The aerobic respiratory chain of *Escherichia coli*: from genes to supercomplexes

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In spite of the large number of reports on the aerobic respiratory chain of *Escherichia coli*, from gene transcription regulation to enzyme kinetics and structural studies, an integrative perspective of this pathway is yet to be produced. Here, a multi-level analysis of the aerobic respiratory chain of *E. coli* was performed to find correlations between gene transcription, enzyme activity, growth dynamics, and supercomplex formation and composition. The transcription level of all genes encoding the aerobic respiratory chain of *E. coli* varied significantly in response to bacterial growth. Coordinated expression patterns were observed between the genes encoding NADH : quinone oxidoreductase and complex I (NDH-1), alternative NADH : quinone oxidoreductase (NDH-2) and cytochrome *bd* I, and also between *sdhA* and *appC*, encoding succinate dehydrogenase and cytochrome *bd* II, respectively. In general, the rates of the respiratory chain activities increased from mid-exponential to late-stationary phase, with no significant further variation occurring until the mid-stationary phase. Multi-level correlations between gene transcription, enzyme activity and growth dynamics were also found in this study. The previously reported NADH dehydrogenase and formate : oxygen oxidoreductase supercomplexes of *E. coli* were already assembled at mid-exponential phase and remained throughout growth. A new succinate oxidase supercomplex composed of succinate dehydrogenase and cytochrome *bd* II was identified, in agreement with the suggestion provided by the coordinated transcription of *sdhA* and *appC*.

INTRODUCTION

The respiratory chains of the facultatively anaerobic, Gram-negative bacterium *Escherichia coli* have been intensively studied since the early 60s of the last century (Hendler & Burgess, 1972; Ingledew & Poole, 1984; Price & Driessen, 2010). Many studies of the structure, kinetics and transcriptional regulation of both the aerobic and anaerobic electron transfer chains, which consider either the whole pathway or the individual enzymes, have been reported separately, but an overall analysis of the respiratory pathway is lacking.

The aerobic electron transfer chain of *E. coli* is located in the cytosolic membrane and comprises three major primary dehydrogenases, e.g. a succinate : quinone oxidoreductase (SDH) (Kasahara & Anraku, 1974) and two NADH dehydrogenases (see Melo et al., 2004 for a review), a type I NADH : quinone oxidoreductase (complex I or NDH-1), a type II NADH : quinone oxidoreductase (NDH-2) (Bragg & Hou, 1967), three ubiquinol : oxygen oxidoreductases, and cytochromes *b* (Minagawa *et al.*, 1992), *bd* and *bd* II (Sturr *et al.*, 1996; Unden & Bongaerts, 1997). The apparent redundancy of the NADH dehydrogenases and oxygen reductases, which is also observed in other prokaryotes, may provide this bacterium with the required robustness to adjust to a wide range of environmental conditions (Pereira *et al.*, 2004), like temperature, carbon source or oxygen availability.

**Abbreviations**: BN-PAGE, blue native PAGE; DA-NADH, deamino-NADH; DCPIP, dichlorophenol indophenol; ES, early stationary phase; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LS, late-stationary phase; ML, mid-log phase; MS, mid-stationary phase; NBT, nitro blue tetrazolium; NDH-1, NADH : quinone oxidoreductase or complex I; NDH-2, alternative NADH : quinone oxidoreductase; qRT-PCR, quantitative real-time PCR; SDH, succinate : quinone oxidoreductase.

Two supplementary tables are available with the online version of this paper.
The supramolecular organization of the aerobic respiratory chain in supercomplexes has already been studied (see Dudkina et al., 2010 for a review), namely in bacteria such as Paracoccus denitrificans (Stroh et al., 2004) and Aquifex aeolicus (Prunetti et al., 2010), and more recently in the cytoplasmic membrane of E. coli (Sousa et al., 2011). In the last, supercomplexes composed by NDH-1 and NDH-2, and by the aerobic formate dehydrogenase (FdoGHI) (Benoit et al., 1998) and cytochrome bo3 have been described.

The expression of the genes encoding the aerobic respiratory chain of E. coli depends on external factors, such as oxygen availability (Gunsalus, 1992), via the two-component ArcBA system (anoxic redox control), which senses oxygen availability and triggers gene transcription (Bekker et al., 2011; Rolfe et al., 2011), or the type of electron acceptors available, and this determines the transcription of the terminal reductases, for instance via the fumarate nitrate regulator, FNR (Spiro & Guest, 1990).

In addition, the expression of respiratory chain components varies according to the phase of bacterial growth (Wackwitz et al., 1999), due to the effect of many other transcriptional regulators (Keseler et al., 2011), whose expression is also dependent on the growth phase. Examples include Fis (Jackson et al., 2004), RpoS (RNA polymerase sigma factor) and IHF (integration host factor) (Unden & Bongaerts, 1997).

In the present work, we have performed a quantitative analysis of the transcription and activity of the E. coli aerobic respiratory chain components in the mid-exponential (mid-log; ML), as well as the early (ES), mid- (MS) and late stationary (LS) phases of growth, to generate a comprehensive view of the events involving the aerobic respiratory chain pathway during the growth of this bacterium. Furthermore, the timing of assembly of respiratory chain supercomplexes during the growth of E. coli was investigated.

**METHODS**

**Bacterial growth.** Wild-type E. coli cells K-12 (ATCC 23716) were grown aerobically in Luria–Bertani (LB) medium (Lennox, 1955) in 500 ml flasks with 100 ml bacterial culture, at 37 °C and 150 r.p.m. An overnight inoculum was prepared, 1 % of which was used to inoculate a new culture to be monitored. Bacterial growth was monitored by following OD600, leading to the generation of a typical growth curve from at least three independent cultures, and allowing the determination of the ML, ES, MS and LS (Fig. 1). Growth curves were fitted with the Richards growth model, using the package “grofit” for the R Language for Statistical Computing (Kahm et al., 2010). Samples from ML, ES, MS and LS were harvested and used for RNA isolation, cDNA synthesis and quantitative real-time PCR (qRT-PCR).

**Membrane preparation, solubilization and analysis.** Cells from E. coli were suspended in 50 mM MES, pH 6.0, disrupted in a French press (6000 p.s.i.; 414 MPa), and submitted to a low-speed centrifugation (14 000 g, 15 min) to pellet intact cells and cell debris. The supernatant was then ultracentrifuged (138 000 g, 2 h), and the membrane fraction was obtained in the pellet, suspended in the same buffer, aliquotted, frozen in liquid nitrogen and stored at −80 °C until used. Protein concentration was determined using the bicinchoninic acid (BCA) protein assay from Pierce (Smith et al., 1985). Membrane solubilization was performed with 6 g digitonin per gram of protein, in a buffer containing 50 mM NaCl, 5 mM aminocaproic acid, 1 mM EDTA, 2 mM PMSF and 50 mM imidazole/HCl, pH 6, as previously described (Sousa et al., 2011). Solubilized membranes (150 µg per lane) were resolved in a 3–10 % acrylamide gradient blue native-PAGE (BN-PAGE) gel (Krause & Seelert, 2008; Schägger & von Jagow, 1991; Wittig et al., 2006), and NADH:NBT (nitro blue tetrazolium) and succinate:NBT oxidoreductase activities were detected in the gel (Zerbetto et al., 1997).

Solubilized membranes (12.5 µg) were also applied to the top of a continuous sucrose gradient (0.3–1.5 M) in a buffer containing 15 mM Tris/HCl, pH 7, 20 mM KCl and 0.2 % digitonin, resolved by ultracentrifugation at 4 °C (20 h, 150 000 g) (Dudkina et al., 2005; Sousa et al., 2011) and collected in 1 ml fractions.

Membrane samples (50 or 100 µg) were resolved by SDS-PAGE (Laemmli, 1970), transferred onto a PVDF membrane essentially according to (Towbin et al., 1979), and detected with polyclonal antibodies against subunits of respiratory chain complexes.

**RNA isolation, cDNA synthesis and quantitative real-time PCR (qRT-PCR).** RNA was extracted from 2.4 × 10⁸ cells harvested at ML, ES, MS and LS using the Aurum Total RNA Mini kit (Bio-Rad), essentially following the manufacturer’s instructions with the exception that the period of incubation with DNase I was extended to 45 min.

Isolated RNA was analysed by agarose gel electrophoresis, and spectrophotometrically in a NanoDrop instrument (Thermo) using the ratios A260/A280 and A260/A230, and the 350–220 nm absorption spectrum to assess integrity and purity. The absence of DNA in the
purified RNA samples was also verified by PCR with primers nuoCDrev and nuoCDfwd (Table 1).

Reverse transcription and qRT-PCR were performed with the iScript Select cDNA Synthesis kit (Bio-Rad) and SsoFast EvaGreen Supermix (Bio-Rad), respectively, following the manufacturer’s instructions, and using 1 μg RNA and 200 ng cDNA as appropriate. Purity and concentration of cDNA were evaluated as described for cDNA.

qRT-PCR data were collectively analysed to determine relative gene expression, using the Pfaffl method with correction for primer efficiency (Pfaffl, 2001).

Primer design, optimization and efficiency. The primers used in qRT-PCR experiments (Table 1) were designed using the Primer3 algorithm (Rozen & Skaletsky, 2000), and the reaction conditions were previously optimized in PCRs with genomic DNA. Primer efficiency was determined by qRT-PCR of five sequential dilutions of cDNA. The obtained threshold cycles were plotted against the log values of the corresponding cDNA concentrations, and primer efficiency was calculated based on the trend line slope.

Enzyme activities. NADH: potassium ferricyanide and succinate: DCPIP (dichlorophenol indophenol) oxidoreductase activities were determined spectrophotometrically by following the oxidation of deaminio-NADH (DA-NADH) and NADH at 340 nm (ε_{340nm} = 6.22 mM⁻¹ cm⁻¹), in a reaction buffer containing 100 mM MOPS, pH 7.2, 250 μM DA-NADH or NADH and 250 μM K,[Fe(CN)]₆ (Hatefi, 1978b), and by following the phenazine methosulfate (PMS)-coupled reduction of DCPIP at 578 nm at 37 °C (ε_{578nm} = 20.5 mM⁻¹ cm⁻¹), in a reaction mixture containing 100 mM MOPS, pH 7.2, 0.05 mM PMS, 0.05 mM DCPIP and 20 mM succinate (Hatefi, 1978a), respectively.

Oxygen uptake due to (DA)-NADH, succinate and quinol oxidation was monitored in a Rank Broths oxygen electrode (Hansatech) at 37 °C. The oxidation of NADH and succinate was measured in a buffer containing 50 mM MOPS, pH 7.2, and 1 mM NADH or 20 mM succinate, respectively. Quinol:oxygen oxidoreductase activity was assayed in a reaction mixture containing 100 mM MOPS, pH 7.2, 50 mM KCl, 0.5 mM EDTA, 5.7 mM DTT and 80 μM coenzyme Q₁. All assays were ended by the addition of 0.5 or 2.5 mM KCN, an inhibitor of haem-copper oxidases.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS). Bands with succinate:NBT oxidoreductase activity resulting from BN-PAGE analysis of digitonin-solubilized membranes were excised from the gel, in-gel digestion was performed, and peptides were extracted and vacuum-dried. The sucrose gradient fractions were buffer-exchanged into 25 mM ammonium bicarbonate, pH 7.5, using Amicon Ultra-0.5 centrifugal filters (10 kDa MWCO, Millipore) through three consecutive concentration and resuspension cycles. Samples were loaded onto a 1D-PAGE gel and electrophoresed briefly to run the proteins into the stacking gel. Bands were excised and treated as above. Tryptic peptides were resuspended in 0.1 % (v/v) trifluoroacetic acid and analysed by nanoflow liquid chromatography (Easy-nLC, Thermo Fisher Scientific) coupled online with an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific). All of the samples were resolved on a 100 μm inner diameter × 360 μm outer diameter × 20 cm long capillary column (Polyimicro Technologies), which was slurry-packed in house with a 5 μm, 30 nm pore-size C-18 silica-bonded stationary phase (Jupiter, Phenomenex). Following precursor and analytical column equilibration, each sample was loaded onto a 2 cm reversed-phase (C-18) precolumn (Thermo Fisher Scientific) at 2 μl min⁻¹ for 6 min with mobile phase A (0.1 % (v/v) formic acid in water). Peptides were eluted at a constant flow rate of 200 nl min⁻¹ by development of a linear gradient of 0.33 % min⁻¹ mobile phase B (0.1 % formic acid in acetonitrile) for 120 min and then to 95 % B for an additional 15 min. The column was washed for 15 min at 95 % B and then quickly brought to 100 % A for the next sample injection. The LTQ-Orbitrap XL mass spectrometer was configured to collect high-resolution (R = 60 000 at m/z 400) broadband mass spectra (m/z 375–1800) using the lock mass feature for the polydimethylcyclosiloxane (PCM) ion generated in the electrospray process (m/z 445.12002).

Protein identification. Tandem mass spectra were searched against UniProt Escherichia coli protein databases (07/2011) from the European Bioinformatics Institute (http://www.ebi.ac.uk/integ8/EBI-Integr8-HomePage.do;jsessionid=624E6C5BA922A73EC29EF3F7F4262E02) using Mascot Daemon (Matrix Science). The data were searched with a precursor mass tolerance of 10 p.p.m. and a fragment ion tolerance of 0.8 Da. Methionine oxidation (+15.99492 Da) was set as a dynamic modification, and a maximum of two missed cleavages were allowed. An automatic decoy search was performed on all raw files, and peptides were filtered using an ion score cut-off of 25, resulting in a

Table 1. Oligonucleotides used in qRT-PCR experiments, genes whose expression was analysed, and encoded proteins

<table>
<thead>
<tr>
<th>Gene</th>
<th>Oligonucleotide</th>
<th>Sequence (5’–3’)</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>appC</td>
<td>AppCRev</td>
<td>GAAAGGCCAGTTGTCCGTGTGTT</td>
<td>Cytochrome bdII O₂ reductase</td>
</tr>
<tr>
<td></td>
<td>AppCFwd</td>
<td>GGTGGTTTATGACCGAGTTG</td>
<td></td>
</tr>
<tr>
<td>cydA</td>
<td>CydBRev</td>
<td>GTCGGTGAGAACCAGACGGAGGT</td>
<td>Cytochrome bdI O₂ reductase</td>
</tr>
<tr>
<td></td>
<td>CydAFwd</td>
<td>GTTATCGGCTGAGAAAGGCTGA</td>
<td></td>
</tr>
<tr>
<td>cyoB</td>
<td>CyoBRev</td>
<td>GTTTATTGTCAGGCAGGTCAG</td>
<td>Cytochrome bo3 O₂ reductase</td>
</tr>
<tr>
<td></td>
<td>CyoBFwd</td>
<td>TGGCATTATTTTGGAGGTCCTG</td>
<td></td>
</tr>
<tr>
<td>ndh</td>
<td>NdhRev</td>
<td>ACACCTTGGCGTTAGAATCTGT</td>
<td>NDH-2</td>
</tr>
<tr>
<td></td>
<td>NdhFwd</td>
<td>CGAAGAAGGTCGTCGTTT</td>
<td></td>
</tr>
<tr>
<td>nuoCD</td>
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<td>NDH-1</td>
</tr>
<tr>
<td></td>
<td>NuoCDrev</td>
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<td></td>
</tr>
<tr>
<td>nuoF</td>
<td>NuoFRev</td>
<td>CGGAGATGACGTACCTTCCAC</td>
<td>NDH-1</td>
</tr>
<tr>
<td></td>
<td>NuoFwd</td>
<td>GCTGTTAATGGCGGATGAATGT</td>
<td></td>
</tr>
<tr>
<td>16S rRNA</td>
<td>16SRev</td>
<td>TACCCGGGCTGTCGCGCAC</td>
<td>Ribosome</td>
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<tr>
<td></td>
<td>16Sfwd</td>
<td>TGGAAGTATTGACCTGGTCAG</td>
<td></td>
</tr>
<tr>
<td>sdhA</td>
<td>SdhARev</td>
<td>CAACCACATTTCGCGCTTCTCT</td>
<td>SDH</td>
</tr>
<tr>
<td></td>
<td>SdhAFwd</td>
<td>ATTCCGGTTATCCCAAACCTGTC</td>
<td></td>
</tr>
</tbody>
</table>
false peptide discovery rate of <1%. In cases where peptides were identified in more than one protein sequence in the database, protein identifications were compiled based on the highest score UniProt accession number listed in the protein match results for each peptide.

Statistical analyses. The correlations between growth, enzyme activities and transcription fold changes were estimated by the Kendall rank correlation coefficient, \( \tau \) (Kendall, 1938). This coefficient can be interpreted as the probability of two observables having the same sequence of ranks. Perfect correlation between two variables is given by \( \tau = 1 \), indicating an identical sequence of ranks, e.g. 4-3-2-1, in both variables. Perfect anti-correlation is given by \( \tau = -1 \), which can be obtained, e.g. by having a rank sequence 4-3-2-1 in one variable and 1-2-3-4 in the other one. The ranks of each observable were determined by pairwise \( t \) tests for the equality of mean values at each time point. In the case of a non-significant difference, a tie of ranks was assumed. The corresponding mean values at each time point. In the case of a non-significant difference, a tie of ranks was assumed. The corresponding mean values at each time point. In the case of a non-significant difference, a tie of ranks was assumed.

\[
\sqrt{1 - \tau^2} \leq |t| < 0.9 \text{ were accepted as weak correlations.}
\]

\[
|t| = \pm 0.667 \text{ indicates that the rank sequences of both series were in perfect concordance with the exception of a single pair of values. Therefore, values in the range } 0.667 \leq |t| < 0.9 \text{ were accepted as weak correlations.}
\]

RESULTS

Growth curve and sampling

The aerobic respiratory chain of \textit{E. coli} was studied during bacterial growth, with the purpose of investigating the timing of assembly of its supercomplexes, and to establish eventual correlations between their composition, and gene transcription and enzyme activity profiles.

The aerobic growth curve of \textit{E. coli} K-12 in LB medium was obtained, and times of sampling for further analysis covering all growth stages were established, namely ML, ES, MS and LS (Fig. 1).

Transcription analysis of respiratory chain components

The relative expression of genes encoding the aerobic electron transfer chain enzymes (Table 1) was analysed by qRT-PCR, in mRNA extracted from aerobically grown \textit{E. coli} at the above-mentioned stages, to establish the transcription variation profile of its components. Primer efficiencies were determined and used in the calculation of relative expression ratios, allowing the comparison of transcript abundance between the different genes (Pfaffl, 2001).

Notable variations in the amounts of respiratory chain transcripts were observed as growth progressed (Fig. 2). According to the variation profile of gene transcription, two groups emerged. One was composed of \textit{nuoF}, \textit{ndh} and \textit{cydA}, which presented the highest levels of transcripts at ML, and decreased in the subsequent phases of growth. It is noteworthy that \textit{nuoF} and \textit{cydA} are the most abundant transcripts at ML. The second group comprised \textit{appC}, \textit{cyoB} and \textit{sdhA}, whose transcriptional levels were minimal at ML. In the next stages of growth, \textit{sdhA} and \textit{appC} displayed a similar variation profile (Fig. 2).

The similarities in temporal development of gene transcription during bacterial growth were quantified by Kendall’s \( \tau \) coefficient. Almost perfect positive correlations were observed between the transcription profiles of \textit{nuoF} and \textit{ndh} and also between \textit{nuoF} and \textit{cydA} (Fig. 3). Moreover, a weaker but still important positive correlation between the expression of \textit{ndh} and \textit{cydA} was in evidence. The transcription of \textit{sdhA} and \textit{appC} genes presented a perfect positive correlation (\( \tau = 1 \); Fig. 3). The expression of \textit{cyoB} did not correlate with any of the respiratory chain genes analysed.

![Fig. 2. Relative transcription levels between each aerobic respiratory chain gene and the most expressed gene/condition (ML-NuoF), normalized to the 16S rRNA reference gene. The assays were conducted in triplicate biological samples. The relative amount of each transcript was calculated using the Pfaffi method with correction for efficiency of primer amplification, to allow comparison of the relative expression between the different genes investigated. Error bars, SEM, calculated following the rules of error propagation (Vandesompele et al., 2002). White, black, dark-grey and light-grey bars correspond to ML, ES, MS and LS, respectively.](http://mic.sgmjournals.org)
Enzyme activity and protein content of respiratory chain components

Respiratory chain activities were determined for the individual enzymes and the whole electron transfer pathway, in membranes isolated from E. coli cells harvested at the selected stages of growth, namely ML, ES, MS and LS.

The rate of oxygen consumption due to NADH oxidation, which accounts for the two NADH dehydrogenases and the three quinol : oxygen reductases, did not present dramatic changes during growth, being slightly smaller at MS than during the rest of growth (Fig. 4a). This activity was ~90 % inhibited by KCN at all growth stages. In contrast, when using the NDH-1-specific substrate DA-NADH, the rate of oxygen consumption was minimal at ML, doubled at ES and remained unchanged until the end of growth. KCN inhibition of the oxygen uptake due to DA-NADH oxidation was ~80 %, presenting a decrease of 10 % in comparison with NADH oxidation. In addition, at each growth stage, the rate of oxygen consumption by E. coli membranes respiring DA-NADH was less than half than when NADH was the respiratory substrate (Fig. 4a). NDH-1 and NDH-2 activities, or NDH-1 activity alone, were assessed by measuring NADH : or DA-NADH : K3[Fe(CN)6] oxidoreductase activities, respectively. Both activities nearly doubled from ML to ES phase and remained constant throughout stationary phase, according to the intensity of cross-reactivity against the SdhA subunit. Cytochrome bdI, in agreement with the literature (Kita et al., 1984), is predominant in stationary phase and nearly absent in the exponential phase, based on the immunodecoration of the CydB subunit. The content of NDH-1 was higher at ES, although the overall variation was not substantial, as inferred from the detection of the NuoI subunit (Fig. 5).

Correlation between bacterial growth, enzyme activity and gene expression

Envisaging the acquisition of an integrated picture of respiratory chain-related events during growth, the full set of data obtained via the different methodological
approaches was analysed using the Kendall $\tau$ correlation coefficient (Fig. 3).

With the exception of the NADH:O$_2$ oxidoreductase activity, all other primary dehydrogenase activities analysed were positively correlated with each other. Perfect positive correlations were verified between the DA-NADH:O$_2$ and DA-NADH:K$_3$[Fe(CN)$_6$] oxidoreductase activities, and almost perfect correlations between succinate:O$_2$ and NADH:K$_3$[Fe(CN)$_6$] oxidoreductase activities. The remaining dehydrogenase activities presented weak, but important, positive correlations between each other. Quinol:oxygen oxidoreductase activity also showed a positive correlation with DA-NADH:O$_2$ and DA-NADH:K$_3$[Fe(CN)$_6$] oxidoreductase activities. Furthermore, all dehydrogenase activities, with the exception of NADH:O$_2$ oxidoreductase, showed positive correlations with the progression of growth, as inferred from OD$_{600}$. The OD$_{600}$ values, in turn, correlated negatively with the expression of $nuoF$, $ndh$ and $cydA$. With the exception of $cyoB$, a negative correlation between the transcription profiles of all genes encoding the respiratory chain and enzyme activities was observed. In detail, $sdhA$ and $appC$ expression correlated negatively with NADH:O$_2$ oxidoreductase activity, while the expression of $nuoF$, $cydA$ and $ndh$ exhibited a negative correlation with the remaining oxidoreductase activities, including succinate oxidoreductase (Fig. 3).

Supramolecular assemblies

The presence of the supercomplexes of the $E. coli$ respiratory chain described previously (Sousa et al., 2011) was studied during bacterial growth to establish the growth phase in which these supramolecular structures are assembled. With this purpose, BN-PAGE-resolved membranes obtained from cells collected at ML, ES, MS and LS were probed for NADH:NBT and succinate:NBT oxidoreductase activities (Fig. 3). The remaining dehydrogenase activities presented weak, but important, positive correlations between each other. Quinol:oxygen oxidoreductase activity also showed a positive correlation with DA-NADH:O$_2$ and DA-NADH:K$_3$[Fe(CN)$_6$] oxidoreductase activities. Furthermore, all dehydrogenase activities, with the exception of NADH:O$_2$ oxidoreductase, showed positive correlations with the progression of growth, as inferred from OD$_{600}$. The OD$_{600}$ values, in turn, correlated negatively with the expression of $nuoF$, $ndh$ and $cydA$. With the exception of $cyoB$, a negative correlation between the transcription profiles of all genes encoding the respiratory chain and enzyme activities was observed. In detail, $sdhA$ and $appC$ expression correlated negatively with NADH:O$_2$ oxidoreductase activity, while the expression of $nuoF$, $cydA$ and $ndh$ exhibited a negative correlation with the remaining oxidoreductase activities, including succinate oxidoreductase (Fig. 3).
spectrometry analysis of this band (Table S1) confirmed that it contains NDH-1 and NDH-2.

Besides the trimeric (band 7) and monomeric forms of SDH (data not shown), two new homo- or hetero-oligomerizations of this enzyme were visualized by in gel activity of succinate:NBT oxidoreductase activity in membranes resolved by BN-PAGE (Fig. 6b, bands 5 and 6), which were also observed in membranes harvested from ML to LS. Peptides of SDH subunits A and B were identified in bands 5–7 upon mass spectrometry analysis (Table S1).

Possible partners of the new oligomerizations containing NDH-1 and NDH-2, and SDH, were investigated by detecting NADH:NBT and succinate:NBT activity in membranes of E. coli strains devoid of NDH-1 (DnuoB) and NDH-2 (Dndh) from cells collected at LS (c). Bands 1, 3 and 4 were previously sequenced (Sousa et al., 2011). Peptides from NDH-1 and NDH-2 were retrieved from LC-MS/MS analysis of band 2a, and of SDH in bands 5 and 6. The molecular mass of the detected bands was estimated using known membrane protein markers from bovine or chicken heart mitochondria, as described by Wittig et al. (2010).

sdhA and appC transcription presented a perfect positive correlation (Fig. 2). To investigate an eventual supramolecular association of SDH and cytochrome bdII, membranes from the MB37 strain of E. coli, where complex I, and bdI and bo3, cytochrome oxidases are inactivated (Bekker et al., 2009), were solubilized, resolved in a 1.5–0.3 M sucrose gradient, and characterized regarding the distribution of succinate:oxygen and quinol:oxygen oxidoreductase activities. As expected, the quinol:oxygen oxidoreductase activity was present from the heaviest to lightest gradient fractions. In contrast, succinate:oxygen oxidoreductase activity was only observed in the heaviest fractions, F1 and F2 (Fig. 7). Upon detergent solubilization, the detection of this activity is only possible if intercomplex interactions are preserved between succinate dehydrogenase and an oxygen reductase, thus indicating the presence of a succinate oxidase supercomplex. Since the sole oxygen reductase expressed in strain MB37 is cytochrome bdII, the assembly of a supercomplex containing SDH and cytochrome bdII was confirmed. Furthermore, direct molecular evidence of the presence of SDH and cytochrome bdII in fractions F1 and F2 of the MB37 gradient was obtained by LC-MS/MS analysis of these fractions. Peptides of all subunits of each complex (SdhABCD and AppAB) were identified in these fractions (Table S2), confirming the composition of the supercomplex identified here, which contains SDH and cytochrome bdII, as suggested by the correlation observed between the gene transcription data.


**DISCUSSION**

The work presented herein used an innovative approach in integrating data corresponding to different levels of cell metabolism, growth dynamics, gene expression, protein expression and activity, and supramolecular assembly to provide a global overview of the aerobic respiratory pathway of *E. coli*.

Notable differences in the relative expression of genes encoding aerobic respiratory chain components were observed: changes of almost one order of magnitude from one growth stage to the next were detected, being generally more pronounced in the transition from the early exponential phase (MS) to the stationary phase (LS) (Fig. 2). The possibility that, besides the growth phase, the variation of oxygen concentration during growth also affected gene expression should not be disregarded. Although there is always some oxygen present during growth (Van Suijdam *et al.*, 1978), it is likely that growth at the latter stages of growth (MS and LS) is limited by the oxygen supply. The transcription level variation of *sdhA*, *cydA* and *cydA* genes during growth did not follow the trend described for progressive oxygen concentration decrease reported by Rolfe *et al.* (2011). It can thus be assumed that although the oxygen concentration in the cultures was low, it was almost unchanged during the sample collection period.

In an array constructed with cDNA prepared from mRNA from *E. coli* K-12/MG1655 cells grown aerobically in minimal medium M9 supplemented with glucose (Wei *et al.*, 2001), other authors verified that globally there are no dramatic variations in gene expression between the exponential and ES phases. However, in agreement with our results, the genes involved in the aerobic respiratory chain presented a decrease in expression from ML to stationary phase.

The transcription of the NADH dehydrogenases encoding genes *nuoF* and *ndh* was positively correlated (Fig. 3), in agreement with the previously identified NADH dehydrogenase supercomplex that contains NDH-1 and NDH-2 (Sousa *et al.*, 2011). The abundance of *nuoF* transcripts was always larger than that of *ndh*, in agreement with earlier studies (Wackwitz *et al.*, 1999); in addition, and in agreement with those authors, the relative expression of *ndh* was highest at ML.

The relative expression of *cydA* also displayed a positive correlation with *nuoF* and *ndh*, suggesting that cytochrome *bdII* could be a preferential intermediate in oxygen reduction by the electrons that result from NADH oxidation by the NADH dehydrogenase supercomplex. The progressive decrease of the transcription level of *cydA* is in contradiction with earlier reports, in which the expression of the *cyd* operon increased when *E. coli* cultures entered the stationary phase, presumably due to decreased oxygen availability (Georgiou *et al.*, 1988). However, this could still be explained by considering that this gene is negatively regulated by the histone-like protein H-NS under aerobic conditions (Govantes *et al.*, 2000), and that this repressor is antagonized by Fis, which is abundant at the early exponential phase (Falconi *et al.*, 1993).

Perfect positive correlation was observed between the transcription profiles of *sdhA* and *appC*, suggesting an alternative electron transfer pathway composed of succinate:quinone oxidoreductase and cytochrome *bdII* oxygen reductase. This suggestion was further supported by the detection of succinate:oxygen oxidoreductase activity in fractions 1 and 2 of the *E. coli* MB37 strain sucrose gradient (Fig. 7), in which cytochrome *bdII* is the sole oxygen reductase present, and was confirmed by MS analysis of the above-mentioned fractions.

We have recently described a supramolecular assembly of formate dehydrogenase (Fdo) and cytochrome *boIII* in the respiratory chain of *E. coli* (Sousa *et al.*, 2011). The existence of these selective associations of the respiratory chain may account for the apparent redundancy of the respiratory chain enzymes observed in this bacterium and others. In terms of energy conservation, these combinations produce proton motive forces with different $H^+/e^−$ ratios, having a value of 3 in the case of the association of NDH-1 ($2H^{+}/e^−$), NDH-2 ($0H^{+}/e^−$) and cytochrome *bdII* ($1H^{+}/e^−$) (Puustinen *et al.*, 1991), and of 1 in the case of SDH ($0H^{+}/e^−$) (Cecchini *et al.*, 2003) and cytochrome *bdII* ($1H^{+}/e^−$) (Borisov *et al.*, 2011). Concerning the association between formate dehydrogenase and cytochrome *boIII*, and considering that there is no information about the eventual proton motive force generated by formate dehydrogenase, the $H^+/e^−$ ratio will be at least 2 (Puustinen *et al.*, 1989). In light of the available knowledge, there is no reasonable hypothesis to explain the physiological significance of the *E. coli* supercomplexes described.

Enzyme activities were also analysed by searching for correlations in their rate variation profile during bacterial growth. With the exception of NADH:oxygen oxidoreductase activity, the respiratory chain activities presented positive correlations with each other (Fig. 3). NADH:oxygen oxidoreductase activity is an outlier to this group, a fact that could be related to the presence of other enzymes with NADH:oxygen oxidoreductase activity (e.g. WrbA, YhdH and QOR; Bekker *et al.*, 2009) in the respiratory chain of *E. coli*.

It is worth mentioning that the highest transcription levels of *nuoF* and *ndh* observed at ML did not lead to a higher NADH and DA-NADH oxidoreductase activity or NDH-1 content. In fact, it was at LS that most genes presented lower transcription levels, but eventual corresponding decreases in enzyme activities were not observed. Such discrepancies between gene expression and enzyme activity could be due to partial mRNA degradation, a mechanism that results in modulation of gene transcription and which is widespread in bacteria (Bastet *et al.*, 2011). Another explanation could be regulation at the translational level, as proposed by Kramer *et al.* (2010), which seems to be the
case for cytochrome \textit{bdII}, whose protein levels are maximal at stationary phase. According to those authors, cytochrome \textit{bdII}-encoding genes have multiple levels of regulation, namely the transcriptional (see above) and translational levels. The primary dehydrogenase whose transcripts are most abundant at ES and MS is SDH, suggesting that it plays an important metabolic role during entry into stationary phase that is not necessarily related to the electron transfer chain.

Moreover, the negative correlations observed between the expression of \textit{sdhA} and \textit{appC} and NADH : \textit{O$_2$} oxidoreductase activity, and between that of \textit{nuoF}, \textit{cydA} and \textit{ndh} and the remaining oxidoreductase activities (Fig. 3), including succinate oxidoreductase, suggest that the aerobic respiratory chain of \textit{E. coli} is a multi-level tightly regulated pathway.

**Conclusions**

This is believed to be the first comprehensive study of the aerobic respiratory chain of \textit{E. coli} during bacterial growth, from gene transcription to enzyme activity and supramolecular organization.

Correlations between the aerobic respiratory chain components of this bacterium were observed at two levels: gene transcription and enzyme activity. The established correlations allowed the association of \textit{nuoF}, \textit{ndh} and \textit{cydA} in one group and of \textit{sdhA} and \textit{appC} in another, representing preferential enzyme partners in the electron transfer chain of \textit{E. coli}, at least partially organized in supercomplexes, and these predictions were supported by experimental data.

Furthermore, multi-level correlations were identified that related gene expression to enzyme activity and growth dynamics, suggesting a tight and complex regulation of the aerobic respiratory pathway.

It was shown that the previously described NADH dehydrogenase and formate : oxygen oxidoreductase supercomplexes are already formed at ML and are present until stationary phase (Fig. 6a). A novel supramolecular assembly containing succinate dehydrogenase and cytochrome \textit{bdII} was described.

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