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ORIGINAL PAPER

# Component identification of electron transport chains in curdlan-producing *Agrobacterium* sp. ATCC 31749 and its genome-specific prediction using comparative genome and phylogenetic trees analysis

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**Abstract** *Agrobacterium* sp. ATCC 31749 (formerly named *Alcaligenes faecalis* var. *myxogenes*) is a non-pathogenic aerobic soil bacterium used in large scale biotechnological production of curdlan. However, little is known about its genomic information. DNA partial sequence of electron transport chains (ETCs) protein genes were obtained in order to understand the components of ETC and genomic-specificity in *Agrobacterium* sp. ATCC 31749. Degenerate primers were designed according to ETC conserved sequences in other reported species. DNA partial sequences of ETC genes in *Agrobacterium* sp. ATCC 31749 were cloned by the PCR method using degenerate primers. Based on comparative genomic analysis, nine electron transport elements were ascertained, including NADH ubiquinone oxidoreductase, succinate dehydrogenase complex II, complex III, cytochrome *c*, ubiquinone biosynthesis protein ubiB, cytochrome *d* terminal oxidase, cytochrome *bo* terminal oxidase, cytochrome *cbb*$_3$-type terminal oxidase and cytochrome *caa*$_3$-type terminal oxidase. Similarity and phylogenetic analyses of these genes revealed that among fully sequenced *Agrobacterium* species, *Agrobacterium* sp. ATCC 31749 is closest to *Agrobacterium tumefaciens* C58. Based on these results a comprehensive ETC model for *Agrobacterium* sp. ATCC 31749 is proposed.

**Keywords** *Agrobacterium* sp. · Electron transport chains · Genomic-specificity analysis · Comparative genomic analysis · Curdlan

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**Abbreviations**

| | |
|---|---|
| Cyt *bo* | Cytochrome *bo* terminal oxidase |
| Cyto-*caa*$_3$ | Cytochrome *caa*$_3$-type terminal oxidase |
| Cyto-*cbb*$_3$ | Cytochrome *cbb*$_3$-type terminal oxidase |
| Cyt *d* | Cytochrome *d* terminal oxidase |
| Cyt *bd* | Cytochrome *bd* terminal oxidase |
| ETC | Electron transport chain |
| MDH | Malate dehydrogenase |
| NQ | NADH Ubiquinone oxidoreductase |
| *Idh* | Isocitrate dehydrogenase |
| *galU* | UTP-Glucose-1-phosphate uridylyltransferase |
| *glmM* | Phosphoglucosamine mutase |
| SDH | Succinate dehydrogenase |
| UbiB | Ubiquinone biosynthesis protein ubiB |
| Ubp | Ubiquinone biosynthesis protein |
| PTS | Phosphotransferase system |
| PYR | Pyruvate |
| bc$_1$ | Cytochrome *bc*$_1$ complex |
| Cyto-C | Cytochrome *c* |

## Introduction

Curdlan is a high molecular weight polymer of glucose which consists of $\beta$-(1,3)-linked glucose residues. It has been estimated that the average degree of polymerization is around 450. Under NaOH solution (>0.2 M), curdlan is completely soluble; after neutralization, it returns to the insoluble state [14]. Curdlan can be produced industrially in the most concentrated form of beta-glucan from glucose and other cheap carbon sources [44], and is the third microorganism-fermented polysaccharide to be approved by the FDA. Curdlan has been used in the food industry as an additive to improve the physical texture of food (textural, thickening and gelling agent in various applications). In addition to its numerous properties described in the literature, curdlan and other beta-glucans have been shown in recent years to prevent intestinal cancer [20, 29] and to enhance the immune response of humans [15, 41]. Therefore, curdlan is becoming increasingly important to the pharmaceutical industry because of its potential application as a therapeutic.

*Agrobacterium* sp. ATCC 31749 (formerly named *Alcaligenes faecalis* var. *myxogenes*) is a non-pathogenic aerobic soil bacterium that has gained considerable interest because of its use in large scale biotechnological production of curdlan [24] and its uniqueness in possessing a highly efficient UDP-glucose regeneration system [34]. The high flux of UDP-glucose regeneration in *Agrobacterium* sp. ATCC 31749 can be attributed to the fact that it is able to couple energy production and carbon metabolism [34]. The carbon metabolism mechanisms for UDP-glucose have been studied [21, 26]. However, there is little information concerning energy production and genomic information in *Agrobacterium* sp. ATCC 31749, especially its ATP regeneration system. Electron transport chains (ETC) serve as the energy pump of bacteria for regenerating ATP through oxidative phosphorylation. Therefore, research on ETC components and its gene information in *Agrobacterium* sp. ATCC 31749 should help us to exploit its unique metabolic capability for UDP-glucose regeneration during curdlan synthesis at the gene level.

Up to now, research on *Agrobacterium* sp. ATCC 31749 has been focused on curdlan synthetic process analysis [17], glucose metabolic network [51] and precursor engineering [23, 43]. However, little work has been done on ETC in *Agrobacterium* sp. ATCC 31749 itself. The traditional method for detection and identification of ETC components is based on the extraction of ETC proteins and their chromatography analysis [40]. This procedure is time-consuming and requires extensive training and equipment. Moreover, it can often be difficult to distinguish between species having similar protein chromatograph characteristics. Therefore, an accurate, rapid and reliable identification method for ETC components is required.

Recently, numerous studies have demonstrated the enormous value of sequence information from closely related species for aiding in the annotation of coding and non-coding features of a reference genome [5, 10, 33]. Comparative sequence analysis provides a rich source of information for improving gene annotations, assigning gene functions, identifying potential regulatory regions and building metabolic models [5]. The use of PCR can be combined with comparative genomic analysis in order to detect the primary components of ETC and predict the genomic specificity in micro-organisms.

The genomic sequence of *A. tumefaciens* C58 (ATCC 33970) has been available since 2001 [48]. More recently, the genome sequences of *A. vitis* S4 and *A. radiobacter* K84 have become available as well [38]. However, sequenced genomes of *Agrobacterium* species revealed that they have substantial gene content differences between them (Fig. 1). So, it is natural to ask which of these three species of *Agrobacterium* is the closest relative to *Agrobacterium* sp. ATCC 31749. Answering this question should help in the study of ETC components of *Agrobacterium* sp. ATCC 31749.

The aims of this work were to (1) clone and identify ETC genes in the *Agrobacterium* sp. ATCC 31749; (2) compare the sequences thus generated to the sequences of *A. tumefaciens* C58, *A. vitis* S4 and *A. radiobacter* K84 and others; and then (3) predict which fully sequenced genome is closest to *Agrobacterium* sp. ATCC 31749.

Partial sequences of ETC genes from *Agrobacterium* sp. ATCC 31749 were cloned with degenerate primers from

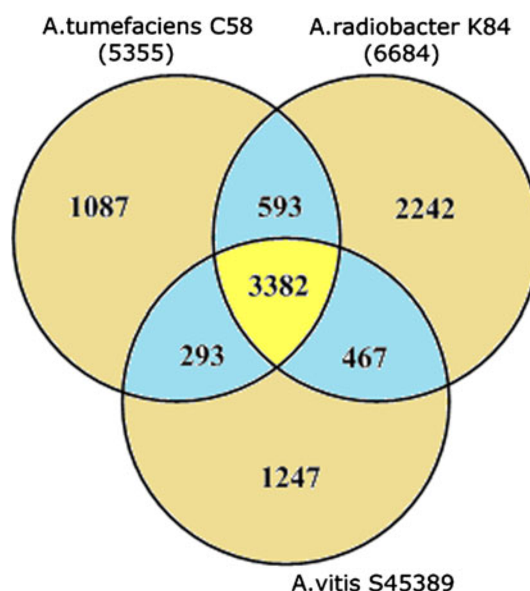


**Fig. 1** Venn diagram of protein coding genes of the genomes in *Agrobacterium tumefaciens* C58, *A. vitis* S4 and *A. radiobacter* K84 (from http://agro.vbi.vt.edu/public)

*A. tumefaciens* C58, *A. vitis* S4, *A. radiobacter* K84 and other species. With these PCR products, the components of the ETC and the potential electric transfer pathways in *Agrobacterium* sp. ATCC 31749 were proposed, and the genomic specificity based on comparative genomic analysis was identified.

## Materials and methods

### Bacterium and culture conditions

The curdlan-producing strain *Agrobacterium* sp. ATCC 31749 was used in this study. The strain was grown in a 250 mL flask containing 60 mL modified minimal medium (pH 7.0) consisting of $KH_2PO_4$ (1.74 g/L), $MgSO_4{\cdot}7H_2O$ (0.05 g/L), extract yeast (1.0 g/L) and glucose (20 g/L). The flask was shaken at 200 rpm at 30°C for 17–18 h.

### DNA extraction and purification

Chromosomal DNA was extracted with phenol/chloroform method (http://userpages.umbc.edu/~jwolf/m1.htm). DNA quantity was assessed by running samples on 1% agarose gel with electrophoresis method. Chromosomal DNA was further purified by phenol-chloroform-isoamyl alcohol extraction by the method of Wheatcroft and Watson [46]. Purified DNA was precipitated with ethanol, resuspended in sterile deionised water at about 1 mg/mL, based on optical density at 260 nm, and stored at −22°C until use.

### Degenerate primer design of ETC genomic sequences

The genomic sequences of select species which are related to the curdlan-producing strains (see Table 1) were chosen as model templates to design degenerate primers based on comparative analysis (data not shown). We used DNAMAN software for sequence alignment. Primers for amplification of different ETC proteins were designed with Primer 3.0 (http://frodo.wi.mit.edu/primer3/) based on the results of sequence alignments. The primer sequences are shown in Table 2 and the positions of the genes are shown in Fig. 2. All primers were synthesised by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China).

### PCR amplification and sequencing of ETC genes

The reaction was performed in a total volume of 50 µL consisting of 5 µL 100 ng DNA sample, 5 µL 10 × PCR buffer,

**Table 2** The degenerate primers designed for PCR amplifying various enzymes in electron transport chains

| ETC enzymes name | Degenerate primer |
|---|---|
| NQ | F:TTCACGGAAATCAATGACGA |
| | R:GACAGCCCGGAACGTAGATA |
| SDH | F:AACTCGCTCTCCCCAAGAAT |
| | R:GATCCTCATGGCTCTGCTTC |
| UbiB | F:TTTTGCAGCATCACCAGTTC |
| | R:GTCGGATATCGAGGGACTGA |
| Cyt *d* | F:TATGTCATGACCGGCCGCAC |
| | R:GAACAGCGTCGGCACATGC |
| Cyt *bo* | F:ACCATTCCAGCGTACGGGCG |
| | R:GGCGGCAATCCGGTGATGT |
| Cyto-$cbb_3$ | F:CGATGGTGGCGAGCCAGAAGTG |
| | R:ATGGTGCAGTGGTGGTACGG |
| Cyto-$caa_3$ | F:CCACAAGGACATCGGCACG |
| | R:GCCGACCGTGTACATGTGGTG |
| MDH | F:GTGGGCTGACCGAGAATTT |
| | R:CGTGCCCTATAACCGTGTTC |
| Cythchrom bc1 complex | F:GGTGAATGGATCCAGCAGTT |
| | R:CACGAACAGCCAGAAGAACA |
| Cytochrome *c* | F:ACGGTTTTCGTCCTGATGTC |
| | R:TCCGTTTCCTTCTTCACACC |

**Table 1** The chosen genomic sequence of these species related to the curdlan-producing strains

| Bacterial source | Source references | Chosen target genomic |
|---|---|---|
| *Agrobacterium* sp. 10C3 | Harada and Harada (1996) and Nakanishi et al. (1976) | *A. tumefaciens* C58 |
| *Agrobacterium* sp. ATCC 31749 | Stasinopoulos et al. (1999) | *A. vitis* S4 |
| | Nakanishi et al. (1976) | *A. radiobacter* K84 |
| *A. radiobacter* IFO12607, 12665,13127,13256 | Nakanishi et al. (1976) | *Rhizobium etli* CIAT 652 |
| *A. rhizogenes* IFO13259 | Ghai et al. (1981) | |
| *Rhizobium trifolii* J60 | Footrakul et al. (1981) | |
| *Rhizobium* sp. TISTR 64B | Buller (1990) | |
| *Cellulomonas* sp. *C. flavigena* KU | Kenyon and Buller (2002) | |

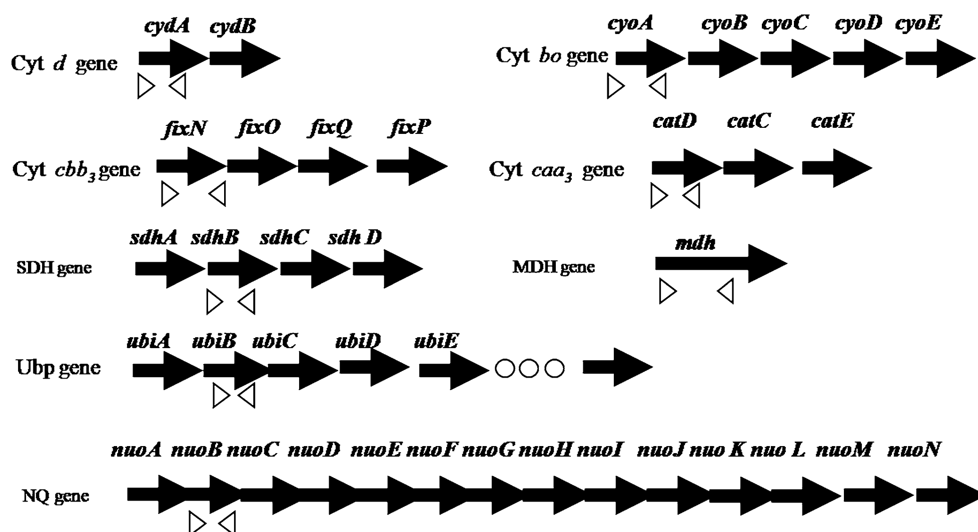


**Fig. 2** Proposed genomic organization of ETC genes in *Agrobacterium* sp. ATCC 31749 based on the genomic information in *Agrobacterium tumefaciens* C58. PCR target regions are indicated between (▷ ◁)

5 µL 200 µmol/L dNTPs, 0.5 µL 0.2 µmol/L primer (each primer) and 0.25 µL 5U/µL Taq polymerase (TaKaRa, Dalian, China), adjusted the total volume to 50 µL with sterilized distilled water. A negative control, consisting of all the reaction components except template DNA, was also included for each amplification. PCR conditions of different ETC genes are shown in Table 3. PCR products were visualized using 1.0% agarose gel stained with ethidium bromide. All amplified products were purified using TIANquick Midi Purification Kit (Tiangen, Beijing, China) according the protocol and then sent to Takara (Dalian, China) for sequencing analysis, the sequencing primers are shown in Table 2.

ETC Genes conservation sequences accession number

The nucleotide sequences of ETC genes in the *Agrobacterium* sp. ATCC 31749 strain reported in this paper have been deposited in GenBank. Their accession numbers are as follows: putative succinate dehydrogenase complexII: accession number GQ428124; putative NADH ubiquinone oxidoreductase chain B partial sequence: GQ428125; putative ubiquinone biosynthesis protein *ubiB* partial DNA sequence: GQ428126; putative cytochrome $cbb_3$-type terminal oxidase *fixN* chain partial sequence: GQ428128; putative cytochrome *d* terminal oxidase protein subunit I partial sequence: GQ428129; putative cytochrome *bo* terminal oxidase subunit I sequence: GQ428130; putative cytochrome $caa_3$-type terminal oxidase chain I partial sequence: GQ428131; putative cytochrome III complex cytochrome B subunit partial sequence HM543454; cytochrome *c* partial sequence HM543455.

Comparative genomics analysis

The search of the homologous nucleotide sequences of different cloned ETC genes was carried out with the BLAST algorithm provided by NCBI [2]. For comparative genome analysis, the sequences of different ETC proteins of *Agrobacterium* strains were downloaded from GenBank.

**Table 3** Optimized PCR protocol for putative different electron transport chains enzymes in *Agrobacterium* sp. ATCC 31749

| Targets | Cycles | Initial denaturation t (min) | Denaturation time (s) | Annealing | | Extension *t* (s) | PCR products size (bp) |
|---|---|---|---|---|---|---|---|
| | | | | T (°C) | *t* (s) | | |
| *NuoB* | 35 | 3 | 60 | 56 | 45 | 60 | 436 |
| *sdhB* | 30 | 3 | 60 | 54 | 45 | 60 | 474 |
| *ubiB* | 35 | 3 | 60 | 58 | 45 | 60 | 412 |
| *cydA* | 35 | 4 | 60 | 54 | 45 | 120 | 1,100 |
| *cyoA* | 35 | 2 | 60 | 60 | 45 | 90 | 790 |
| *fixN* | 30 | 3 | 60 | 56 | 45 | 90 | 600 |
| *catD* | 35 | 3 | 60 | 56 | 45 | 90 | 800 |
| *mdh* | 35 | 3 | 60 | 56 | 45 | 60 | 453 |
| *cycM* | 35 | 3 | 60 | 57 | 45 | 60 | 509 |
| *fbcB* | 35 | 3 | 60 | 55 | 45 | 60 | 546 |

Phylogenetic analyses

We used the supermatrix approach for constructing the phylogenetic tree [49]. The tree based on the concatenation of all sequences was obtained using the server at www.phylogeny.fr [13]. It uses MUSCLE for multiple alignment, GBLOCKS for multiple alignment automated trimming, and PHYML for tree building.

## Results

Ten predicted ETC genes in *Agrobacterium* sp. ATCC 31749 were successfully amplified by PCR (Fig. 3) under the conditions described in the "Materials and methods". The genes are the following:

NADH ubiquinone oxidoreductase

The proton-pumping NADH, ubiquinone oxidoreductase (NQ, Complex I), is the first respiratory chain complex in many bacteria [1, 25]. In general, bacterial NQ consists of 14 different subunits [36]. We obtained subunit B of NQ (*NuoB*).

Succinate dehydrogenase

Succinate dehydrogenase (SDH, Complex II) is an indispensable enzyme involved in the Krebs cycle and aerobic respiration. Usually it contains four subunits [8, 45]. We amplified the *sdhB* subunit of the succinate dehydrogenase gene.

Ubiquinone biosynthesis protein

Ubiquinone is an essential electron carrier in prokaryotes. Ubiquinone biosynthesis involves at least nine reactions. In the present work we mainly focused on identifying the ubiquinone biosynthesis protein (UbiB). The *ubiB* gene was thought to encode 2-octaprenylphenol hydroxylase, which catalyzes the first hydroxylation step in the ubiquinone biosynthesis pathway in bacteria [27, 30].

Complex III

Complex III (cytochrome $bc_1$ complex), a component of the electron transport chain, was encoded by *fbcFBC* genes in *Agrobacterium* sp. The sequence obtained was that cytochrome III complex cytochrome B subunit partial sequence (*fbcB*).

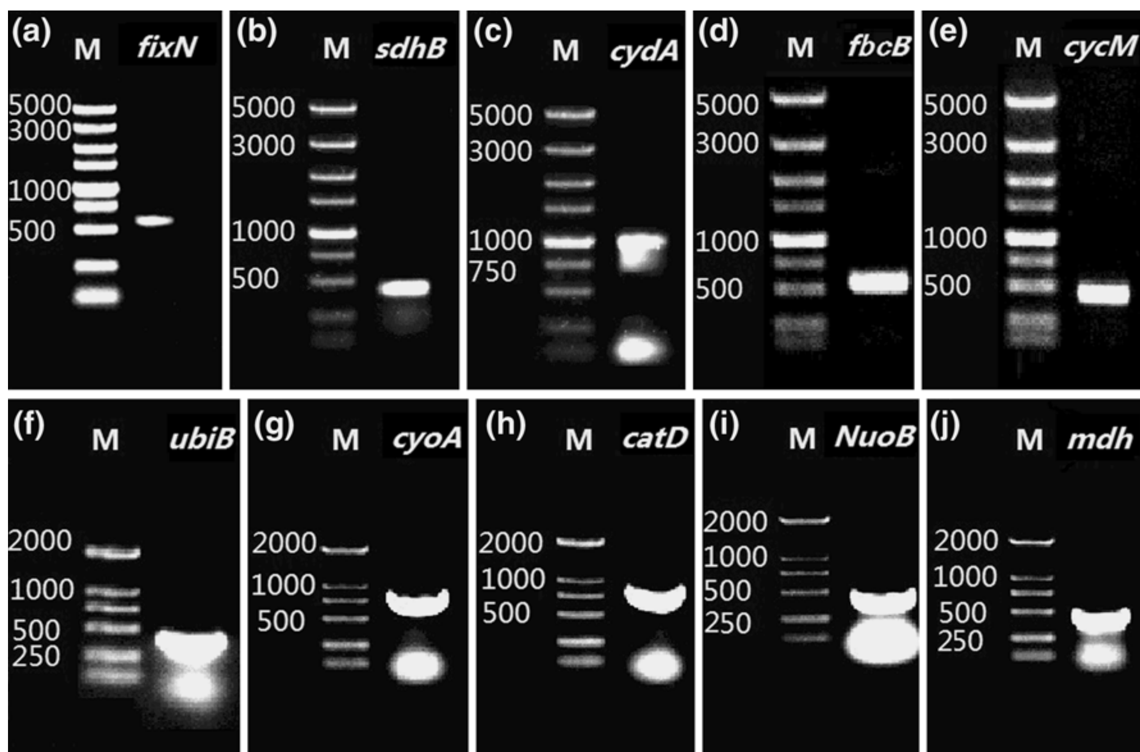


**Fig. 3** PCR detection of the ETC component genes in the genome *Agrobacterium* sp. ATCC 31749. Agarose gel analysis of products of PCR reactions were performed with total DNA as template and the primers listed in Table 2. The abbreviations of gene names are indicated above each *lane*. "*M*" indicates DNA molecular size marker (150 bp DNA Ladder, Takara)

### Cytochrome *c*

Cytochrome *c* is an essential component of the electron transport chain. It transfers electrons between Complexes III and IV. In bacteria, cytochrome *c* is encoded by *cycM c* [6]. The partial sequence obtained in this study was *cycM*.

### Cytochrome *d* terminal oxidase

The cytochrome *d* terminal oxidase (Cyt *d*) is the only well characterised bacterial terminal oxidase that is unrelated to the heme-copper oxidase superfamily and its function as a quinol oxidase. The genes *cydAB* involved in the expression of Cyt *d* have been identified in *Escherichia coli* and other species [18, 39]. The sequence obtained was that of *cydA*.

### Cytochrome *bo* terminal oxidase

Cytochrome *bo* terminal oxidase (Cyt *bo*) is a terminal oxidase encoded by the *cyoABCDE* operon [9, 28] that participates in the respiratory chain and is expressed under high oxygen growth conditions [47]. The sequence obtained was that of Cyt *bo* subunit sequence I (*cyoA*).

### Cytochrome $cbb_3$-type oxidase

Cytochrome $cbb_3$-type oxidase (Cyto-$cbb_3$) is a member of the heme copper oxidase superfamily. It is composed of four subunits and encoded by the *fixNOQP* operon [31]. The sequence obtained was that of subunit I (*fixN*) of cytochrome $cbb_3$-type oxidase.

### Cytochrome $caa_3$-type oxidase

Cytochrome $caa_3$-type oxidase (Cyto-$caa_3$) has a cytochrome *c* domain fused to subunit II, which is one of the highly conserved subunits found in other members of this family [35]. The sequence obtained was that of *catD* of cytochrome $caa_3$-type c oxidase.

### Malate dehydrogenase

We also obtained a PCR fragment of a putative malate dehydrogenase (MDH) gene (*mdh*).

For all sequences above, we verified that the highest BLAST similarity was against *Agrobacterium tumefaciens* C58 genes (Table 4), and the similarity values were all highly significant (*E*-values of 0). Using the above generated sequences, except for *cycM*, *cydA* and *mdh* (there were no sequences for *R. etli* and *S. meliloti*; in the supermatrix approach, all organisms must have all genes present), plus the already available sequence for a gene encoding a phosphatidylserine synthase (GenBank accession AF410774.1), plus sequences for genes *idh* (GenBank accession GU993999), *galU* (GenBank accession GU993998), we built a phylogenetic tree using ortholog sequences identified by BLAST. We used ortholog sequences from six related members of the *Rhizobiales* order. The result is shown in Fig. 4. The tree shows that *Agrobacterium* sp. ATCC 31749 groups together with *A. tumefaciens* C58, with good bootstrap support. In comparison with the species tree generated by Slater et al. [38], the main discrepancy is in the positions of *Sinorhizobium meliloti* and *A. vitis* S4. We attribute this discrepancy to the fact that Slater et al. used many more gene sequences to build their tree than we did. Given the short branch lengths of the *Agrobacterium* sp. ATCC 31749—*A. tumefaciens* C58 clade in our tree and the good bootstrap support for that clade, we believe that our conclusion that *A. tumefaciens* C58 is the closest fully sequenced genome to *Agrobacterium* sp. ATCC 31749 is robust. The tree suggests that *Agrobacterium* sp. ATCC 31749 is possibly a representative of the biovar 1 category of *Agrobacteria*.

In addition, we note that BLAST alignments suggest that our sequence for the gene *fixN* has one frameshift, possibly due to sequencing error.

## Discussion

This study focused on ETC genes in *Agrobacterium* sp. ATCC 31749 and their relationship to other *Agrobacterium* species genes. One of our main findings is that *Agrobacterium* sp. ATCC 31749 is very close to *A. tumefaciens* C58 compared with other species. This finding will facilitate additional studies of *Agrobacterium* sp. ATCC 31749 at the gene level.

### Proposed pathway map of ETC in *Agrobacterium* sp. ATCC 31749

Bioenergetics studies have suggested the respiratory chain complexes have specificities that vary from species to species [50]. Therefore, we found that it is necessary to investigate ETC genes of *Agrobacterium* sp. ATCC 31749. Usually the ETC is composed of complexes I (NQ), II (SDH), III ($bc_1$ complex-quinol oxidase), and IV (cytochrome *c* terminal oxidase, including Cyt $caa_3$ and Cyt $cbb_3$) as well as other terminal oxidases. The present work has identified genes from complexes I, II, III and IV in *Agrobacterium* sp. ATCC 31749.

For identification, the existence of ubiquinone in *Agrobacterium* sp. ATCC 31749, proposed partial sequence of *ubiB* (GQ428126), the biosynthesis genes of ubiquinone in *A. tumefaciens* C58, was cloned and identified from *Agrobacterium* sp. ATCC 31749. In addition, *A. tumefaciens*

**Table 4** The homology analysis of various nucleotide sequences from different species

| Gene 1 (*As.* ATCC 31749) | Alignment length | Gene 2 | Protein name | Source | Identities | Average identity | *E* value |
|---|---|---|---|---|---|---|---|
| *NuoB* | | | | | | | |
| gb\|GQ428125.1\| | 364 | gb\|AE007869.2\| | NQ | *At.*C58 | 364 | 1.0 | 0 |
| | | gb\|CP000133.1\| | NQ | *Re.*CFN 42 | 317 | 0.87 | 1E-112 |
| | | gb\|CP001622.1\| | NQ | *Rl.*WSM1325 | 316 | 0.86 | 2E-118 |
| | | gb\|CP001074.1\| | NQ | *Re.*CIAT652 | 314 | 0.86 | 2E-117 |
| | | dbj\|AP009384.1\| | NQ | *Ac.*ORS 571 | 313 | 0.86 | 2E-117 |
| | | gb\|CP000628.1\| | NQ | *Ar.* K84 | 312 | 0.85 | 1E-114 |
| | | gb\|CP000633.1\| | NQ | *Av.*S4 | 311 | 0.85 | 2E-113 |
| | | gb\|CP001578.1\| | NQ | *Bm.*CCM 4915 | 308 | 0.85 | 8E-111 |
| | | dbj\|BA000012.4\| | NQ | *Bi.* ATCC9039 | 308 | 0.85 | 8E-111 |
| | | emb\|AL591688.1\| | NQ | *Sm.*1021 | 309 | 0.84 | 3E-110 |
| | | gb\|CP000758.1\| | NQ | Oa. 49188 | 303 | 0.83 | 9E-104 |
| | | gb\|CP000661.1\| | NQ | *Rs.*ATCC 17025 | 289 | 0.80 | 2E-87 |
| *sdhB* | | | | | | | |
| gb\|GQ428124.1\| | 422 | gb\|AE007869.2\| | SDH | *At.*C58 | 422 | 0.97 | 0 |
| | | emb\|AL591688.1\| | SDH | *Sm.*1021 | 363 | 0.86 | 7E-138 |
| | | gb\|CP000628.1\| | SDH | *Ar.* K84 | 365 | 0.85 | 5E-133 |
| | | gb\|CP001074.1\| | SDH | *Re.*CIAT652 | 361 | 0.84 | 1E-127 |
| | | gb\|CP000633.1\| | SDH | *Av.*S4 | 345 | 0.81 | 2E-112 |
| *ubiB* | | | | | | | |
| gb\|GQ428126.1 | 412 | gb\|AE007869.2\| | ubiB | *At.*C58 | 412 | 0.99 | 0 |
| | | gb\|CP000628.1\| | PH | *Ar.* K84 | 411 | 0.86 | 2E-121 |
| | | emb\|AL591688.1\| | ubiB | *Sm.*1021 | 411 | 0.82 | 9E-95 |
| | | gb\|CP001016.1\| | ubiB | *Bi.* ATCC9039 | 352 | 0.74 | 2E-31 |
| | | gb\|CP000633.1\| | PH | Av.S4 | 333 | 0.81 | 2E-105 |
| *mdh* | | | | | | | |
| gb\|GQ428127.1\| | 453 | gb\|AE007870.2\| | MDH | *At.*C58 | 446 | 0.96 | 0 |
| | | dbj\|AP009384.1\| | MDH | *Ac.ORS 571* | 395 | 0.71 | 3E-47 |
| | | gb\|CP000662.1\| | MDH | *Rs.*ATCC 17025 | 403 | 0.71 | 3E-42 |
| *cydA* | | | | | | | |
| gb\|GQ428129.1\| | 785 | AE007870.2\| | Cytd | *At.*C58 | 771 | 0.97 | 0 |
| | | gb\|CP001077.1\| | Cytbd | Re.CIAT652 | 646 | 0.84 | 0 |
| | | gb\|CP000629.1\| | Cytd | *Ar.* K84 | 655 | 0.83 | 0 |
| | | gb\|CP000759.1\| | Cytbd | *Oa.* 49188 | 617 | 0.78 | 0 |
| | | gb\|CP001579.1\| | Cytd | *Bm.*CCM 4915 | 616 | 0.78 | 0 |
| | | gb\|AE008918.1\| | Cytd | *Bm.*16 M | 616 | 0.78 | 0 |
| | | dbj\|AP009384.1\| | Cytd | *Ac.*ORS 571 | 623 | 0.81 | 0 |
| *cyoA* | | | | | | | |
| gb\|GQ428130.1\| | 790 | gb\|AE007869.\| | Cytbo | *At.*C58 | 791 | 0.98 | 0 |
| | | gb\|CP000874.\| | Cytbo | *Rs.*NGR234 | 786 | 0.85 | 0 |
| | | emb\|AL591985\| | Cytbo | *Sm.*1021 | 786 | 0.84 | 0 |
| | | gb\|CP000628.1\| | Cytbo | *Ar.* K84 | 786 | 0.83 | 0 |
| | | gb\|CP001075.1\| | Cytbo | *Re.*CIAT652 | 788 | 0.83 | 0 |
| | | gb\|CP000634.1\| | Cytbo | *Av.*S4 | 787 | 0.82 | 0 |
| | | dbj\|BA000012.4\| | Cytbo | *Ml.* 303099 | 787 | 0.82 | 0 |

**Table 4** continued

| Gene 1 (*As.* ATCC 31749) | Alignment length | Gene 2 | Protein name | Source | Identities | Average identity | *E* value |
|---|---|---|---|---|---|---|---|
| *fixN* | | | | | | | |
| gb\|GQ428128.1\| | 521 | gb\|AE007869.2\| | Cyto-$cbb_3$ | *At.*C58 | 521 | 0.99 | 0 |
| | | gb\|CP000138.1\| | Cyto-$cbb_3$ | Re.CFN 42 | 464 | 0.88 | 0 |
| | | gb\|AE006469.1\| | Cyto-$cbb_3$ | *Sm.*1021 | 463 | 0.87 | 0 |
| | | gb\|CP001389.1\| | Cyto-$cbb_3$ | *Rs.*NGR234 | 455 | 0.86 | 2E-178 |
| | | gb\|CP000633.1\| | Cyto-$cbb_3$ | *Av.*S4 | 454 | 0.86 | 2E-177 |
| | | gb\|CP001076.1\| | Cyto-$cbb_3$ | *Re.*CIAT652 | 447 | 0.85 | 4E-167 |
| | | emb\|CU234118.1\| | Cyto-$cbb_3$ | *Bs.*ORS278 | 435 | 0.83 | 1E-155 |
| | | gb\|CP001196.1\| | Cyto-$cbb_3$ | *Oc.*OM5 | 434 | 0.84 | 1E-154 |
| | | gb\|CP000489.1\| | Cyto-$cbb_3$ | *Pd.*PD1222 | 443 | 0.83 | 1E-154 |
| | | gb\|CP000356.1\| | Cyto-$cbb_3$ | *Sal.*RB2256 | 438 | 0.83 | 7E-152 |
| *catD* | | | | | | | |
| gb\|GQ428131.1\| | 796 | gb\|AE007869.2\| | Cyto-$caa_3$ | *At.*C58 | 790 | 0.99 | 0 |
| | | gb\|CP001074.1\| | Cyto-$caa_3$ | *Re.*CIAT652 | 688 | 0.88 | 0 |
| | | gb\|CP001622.1\| | Cyto-$caa_3$ | *Rl.*WSM1325 | 685 | 0.87 | 0 |
| | | emb\|AL591688.1\| | Cyto-$caa_3$ | *Sm.*1021 | 695 | 0.87 | 0 |
| | | gb\|CP000133.1\| | Cyto-$caa_3$ | *Re.*CFN 42 | 681 | 0.87 | 0 |
| | | gb\|CP000628.1\| | Cyto-$caa_3$ | *Ar.* K84 | 675 | 0.86 | 0 |
| | | gb\|CP000633.1\| | Cyto-$caa_3$ | *Av.*S4 | 677 | 0.85 | 0 |
| | | dbj\|BA000012.4\| | Cyto-$caa_3$ | *Ml.* 303099 | 673 | 0.86 | 0 |
| | | gb\|CP000758.1\| | Cyto-$caa_3$ | *Oa.* 49188 | 658 | 0.83 | 0 |
| | | gb\|CP000911.1\| | Cyto-$caa_3$ | *Bs.* 23445 | 657 | 0.83 | 0 |
| | | gb\|CP000908.1\| | Cyto-$caa_3$ | *Me.*PA1 | 640 | 0.81 | 0 |
| | | gb\|CP001389.1\| | Cytbo | *R s.* NGR234 | 688 | 0.87 | 0 |
| *cycM* | | | | | | | |
| gb\|HM543455\| | 409 | gb\|AE007869.2\| | Cyto-C | *At.*C58 | 409 | 0.99 | 0 |
| | | gb\|CP000738.1\| | Cyto-C | *Sm.* WSM419 | 293 | 0.72 | 5E-21 |
| | | gb\|CP000633.1\| | Cyto-C | *Av.*S4 | 393 | 0.69 | 7E-73 |
| | | gb\|CP000628.1\| | Cyto-C | *Ar.* K84 | 308 | 0.69 | 1E-27 |
| *fbcB* | | | | | | | |
| gb\|HM543454\| | 489 | gb\|AE007869.2\| | $bc_1$ | *At.*C58 | 489 | 0.98 | 0 |
| | | gb\|CP001389.1\| | $bc_1$ | *R s.* NGR234 | 488 | 0.88 | 4E-174 |
| | | emb\|AL591688.1\| | $bc_1$ | *Sm.*1021 | 488 | 0.87 | 2E-171 |
| | | gb\|CP000628.1\| | $bc_1$ | *Ar.* K84 | 482 | 0.85 | 5E-153 |
| | | gb\|CP000633.1\| | $bc_1$ | *Av.*S4 | 482 | 0.84 | 2E-146 |

*Agrobacterium radiobacter* K84: *Ar.* K84; *Sinorhizobium meliloti* 1021: *S. meliloti* 1021; *Agrobacterium* sp. ATCC 31749: As. ATCC 31749; *Beijerinckia indica subsp. indica* ATCC 9039: *Bi.* ATCC9039; *Azorhizobium caulinodans* ORS 571: *Ac.*ORS 571; *Rhodobacter sphaeroides* ATCC 17025: *Rs.*ATCC 17025; *Rhizobium* sp. NGR234: *Rs.*NGR234; *Sinorhizobium meliloti* 1021: *Sm.*1021; *Rhizobium etli* CIAT 652: *Re.*CIAT652; *Agrobacterium vitis* S4: *Av.*S4; *Rhizobium etli* CFN 42: *Re.*CFN 42; *Rhizobium leguminosarum bv. trifolii* WSM1325: *Rl.*WSM1325; *Azorhizobium caulinodans* ORS 571: *Ac.*ORS 571; *Brucella microti* CCM 4915: *Bm.*CCM 4915; *Ochrobactrum anthropi* ATCC 49188: *Oa.* 49188; *Brucella microti* CCM 4915: *Bm.*CCM 4915; *Brucella melitensis* 16 M: *Bm.*16 M; *Azorhizobium caulinodans* ORS 571: *Ac.*ORS571; *Bradyrhizobium* sp.ORS278: *Bs.*ORS278*; Oligotropha carboxidovorans* OM5: *Oc.*OM5; *Paracoccus denitrificans PD*1222: *Pd.*PD1222; *Sphingopyxis alaskensis* RB2256: *Sal.*RB2256; *Mesorhizobium loti* MAFF303099: *Ml.* 303099; *Brucella suis* ATCC 23445: *Bs.* 23445; *Methylobacterium extorquens* PA1: *Me.*PA1; *Rhizobium* sp. NGR234: *R s.* NGR234; *Sinorhizobium medicae* WSM419:*Sm.* WSM419

C58 has the gene we have identified in *Agrobacterium* sp. ATCC 31749 that codes for ubiquinone oxidoreductase (accession number GQ428125). This gene catalyses the transfer of electrons from NADH to ubiquinone, thus lending further support to the presence of ubiquinone in *Agrobacterium* sp. ATCC 31749.

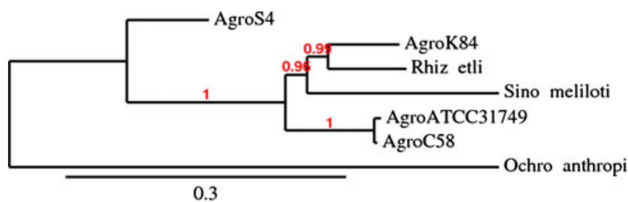


**Fig. 4** Maximum likelihood phylogenetic tree built for seven *Rhizobiales* species using 10 gene sequences (see "Materials and methods"). The numbers at the nodes are bootstrap confidence levels obtained for 1,000 replicates. AgroS4: *Agrobacterium vitis* S4; AgroK84: *Agrobacterium radiobacter* K84; Rhiz etli: *Rhizobium etli* CFN 42; Sino meliloti: *Sinorhizobium meliloti* 1021; AgroATCC31749: *Agrobacterium* sp. ATCC 31749; AgroC58: *Agrobacterium tumefaciens* C58; Ochro anthropi; *Ochrobactrum anthropi* ATCC 49188

Although the electron transfer chain order of *Agrobacterium* sp. has not been determined using the flow-flash technique [16], some electron transfer schemes have been proposed for these bacteria [7, 32, 50]. These identified electron transfer chains allowed us to reconstruct the ETC order in *Agrobacterium* sp. ATCC 31749. In Fig. 5 we propose a comprehensive model for ETCs in *Agrobacterium* sp. ATCC 31749, based on the ETC gene sequences here presented, gene information from of *A. tumefaciens* C58 [37], and ETC order reported in other species. The identification of ETC genes in *Agrobacterium* sp. ATCC 31749 has revealed that this species contains almost all the terminal oxidases (or $bc_1$ complex-quinol oxidase) complexes mentioned in the model. This raises the question as to why multiple terminal oxidases are needed.

ETC elements can be more exactly identified at the protein level rather than the DNA level. Therefore, we plan to identify the ETC elements described here at the protein level as follow-up work.

### Comparison of the ETC elements found in ATCC 31749 to *Escherichia coli* and related bacterial species

So far, *Escherichia coli* is one of a small number of bacteria whose ETC elements have been subject to in-depth investigation. The results obtained here revealed that the ETC in *Agrobacterium* sp. ATCC 31749 is different from that in *Escherichia coli*, which has no cytochrome *c*, mitochondrial complex III-equivalent (cytochrome $bc_1$ complex) or complex IV (cytochrome *c* terminal oxidase). *Escherichia coli* has only cytochrome b*o*- and *d*-complex, which can directly reduce oxygen to water and generate, concomitantly, an electrochemical proton gradient across the membrane [3]. The energetic efficiency of cytochrome $cbb_3$-type and cytochrome $caa_3$-type terminal oxidase complexes in the respiratory chain is higher than cytochrome *d*-complex and cytochrome *bo*-complex [4, 11, 12]. Therefore, the fact that the cytochrome $cbb_3$- and $caa_3$-type terminal oxidase complexes exist in *Agrobacterium* sp. ATCC 31749 should be one reason for its uniqueness in possessing a highly efficient UDP-glucose regeneration system.

To our knowledge, this is the first report of the ETCs of *Agrobacterium* sp. ATCC 31749. It should be noted that the database matches results of ETC genes appear to be in

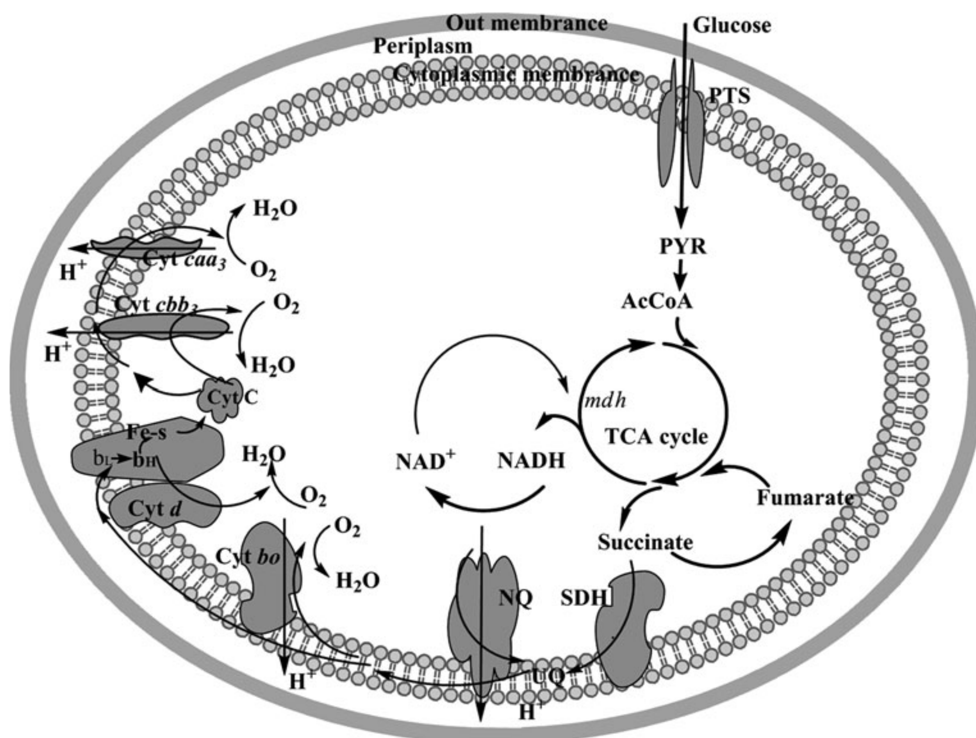


**Fig. 5** Proposed model of respiratory electron transport chains in *Agrobacterium* sp. ATCC 31749

contrast to our previous speculation that the ETCs should be conserved in all bacterial species. The results suggest that the ETC elements in *Agrobacterium* sp. ATCC 31749 are conserved only in related bacterial species, e.g. *A. radiobacter* K84, *A. vitis* S4, *S. meliloti* 1021, and *R. etli* CFN 42 (Table 4). The ETC genes in *Agrobacterium* sp. ATCC 31749 all had the highest similarity (measured by percent identity) to those in *A. tumefaciens* C58, compared with other bacterial species presented in this work. These results led to the phylogeny in Fig. 4. It is interesting to note that *Agrobacterium* sp. ATCC 31749 is not a plant pathogen, whereas *A. tumefaciens* C58 is. If both belong to the biovar 1 category of *Agrobacteria*, as appears likely from Fig. 4, then this is further evidence that the most recent common ancestor of biovar 1 organisms was a non-pathogen, as speculated by Setubal et al. [37].

Potential contribution to exploring the mechanisms of curdlan synthesis and secretion

The finding that the genome of *Agrobacterium* sp. ATCC 31749 is very similar to *A. tumefaciens* C58 gives us a new model organism for exploring the mechanisms of curdlan synthesis and secretion. Note that our work suggests that *A. tumefaciens* C58 has all genes necessary for curdlan production (*crdASC*) [19], and is the only other sequenced *Agrobacterium* species to have them. However, we are not aware of any work reporting that *A. tumefaciens* C58 can produce curdlan. Why not?

Curdlan can be produced by *Agrobacterium* sp. ATCC 31749 only under nitrogen-limited conditions [22]. Therefore, one possible explanation for the lack of curdlan production reports in *A. tumefaciens* C58 is that it has never been cultured under nitrogen-limited conditions. Even if *A. tumefaciens* C58 cannot produce curdlan under nitrogen-limited conditions, *A. tumefaciens* C58 will be a useful model organism for the exploration of mechanisms for curdlan synthesis and regulation through careful genomic comparisons between *A. tumefaciens* C58 and *Agrobacterium* sp. ATCC 31749. Having the full genomic sequence of *Agrobacterium* sp. ATCC 31749 should be an invaluable tool in this respect.

Most of the polysaccharides produced by bacteria and fungi are insoluble [42], thus making it difficult to explore polysaccharide secretion mechanisms in bacteria in vitro. Using whole genome comparisons should help us in understanding control mechanisms of polysaccharides in bacteria in general and in *Agrobacterium* sp. ATCC 31749 in particular.

The ETC model resulting from our analysis provides the basis for discovering the reason for the unique metabolic capability for UDP-glucose regeneration in *Agrobacterium* sp. ATCC 31749, and therefore discovering how to tap its biotechnological potential. It will also allow further studies of the bioenergetics and electron transfer mechanisms from ATP regeneration at the gene level.

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