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The composition of the *Bacillus subtilis* aerobic respiratory chain supercomplexes

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Abstract *Bacillus subtilis* has a bifurcated respiratory chain composed of a cytochrome branch and a quinol oxidase branch. The respiratory complexes of this bacterium have been elucidated mostly by the analysis of the genome and by the isolation of individual complexes. The supramolecular organization of this respiratory chain is not known. In this work, we have analyzed the organization of the supercomplex in membranes isolated from *B. subtilis* grown in aerobic conditions in a medium with 3 % succinate. We used two different native electrophoretic techniques, clear native electrophoresis (CNE) and blue native electrophoresis (BNE). Using a heme-specific stain and Coomassie blue stain with in-gel activity assays followed by mass spectrometry, we identified the proteins resolved in both the first and second dimensions of the electrophoreses to detect the supercomplexes. We found that complexes b_6c and caa_3 form a very high molecular mass supercomplex with the membrane-bound cytochrome c_{550} and with ATP synthase. Most of the ATP synthase was found as a monomer. Succinate dehydrogenase was identified within a high molecular band between F_0F_1 and F_1 and together with nitrate reductase. The type-2 NADH dehydrogenase was detected within a low molecular mass band. Finally, the quinol oxidase aa_3 seems to migrate as an oligomer of high molecular mass.

Keywords *Bacillus subtilis* · Respiratory chain · Supercomplexes

Abbreviations

CNE	Clear native electrophoresis
BNE	Blue native electrophoresis
SDH	succinate dehydrogenase
NADH DH	NADH dehydrogenase

Introduction

Bacillus subtilis is a rod-shaped, Gram-positive bacterium that inhabits the soil and can grow as an aerobe, or under anoxic conditions, it can employ either a fermentative metabolism or a respiratory type using nitrate or nitrite as terminal electron acceptors (Sonenshein et al. 2002). The aerobic respiratory chain of this bacterium is composed of several dehydrogenases, namely type-2 NADH dehydrogenase (NADH DH), succinate dehydrogenase (SDH) and glycerol 3-phosphate dehydrogenase, which reduce a menaquinone pool. Several respiratory complexes are involved in menaquinol oxidation. Electrons can flow to a b_6c complex, a membrane-bound cytochrome c_{550} and a cytochrome caa_3 oxidase (Lauraeus and Wikström 1993). Alternatively, menaquinol oxidases can transfer electrons directly to oxygen. *B.*

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subtilis has three quinol oxidases: an aa_3-600 oxidase, a *bd* oxidase and a *bb'* type oxidase (Azarkina et al. 1999).

The genes encoding the proteins of the oxidative phosphorylation and the alternative respiratory chains of *B. subtilis* have been identified in the complete genome (Kunst et al. 1997). The genome contains at least 37 open reading frames encoding polypeptides with similarity to proteins involved in oxidative phosphorylation and to alternative membrane oxidases (KEGG orthology). It is important to keep in mind the salient features of the *B. subtilis* respiratory chain. Protein YjlD is the type-2 NADH dehydrogenase (EC 1.6.99.3). SDH (EC 1.3.99.1) has three subunits: SdhA is the FAD-binding protein, SdhB is the iron-sulfur protein, and SdhC has two *b* hemes. SDH can alternate between two activities in *B. subtilis*: fumarate reductase and succinate dehydrogenase (Schnorpfeil et al. 2001). The menaquinol:cytochrome reductase (EC 1.3.99.18) or b_6c complex has unusual characteristics; it has three subunits, including a Rieske-type iron-sulfur protein, QcrA, a b_6 cytochrome (QcrB) with high similarity to the photosynthetic b_6 and a third subunit, QcrC, with two domains. The N-terminal domain of QcrC is homologous to subunit IV of the b_6f complexes of cyanobacteria and chloroplast, while the C-terminal domain is a *c*-type cytochrome (Yu et al. 1995; Yu and Le Brun 1998). The caa_3 oxidase (also known as caa_3-605, EC 1.9.3.1) has four subunits in cluster CtaCDEF. CtaC is a *c*-type cytochrome. The N-terminal domain of CtaC is homologous to subunit II of the cytochrome *c* oxidase from *Paracoccus denitrificans*, and the C-terminal domain is a *c*-type cytochrome. The quinol oxidase aa_3 (EC 1.10.3.12) has four subunits in cluster QoxABCD. This aa_3-600 oxidase pumps protons and is essential for growth (Winstedt and von Wachenfeldt 2000). The other two quinol oxidases do not pump protons but are required for growth depending on the oxygen concentration (Azarkina et al. 1999). Finally, there are two *c*-type cytochromes: c_{550} and c_{551}. The first contains a transmembrane helix, and the second is attached to a phospholipid (Bengtsson et al. 1999).

The association of the respiratory chain complexes to form supercomplexes has been observed in mitochondrial and bacterial respiratory chains. It has been claimed that the formation of supercomplexes lends kinetic advantages, such as the channeling of substrates and products to increase the efficiency of the electron transport and to prevent excess oxygen radical formation (Lenaz and Genova 2009). Supercomplexes also have a role in the stability and assembly of the individual complexes (Stroh et al. 2004).

Supercomplexes in the bacterial respiratory chains have been observed in Gram-positive bacteria including the thermophilic bacterium PS3, from which a quinol oxidase supercomplex containing the *bc*-type complex and the caa_3 oxidase was isolated. The isolation of this active supercomplex was achieved after the solubilization of the membranes with the detergent heptyl thioglucoside (Kutoh and Sone 1988). Triton X-100 dissociated the supercomplex; therefore, the use of milder detergents was recommended (Sone et al. 1987; Tanaka et al. 1996). Moreover, a cytochrome bc_1-aa_3 supercomplex with quinol oxidase activity was isolated from the Gram-positive bacterium *Corynebacterium glutamicum* (Niebisch and Bott 2003). In addition, using the non-ionic detergent dodecyl maltoside, this supercomplex was isolated from the Gram-negative bacterium, *P. denitrificans* (Berry and Trumpower 1985). More recently, isolated membranes from *P. denitrificans* were solubilized with digitonin and analyzed by blue native (BN) electrophoresis. An NADH oxidase supercomplex comprised of $I_1III_4IV_4$ was obtained and named the "respirasome" (Stroh et al. 2004). Moreover, supramolecular complexes were found in the aerobic respiratory chain of *Escherichia coli*. The membranes isolated from this bacterium contained several supercomplexes, including one formed by the type-2 NADH:quinone oxidoreductase, the *bd* and bo_3 quinol:oxygen reductases and the aerobic formate dehydrogenase (Sousa et al. 2011).

A characterization of the formation of supercomplexes in *B. subtilis* membranes has been lacking. Here, we present a detailed analysis using native electrophoreses together with various staining procedures and mass spectrometry of the respiratory chain in *B. subtilis*. We found a main supercomplex formed by caa_3 oxidase, the b_6c complex and cytochrome c_{550} with the ATP synthase. A supercomplex formed by SDH with nitrate reductase (a proton-pumping enzyme) and the quinol oxidase aa_3 was also discovered. Type-2 NADH dehydrogenase was observed as a single protein that does not associate with any of the other complexes. Finally, a menaquinol oxidase oligomer was detected.

Materials and methods

Strain, cell growth and membrane preparation

B. subtilis 168 was obtained from the *Bacillus* Genetic Stock Center (Ohio State University) and grown overnight on LB plates. Cells were grown in a super-rich liquid media (SRM) as reported by (Henning et al. 1995). The one-liter cultures were inoculated with 100 mL starter cultures prepared from *B. subtilis* freshly grown on LB media plates. The cultures were grown for 23 h. at 37 °C in an orbital shaker at 250 rpm. The cells were harvested by centrifugation in a GSA rotor (Sorvall) at 2,600 x*g* for 10 min when the O.D. at 600 nm reached a value of 3.5–5.5. Finally, they were washed with 100 mM K phosphate buffer, pH 6.6, and centrifuged at 2,600 x*g* in a SS-34 rotor (Sorvall). The bacteria were stored as pellets at −20 °C.

Plasma membranes were isolated following a combined protocol from (Henning et al. 1995) and (Hägerhäll et al. 1992). Briefly, 30 g of *B. subtilis* were thawed in a water bath at 30 °C and suspended in 50 mM K phosphate buffer,

pH 8.0. Lysozyme was added to a final concentration of 0.3 g/L together with 0.01 g/L of DNAse and RNAse. The protease inhibitors AEBSF (4-(2-aminoethyl)benzensulfonyl fluoride hydrochloride) at a final concentration of 0.1 mM and a mixture of 5 mM ε-aminocaproic acid and 2 mM Na_2 EDTA were added at this point. Cells were incubated for 15 min at 37 °C and 140 rpm, and next, $MgSO_4$ was added to a final concentration of 10 mM. Cells were incubated for 30 min at 37 °C, and Na_2 EDTA was added at a final concentration of 15 mM (from a 0.3 M solution, pH 7.4). Two min later, $MgSO_4$ was added at a final concentration of 20 mM. The unlysed cells were pelleted at 2,600 x*g* for 20 min at 4 °C in a Sorvall GSA rotor. The pooled supernatants were centrifuged again at 19,696 x*g* for 42 min in the GSA rotor. The supernatant was discarded, and the pellet was suspended in 40 mL 0.1 MK phosphate, pH 6.6, and centrifuged in a Sorvall SS-34 rotor at 20,217 x*g* for 15 min at 4 °C. The pellet was suspended in a minimum volume, and an equal volume of glycerol was added together with AEBSF to a final concentration of 0.1 mM. Isolated membranes were stored at −20 °C, and remained active during the next 6 months.

Protein quantification and cytochrome concentration measurement

The total protein content of the isolated membranes was determined (Lowry et al. 1951; Markwell et al. 1978). The cytochrome identification and the determination of the cytochrome concentration were performed with an Aminco Dw2a™ UV-visible spectrophotometer with the OLIS DW2 conversion and OLIS software (Bogart, GA USA). A base line spectrum was recorded in 50 mM K phosphate buffer, pH 7.0, over the range 400−650 nm. Membranes were diluted to a protein concentration of 2.5 mg/mL in the cuvette. Difference spectra (dithionite-reduced minus oxidized) were obtained. The extinction coefficients used for the quantification of the different cytochromes were: 19 mM^{-1} cm^{-1} for cytochrome $c + c_1$ (at 550 nm), 20 mM^{-1} cm^{-1} for cytochrome *b* (at 560 nm), and 16.5 mM^{-1} cm^{-1} for cytochrome *a* (at 600 nm) as reported (de Vrij et al. 1987).

Clear native electrophoresis (CNE)

CNE was performed as described (Wittig et al. 2007). Briefly, *B. subtilis* membranes were solubilized in 4 g of digitonin/g of protein using a 20 % solution of digitonin. After centrifugation, a sample of the supernatant was taken to record spectra and to determine the cytochrome concentration. Protein amounts ranging from 0.132 mg to 0.5 mg were loaded on a 4–13 % polyacrylamide gradient gel. Electrophoresis was performed as described for Clear Native Electrophoresis type 1 (CNE-1) by the authors.

Blue native electrophoresis (BNE)

BN electrophoresis was performed as described in the literature with some modifications (Wittig et al. 2006; Wittig and Schägger 2008). Briefly, membranes from *B. subtilis* were solubilized with six different concentrations of n-dodecyl-β-D-maltoside (DDM) from a 10 % solution. The DDM/protein ratios were: 0.6, 1.0, 1.5, 2.0 and 2.5 (g/g). The protease inhibitor 0.1 mM AEBSF and a protease inhibitor cocktail (Sigma-Aldrich, St. Louis MO USA) were added together with the detergent. A sample of the supernatant was taken to obtain a spectrum and determine the cytochrome concentration. Each cytochrome was loaded at an amount of 0.2 nmol (approximately 2.4 mg of protein) onto a 4–13 % polyacrylamide gradient gel and the gel was stained for cytochrome staining after the electrophoresis was complete. The stacking gel contained 4 % (w/v) acrylamide. The electrophoresis was performed at a constant temperature (0–6 °C) and the current was limited to 25 mA.

Gel staining

In-gel catalytic assay

CNE gels were stained to detect the catalytic activity of each complex according to the technique described for the detection of mitochondrial respiratory complexes (Wittig et al. 2007). To detect NADH dehydrogenase, a strip of the gel was cut and immersed in 15 mL buffer I: 5 mM Tris-HCl, pH 7.4, NADH (0.01 % final) and 2.5 mg/mL nitrotetrazolium blue (NTB). The reaction was stopped after a 10- to 15- min incubation. For SDH, a strip of the gel was incubated in 15 mL buffer II: 5 mM Tris-HCl, pH 7.4, 20 mM sodium succinate, 0.2 mM PMS (stock prepared in DMSO), and 2.5 mg/mL NTB. The reaction was stopped after 20–30 min. For the b_6c complex, a separate strip of the gel was incubated in 15 mL buffer III: 50 mM sodium phosphate, pH 7.2 and 0.5 mg/mL diaminobenzidine. The reaction was stopped after 1–2 h. For complex IV, a separate strip of the gel was incubated in 15 mL buffer IV: 50 mM sodium phosphate, pH 7.2, 0.5 mg/mL diaminobenzidine, and 50 μM cytochrome *c*. The reaction was stopped after 1–2 h. For F_0F_1 ATP synthase, a strip of the gel was incubated in 15 mL of preincubation buffer: 35 mM Tris-HCl, 270 mM glycine, pH 8.3 for 2 h. Afterward, it was incubated in buffer V: 35 mM Tris-HCl, 270 mM glycine, 14 mM $MgSO_4$, 0.2 % $Pb(NO_3)_2$, and 8 mM ATP. The reaction was monitored until a white precipitate formed (after 1–2 days).

TMBZ staining

After 2D-SDS-PAGE, the gels were stained with TMBZ (N,N,N′,N′-tetramethylbenzidine) using the method described by (Thomas et al. 1976). To achieve good staining of the subunits

with a covalently bound heme, a spectrum of the solubilized fraction prepared for BN electrophoresis or CNE was acquired. We estimated experimentally that 0.2 nmol of cytochrome would be sufficient to achieve good TMBZ staining.

Coomassie blue and silver staining

After the TMBZ staining, Coomassie blue staining was performed according to Laemmli (Laemmli 1970). 2D-CNE-SDS-PAGE gels were silver stained as described at http://msf.ucdavis.edu/silverstaining_protocols.html.

Determination of protein apparent molecular mass

To estimate an apparent molecular mass for the protein bands resolved by the different types of electrophoreses, appropriate molecular mass markers were used (Wittig et al. 2010). The scanned image of each gel was analyzed with the software Image Gauge 4.0 version Fuji Film.

Mass spectrometry analysis

The proteins to be analyzed by mass spectrometry (MS) were cut from gels of 1D-BNE, 1D-CNE, 2D-BNE-PAGE or 2D-CNE-PAGE. The gel fragments were washed with water and then with 50 % (v/v) acetonitrile in water, with acetonitrile mixed with 100 mM ammonium bicarbonate (1:1), and with 100 % acetonitrile as described (Lino et al. 2006). The proteins in the gel fragments were then reduced, carbamidomethylated, and digested with trypsin, and the resulting peptides were extracted from the gel as described (Shevchenko et al. 1996).

These peptides were purified and concentrated using resin-loaded tips (Zip Tip, Millipore, Billerica, MA USA) and mixed with matrix (α-cyano-4-hydroxycinnamic acid) before being analyzed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-ToF MS) in an Autoflex III Smartbeam instrument (Bruker Daltonics Inc. Billerica, MA USA) in reflectron mode. The mass lists obtained were compared with theoretical lists obtained from databases (mainly SwissProt) using the online program Mascot (from Matrix Science at http://www.matrixscience.com/search_form_select.html).

Some selected proteins were analyzed by sequencing their peptides. These were separated by HPLC in an Accela (ThermoFisher Scientific, San Jose, CA USA) instrument with a Picofrit C18 column, run with a linear 3–70 % acetonitrile gradient in 0.1 % formic acid (over 15 min with a flow rate of 600 nl/min). The resolved peptides were analyzed in an LTQ-Velos ion-trap mass spectrometer (Thermo Fisher Waltham MA USA) with a nanospray ion source. The most abundant peptides were fragmented by CID and pulsed CID (PQD) in a cycle producing approximately one fragmentation spectrum per s. The spectrometer was controlled by Xcalibur 2.1 software. The data were analyzed using Proteome Discoverer 1.2 software with the Mascot 2.2 algorithm, comparing them with the theoretical spectra of the NCBInr or SwissProt databases using the following search parameters: taxonomy, *B. subtilis*; constant modifications, carbamidomethylated C; variable modifications, oxidized Methione; and tolerances, 1.2 Da for precursor ions and 0.6 Da for fragmentation products. The sequences of some peptides were also obtained manually with Xcalibur 2.1 software and compared with those in databases using the MS-Blast search program at http://genetics.bwh.harvard.edu/msblast/ (Shevchenko et al. 2001).

Results

The cytochrome content of membranes isolated from *B. subtilis*

B. subtilis was grown in aerobic conditions and in SRM (Henning et al. 1995) to induce the respiratory chain. Figure 1 contains the differential spectrum (dithionite-reduced minus oxidized) that shows the type and content of the cytochromes of this membrane. Three alpha peaks are seen; at 550 nm, we identified the alpha-band of *c*-type cytochromes. This peak is higher than the *b* and *a* alpha peaks, indicating that cytochrome *c* is the most abundant type of cytochrome. The alpha peaks at 558–560 nm were attributed to the *b*-type cytochromes. At 600 nm, the alpha peak corresponds to the *a*-type

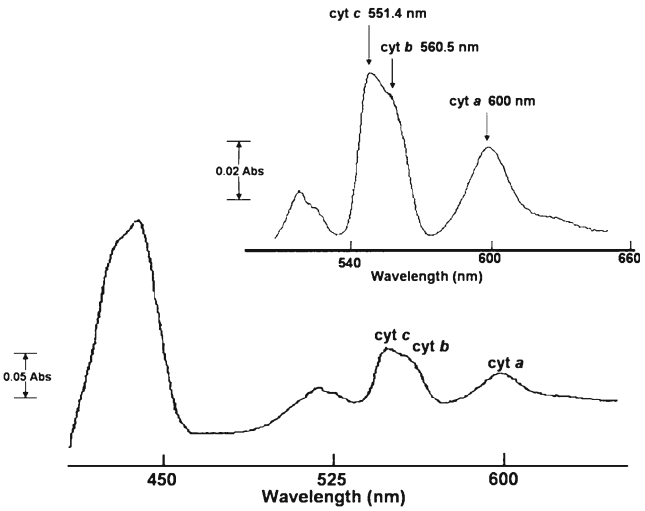


Fig. 1 Differential spectra (dithionite-reduced minus oxidized) of the membranes isolated from *B. subtilis*. The concentration of each cytochrome was determined using 2.06 mg of membrane protein in the cuvette. The amount of cytochromes was estimated using previously reported extinction coefficients (de Vrij et al. 1987). The relative amounts were as follows: cytochromes $c+c_1$ (551.4 nm) were present at 1.44 nmol per mg of membrane protein; cytochromes type *b* (560.5 nm) were present at 0.58 nmol per mg of membrane protein; and cytochromes type *a* (600 m) were present at 0.74 nmol per mg of membrane protein

cytochromes. The relative concentrations of the cytochromes (expressed in nmoles of cytochrome/mg protein) were as follows: 1.44 for *c*-type cytochromes, 0.58 of *b*-type cytochromes, and 0.74 for *a*-type cytochromes.

Identification of the respiratory chain supercomplexes. CNE of the digitonin-solubilized *B. subtilis* membranes

Figure 2a shows the in-gel catalytic activity staining used to detect the mitochondrial respiratory complexes I-V (Wittig et al. 2007). Five bands of activity were observed. A fast mobility band was obtained for an NADH dehydrogenase type-2 (NADH DH type-2); type 1 is absent in *B. subtilis* (Björklöf et al. 2000; Kunst et al. 1997). A major band that likely represents an oligomeric SDH was detected at the middle of the strip. In-gel activity was observed for the complexes b_6c and caa_3 in the same region of the 1D gel (with slow mobility); the overlap could be interpreted as a supercomplex association between the b_6c and caa_3 complexes. A conspicuous band of ATP synthase in-gel activity was observed (marked as V) along with several diffuse bands. Based on the amount of membrane protein required

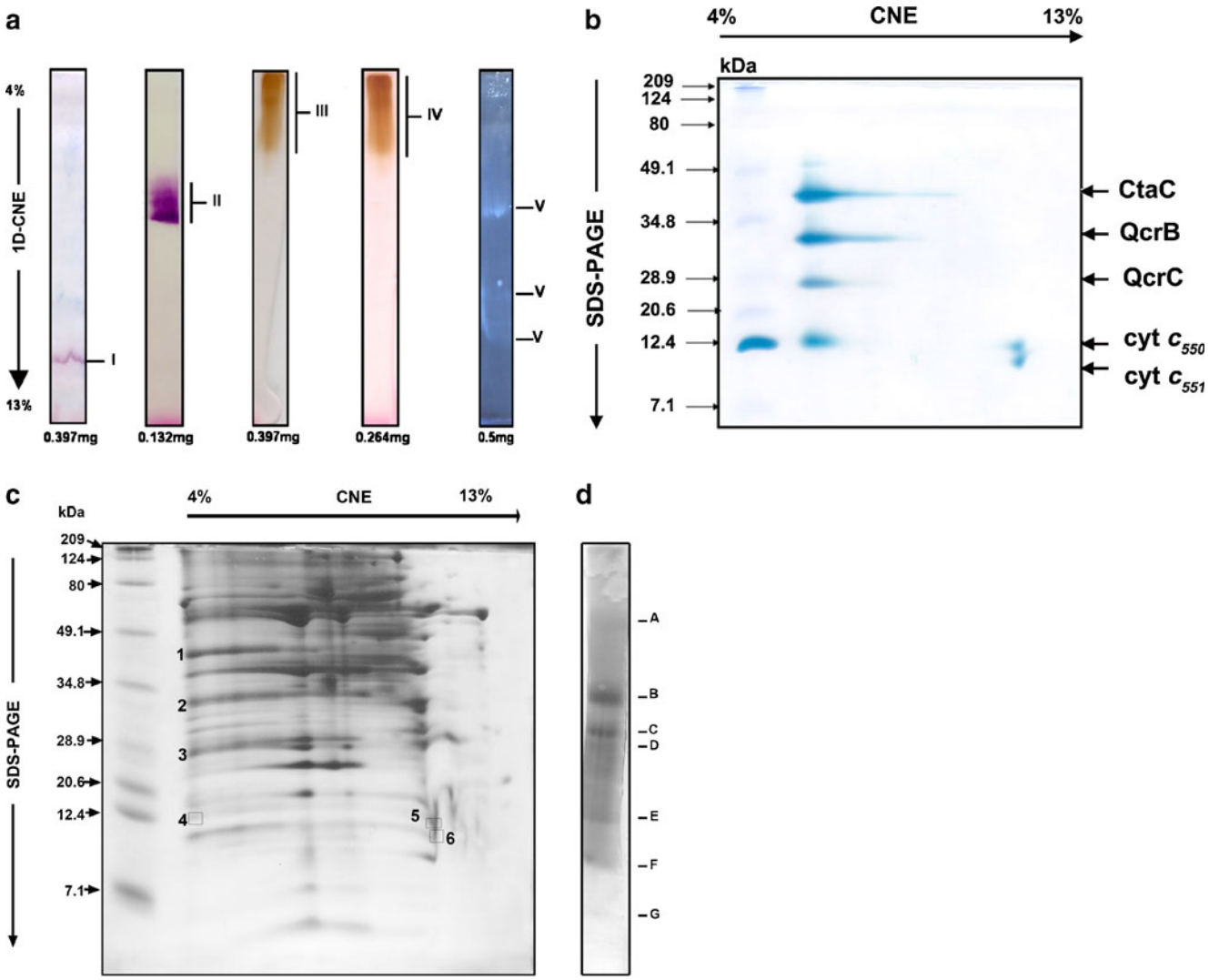


Fig. 2 In-gel catalytic assays for complexes I-V. **a** CNE analysis of *B. subtilis* membranes solubilized with 4 g digitonin/g membrane protein. The amount of protein loaded is shown in the figure. **b** Cytochrome detection by TMBZ staining after the 2D-CNE-SDS-PAGE analysis using 2.4 mg of protein for CNE. **c** The cytochrome subunits resolved by CNE (2.4 mg protein per lane) were identified by mass spectrometry. The identified proteins are marked (1 through 6) in the 2nd dimension of the SDS-PAGE analysis. Table 1 shows the identification determined for each protein by mass spectrometry. **d** Proteins contained within selected bands resolved by 1D CNE (2.4 mg protein per lane) were identified by mass spectrometry. Table 2 shows the proteins identified in each band. The gel strip was stained with Coomassie blue

to allow the detection of the activity of each complex, we presume that the amount of the complexes will be SDH >cytochrome caa_3 oxidase >b_6c complex>>NADH DH-type-2.

In addition to the five strips used for the detection of in-gel activity, one strip was analyzed by 2D-SDS-PAGE electrophoresis and stained with TMBZ (Fig. 2b) to reveal the proteins containing covalently bound hemes. Four bands appeared at the left side of the 2D gel with apparent molecular masses of 43, 32, 24 and 15.25 kDa. The known proteins containing *c*-type hemes with those masses include CtaC, the subunit II of caa_3 oxidase (calculated mass from the genome of 40.1 kDa), as the 43 kDa protein and QcrC from the b_6c complex (with a calculated mass of 28.1 kDa) as the 32 kDa band. QcrB (with a calculated mass of 25.5 kDa) is the 24 kDa protein, and cytochrome c_{550} (with a calculated mass of 12.8 kDa) is the band at 15.25 kDa. In addition, two high mobility bands at the right side of the 2D electrophoresis gel stained with TMBZ correspond to the dissociated small, membrane-bound cytochromes, c_{550} and c_{551} (calculated mass of 11.9 kDa). A faint band running at a higher molecular mass was observed, which probably represents CtaC with an associated c_{550}. The 2D gel was silver stained (Supplementary Fig. 1 and Supplementary Table 1, including the apparent molecular masses of the spots) and prepared for mass spectrometry analysis. Unfortunately, we found that the proteins in the TMBZ-stained bands used to prepare them for mass spectrometry analysis were not hydrolyzed by trypsin and therefore could not be identified from this gel.

We repeated the 2D analysis using a CNE gel loaded with more protein (2.4 mg per lane) and stained with Coomassie blue (Fig. 2c) to clearly identify the cytochrome subunits detected in the previous gels. Table 1 contains the mass spectrometry analysis of spots 1 through 6 from this second 2D analysis, which confirmed the identity of the cytochromes visualized with TMBZ and their positions at high molecular mass in the CNE gel: CtaC from oxidase caa_3 and cytochrome c_{550} (spot 1), QcrC from the b_6c complex (spot 2) and QcrA and QcrB from the b_6c complex (in spot 3). The small cytochromes were identified in spot 4 (cytochrome c_{550}) and spots 5 and 6 (cytochrome c_{551}). We also analyzed three bands of the CNE gel strip (bands A, E and F), where we identified the presence of the complexes b_6c, caa_3 and the small cytochromes (shown in Fig. 2d and in Table 2). Band A contained the b_6c complex (identified by the presence of QcrB), caa_3 oxidase (identified by the presence of CtaC) and ATP synthase (identified by the presence of ATPβ). Band E contained the cytochromes c_{550} and c_{551} and the subunit SdhA from the SDH complex and NADH dehydrogenase (protein YjlD). Band F also contained cytochromes c_{550} and c_{551}. Furthermore, in this band, we identified ATP synthase (subunits α and β), NADH dehydrogenase (protein YjlD) and SDH (subunit SdhA).

The high amount of protein loaded on the 1D gel (Fig. 2d) allowed the identification of the cytochrome subunits, including the smaller subunits (c_{550} and c_{551}). A second analysis with less protein allowed us to identify other proteins that could form oligomers. Using the spots of the 2D gel as fingerprints, we defined the range of supercomplexes marked in the 1D-CNE gel strip with capital letters from A through E (Fig. 3 and Table 3). Band A contained ATP synthase identified in the spots 1, 2 and 6 (subunits α, β and b), cytochrome caa_3 (CtaC identified in spot 3), quinol oxidase aa_3 (QoxA identified in spot 4) and the b_6c complex (QcrA and B identified in spot 5). Band B also contained the ATP synthase (subunits α, β, γ, δ and b identified in spots 7, 8, 9, 11, and 12, respectively); the quinol oxidase aa_3 was identified by the presence of QoxA in spot 10. Band C contained SDH (subunits SdhA and B identified in spots 13 and 14, respectively). Band D contained quinol oxidase aa_3 (QoxA identified in spot 15) and ATP synthase (subunits α and β), and band E contained

Table 1 Mass spectrometry analysis of the proteins identified in the 2D-CNE-SDS-PAGE analysis that included the 1D CNE of an overloaded lane (from Fig. 2c)

Spot number (2D)	ID (only respiratory chain proteins are listed)	Predicted Mr	Score	Number of peptides	Coverage
1	*ctaC*=cytochrome caa_3 oxidase (subunit II)	40,100	489.23	9	24.44
1	*cccA*=cytochrome c_{550}	12,800	77.13	3	29.17
2	*qcrC*=menaquinol:cytochrome *c* oxidoreductase (cytochrome *cc* subunit)	28,100	352.24	8	23.14
3	*qcrA*=menaquinol:cytochrome *c* oxidoreductase (iron-sulfur subunit)	18,700	690.94	13	82.63
3	*qcrB*=cytochrome b_6	25,500	375.37	6	29.46
3	*atpB*=F_0F_1 ATP synthase subunit delta	20,000	82.69	2	9.94
4	*cccA*=cytochrome c_{550}	12,800	121.49	4	29.17
5	*cccB*=cytochrome c_{551}	11,900	130.62	2	25.89
6	*cccB*=cytochrome c_{551}	11,900	149.34	2	25.89

Table 2 The mass spectrometry analysis of a 1D-CNE gel (from Fig. 2d)

Band (1D)	ID[a] (only respiratory chain proteins are listed)	Predicted Mr	Score	Number of peptides	Coverage
A	menaquinol:cytochrome *c* oxidoreductase (iron-sulfur subunit)	16,200	208.53	6	39.58
A	cytochrome b_6	25,470	179.55	4	18.75
A	cytochrome caa_3 oxidase (subunit II)	40,100	170.14	4	12.36
A	cytochrome-*c* oxidase subunit I	68,900	70.58	2	4.03
A	F_0F_1 ATP synthase subunit beta	51,400	69.73	2	5.50
E	succinate dehydrogenase flavoprotein subunit	65,110	341.38	11	19.62
E	NADH dehydrogenase	41,900	302.44	7	26.53
E	cytochrome c_{551}	11,900	137.36	2	25.89
E	cytochrome c_{550}	12,800	89.21	3	29.17
F	F_0F_1 ATP synthase subunit alpha	54,600	1102.40	41	49.40
F	F_0F_1 ATP synthase subunit beta	51,400	902.38	23	46.30
F	NADH dehydrogenase	41,900	274.62	8	25.51
F	succinate dehydrogenase flavoprotein subunit	65,100	218.96	7	16.72
F	F_0F_1 ATP synthase subunit gamma	31,600	158.75	3	12.20
F	cytochrome c_{551}	11,900	130.68	2	25.89
F	cytochrome c_{550}	12,800	55.44	2	20.83

[a] *ID* identity of the protein

nitrate reductase (spot 16, subunit NarG). The molecular masses of the supercomplexes are discussed below.

The components of the main supercomplexes in bands observed by BN electrophoresis

ATP synthase, b_6c-caa_3

Using CNE and in-gel catalytic assays, we identified several bands of ATPase activity presumably representing different oligomeric forms of the complex. To compare these oligomeric forms with those of other bacteria, we solubilized *B. subtilis* membranes with DDM. A proper working concentration of DDM was found by varying the ratio of DDM to mg of membrane protein (0.6, 1.0, 1.5, 2.0 and 2.5 mg of DDM per mg of membrane protein, Supplementary Fig. 2). At 0.6 mg of DDM/mg of membrane protein, we observed a pattern of Coomassie bands similar to those observed in the CNE analysis (Fig. 4). The identification by mass spectrometry of the individual subunits separated by 2D-SDS-PAGE is shown in Table 4. Seven protein bands were resolved by 1D-BNE (bands A to G). Four (bands A, C, E and G) contained ATP synthase identified by the α and β subunits at the expected molecular masses (spots 1, 5, 5′ and 5″ in the 2D gel). The migration of the four forms of the ATP synthase in the 1D-BNE gel is indicated in the lower part of the 2D gel. The mobility of these four bands could indicate a possible supercomplex composed by ATP synthase and the complexes b_6c and caa_3 (band A), a monomeric ATP synthase (band C), F_1 (band E) and $\alpha_1\beta_1$ subunits (band G).

The supercomplex b_6c-caa_3

The subunits of other complexes were identified in the BNE gel; the QcrA (spot 3) and QcrB (spot 4) subunits of the b_6c complex were located in bands A and B of the 1D gel at a range

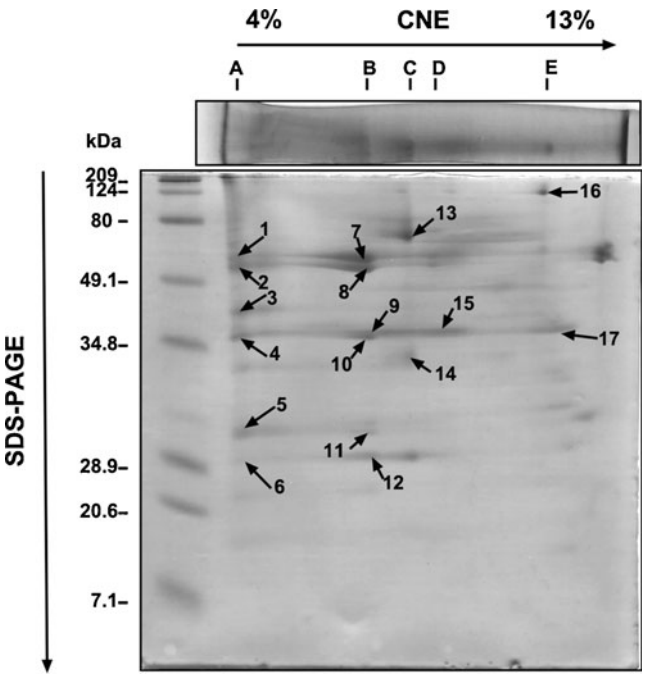


Fig. 3 CNE analysis using 1 mg per lane and analyzed in the 2nd dimension by SDS-PAGE. The proteins identified by mass spectrometry (marked 1 through 17) are shown in Table 3

Table 3 Mass spectrometry identification of proteins from the 2D-CNE-SDS-PAGE analysis (from Fig. 3)

Spot number	Band (1D)	ID (gene and protein name)	Predicted Mr	Score	Number of peptides	Coverage
1	A	*atpA*=ATP synthase subunit alpha	54679	68	7	18 %
2	A	*atpD*=ATP synthase subunit beta	51388	86	9	24 %
3	A	*ctaC*=Cytochrome c oxidase subunit 2	40354	90	11	27 %
3	A	*atpD*=ATP synthase subunit beta	51388	70	9	24 %
4	A	*qoxA*=Quinol oxidase subunit 2	36316	54	6	21 %
5	A	*qcrA*=Menaquinol-cytochrome *c* reductase iron-sulfur subunit	18952	55	5	41 %
5	A	*qcrB*=Menaquinol-cytochrome *c* reductase cytochrome *b* subunit	25528	52	5	25 %
6	A	*atpF*=ATP synthase subunit b	19196	59	7	27 %
7	B	*atpA*=ATP synthase subunit alpha	54679	69	7	20 %
8	B	*atpB*=ATP synthase subunit beta	51388	81	9	30 %
9	B	*atpG*=ATP synthase gamma chain	31635	104	11	34 %
10	B	*qoxA*=Quinol oxidase subunit 2	36316	72	7	24 %
11	B	*atpH*=ATP synthase subunit delta	19970	92	8	30 %
12	B	*atpF*=ATP synthase subunit b	19196	77	6	32 %
13	C	*sdhA*=Succinate dehydrogenase flavoprotein subunit	65395	144	13	25 %
14	C	*sdhB*=Succinate dehydrogenase iron-sulfur subunit	29026	29	3	
15	D	*qoxA*=Quinol oxidase subunit 2	36316	85	6	30 %
16	E	*narG*=Nitrate reductase alpha chain	139411	82	11	11 %
17	E	*atpG*=ATP synthase gamma chain	31635	66	7	29 %

of molecular masses between those of F_0F_1 and F_1. Oxidase caa_3 was also found in bands A and B where subunit CtaC (spots 2 and 2′) was identified. We propose that band B contains the supercomplex b_6c-caa_3 with a molecular mass of approximately 796 kDa, using the proposed oligomeric forms of ATP synthase located in bands C and E as reference molecular masses.

Other supercomplexes detected: SDH-Nar and ATP synthase-aa_3

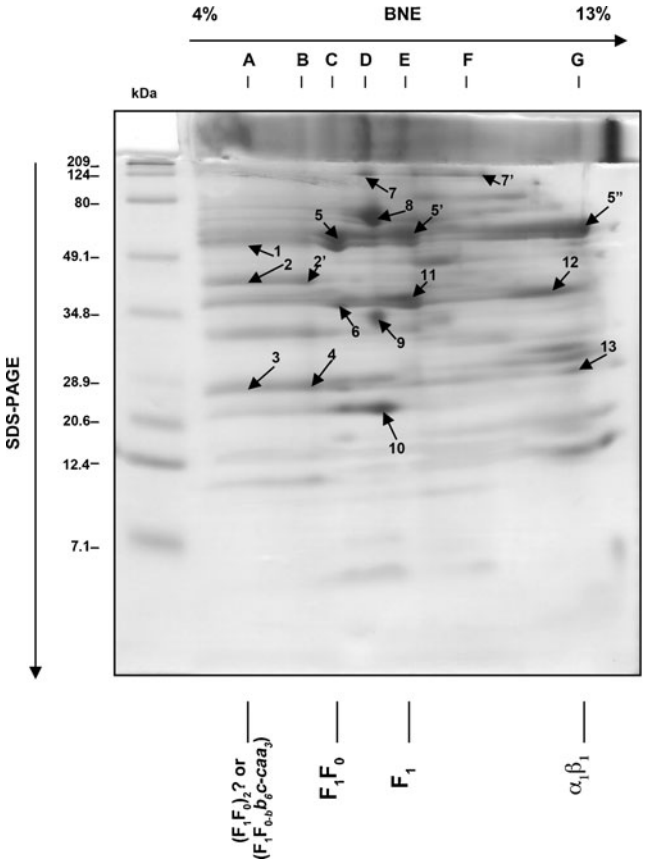
SDH was found in band D where the subunits SdhA (spot 8) and SdhB (spot 9) were identified (Table 4). SDH migrated between bands C and E where a monomeric ATP synthase and F_1 were located, respectively. A molecular mass between 530 and 382 kDa, which is higher than monomeric SDH (117 kDa), can be estimated. In this same band, NarG, a subunit of nitrate reductase, was identified (spot 7). A supercomplex composed of SDH-nitrate reductase is proposed. Band E contained the quinol oxidase aa_3 identified by the presence of subunit QoxA (spot 11), where F_1 was also identified. Another supercomplex with ATP synthase and oxidase aa_3 with a molecular mass higher than 382 kDa is proposed. Band F contained nitrate reductase, identified by the presence of subunit NarG (spot 7′). A molecular mass of 244 kDa is proposed based on bands E and G as reference molecular masses.

Cytochrome c_{550} is part of the supercomplex b_6c-caa_3

A twin 2D-SDS-PAGE was stained with TMBZ to corroborate the localization of the cytochrome subunits with a covalently bound heme (Supplementary Fig. 3A). Five bands were conspicuously stained and assigned to the known *c*-type cytochromes of *B. subtilis*. The supercomplex b_6c-caa_3 was detected at different molecular masses revealing several stoichiometries of the supercomplex. TMBZ staining also reveals that cytochrome c_{550} is part of the supercomplex. Another band below cytochrome c_{550} was assigned to cytochrome c_{551}; this cytochrome seems to bind caa_3 and b_6c at the low molecular mass assumed from the band that appears at the middle of the 2D gel. At a higher DDM concentration (1.5 mg/mg membrane protein, Supplementary Fig. 3B and C), cytochrome c_{551} seems to shift its mobility and runs at a lower molecular mass indicating that not all the cytochrome is part of the supercomplex.

The molecular mass determination of the supercomplexes

We determined the molecular mass of the identified bands on the BNE gel using a previously described method (Wittig et al. 2010). Each band was assigned to a supercomplex using the information obtained from the analysis of the BNE and CNE gels by mass spectrometry (Fig. 5 and Table 5). To identify



◀ **Fig. 4** 2D-BNE-SDS-PAGE analysis using 0.6 mg DDM/mg membrane protein. The proteins identified by mass spectrometry are numbered from 1 to 13. Table 4 shows the mass spectrometry identification of these proteins

each mitochondrial complex, a 2D analysis was run (data not shown). Table 5 shows the bands from A to G observed on the first BNE gel of *B. subtilis* membranes, the composition of each band (from the 2D analysis and mass spectrometry identification), the molecular mass obtained by the mobility of each band (*Mapp*) and the calculated mass (*Mcalc*). In four bands, subunits of the ATP synthase were identified: band A, which had the highest molecular mass (1,242 kDa), contained ATPase and the complexes caa_3 and b_6c were found, band C, with a molecular mass of 635 kDa, contained the second form of ATPase, band E at 376 kDa contained a third population of ATPase and the aa_3 quinol oxidase, and finally, band G at 109 kDa, which was the lowest molecular mass, contained ATPase. Other respiratory complexes were identified in the same gel. The complexes caa_3 and b_6c were found over a range of molecular masses from 1,242 to 515 kDa (bands A to D). Specifically, bands A and B contained the highest molecular mass supercomplexes and were also positive for TMBZ staining. In the range between 1,242 and 400 kDa, we also found the quinol oxidase aa_3. It is possible that the oxidase aa_3 will form a supercomplex with ATP synthase because it was identified in bands A and E. Band E

Table 4 Mass spectrometry identification of proteins from the 2D-BNE-SDS-PAGE analysis (Fig. 4)

Spot Number (2D)	Complex (1D)	ID (gene and protein name)	Theoretical M_r	Score	Number of peptides	Coverage
1	A	*atpD*=ATP synthase subunit beta	51388	76	9	24 %
2	A	*ctaC*=Cytochrome c oxidase subunit 2	40354	69	8	18 %
2′	B	*ctaC*=Cytochrome c oxidase subunit 2	40354	119	10	21 %
3	A	*qcrB*=Menaquinol-cytochrome c reductase cytochrome *b* subunit	25528	46	5	25 %
4	B	*qcrA*=Menaquinol-cytochrome *c* reductase iron-sulfur subunit	18952	56	6	44 %
5	C	*atpA*=ATP synthase subunit alpha	54679	160	19	39 %
5′	E	*atpA*=ATP synthase subunit alpha	54679	214	21	42 %
5″	G	*atpA*=ATP synthase subunit alpha	54679	135	15	33 %
6	C	*atpG*=ATP synthase gamma chain	31635	102	11	40 %
7	D	*narG*=Nitrate reductase alpha chain	139411	222	32	26 %
7′	F	*narG*=Nitrate reductase alpha chain	139411	210	26	21 %
8	D	*sdhA*=Succinate dehydrogenase flavoprotein subunit	65395	255	26	44 %
9	D	*sdhB*=Succinate dehydrogenase iron-sulfur subunit	29026	105	12	39 %
10	D	*atpF*=ATP synthase subunit b	19196	102	9	46 %
11	E	*qoxA*=Quinol oxidase subunit 2	36316	121	10	35 %
12	G	*atpG=ATP synthase gamma chain*	31635	102	12	41 %
13	G	*atpH*=ATP synthase subunit delta	19970	90	8	34 %

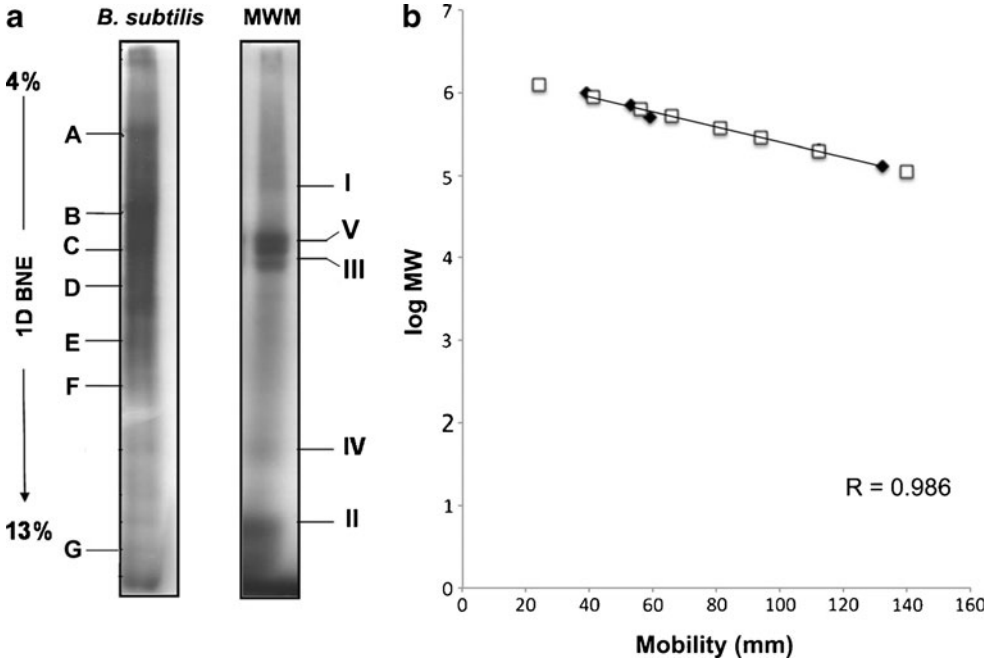


Fig. 5 Molecular mass determination using a chicken heart crude membrane preparation. **a** Two lanes of the same gel were compared in terms of the number of bands (indicated by *capital letters*) observed after the resolution of a *B. subtilis* sample (1 mg membrane protein in the lane) with 1.5 mg DDM/mg membrane protein and a chicken heart homogenate sample (indicated by *Roman numbers*) with 0.08 mg DDM/mg protein. **b** Mass estimation of the supercomplexes from *B. subtilis*. Squares: *B. subtilis* proteins; black diamonds: chicken heart complexes. Table 5 contains the assignment of molecular mass to each band

could contain an F_1 with a lower amount of subunits α and β. Band D contained SDH and nitrate reductase with a molecular mass of 515 kDa; we propose that these two complexes could be a supercomplex. Nitrate reductase was also identified in band F with a molecular mass of 286 kDa.

Discussion

Some components of the *B. subtilis* aerobic respiratory chain can be identified from the spectral analysis of its membranes

B. subtilis grown in aerobic conditions with succinate as the carbon source expresses its aerobic respiratory chain, making the identification of the supercomplexes by native electrophoresis possible. A differential spectral analysis revealed the enrichment of various types of cytochromes in the plasma membrane. There are four *c*-type cytochromes (all identified in the present work) that contribute to the absorption peak at 550 nm: membrane-bound c_{550} and c_{551} (which distribute electrons between the b_6c complex and the cytochrome *c* oxidase), QcrC of the b_6c complex and CtaC of the caa_3 oxidase complex (the nomenclature used here corresponds to that used in the reported genome (Kunst et al. 1997)). The *b*-type hemes are attributed to SdhC from SDH, QcrB from the b_6c complex and to the *b* hemes of nitrate reductase (NarG, identified in this work). Finally, two oxidase subunits contribute to the absorption peak at 600 nm: CtaD of the caa_3-605 cytochrome oxidase and QoxA of the aa_3-600 quinol oxidase (also identified in this work). A shoulder at 630 nm could indicate the presence of cytochrome *d* of the cytochrome *bd* oxidase (Winstedt and von Wachenfeldt 2000).

The complexes b_6c and caa_3 form a supercomplex integrated with the membrane-bound cytochrome c_{550}

An oxidoreduction sequence diagram of the respiratory chain will predict in principle the associations between respiratory complexes (Azarkina et al. 1999). We can expect that the electron donors to menaquinone-7 (namely, the type-2 NADH dehydrogenase and SDH) will associate with

Table 5 *Mapp*, the apparent masses of bands A-G after BNE of *B. subtilis* membrane proteins using membrane molecular mass markers. *Mcalc,* calculated masses according to stoichiometry of each complex

Bands (1D-BN)	Composition:	*Mapp* (kDa)	*Mcalc* (kDa)	Remarks
A	(F_1F_0)-$(b_6c)_4$- $(c_{550})_4$- $(caa_3)_2$	1242	1161	
B	$(b_6c)_4$- $(c_{550})_4$- $(caa_3)_3$	870	869	
C	F_1F_0	635	775	
D	$(Nar)_1$ $(SDH)_2$	515	453	
E	$F_1(\alpha_1\ \beta_1)$-aa_3	376	316	aa_3 was identified in this band.
F	Nar monomeric	286	219	
G	1α:1β	109	106	

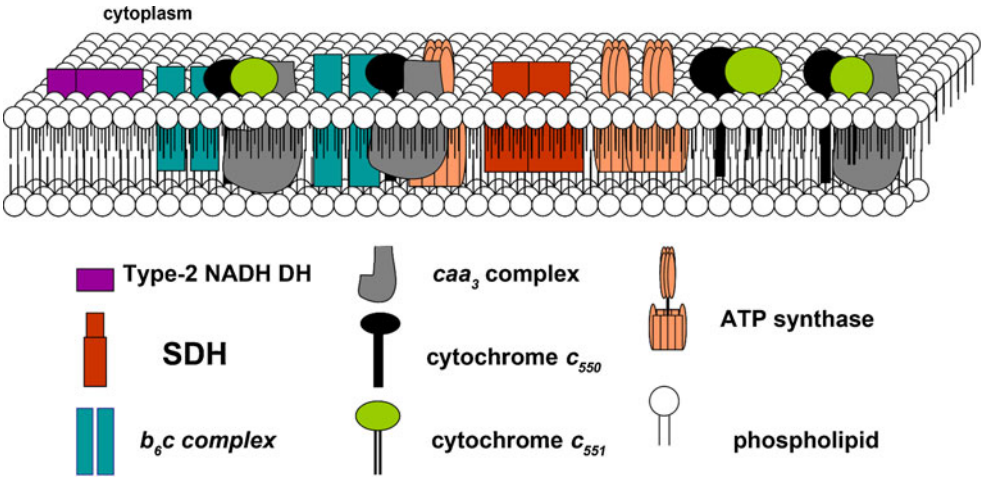


Fig. 6 A representation of the *B. subtilis* respiratory chain supercomplexes based on our results. Individual complexes are shown below the membrane for identification

the menaquinol electron acceptors (the b_6c complex, the quinol oxidases and nitrate reductase). Moreover, the b_6c complex and cytochrome *c* oxidase caa_3 are expected to associate together with the electron donors. The discussion will first address the most abundant supercomplex formed by the b_6c complex and the caa_3 oxidase (Fig. 6).

The in-gel activity analysis revealed that the b_6c and the caa_3 complexes were at the same position on the CNE gel strips, a position that indicates a high molecular mass. Furthermore, the 2D-CNE-SDS-PAGE strips stained with TMBZ showed that four *c*-type cytochrome bands are contained within this high molecular mass supercomplex. The staining revealed the association of the complexes b_6c and caa_3 and the presence of cytochrome c_{550} within the supercomplex. Mass spectrometry identified both complexes in the first dimension analysis by CNE (Fig. 2d, Table 2). With this evidence, we can postulate the existence of a supercomplex formed by the oligomeric b_6c-caa_3 complexes and cytochrome c_{550}, i.e., $caa_3(b_6c)_2caa_3$.$caa_3(b_6c)_2caa_3$. Some c_{550} was stained at the right edge of the gel together with c_{551}, indicating the release of these cytochromes from the supercomplex. A similar supercomplex was found in the thermophilic *Bacillus* PS3 (Tanaka et al. 1996). The isolation of a supercomplex formed by the bc_1 complex (complex III) and the cytochrome *c* oxidase aa_3 (complex IV) from *P. denitrificans* was reported (Berry and Trumpower 1985). This supercomplex contained the small, membrane-bound cytochrome c_{552}. The supercomplex formed by these complexes, and c_{552} was also analyzed by BNE and with specific antibodies against the subunits of the bc_1 and aa_3 complexes as well as to c_{552} (Stroh et al. 2004). The calculated molecular mass of the supercomplex was 984 kDa for a supercomplex with a stoichiometry of III_4IV_4 and 722 kDa for a supercomplex with a stoichiometry of III_4IV_2. The intimate relationship between the bc_1 complex and the cytochrome *c* oxidase has been demonstrated in the bacterium *C. glutamicum*. Furthermore, when trying to purify either of the complexes using His-tagged subunits, both complexes were pulled down and could not be separated (Kurokawa and Sakamoto 2005; Niebisch and Bott 2003).

BNE analysis together with TMBZ staining confirmed the formation of this high molecular mass supercomplex, when using either 0.6 or 1.5 mg of DDM per mg of membrane protein. The four bands identified as the components of the supercomplex are shown in the 2D analysis over a range of molecular masses from 1,242 kDa to 500 kDa, until the caa_3 and cytochrome c_{550} seem to separate from the b_6c (Supplementary Fig. 3). We determined that the molecular mass of the supercomplex found in band B is 870 kDa. The b_6c complex is proposed to exist as a dimer because only as dimers are the *bc*-type complexes active (Castellani et al. 2010). The calculated molecular mass of the b_6c complex dimer is 145 kDa and that of dimeric cytochrome *c* oxidase is 290 kDa; thus, the minimum molecular mass of the respiratory supercomplex must be approximately 450 kDa to be composed of caa_3-$(b_6c)_2$-caa_3; therefore, the molecular mass of 871 kDa indicates two units of this supercomplex. A molecular mass of approximately 900 kDa was proposed for the *P. denitrificans* supercomplex with a stoichiometry of III_4IV_4. Cytochrome c_{552} was observed to favor supercomplex formation in *P. denitrificans*, and the number of supercomplex structures was reduced in the absence of this cytochrome (Stroh et al. 2004). We propose that the cytochrome c_{550} of *B. subtilis* has the same role because it is a permanent component of the supercomplex.

ATP synthase is a monomer in *B. subtilis* membranes

ATP synthase appeared at various molecular masses in both types of native electrophoresis used in this work. To obtain a

positive identification of a band containing the ATP synthase, we required that two criteria be met: the observation of in-gel activity and the identification by mass spectrometry of at least one subunit of the ATP synthase. Three bands of ATPase activity were observed via in-gel activity. The heaviest protein-complex band with ATPase activity had a molecular mass of 635 kDa. This mass fits that of a monomeric ATP synthase F_0F_1. *B. subtilis* ATP synthase has eight subunits coded by the genes *atpA-H*; an additional gene (*AtpI*) encodes a protein that directs the assembly of F_0 (Santana et al. 1994).

The molecular weight of the second band exhibiting ATPase activity in the CNE gel strip and containing subunits identified by mass spectrometry was 382 kDa, which fits that of an F_1. Finally, the third band with ATPase activity and identified α and β subunits was assigned a molecular mass of approximately 109 kDa. This value corresponds to the molecular weight of a heterodimer formed by α and β subunits.

An association of ATP synthase with the other respiratory complexes has been proposed for *B. pseudofirmus*. The ATP synthase was found to interact with the oxidase caa_3 *in vitro*, suggesting that this interaction may contribute to confined proton transfer during alkaliphilic oxidative phosphorylation at high pH (Liu et al. 2007). As was previously mentioned, the mass spectrometry analysis of band A from CNE and BNE gel strips revealed the presence of ATP synthase via the identification of subunits α and β together with the subunits of the b_6c and caa_3 complexes. The supercomplex b_6c-caa_3 directly provides the protons for ATP synthesis. Another possible interaction of ATP synthase with the components of the flagellar apparatus was recently observed in *E. coli* (Zarbiv et al. 2011).

SDH was observed as an oligomer

The in-gel activity of SDH was observed at the middle of the CNE gel strip, and SDH was identified by mass spectrometry in the range between 550 kDa and 383 kDa, where the bands containing F_0F_1 and F_1 were located. A molecular mass of 515 kDa was observed on the BNE gel strip using the mitochondrial complexes as molecular mass markers. Nitrate reductase was identified in the same band, by mass spectrometry of the 2D analysis (Fig. 4, Table 4). A stoichiometry of SDH_2Nar_1 (with a calculated mass of 453 kDa) could fit the observed mass. The SDH of *E. coli* membranes has been characterized as a trimer (Sousa et al. 2011). It is possible that the supercomplex could be formed by a trimeric SDH and a monomeric nitrate reductase. Nitrate respiration could be coupled to the menaquinol produced by SDH to eliminate the excess reducing power (Richardson 2000). A stoichiometry of SDH_3Nar_1 will result in a calculated molecular mass of 570 kDa higher than the apparent molecular mass (Table 5). Another possible supercomplex could be formed by the SDH and quinol oxidase aa_3 identified in spots 10 and 15 after the 2D analysis (Fig. 3). In our laboratory, we have not been able to separate SDH from the oxidase aa_3 by ionic exchange chromatography.

The complex aa_3 was the quinol oxidase found in this membrane

The analysis revealed the presence of quinol oxidase aa_3 in band A, and it was identified in spot 4 with mass spectrometry (Fig. 3, Table 3). This quinol oxidase is essential for aerobic growth, as shown previously (Winstedt and von Wachenfeldt 2000). The presence of this quinol oxidase at a very high molecular mass could indicate an association with ATP synthase, because QoxA was identified in band A. As mentioned before for cytochrome caa_3 oxidase, oxidase aa_3 pumps protons useful for ATP synthesis.

Type 2 NADH dehydrogenase

Type 2 NADH dehydrogenase was identified by in-gel activity within a band of low molecular mass and confirmed by mass spectrometry (Fig. 2a and d). Bands E and F were assigned to the gene *yjlD* with a predicted mass of the gene product of 41.8 kDa (Fig. 2d; Table 2). YjlD exhibits a 57 % similarity to *B. pseudofirmus* Ndh2-1 (named NDH-2A), which is proposed to be the electron donor to the respiratory chain (Liu et al. 2008). A mass of approximately 200 kDa is estimated because the protein appeared smaller than the nitrate reductase band (219 kDa for the monomer) in the 1D gel. In our analysis, we were not able to detect an association of the NADH DH with other respiratory complexes.

The flexibility of the respiratory chain of *B. subtilis* can be seen in the supercomplexes found in this work. It will be interesting to analyze the supercomplex composition of *B. subtilis* growing in varying aerobic conditions.

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References

Azarkina N, Siletsky S, Borisov V, von Wachenfeldt C, Hederstedt L, Konstantinov AA (1999) A cytochrome *bb*'-type quinol oxidase in *Bacillus subtilis* strain 168. J Biol Chem 274(46):32810–32817

Bengtsson J, Rivolta C, Hederstedt L, Karamata D (1999) *Bacillus subtilis* contains two small c-type cytochromes with homologous heme domains but different types of membrane anchors. J Biol Chem 274(37):26179–26184

Berry EA, Trumpower BL (1985) Isolation of ubiquinol oxidase from *Paracoccus denitrificans* and resolution into cytochrome bc_1 and cytochrome c-aa_3 complexes. J Biol Chem 260(4):2458–2467

Björklöf K, Zickermann V, Finel M (2000) Purification of the 45 kDa, membrane bound NADH dehydrogenase of *Escherichia coli* (NDH-2) and analysis of its interaction with ubiquinone analogues. FEBS Lett 467(1):105–110

Castellani M, Covian R, Kleinschroth T, Anderka O, Ludwig B, Trumpower BL (2010) Direct demonstration of half-of-the-sites reactivity in the dimeric cytochrome bc_1 complex: enzyme with one inactive monomer is fully active but unable to activate the second ubiquinol oxidation site in response to ligand binding at the ubiquinone reduction site. J Biol Chem 285(1):502–510. doi:10.1074/jbc.M109.072959

de Vrij W, van den Burg B, Konings WN (1987) Spectral and potentiometric analysis of cytochromes from *Bacillus subtilis*. Eur J Biochem 166(3):589–595

Hägerhäll C, Aasa R, von Wachenfeldt C, Hederstedt L (1992) Two hemes in *Bacillus subtilis* succinate:menaquinone oxidoreductase (complex II). Biochemistry 31(32):7411–7421

Henning W, Vo L, Albanese J, Hill BC (1995) High-yield purification of cytochrome aa_3 and cytochrome caa_3 oxidases from *Bacillus subtilis* plasma membranes. Biochem J 309(Pt 1):279–283

Kunst F, Ogasawara N, Moszer I, Albertini AM, Alloni G, Azevedo V, Danchin A (1997) The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. Nature 390(6657):249–256. doi:10.1038/36786

Kurokawa T, Sakamoto J (2005) Purification and characterization of succinate:menaquinone oxidoreductase from *Corynebacterium glutamicum*. Arch Microbiol 183(5):317–324. doi:10.1007/s00203-005-0775-8

Kutoh E, Sone N (1988) Quinol-cytochrome c oxidoreductase from the thermophilic bacterium PS3. Purification and properties of a cytochrome $bc_1(b_6f)$ complex. J Biol Chem 263 (18):9020–9026

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227(5259):680–685

Lauraeus M, Wikström M (1993) The terminal quinol oxidases of *Bacillus subtilis* have different energy conservation properties. J Biol Chem 268(15):11470–11473

Lenaz G, Genova ML (2009) Structural and functional organization of the mitochondrial respiratory chain: a dynamic super-assembly. Int J Biochem Cell Biol 41(10):1750–1772

Lino B, Carrillo-Rayas MT, Chagolla A, González de la Vara LE (2006) Purification and characterization of a calcium-dependent protein kinase from beetroot plasma membranes. Planta 225 (1):255–268. doi:10.1007/s00425-006-0343-8

Liu J, Krulwich TA, Hicks DB (2008) Purification of two putative type II NADH dehydrogenases with different substrate specificities from alkaliphilic *Bacillus pseudofirmus* OF4. Biochim Biophys Acta 1777(5):453–461. doi:10.1016/j.bbabio.2008.02.004

Liu X, Gong X, Hicks DB, Krulwich TA, Yu L, Yu CA (2007) Interaction between cytochrome caa_3 and F_1F_0-ATP synthase of alkaliphilic *Bacillus pseudofirmus* OF4 is demonstrated by saturation transfer electron paramagnetic resonance and differential scanning calorimetry assays. Biochemistry 46(1):306–313. doi:10.1021/bi0619167

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193 (1):265–275

Markwell MA, Haas SM, Bieber LL, Tolbert NE (1978) A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal Biochem 87(1):206–210

Niebisch A, Bott M (2003) Purification of a cytochrome bc-aa_3 supercomplex with quinol oxidase activity from *Corynebacterium glutamicum*. Identification of a fourth subunity of cytochrome aa_3 oxidase and mutational analysis of diheme cytochrome c_1. J Biol Chem 278(6):4339–4346. doi:10.1074/jbc.M210499200

Richardson DJ (2000) Bacterial respiration: a flexible process for a changing environment. Microbiology 146(Pt 3):551–571

Santana M, Ionescu MS, Vertes A, Longin R, Kunst F, Danchin A, Glaser P (1994) *Bacillus subtilis* F_0F_1 ATPase: DNA sequence of the atp operon and characterization of atp mutants. J Bacteriol 176 (22):6802–6811

Schnorpfeil M, Janausch IG, Biel S, Kröger A, Unden G (2001) Generation of a proton potential by succinate dehydrogenase of *Bacillus subtilis* functioning as a fumarate reductase. Eur J Biochem 268:5. doi:10.1046/j.1432-1327.2001.02202.x

Shevchenko A, Sunyaev S, Loboda A, Bork P, Ens W, Standing KG (2001) Charting the proteomes of organisms with unsequenced genomes by MALDI-quadrupole time-of-flight mass spectrometry and BLAST homology searching. Anal Chem 73(9):1917–1926

Shevchenko A, Wilm M, Vorm O, Mann M (1996) Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. Anal Chem 68(5):850–858

Sone N, Sekimachi M, Kutoh E (1987) Identification and properties of a quinol oxidase super-complex composed of a bc_1 complex and cytochrome oxidase in the thermophilic bacterium PS3. J Biol Chem 262(32):15386–15391

Sonenshein AL, Hoch JA, Losick R (2002) Bac*illus subtilis and its closest relatives.* From genes to cells. Washington, DC: ASM Press.

Sousa PM, Silva ST, Hood BL, Charro N, Carita JN, Vaz F, Penque D, Conrads TP, Melo AM (2011) Supramolecular organizations in the aerobic respiratory chain of *Escherichia coli*. Biochimie 93 (3):418–425. doi:10.1016/j.biochi.2010.10.014

Stroh A, Anderka O, Pfeiffer K, Yagi T, Finel M, Ludwig B, Schägger H (2004) Assembly of respiratory complexes I, III, and IV into NADH oxidase supercomplex stabilizes complex I in *Paracoccus denitrificans*. J Biol Chem 279(6):5000–5007. doi:10.1074/jbc.M309505200

Tanaka T, Inoue M, Sakamoto J, Sone N (1996) Intra- and inter-complex cross-linking of subunits in the quinol oxidase supercomplex from thermophilic *Bacillus* PS3. J Biochem 119(3):482–486

Thomas PE, Ryan D, Levin W (1976) An improved staining procedure for the detection of the peroxidase activity of cytochrome P-450 on sodium dodecyl sulfate polyacrylamide gels. Anal Biochem 75 (1):168–176

Winstedt L, von Wachenfeldt C (2000) Terminal oxidases of *Bacillus subtilis* strain 168: one quinol oxidase, cytochrome aa_3 or cytochrome *bd*, is required for aerobic growth. J Bacteriol 182 (23):6557–6564

Wittig I, Beckhaus T, Wumaier Z, Karas M, Schägger H (2010) Mass estimation of native proteins by blue native electrophoresis: principles and practical hints. Mol Cell Proteomics 9(10):2149–2161. doi:10.1074/mcp.M900526-MCP200

Wittig I, Braun HP, Schägger H (2006) Blue native PAGE. Nat Protoc 1(1):418–428. doi:10.1038/nprot.2006.62

Wittig I, Karas M, Schägger H (2007) High resolution clear native electrophoresis for in-gel functional assays and fluorescence studies of membrane protein complexes. Mol Cell Proteomics 6 (7):1215–1225. doi:10.1074/mcp.M700076-MCP200

Wittig I, Schägger H (2008) Features and applications of blue-native and clear-native electrophoresis. Proteomics 8(19):3974–3990. doi:10.1002/pmic.200800017

Yu J, Hederstedt L, Piggot PJ (1995) The cytochrome *bc* complex (menaquinone:cytochrome *c* reductase) in *Bacillus subtilis* has a nontraditional subunit organization. J Bacteriol 177(23):6751–6760

Yu J, Le Brun NE (1998) Studies of the cytochrome subunits of menaquinone:cytochrome *c* reductase (*bc* complex) of *Bacillus subtilis*. Evidence for the covalent attachment of heme to the cytochrome b subunit. J Biol Chem 273(15):8860–8866

Zarbiv G, Li H, Wolf A, Cecchini G, Caplan SR, Sourjik V, Eisenbach M (2011) Energy complexes are apparently associated with the switch-motor complex of bacterial flagella. J Mol Biol. doi:10.1016/j.jmb.2011.12.027