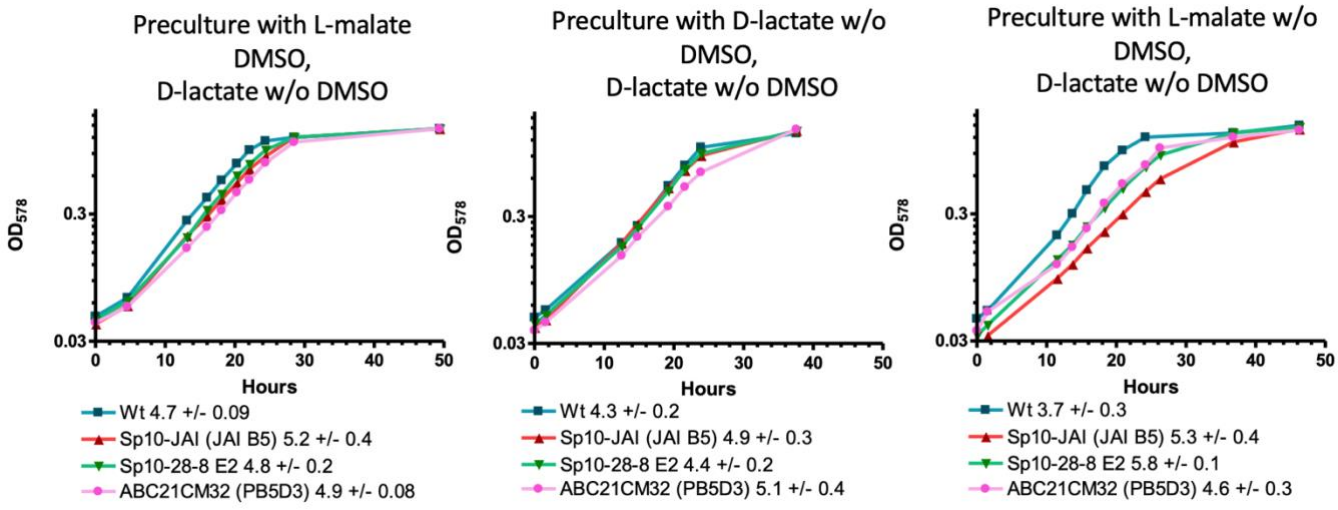


Figure 3.7: Phototrophic growth of wild type and complex I mutant strains with D-lactate as the carbon source. The graph to the left shows an experiment where DMSO is included in both the preculture and the experiment and was performed 5/25. The graph in the middle shows an experiment where DMSO is neither in the experiment nor the preculture, and the preculture contains D-lactate and was performed 5/27. The graph to the right shows an experiment where DMSO is neither in the experiment nor the preculture, and the preculture contains L-malate and was performed 5/29. The numbers next to the strain names are the calculated growth rate +/- the standard deviation.

None of the mutants grew slower than the wild type when they were grown aerobically with L-lactate as the carbon source, as shown in figure 3.8.

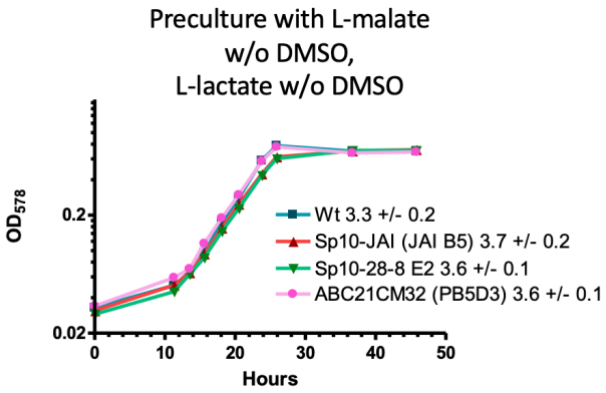


Figure 3.8: Aerobic growth of wild type and complex I mutant strains with D-lactate as the carbon source. The graph shows an experiment where DMSO is neither included in the experiment nor the preculture and was performed 5/29. The numbers next to the strain names are the calculated growth rate +/- the standard deviation.

The complex I_E mutant strains grew significantly slower, and the complex I_A mutant strain grew slightly slower than the wild type when they were grown anaerobically in the light with L-lactate as the carbon source, as shown in figure 3.9.

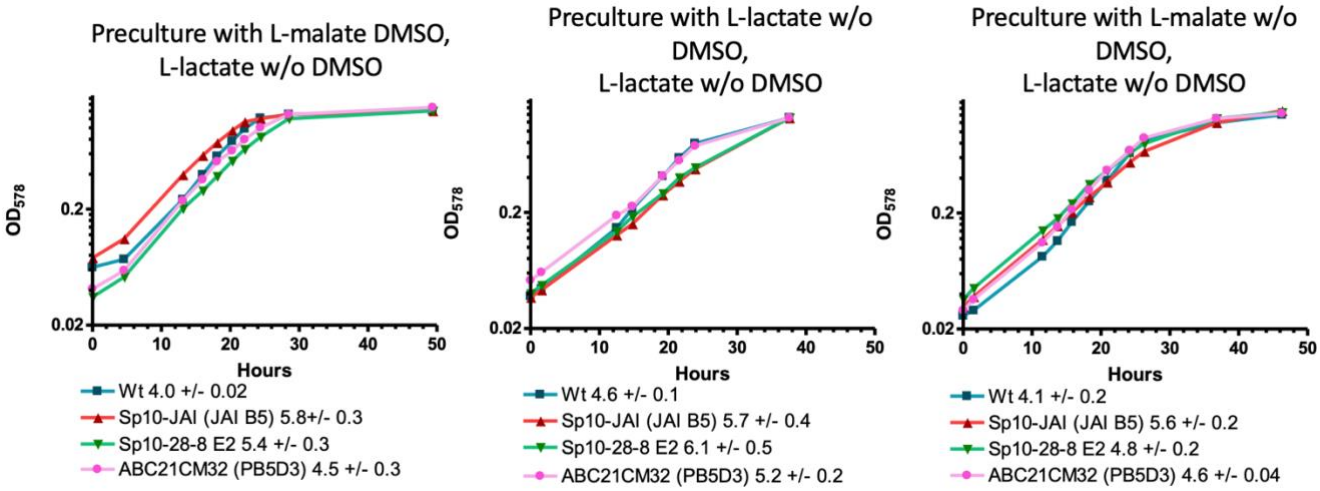


Figure 3.9: Phototrophic growth of wild type and complex I mutant strains with D-lactate as the carbon source. The graph to the left shows an experiment where DMSO is included in both the preculture and experiment and was performed 5/25. The graph in the middle shows an experiment where DMSO is neither in the experiment nor the preculture, and the preculture contains L-lactate and was performed 5/27. The graph to the right shows an experiment where DMSO is neither in the experiment nor the preculture, and the preculture contains L-malate and was performed 5/29. The numbers next to the strain names are the calculated growth rate +/- the standard deviation.

All mutants grew slower than the wild type when they were grown aerobically with acetate as the carbon source. When they were grown phototrophically, they showed the same growth rate as the wild type, as shown in figure 3.10.

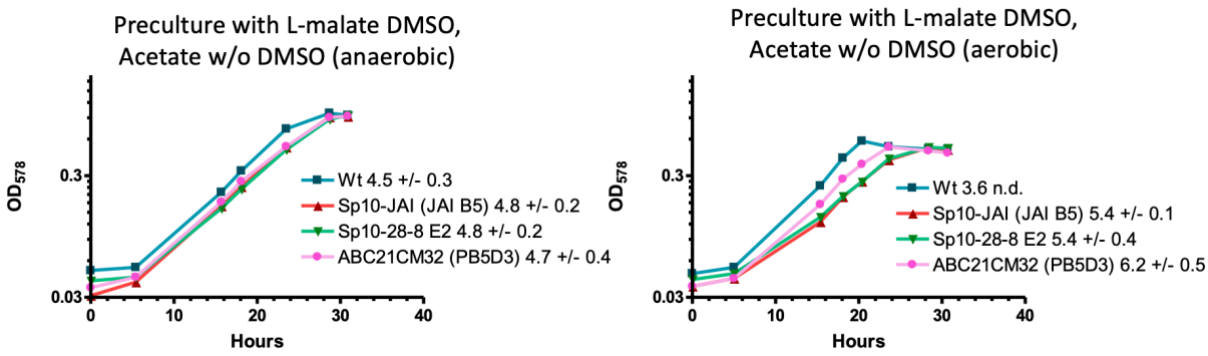


Figure 3.10: Phototrophic growth (left) and aerobic growth (right) of wild type and complex I mutant strains with acetate as the carbon source, performed 4/21. Both graphs show experiments where DMSO is present in the preculture, but not in the experiment. The numbers next to the strain name are the calculated growth rate +/- the standard deviation. N.d. stands for not determined and is given when the standard deviation is too high to give an accurate growth rate.

The final experiment, shown in figure 3.11, was with D-lactate w/o DMSO, and the preculture was also D-lactate w/o DMSO. In this experiment, the cells had been transferred to D-lactate twice before the experiment, to prevent DMSO carryover from the previous nine transfers. All mutants grew slower than the wild type and they also grew slower than when the cells had only been transferred to D-lactate once, like in figure 3.7.

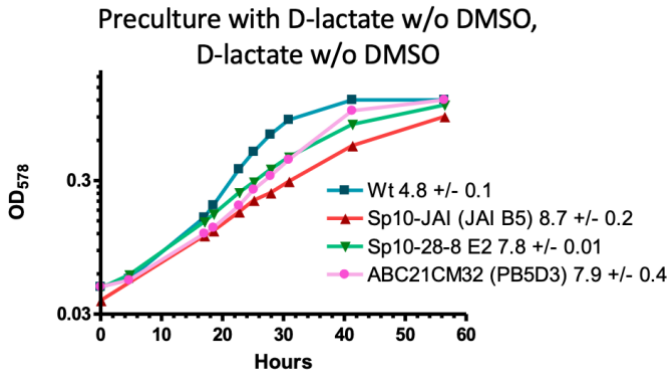


Figure 2.11: Phototrophic growth of wild type and complex I mutant strains with D-lactate as the carbon source. DMSO is neither present in the experiment nor the preculture. The numbers next to the strain name are the calculated growth rate +/- the standard deviation.

4 Discussion

4.1 Transposon mutagenesis

A transposon mutagenesis experiment was done to see if any of the random mutations obtained would affect *C. sphaeroides´* ability to grow with any of the chosen carbon substrates. As shown in table 3.1, the mutation rate is higher when grown with MM L-malate than with MM acetate. A possible explanation for this is that *C. sphaeroides* generally grows slower with acetate than with L-malate, and a slower growth rate with acetate can lead to a decrease in conjugation and transposition efficiency. Another explanation is the technique. The plating of the cells was done by two different people for the two different types of plates. If one is more "aggressive" with the plating, fewer cells will grow.

Not much is known about the genes where the transposon was inserted in Sp22EF-138, Sp22RG-53 or Sp22VRL-60.

The gene interrupted in Sp22RG-53 is annotated to encode a probable peptidase, which catalyzes proteolysis, meaning that it breaks proteins into smaller peptides or single amino acids.

The gene interrupted in Sp22VRL-60 encodes a membrane protein. The gene is located directly next to genes that code for a putative tartrate dehydratase alpha subunit and is likely transcribed with it. Tartrate dehydratase is an enzyme that takes part in glyoxylate and dicarboxylate metabolism.

It is unknown if the genes interrupted have an effect on the use of the carbon substrates tested in this project.

To determine if the interrupted genes do have a significant effect on *C. sphaeroides*' ability to use acetate as a carbon source, the phenotype should be seen again in a different experiment, either by performing a clean in-frame deletion of the gene or by mapping another transposon to the same gene on a different mutant.

4.2 In-frame deletion of *nuoE* on the gene cluster for complex I_E

A clean in-frame deletion of *nuoE* on the gene cluster coding for complex I_E was performed to see what impact that will have on the assimilation of different carbon substrates.

In theory, there should be a 50 % chance to obtain an upstream- or downstream crossover once the plasmid has been introduced into the cell, because the flanking regions of both the upstream- and downstream crossovers are the same length (1.3 kb). However, based on the results shown in table 3.4, there seems to be a favor in crossovers at the upstream flanking

region. One single crossover behaved like a wild-type *C. sphaeroides*. One explanation for this is that only 8 out of hundreds of colonies were picked. If the population was bigger than 8, a more accurate crossover rate could have been determined.
The PCR worked with colonies, but it did not work as well with isolated DNA. A possible explanation for this is that the volume-to-concentration ratio is wrong. However, the PCR still did not work when the concentration was measured, and the volume was adjusted. Another theory is that the genomic DNA should be eluted with water instead of elution buffer in 2.3.5.1. This is because the elution buffer contains EDTA, which can cause an interruption in the PCR reaction because it can bind Mg^{2+} which functions as a cofactor for DNA polymerase activity by enabling the incorporation of dNTPs during polymerization. This possibility was not tested out due to lack of time.
The next step would be to obtain a double crossover mutant, and then determine the genotype, using PCR and the phenotype, by patching them on plates with different carbon sources.

4.3 Complex I_A and complex I_E mutant strains

Because in-frame deletion strains were not available, three transposon mutants were phenotyped with growth experiments.
The mutants grew slower aerobically and with the same efficiency as the wild type anaerobically, with L-malate as the carbon source. Spero et al. argue that complex I_A mutants grow slower with all the tested carbon substrates when they are grown aerobically, which is consistent with the complex I_A mutant in this thesis. However, their complex I_E mutant behaved the same as wild type, whereas the complex I_E mutant in this project also grew slower with L-malate aerobically. A reason for this could be that the gene *nuoN* gene in the gene cluster coding for complex I_E is more important for aerobic growth than the gene *nuoG*, which was interrupted in the paper of Spero et al., is. [13]

The complex I_E mutants grew significantly slower than the wild type with succinate when the preculture did not include DMSO, both aerobically and anaerobically. In this case, it appears that the preculture for the experiment matters, and to get an accurate result, the preculture should not contain DMSO. The complex I_A mutant grew with the same efficiency as the wild type, regardless of DMSO, both aerobically and anaerobically. This is contradictory to the data in Spero et al.´s paper, as their complex I_A mutant strain grew slower with succinate aerobically, whereas the complex I_E mutant strain had the same growth rate as the wild type.

According to figure 2 in their paper, complex I_A should also have a slower growth rate in succinate, when it is grown anaerobically in the light, which is not the case in this experiment [13].

The mutants grew with the same efficiency as the wild type with D-lactate when they were grown aerobically. This is an especially striking result for the complex I_A mutant strain, which was originally phenotyped to be D-lactate negative. The mutant should be D-lactate negative according to figure 7 in the paper of Spero et al. as well. The complex I_E mutant strains grew slower than the wild type when it is grown anaerobically in the light, and DMSO is not included in the preculture. The complex I_A mutant strain also grows slower than the wild type when the preculture does contain DMSO. According to figure 2 in the paper of Spero et al., all mutants should, however, have a much slower growth rate than it does in this experiment [13].

The mutants grew with the same efficiency as the wild type with L-lactate when they were grown aerobically. This is also inconsistent with the data in Spero et al.´s paper, as the complex I_A mutant should have a slower growth rate than the wild type [13]. The complex I_A mutant strain also grew slower with L-lactate aerobically when the growth experiment was done last year. It is, however, consistent with their data for a complex I_E mutant. The mutants, especially the complex I_E mutants, grew slower than the wild type anaerobically with L-lactate as the carbon source, both when the preculture contained DMSO and when it did not contain DMSO. This is consistent with previous data, but the growth rates are not nearly as slow as the mutants of Spero et al. were.

The growth experiment with acetate did not include a preculture without DMSO. However, even with DMSO in the preculture, all the mutants grew slower than the wild type aerobically. The mutants grew with the same efficiency as the wild type anaerobically in the light. Acetate was not tested in the paper by Spero et al. or when the growth experiment was done last year, so there is no data to compare it to. From this experiment alone, it therefore seems like both complex I_A and complex I_E are important for aerobic growth with acetate.

Overall, the growth rate for the different mutant strains do not differ much from the wild type. A likely explanation for this is that the strains were transferred in media which included DMSO nine times before the growth experiments were done, this can lead to suppression.

The final experiment done with D-lactate anaerobically, where the cells had been transferred to D-lactate without DMSO twice before the experiment, shows that there is a significant difference when the cells had been transferred twice to D-lactate, before the growth experiment with D-lactate was done, as all the mutants grew slower than when it had only been transferred once to D-lactate. The fact that the mutants tended to grow slower when DMSO was not included in the preculture also backs this theory up. There is therefore a significant risk of DMSO carryover when the cells are transferred from a medium which contains DMSO. When the growth experiment for the complex I_A mutant strain was done last year, it was executed right after reviving the mutant from a stock. For future growth experiments, the cells should therefore be tested right after they are revived, or if they must be transferred multiple times, the media should not contain DMSO.

4.4 Conclusion

By looking at the results from the growth experiment, it is concluded that an accurate phenotype was likely not given to the complex I_E mutant (Sp10-28-8 E2/Sp10-JAI), because of the DMSO carryover and suppression that came with transferring the cells multiple times. The complex I_A mutant had already been given a phenotype when it was isolated last year, and this phenotype did not match the results of the growth experiment done in this project. The genes that were interrupted by the transposon insertion in the mutants Sp22EF-138, Sp22RG-53 and Sp22VRL-60 have an unknown function in *C. sphaeroides*' ability to use acetate as a carbon source. This will remain unsolved until the same phenotype and genotype are seen again in a different experiment. The function of *nuoE* in the gene cluster coding for complex I_E remains unsolved until a double crossover mutant can be obtained, and the mutant can be genotyped and phenotyped.

5 Bibliography

[1] "*Rhodobacter sphaeroides* - an overview | ScienceDirect Topics." https://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/rhodobacter-sphaeroides (accessed Apr. 28, 2022).

[2] C. Mackenzie, A. E. Simmons, and S. Kaplan, "Multiple chromosomes in bacteria. The yin and yang of trp gene localization in *Rhodobacter sphaeroides* 2.4.1.," *Genetics*, vol. 153, no. 2, pp. 525–538, Oct. 1999.

[3] A. Suwanto and S. Kaplan, "A self-transmissible, narrow-host-range endogenous plasmid of *Rhodobacter sphaeroides* 2.4.1: physical structure, incompatibility determinants, origin of replication, and transfer functions.," *J Bacteriol*, vol. 174, no. 4, pp. 1124–1134, Feb. 1992.

[4] M. Ahmad, A. Wolberg, and C. I. Kahwaji, "Biochemistry, Electron Transport Chain," in *StatPearls*, Treasure Island (FL): StatPearls Publishing, 2022. Accessed: May 23, 2022. [Online]. Available: http://www.ncbi.nlm.nih.gov/books/NBK526105/

[5] S. H. Zinder and T. D. Brock, "Dimethyl sulfoxide as an electron acceptor for anaerobic growth," *Arch Microbiol*, vol. 116, no. 1, pp. 35–40, Jan. 1978, doi: 10.1007/BF00408731.

[6] "Oxidative phosphorylation | Biology (article) | Khan Academy." https://www.khanacademy.org/_render (accessed Jun. 03, 2022).

[7] U. Kappler and H. Schäfer, "Transformations of Dimethylsulfide," in *The Metal-Driven Biogeochemistry of Gaseous Compounds in the Environment*, P. M. H. Kroneck and M. E. S. Torres, Eds. Dordrecht: Springer Netherlands, 2014, pp. 279–313. doi: 10.1007/978-94-017-9269-1_11.

[8] U. Ermler, G. Fritzsch, S. K. Buchanan, and H. Michel, "Structure of the photosynthetic reaction centre from *Rhodobacter sphaeroides* at 2.65 å resolution: cofactors and protein-cofactor interactions," *Structure*, vol. 2, no. 10, pp. 925–936, Oct. 1994, doi: 10.1016/S0969-2126(94)00094-8.

[9] J. V. Vermaas, A. T. Taguchi, S. A. Dikanov, C. A. Wraight, and E. Tajkhorshid, "Redox Potential Tuning through Differential Quinone Binding in the Photosynthetic Reaction Center of *Rhodobacter sphaeroides*," *Biochemistry*, vol. 54, no. 12, pp. 2104–2116, Mar. 2015, doi: 10.1021/acs.biochem.5b00033.

[10] "Home - *Rhodobacter sphaeroides* 2.4.1." https://genome.jgi.doe.gov/portal/rhosp/rhosp.home.html (accessed Jun. 09, 2022).

[11] A. Galkin, S. Dröse, and U. Brandt, “The proton pumping stoichiometry of purified mitochondrial complex I reconstituted into proteoliposomes,” *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, vol. 1757, no. 12, pp. 1575–1581, Dec. 2006, doi: 10.1016/j.bbabio.2006.10.001.

[12] L. K. Sharma, J. Lu, and Y. Bai, “Mitochondrial Respiratory Complex I: Structure, Function and Implication in Human Diseases,” *Curr Med Chem*, vol. 16, no. 10, pp. 1266–1277, 2009.

[13] M. A. Spero, J. R. Brickner, J. T. Mollet, T. Pisithkul, D. Amador-Noguez, and T. J. Donohue, “Different Functions of Phylogenetically Distinct Bacterial Complex I Isozymes,” *Journal of Bacteriology*, vol. 198, no. 8, pp. 1268–1280, doi: 10.1128/JB.01025-15.

[14] A. Dupuis, A. Peinnequin, E. Darrouzet, and J. Lunardi, “Genetic disruption of the respiratory NADH-ubiquinone reductase of *Rhodobacter capsulatus* leads to an unexpected photosynthesis-negative phenotype,” *FEMS Microbiology Letters*, vol. 148, no. 1, pp. 107–114, Mar. 1997, doi: 10.1111/j.1574-6968.1997.tb10275.x.