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An Investigation into Membrane Bound Redox Carriers Involved in Energy Transduction Mechanism in *Brevibacterium linens* DSM 20158 with Unsequenced Genome

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Abstract *Brevibacterium linens* (*B. linens*) DSM 20158 with an unsequenced genome can be used as a non-pathogenic model to study features it has in common with other unsequenced pathogens of the same genus on the basis of comparative proteome analysis. The most efficient way to kill a pathogen is to target its energy transduction mechanism. In the present study, we have identified the redox protein complexes involved in the electron transport chain of *B. linens* DSM 20158 from their clear homology with the shot-gun genome sequenced strain BL2 of *B. linens* by using the SDS–Polyacrylamide gel electrophoresis coupled with nano LC–MS/MS mass spectrometry. *B. linens* is found to have a branched electron transport chain (Respiratory chain), in which electrons can enter the respiratory chain either at NADH (Complex I) or at Complex II level or at the cytochrome level. Moreover, we are able to isolate, purify, and characterize the membrane bound Complex II (succinate dehydrogenase), Complex III (menaquinone cytochrome *c* reductase cytochrome *c* subunit, Complex IV (cytochrome *c* oxidase), and Complex V (ATP synthase) of *B. linens* strain DSM 20158.

Keywords Redox proteins · Cytochromes · Isolation · Purification · Characterization · Proteomics

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Introduction

Electron transport chains (ETC) are redox reactions that transfer electrons from an electron donor to an electron acceptor. The transfer of electrons is coupled to the translocation of protons across a membrane, producing a proton gradient which is ultimately used for ATP generation. The prokaryotic ETC involves various electron donors and acceptors. Electrons can enter the ETC pathway at various dehydrogenase levels such as the quinone or mobile cytochrome level, whereas the route of entry of electrons in ETC in Brevibacterium flavum is at three points, the NADH level prior to menaquinones, at cytochrome *b*, and at cytochrome *c* point (Shvinka et al. 1979). An individual bacterium can carry out its respiratory chain through different pathways simultaneously. Also, bacteria can use a number of different electron donors; when organic matter is the energy source, the donor may be NADH or succinate, in which case electrons enter the electron transport chain via NADH dehydrogenase (similar to Complex I in mitochondria) or succinate dehydrogenase (similar to Complex II). Other dehydrogenases may be used to process different energy sources: formate

dehydrogenase, lactate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, and H_2 dehydrogenases. These dehydrogenases have been reported in the respiratory chain of Corynebacterium glutamicum which transfers electrons to menaquinones and which ultimately transfer these electrons to oxygen via terminal oxidases (Bott and Niebisch 2003). Quinones are mobile, lipid-soluble carriers that shuttle electrons (and protons) between large, relatively immobile macromolecular complexes embedded in the membrane. Bacteria use ubiquinone (the same quinone that mitochondria use) and related quinones such as menaquinone. Bacteria use a number of different mobile cytochrome electron carriers. Other cytochromes are found within macromolecules such as Complex III and Complex IV. They also function as electron carriers (Nicholls and Ferguson 2002). Brevibacterium linen, a gram positive actinobacterium, is well known for the production of amino acids, lipases, and proteases (Rattray and Fox 1999). Most studies of the organism have been carried out on its secretome, but still studies of its respiratory chain have yet to be attempted in order to understand its bioenergetic metabolism. The reason for the delay in its detailed study may be due to its incomplete genome sequence. Moreover, 16S rDNA sequence analysis also has shown the existence of heterogeneity in genome among different strains of *B. linens* (Bott and Niebisch 2003; Oberreuter et al. 2002). However, the present study has been carried out in order to further understand its ETC through the application of advanced proteomics techniques. To date, proteome analysis employing SDS–Polyacrylamide gel electrophoresis coupled with highly sensitive nano LC–MS/MS mass spectrometry has proven to be the one of the most powerful methods for identification of proteins in complex (Hahne et al. 2008).

Materials and Methods

Microorganism, Inoculum Preparation, Culture Conditions, and Analytical Methods

Brevibacterium linen DSM 20158 was used in the present study. The bacterial culture was maintained on a growth medium (M_1) containing (g/L) tryptone (5.0), yeast extract (5.0), glucose (10.0), NaCl (5.0), K_2HPO_4 (1.0), $MgSO_4.7H_2O$ (0.2), and agar, for 24 h at 37 °C (Rattray and Fox 1999) and then stored at 4 °C. Inoculum was prepared by transferring a loop full of cells from a culture slant to 50 mL of sterilized growth medium (M_1) without agar in an Erlenmeyer flask (250 mL). The 1 L growth medium was inoculated in a 5 L flask at 30 °C and 180 rpm shaking. The cultivation of *B. linens* was performed in 1 L of the above growth medium (M_1) in Erlenmeyer flask (5 L). The culture was harvested at the late exponential phase by centrifugation at 4,000 rpm for 30 min for biomass collection.

Membrane Protein Extracts

Frozen cells (about 40 g in a centrifugally packed state) were suspended in 200 mL of 25 mM Tris HCl buffer (pH 8.3), 1 mM EDTA and protease inhibitor cocktail (buffer A). The suspension was sonicated with a sonic oscillator (Soniprep 150 SANYO UK) at 12–14 kHz for a total period of 15 min with intervals of 1 min at 4 °C. After sonication, the suspension was subjected to centrifugation at 15,000 rpm at 4 °C for 15 min. The supernatant containing the membrane and cytoplasmic proteins was then ultracentrifugated at 35,000 rpm at 4 °C for 60 min. This produced a reddish cell membrane pellet, which was resuspended in buffer A containing 1 % (wt/vol) Triton X 100. The resulting mixture was stirred overnight to solubilize the cell membrane proteins. This suspension was then subjected to ultracentrifugation at 45,000 rpm at 4 °C for 45 min to give a reddish supernatant containing membrane proteins as previously reported (Ahmad et al. 2012; Shabbiri et al. 2010).

Purification of Respiratory Complexes

Reddish supernatant containing total membrane proteins was subjected to ion-exchange chromatography on a Hi-Trap HQ (anion exchange) column (4.0 by 16.0 cm) equilibrated with buffer A containing 1 % (wt/vol) Triton X-100. The column was washed with 1 % Triton X-100 buffer A, and then adsorbed respiratory enzymes were eluted using linear gradient of an increasing concentration of buffer B (25 mM Tris HCl buffer (pH 8.3), 1 mM EDTA, 1.0 M NaCl, and 1 % Triton X-100). The elutes with enzymatic activity were collected and dialyzed against 2 L of buffer A with 1 % Triton X-100 and each subjected separately to gel filtration with a Bio-Gel-P 100 (1.5 by 8.0 cm) column equilibrated with 10 mM Tris–HCl buffer (pH:8.4) containing 1 % Triton X-100 and 0.3 M NaCl. The brownish-red fractions were collected, dialyzed, and lyophilized to appropriate volume for using as purified preparations.

Polyacrylamide Gel Electrophoresis and nLC-ESI MS/MS Analysis

The purified enzymes were subjected to Native PAGE in the presence of Triton X-100 at 4 °C and stained with Coomassie brilliant blue and the modified heme staining reagent. For heme staining, gel was immersed in solution containing 1 % (w/v) ortho-toluidine, 10 % (v/v) glacial acetic acid, 80 % (v/v) methanol, and 1 % (v/v) hydrogen

peroxide, and checked for the presence of heme (Connelly et al. 1958; Kabashima and Sakamoto 2011). Apparent molecular weights of proteins were estimated by the method of Laemmli using SDS-contained polyacrylamide gel electrophoresis (Laemmli 1970). Each purified sample of solubilized membrane proteins was loaded upon 10.0 % SDS-PAGE along with a standard marker proteins ladder.

Each excised Coomassie stained gel band of respiratory enzyme complexes was cut into 1 mm cubes and then subjected to in-gel digestion, using a ProGest Investigator in-gel digestion robot (Genomic Solutions, Ann Arbor, MI) using standard protocols (Shevchenko et al. 1996). In brief, the gel cubes were destained by washing with acetonitrile and subjected to reduction and alkylation before digestion with trypsin at 37 °C. The peptides were extracted with 10 % formic acid and concentrated down to 20 μL using a Speed Vac (ThermoSavant). They were then separated using an UltiMate nanoLC (Dionex) equipped with a PepMap C18 trap & column, using a gradient of increasing acetonitrile concentration, containing 0.1 % formic acid (5–35 % acetonitrile in 180 min, respectively, 35–50 % in a further 30 min, followed by 95 % acetonitrile to clean the column). The eluent was sprayed into a QStar XL tandem mass spectrometer (ABSciex, Foster City, CA) and analyzed in Information Dependent Acquisition (IDA) mode, performing 1 s of MS followed by 3 s MS/MS analyses on the two most intense peaks seen by MS. These masses are then excluded from analysis for the next 60 s. MS/MS data for doubly and triply charged precursor ions was converted to centroid data, without smoothing, using the Analyst QS1.1 mascot.dll data import filter with default settings. The MS/MS data file generated was analyzed using the Mascot 2.1 search engine (Matrix Science, London, UK) against the NCBInr Oct 2010 (12138964 sequences) database with no species restriction. The data was searched with tolerances of 0.2 Da for the precursor and fragment ions, trypsin as the cleavage enzyme, one missed cleavage, carbamidomethyl modification of cysteines as a fixed modification and methionine oxidation selected as a variable modification. The Mascot search results were accepted if a protein hit included at least one peptide with a score above the homology threshold and one above the identity threshold.

Spectroscopy

Absorption spectra of respiratory proteins were recorded by UV–Visible (UV–Vis) spectrophotometry at room temperature in the visible range (380–650 nm), using a quartz cuvette. Each of the three complexes from *Brevibacterium linens* DSM 20158 were suspended in 10 mM Tris–HCl (pH 7.4) containing 1 % Triton X-100 separately. Cytochromes were oxidized with 25 mM potassium ferricyanide solution and reduced by adding 50 mM sodium dithionite. For obtaining ferrohemochrome spectra, the enzyme was suspended in 0.2 N NaOH and 5 % pyridine and then reduced with a small amount of sodium dithionite.

Physical and Chemical Measurements

The content of heme a, heme b, and heme c in the cytochromes was determined on the basis of millimolar extinction coefficients (ε_{mM}) of 26.7, 34.3 $mM^{-1}cm^{-1}$, and 29.2 mM^{-1} at the alpha peak of the pyridine ferrohemochromes of a, b, and c, respectively. Whereas, protein content was determined by the bicinchoninic acid assay protocol (Pierce, Rockford, IL, USA) with bovine serum albumin as a standard (Qureshi et al. 1998a, b).

Complex II (Succinate Dehydrogenase) Activity Assay

Succinate dehydrogenase also known as Complex II activity was measured at room temperature spectrophotometrically (UV–Vis Spectrophotometer. Hitachi U-2001) in time-scanned mode. The oxidation of succinate to fumarate with concomitant transport of an electron to DCIP dye (which act as electron acceptor) was determined by monitoring the decrease in absorbance at 600 nm for 5 min. The reaction mixture contained 9 μL of 0.1 M EDTA, 3 ml of 100 mM sodium phosphate buffer (pH 7.4), 159 μL of 1 mM DCIP, and 60 μL of 0.1 M sodium succinate. Addition of the enzyme (30–40 μL of Complex-II) initiated the reaction (Shabbiri et al. 2010).

Complex III (Menaquinol Cytochrome *c* Reductase) Activity Assay

The Das's method was used for preparing menaquinone samples (Das et al. 1989). In this method, lipids were extracted from whole cells (50 g of cell paste) by stirring with 400 mL of an acetone–methanol (1:1) mixture for 15 h. The extract was filtered to remove cell debris and concentrated to 10 ml at 40 °C. It was then extracted with petroleum ether by using a separating funnel. The resulting layer of petroleum ether was evaporated to dryness. The yellowish residue was dissolved in a small volume of hexane, and purified menaquinone was obtained from it by employing preparative thin-layer chromatography on silica gel GF-coated plates with benzene–hexane (1:1) as the developing solvent. In this system, menaquinone has an R_f value almost 0.7. Vitamin K2 (MK-4) (Sigma Chemical Co., St. Louis, Mo.) was used as a standard. Menaquinone was revealed by brief UV irradiation and eluted from the silica gel with chloroform. The purity of the menaquinone was checked by UV absorption spectroscopy before it was finally dissolved and stored in absolute ethanol or chloroform for further studies. Under the conditions of isolation,

the menaquinone was obtained in the fully oxidized form. Spectroscopic studies of menaquinone were performed by the method of Dunphy and Brodie (1971). The cytochrome *c* subunit of Complex III is also responsible in transferring electrons to the terminal oxidase, also known as Complex IV or cytochrome aa_3. This process can be studied by measuring $TMPDH_2$-oxidase activity using the method of Qureshi et al. (1998a). The standard reaction mixture (3 ml in total volume) contained 50 mM Tris HCl buffer, pH 7.5, 1 mM EDTA, 22 µL of the washed bacterial membrane fraction, and 1.8 mM $TMPDH_2$ (*N*,*N*,*N*',*N*'-tetramethyl-p-phenylenediamine) for assay of $TMPDH_2$-oxidase activity; this activity was measured by following the increase in absorbance at 610 nm Qureshi et al. (1998a).

Complex IV (Cytochrome *c* Oxidase) Activity Assay

To monitor cytochrome *c* oxidase activity, 2 mL reaction mixture contained 25 mM Tris buffer (pH 7.2), 1 mM EDTA, 22 µL of the washed bacterial membrane fraction, and 32 µM horse heart ferrocytochrome *c*. Cytochrome *c* oxidase activity was assayed by following the decrease in absorbance at 550 nm (Qureshi et al. 1998a, b).

Complex V (ATP Synthase) Activity Assay

To monitor the dependence of Complex V activity on NADH or succinate, 0.5 mg of membrane protein was pre-incubated with 2.5 mM NADH in 50 mM Tris–HCl pH 7.0 and 100 mM succinate in 50 mM Tris–HCl pH 7.0, respectively, for 2 min at room temperature (21 °C) in a final volume of 10 mL. After pre-incubation, initiation of ATP synthesis and its kinetics was studied by the luciferin/luciferase assay as described by Tomashek et al. (2004). ATP synthesis was initiated by the addition of 50 µL each of ADP and Pi into the 10 mL of the above pre-incubated solution with succinate. Then, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 µL aliquots were taken out and transferred to the 400 µL of stop solution (1 % trichloroacetic acid, 2 mM EDTA). For measuring ATP amount in each sample, in luminometer cuvette, 100 µL of 50 mM Tris–HCl, 2 mM EDTA, pH 7.0 buffer, and 50 µL of luciferin/luciferase reagent (ATP bioluminescence assay kit CLS II, Roche Applied Science) were added to read the background signal (typically 0.0–0.1 mV) from the luminometer. The peak signal in the luminometer was read by adding the 2 µL of the stopped reaction mixture. The new signal was then read by adding the 10 µL of ATP (1.7 pmol of ATP) as an internal standard. The synthesized amount of ATP was calculated from a standard curve and corrected for quenching based on the internal standard.

Results and Discussion

Purification of Respiratory Complexes from *B. linens* DSM 20158

In order to understand the respiratory mechanism of the aerobic gram positive bacterium, B. linens DSM 20158, it was necessary to isolate and purify the major components of its electron transport mechanism. In bacteria, when the energy source is organic matter, electrons enter the ETC via NADH dehydrogenase (Complex I) or succinate dehydrogenase (Complex II) (White 2007). In the present study, it was found that electrons enter the respiratory chain through Complex II rather than NADH dehydrogenase (Complex I) as confirmed further by the independence of ATP synthase on NADH in *B. linens* DSM 20158.

Succinate dehydrogenase (Complex II) is a membrane bound respiratory complex and is associated with cytochrome *b*, menaquinole cytochrome *c* reductase, cytochrome *c* subunit (Complex III), cytochrome *c* oxidase (Complex IV), and ATP synthase (Complex V), all of which are also membrane bound. Various detergents were surveyed for their utility in the purification of these complexes, and Triton X-100 was found to be the best for solubilization of these complexes in an active form through a simple, single detergent solubilization technique. Following solubilization with Triton X-100 in an active form, these protein complexes were purified by ion-exchange chromatography followed by gel filtration. Purification factors and yields of Complex II are shown in the Table 1, starting from solubilized membranes containing a total of 215 mg of protein and 292 nmol of total heme b. Table 2 is a representative purification of Complex III consisting of menaquinol-cytochrome *c* reductase, cytochrome *c* subunit. The final amount of membrane bound cytochrome *c* was 6.4 nmol/mg of protein. Cytochrome *c* oxidase purification factors are represented by Table 3 showing a final yield of

Table 1 Purification summary of *B. linens* membrane bound cytochrome *b*

Step	Total vol. (ml)	Total Protein (mg)	Total heme b (nmol)	Heme b protein (nmol/mg)	Yield (%)
Solubilized membranes	75	215	292	1.35	100
Hi-Trap HQ (anion exchange)	25	37	123	3.32	42
Gel filtration	12	9.2	46.2	5.02	16

Table 2 Purification summary of *B. linens* cytochrome *c*

Step	Total vol. (ml)	Total protein (mg)	Total heme c (nmol)	Heme c protein (nmol/mg)	Yield (%)
Solubilized membranes	75	215	344	1.6	100
Hi-Trap HQ (anion exchange)	22	39	167	4.3	49
Gel filtration	10	9.8	62.8	6.4	18

Table 3 Purification summary of *B. linens* (Complex IV) cytochrome *c* oxidase

Step	Total vol. (ml)	Total protein (mg)	Total heme aa_3 (nmol)	Heme aa_3 protein (nmol/mg)	Yield (%)
Solubilized membranes	75	215	559	2.6	100
Hi-Trap HQ (anion exchange)	20	41	359.1	8.75	64
Gel filtration	8	9.2	241.6	26.26	43

Table 4 Purification summary of *B. linens* ATP synthase

Step	Total vol. (ml)	Total protein (mg)	Activity (U)	Specific activity (U/mg)	Yield (%)
Solubilized membranes	75	215	156	0.72	100
Hi-Trap HQ (anion exchange)	20	45	105	3.0	7
Gel Filtration	8	8.2	41	5.0	26

43 % of Complex IV. Purification of Complex V was started with solubilized membranes containing a total amount of 215 mg of protein and 156 U of total ATP synthase activity and after purification giving yield of 26 % in the final purification step (Table 4).

Polyacrylamide Gel Electrophoresis and Mass Spectrometric Analysis of Respiratory Complexes

Purified fractions in Triton X-100 at 4 °C were subjected to Native PAGE and stained with either Coomassie brilliant blue or the heme staining reagent. Enrichment of the various complexes is shown in the Fig. 1a–g. Individual subunits from each of the protein complexes were solubilized and separated by 10 % SDS-PAGE as shown in Fig. 2a–d. Each of the pure respiratory complexes were run on SDS-PAGE and gel bands excised and then subjected to trypsin digestion followed by nano LC-ESI MS/MS analysis on a QStar XL and were identified by the MASCOT search algorithm as shown in Table 5. The b-type heme is covalently attached to protein as the band could be seen by heme staining. Complex II sample showed prominent protein stained bands of about 62-, 30-, and 12-kDa in the membrane fraction of B. linens as shown in Fig. 2a. This resembles the type of Complex II found in other gram positive bacteria (Bott and Niebisch 2003; Qureshi et al. 1996). The cytochrome *bc* subunit of the menaquinol cytochrome *c* reductase complex is found in many gram positive bacteria such as Bacillus subtilis and is 22 kDa (Yu et al. 1995). It also has been reported that *Rhodothermus marinus*, a thermohalophilic aerobe, has an unusual Complex III lacking a Rieske-type FeS center (Pereira et al. 1999). Schilling et al. (2006) analyzed the proteome of respiratory complexes (Complex II and III) from bovine and mouse heart mitochondria through SDS-PAGE followed by nLC-ESI MS/MS and MALDI-TOF MS. They found a higher mass spectrometric sequence coverage of these complexes by nLC-ESI MS/MS than MALDI-TOF MS (Schilling et al. 2006). Here, in *B. linens*, menaquinol cytochrome *c* reductase cytochrome *c* subunit was identified by mass spectrometry with a single band of about 25 kDa (Fig. 2b), and cytochrome *c* oxidase was found to be composed of two subunits at 62- and 32-kDa (Fig. 2c) identified by mass spectrometry. F_1F_o-ATP synthase is essential for ATP generation by oxidative phosphorylation. Usually the three components of the membrane integral F_o part are a-, b-, and c-subunits; whereas the five components of the peripheral F_1 part are α-, β-, δ-, γ-, ε-subunits (Bott and Niebisch 2003). However, in the present study, in-gel digestion followed by mass spectrometry of gel bands running at 59, 52, 33, 9, 20, and 7 kDa revealed that ATP synthase (Complex V) consisted of α-, β-, γ-, ε-, b-, and c-subunits, respectively (Fig. 2d). An a-subunit was not identified, possibly due to fusion with subunit c of F_o but this remains to be determined as the process of F_o assembly is not still fully known (Pierson et al. 2011). The delta subunit of the ATP synthase complex also is missing, which may be due to defective F_1F_o-ATP synthase as also

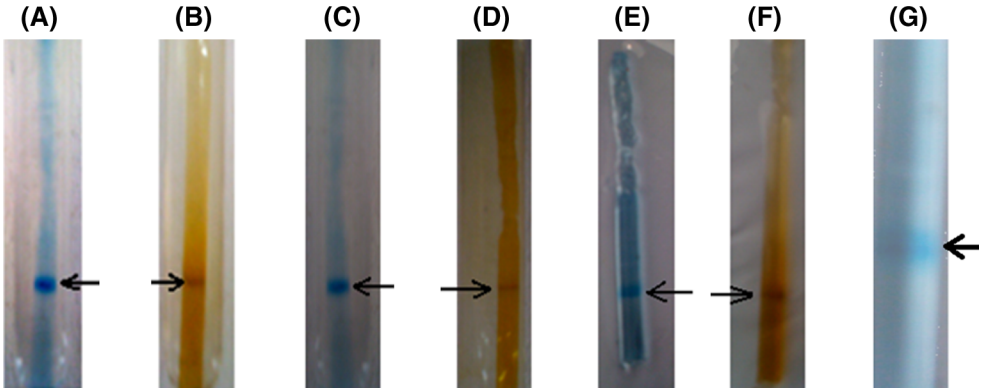

Fig. 1 Native PAGE of Complex II, III, IV, and V **a** CBB staining of Complex II, **b** heme staining of Complex II showing presence of heme b, **c** CBB staining of Complex III, **d** heme staining of Complex III showing presence of heme c, **e** CBB staining of Complex IV, **f** heme staining of Complex IV showing presence of heme aa_3, **g** CBB staining of Complex V

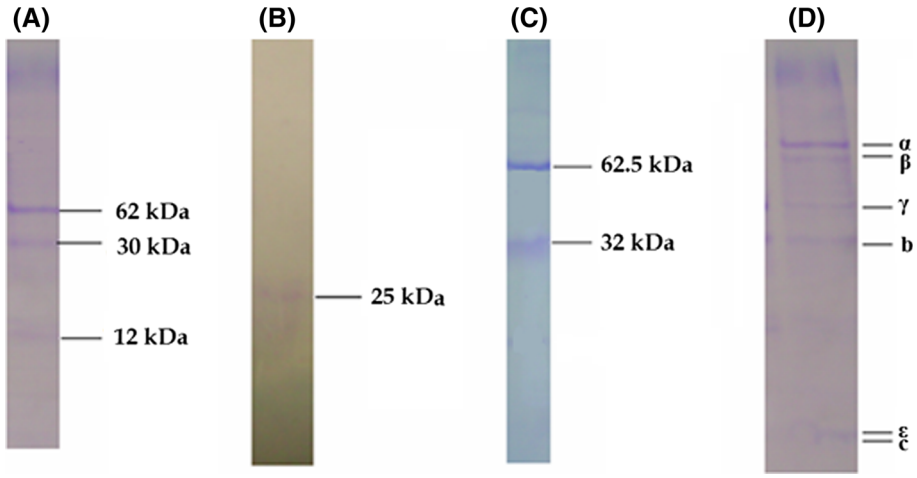

Fig. 2 SDS-PAGE of ETC complexes isolated **a** Complex II, **b** Complex III cytochrome *c* subunit, **c** cytochrome *c* oxidase, and **d** ATP synthase

Table 5 Proteins identified from *B. linens* DSM 20158 by Mascot by similarity to shot-gun genome sequence of *B. linens* BL2

Accession no	Mass	Protein	Fig. reference	Protein score	Peptides matched	% Coverage
gil260907392	64873	succinate dehydrogenase flavoprotein subunit	Fig. 2a	814	11	19
gil260907394	12281	succinate dehydrogenase cytochrome *b* subunit		177	2	27
gil260907391	30445	succinate dehydrogenase iron-sulfur subunit		675	9	39
gil260906797	25927	menaquinol cytochrome *c* reductase cytochrome *c* subunit	Fig. 2b	311	4	30
gil260906804	62800	cytochrome *c* oxidase, subunit I	Fig. 2c	112	2	5
gil497570464	32414	cytochrome *c* oxidase, subunit II		121	3	7
gil260906205	59033	F_0F_1 ATP synthase subunit alpha	Fig. 2d	904	11	30
gil260906206	33263	F_0F_1 ATP synthase subunit gamma		311	5	16
gil260906208	9060	F_0F_1 ATP synthase subunit epsilon		164	2	29
gil260906207	52667	ATP synthase F_1, beta subunit		1329	15	52
gil260906203	20146	ATP synthase F_0, B subunit		361	2	42
gil497569865	7298	ATP synthase, subunit C		261	3	58

reported previously (Bott and Niebisch 2003; Sekine et al. 2001). The above identified proteins are involved in the respiratory mechanism of *B. linens* DSM 20158. Most of these complexes are membrane bound (Bott and Niebisch 2003; Shabbiri et al. 2013).

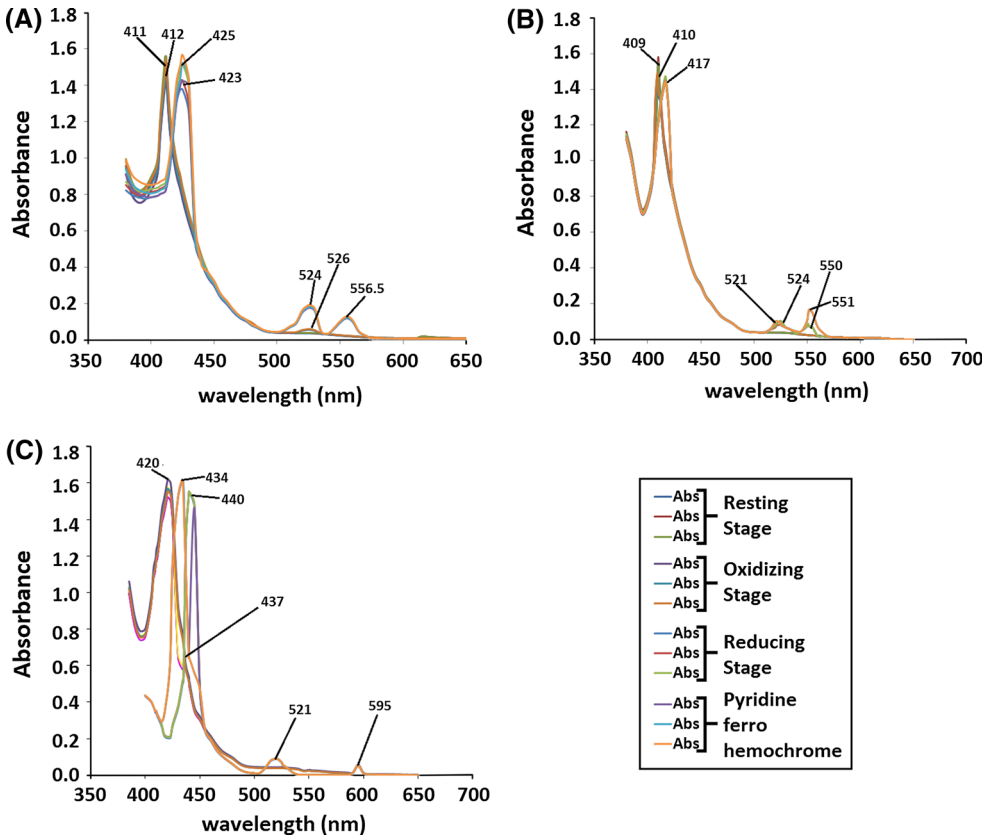

Fig. 3 **a** Oxidized/reduced absorption spectra of Complex II showing the presence of cytochrome *b*. The α-peak at 556.5 nm is specific to heme b. **b** Absorption spectra of Complex III showing the presence of cytochrome *c* subunit. All the measurements were performed in triplicate. **c** Absorption spectra of cytochrome *c* oxidase. The reduced spectrum showed an α-peak 595 nm, β-peak 521 nm, and γ-peak 440 nm that were further confirmed by addition of pyridine ferrohemochrome which shifted γ-peak at 434 nm. All the measurements were performed in triplicate

Spectral Properties of Complex-II

The absorption spectrum of Complex-II is shown in the Fig. 3a. This figure showed a single absorption peak at 411.0 nm that is characteristic peak for cytochrome *b* and was shifted to 412.0 nm in the oxidized state. This shift in absorption peak from resting to oxidizing state was not significant showing that cytochrome *b* was in the oxidized form in resting state. To further confirm the presence of cytochrome *b*, the enzyme was reduced with the addition of sodium dithionite which gave peaks at 556.5, 524, and 423 nm as shown in Fig. 3a. The α-peak at 556.5 nm in the reduced form is a characteristic peak of heme b that was further confirmed by the pyridine ferrohemochrome spectrum. Pyridine ferrohemochrome is a diagnostic test to identify ferroheme containing proteins. The spectral analysis of Complex II suggests that it comprises of a single heme b. This study corroborates with other studies (Ahmad et al. 2012; Qureshi et al. 1996; Shabbiri et al. 2010).

Spectral Properties of Menaquinone

The isolated menaquinone from *B. linens* was analyzed by UV absorption spectroscopy. The re-dissolved menaquinone isolated from the bacteria and commercially available menaquinone (MK) gave identical UV absorption spectra with Amax values at 242, 248, 260, 270, and 330 nm (Dunphy and Brodie 1971). The amount of menaquinone was determined to be about 13.8 nmol. The present study suggests that cytochrome *b* of Complex II could be the electron donor for menaquinones in Complex III.

Spectral Characterization of Membrane Bound Cytochrome *c* 551

Figure 3b depicts the absorption spectra of the membrane bound cytochrome *c* when oxidized by potassium ferricyanide giving a single absorption peak at 409 nm. In comparison, the absorption peak is at 410 nm in the resting state, and when reduced by sodium dithionite shows an α-peak at 551 nm, β-peak at 521 nm, and γ- peak at 417 nm. In the pyridine ferrohemochrome spectrum, the α-peak was shifted to 550 nm whereas the β and γ-peaks remained the same. However, this shift in α-peak was not significant and these results are in agreement with that of Qureshi et al. (1998a, b) for membrane bound cytochrome *c*-551 Qureshi et al. (1998a).

Spectral Properties of Cytochrome *c* Oxidase

The absorption spectra of cytochrome *c* oxidase (Fig. 3c) showed a single absorption peak at 420 nm and a broad

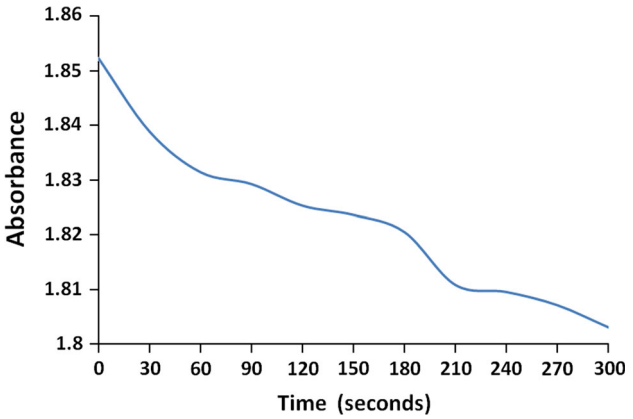

Fig. 4 Redox activity of Complex II. The oxidation of succinate to fumarate with concomitant transport of an electron to DCIP dye was determined by monitoring the decrease in absorbance at 600 nm for 5 min

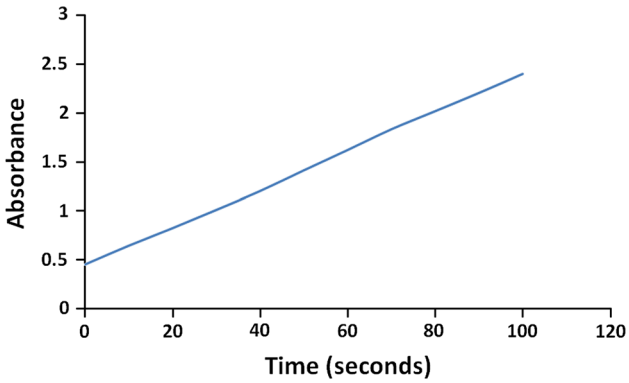

Fig. 5 $TMPDH_2$ activity showing the presence of Complex III

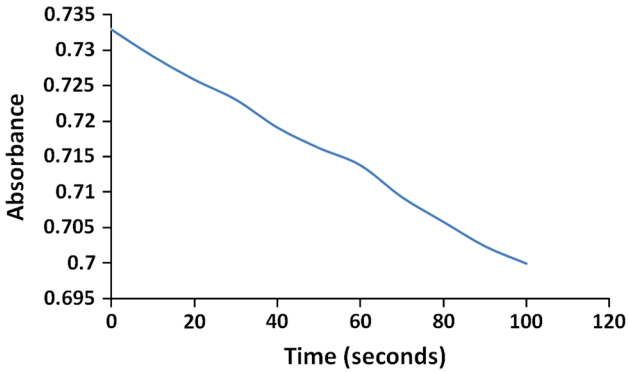

Fig. 6 Redox activity of cytochrome *c* oxidase. 32 μM of horse heart ferrocytochrome *c* was added to purify bacterial membrane fraction containing cytochrome *c* oxidase. A decrease in absorbance at 550 nm was revealed activity of cytochrome *c* oxidase

hump at 595 nm in the resting state, which is consistent with the single absorption peak at 420 nm in the oxidized state. This indicates the auto-oxidizable nature of cytochrome *c* oxidase (Lauraeus et al. 1991). The reduced spectrum showed an α-peak at 595 nm, β-peak at 521 nm, and γ-peak at 440 nm, whereas the pyridine ferrohemochrome spectrum further confirmed the presence of this terminal oxidase by showing an α-peak at 595 nm, β-peak at 521 nm, and γ-peak at 434 nm.

Redox Activity of Complex II

The enzymatic properties of the *B. linens* succinate dehydrogenase were analyzed using succinate as electron donor and DCIP as electron acceptor. *B. linens* succinate dehydrogenase catalyzes the reduction of menaquinone (MK), a low potential quinone, and thus belongs to subclass 3. Succinates, MK reductase of *B. subtilis* (Hederstedt 2002) and MKH2: fumarate reductase of *W. succinogenes*, are also members of this subclass (Kroger et al. 2002). It had been reported previously that usually electron transport from succinate to menaquinone involves two heme b groups (Schirawski and Unden 1998), but in the present study, we have found one heme b group which is associated with succinate dehydrogenase. On the basis of total heme b content, the recovery in the purification was calculated to be about 5.2 nmol/mg of heme b, which yields 15 % of the total solubilized proteins. For further investigation and confirmation of the presence of cytochrome *b* in *B. linens*, energy-linked reduction of cytochrome *b* has been studied when Complex II is fully reduced with a substrate such as succinate. It is well studied that Complex II is a membrane bound respiratory complex and is associated with cytochrome (Waldeck et al. 1997). The artificial electron acceptor DCIP was used to detect the enzymatic properties of *B. linens* succinate dehydrogenase. When sodium succinate (an electron donor) was added to the oxidized enzyme, there was decrease in absorbance of DCIP within 5 min due to the reduction of heme b of the enzyme (Fig. 4). The overall activity was quite prominent and notable.

Assay Showing Presence of Complex III

Spectral analysis of Complex III shows the presence of menaquinones and cytochrome *c*. UV–Vis spectrum (Fig. 5) of Complex III shows the presence of only one heme which is characteristic of cytochrome *c* and this is confirmed by the heme staining of this complex. Usually c-type cytochromes function as physiological electron donors to the terminal enzyme complexes in the electron transport chain. The high $TMPDH_2$ activity also indicated the presence of Complex III and its cytochrome *c* component acts as the electron carrier to the terminal cytochrome *c* oxidase (Fig. 6). Such results were also consistent with the studies performed by Qureshi et al. (1998a).

Redox Activity of Cytochrome *c* Oxidase

The prokaryotes respiratory electron transport system can utilize a variety of terminal oxidases which may vary in oxygen affinity, electron donor specificity, heme, cytochrome type, and metal composition. This diversity may lead to an unevenness of growth conditions, depending upon the availability of energy source and oxygen for different strains of the same bacterium and even for the same strain of the bacterium under different conditions. In *B. linens* BL2, under the present aerobic conditions, we were successful in purifying the cytochrome *c* oxidase as the terminal oxidase and identifying it by mass spectrometry. The possibility remains, however, that other terminal respiratory oxidases also exist in the DSM 20158 strain as the genome of this strain of *B. linens* is not sequenced yet and under these conditions, no significant hit was found for bd type terminal oxidase by mass spectrometry. Although there are some physiological dissimilarities, most terminal oxidases such as mitochondrial aa_3 cytochrome *c* oxidase belong to the superfamily of heme copper oxidases (Garcia-Horsman et al. 1994). It is a well-established fact that cytochrome *c* oxidase species which bind to oxygen receive electrons from cytochrome *c* pass them to a-CuA and then on to the a_3-CuB center and finally to oxygen (Lucas et al. 2011). Many studies have shown that Complex III contains a single heme c of 26 kDa, which shares significant sequence homology with a heme c domain of cytochrome caa_3-type terminal oxidases (Pereira et al. 2001). It also has been reported that a mitochondrial type

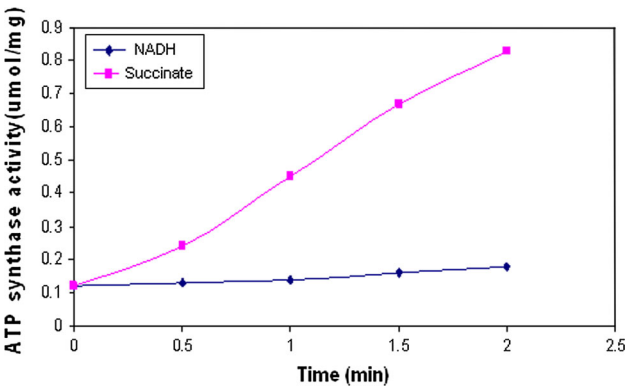

Fig. 7 Effect of NADH and succinate on time course of ATP synthesis. Purified fraction of bacterial ATP synthase was incubated with 2.5 mM NADH and 100 mM succinate, respectively, and monitor for ATP synthesis and relative kinetics by luciferin/luciferase assay

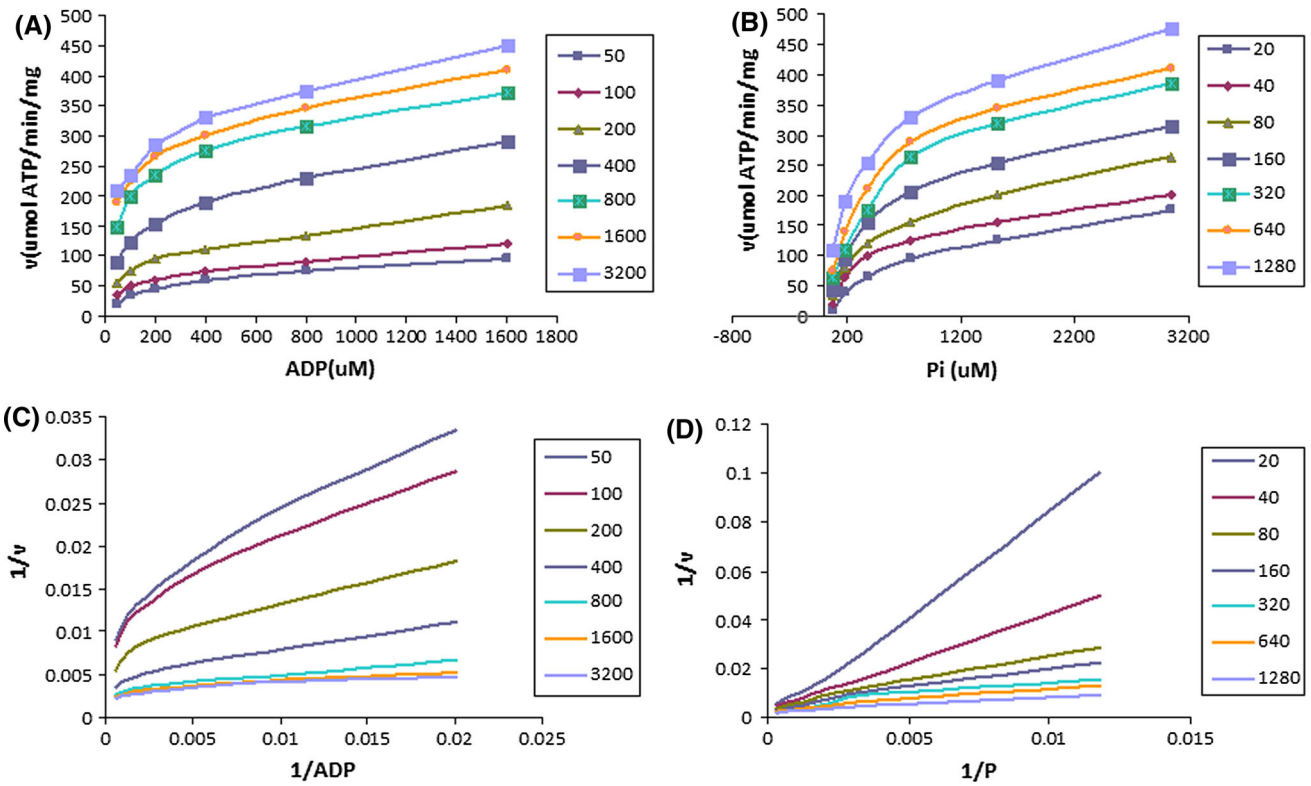

Fig. 8 Velocity data plots for ADP and P_i. A single membrane preparation was used for these assays. The velocity curves are shown for ADP (**a**) and P_i (**b**), while (**c**) and (**d**) represent double reciprocal plots for ADP and P_i, respectively. *Symbol key boxes* show the concentrations for second substrate in μM

mobile cytochrome *c* shuttles electrons from Complex III to Complex IV (Mooser et al. 2006). The *B. linens* isolated membrane fraction showed high-cytochrome *c* oxidase activity (Fig. 6), whereas $TMPDH_2$ activity (Fig. 5) has shown that the Complex III is also present and its cytochrome *c* component acts as the electron carrier to the terminal cytochrome *c* oxidase. This is consistent with the studies of Qureshi et al. (1998b). It is clear that the TMPD reaction monitors the transfer of electron from complex III to complex IV (cytochrome *c* oxidase) of the electron transport chain and bypasses the cytochrome *b* and earlier regions of the chain as supported by McEwen et al. (1985).

Activity of ATP Synthase

It was found in the present study that ATP synthase activity was not enhanced by the pre-incubation with NADH; however, activity was achieved on pre-incubation with 1.25 mM sodium succinate for at least one minute (Fig. 7). In bacteria, where the energy source is the organic matter, electrons enter the ETC via NADH dehydrogenase (Complex I) or succinate dehydrogenase (Complex II) (White 2007). In the present study, it was found that electrons enter the respiratory chain through Complex II rather than NADH dehydrogenase (Complex I). Furthermore, ATP synthase activity is independent of NADH in the case of B. linens DSM 20158. It has been proved by various studies that energization of respiratory chain either with NADH or succinate is successful for kinetic studies (Schirawski and Unden 1998).

Dependence of Velocity on ADP and P_i

Forty-nine ATP synthase assays were carried out to study the effect of ADP and Pi concentrations on the synthesis rate, with a matrix of seven concentrations for each substrate, covering a concentration range of 2 orders of magnitude for each. The primary velocity data are shown in Fig. 8a, b. The affinity of the enzyme for both ADP and P_i depends on the concentration of the other substrate. Figure 8c, d represent the double reciprocal plots. For ADP, the analytical plot appears to be biphasic due to non-linear curve (Fig. 8c), whereas for P_i, the analytical plots appear to be linear (Fig. 8d). The Km^{Pi} ranges from 140 to 460 μM depending upon the concentration of ADP. Due to the biphasic nature of ADP, we have determined $Km^{ADP<166} \sim 15$ uM and $Km^{ADP>166} \sim 70$ uM. Vmax as a function of P_i is almost 400 μM ATP/min/mg of protein, whereas the Vmax for ADP $\leq$ 166 at highest value of P_i (3200 μM) was 352 μM ATP/min/mg of protein, and Vmax for ADP $\geq$ 166 at highest value of P_i (3200 μM) was 510 μM ATP/min/mg of protein. These results are consistent with those of Tomashek et al. (2004).

Conclusions

Brevibacterium linens DSM 20158 is a unsequenced gram positive aerobic organism, used industrially for amino acid L-lysine production. Although many studies have linked the amino acid production by bacteria with its respiratory mechanism, still no work has been carried out in exploring the electron transport chain of this bacterium. The comparative proteome analysis of this strain with the shot-gun genome sequence of the B. linens BL2 gives us information about the involvement of various dehydrogenases, cytochromes, and terminal oxidases in the respiratory mechanism of this bacterium and also shows that there may be more than one pathway involved in carrying out its respiratory mechanism. The major components (Complexes II, III, IV, and V) of the respiratory chain we have identified in the membrane proteins of this strain were as confident matches to those of B. linens BL2. With the help of mass spectrometry, we were able to identify one pathway for the respiratory chain used by *B. linens* DSM 20158 under the specified conditions in the present study and purified them from this bacterial strain. These proteins were further confirmed by their spectroscopic properties, their characterization by SDS–PAGE, and mass spectrometric analysis followed by bioinformatics tools. The exploration of the respiratory chain suggests that amino acid production may be improved by engineering the system of oxidative phosphorylation. In the field of microbial amino acid production, this opinion is also shared by other scientists as well (Bott and Niebisch 2003).

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