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Replacement of a terminal cytochrome *c* oxidase by ubiquinol oxidase during the evolution of acetic acid bacteria

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ABSTRACT

The bacterial aerobic respiratory chain has a terminal oxidase of the heme-copper oxidase superfamily, comprised of cytochrome *c* oxidase (COX) and ubiquinol oxidase (UOX); UOX evolved from COX. *Acetobacter pasteurianus*, an α-Proteobacterial acetic acid bacterium (AAB), produces UOX but not COX, although it has a partial COX gene cluster, *ctaBD* and *ctaA*, in addition to the UOX operon *cyaBACD*. We expressed *ctaB* and *ctaA* genes of *A. pasteurianus* in *Escherichia coli* and demonstrated their function as heme O and heme A synthases. We also found that the absence of *ctaD* function is likely due to accumulated mutations. These COX genes are closely related to other α-Proteobacterial COX proteins. However, the UOX operons of AAB are closely related to those of the β/γ-Proteobacteria (γ-type UOX), distinct from the α/β-Proteobacterial proteins (α-type UOX), but different from the other γ-type UOX proteins by the absence of the *cyoE* heme O synthase. Thus, we suggest that *A. pasteurianus* has a functional γ-type UOX but has lost the COX genes, with the exception of *ctaB* and *ctaA*, which supply the heme O and A moieties for UOX. Our results suggest that, in AAB, COX was replaced by β/γ-Proteobacterial UOX via horizontal gene transfer, while the COX genes, except for the heme O/A synthase genes, were lost.

1. Introduction

Aerobic organisms have the ability to generate energy by means of a respiratory chain that uses oxygen as the terminal electron acceptor. The two major terminal oxidases in the respiratory chain are heme–copper oxidases and *bd*-type oxidases. The heme–copper oxidase superfamily has a binuclear O_2-reducing site consisting of a heme (heme A, O, or B) and a copper (Cu_B) and divided into cytochrome *c* oxidase (COX), which receives electrons from cytochrome *c*, and ubiquinol oxidase (UOX), which takes electrons from ubiquinol. Sequence alignment and phylogenetic analysis show that the heme–copper oxidases are classified as A-, B-, and C-type oxidases [1,2]. The A-type enzymes have two separate proton channels (D-channel and K-channel) to generate a proton-motive force, while the B- and C-type enzymes have only an alternative K-channel and have lower proton-pumping capacity [1,3]. Unlike cbb_3-type cytochrome *c* oxidase (C-type oxidase), A- and B-type oxidases include both COX and UOX, where UOX seems to have been derived from COX by loss of the Cu_A site in subunit II [1]. Especially, in A-type UOX, it has been postulated that a gene duplication event occurred during the evolution of Gram-positive bacterial COX, which yielded UOX, which is thought to have been passed to the Proteobacteria by lateral gene transfer (Fig. 1) [4].

Acetic acid bacteria (AAB) include more than 10 genera, including *Acetobacter*, *Gluconacetobacter* and *Gluconobacter*, and are phylogenetically located in the *Acetobacteraceae* family, order *Rhodospirillales*, class α-Proteobacteria. The respiratory chains of these AAB have been well investigated, since classic aerobic microbial fermentation, such as acetic acid, gluconate or sorbose production, is known to be carried out by linking directly to the respiratory chain [5]. The respiratory chain is uniquely truncated, consisting of quinoprotein or flavoprotein periplasmic dehydrogenases and terminal UOXs [5]. *Acetobacter pasteurianus* IFO 3284 (previously *Acetobacter aceti* IFO 3284) has a cytochrome ba_3 UOX (initially termed cytochrome a_1) [6] or cytochrome bo_3 UOX [7]. These oxidases are comprised of the same proteins but different hemes in the binuclear center (heme A and heme O, respectively) [7,8]. The electronic configuration at the binuclear center of the two enzymes is the same in the oxidized state, but different

Abbreviations: AAB, acetic acid bacteria; COX, cytochrome *c* oxidase; HAS, heme A synthetase; HOS, heme O synthetase; LB, Luria–Bertani; UOX, ubiquinol oxidase

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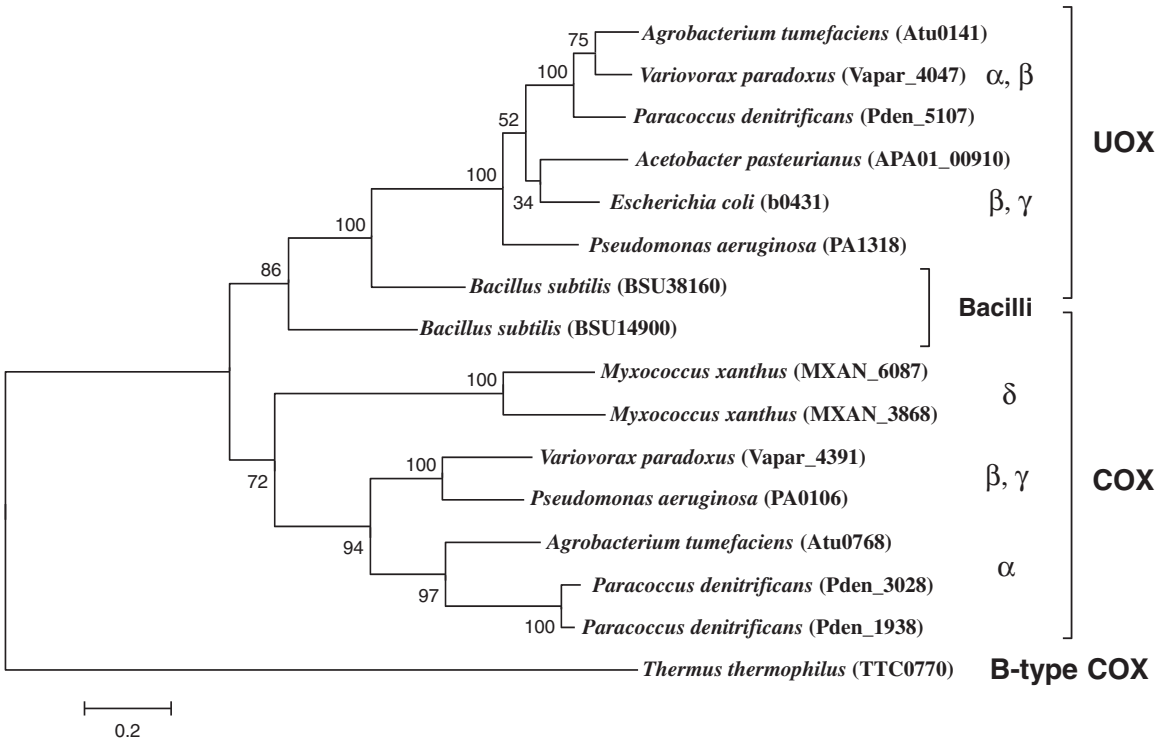

Fig. 1. Maximum-likelihood phylogenetic tree of A-type COX and UOX subunit I in 8 species, *Acetobacter pasteurianus* (APA01_00910), *Agrobacterium tumefaciens* C58 (Atu0141, Atu0768), *Paracoccus denitrificans* (Pden_1938, Pden_3028, Pden_5107), *Variovorax paradoxus* (Vapar_4047, Vapar_4391), *Escherichia coli* K-12 MG1655 (b0431), *Pseudomonas aeruginosa* (PA0106, PA1318), *Myxococcus xanthus* (MXAN3868, MXAN6087) and *Bacillus subtilis* (BSU14900, BSU38160). COX and UOX mean the cytochrome *c* and ubiquinol oxidases, respectively. *Thermus thermophilus* B-type oxidase (TTC0770) was used as the outgroup. *A. pasteurianus*, *A. tumefaciens*, *P. denitrificans* are α − Proteobacteria, while *E. coli* and *P. aeruginosa* are α − Proteobacteria (see Fig. S3). *V. paradoxus* and *M. Xanthus* are β − and δ − Proteobacteria, respectively.

in the reduced ligand-bound state [9]. The cytochrome ba_3/bo_3 gene operon (*cyaBACD*) was cloned first from *A. pasteurianus* 1023 (formerly *A. aceti* 1023) and found later in the genome of *A. pasteurianus* IFO 3283 [10,11]. This cytochrome ba_3/bo_3 of AAB is a member of A-type heme–copper oxidases (Fig. 1).

Heme O is synthesized by the replacement of the vinyl group of heme B with 17-carbon hydroxyethylfarnesyl at position 2 of the porphyrin ring; heme O is converted to heme A by replacing the methyl group at position 8 with a formyl group (see [12] for a review). Heme O was discovered in *Escherichia coli* cytochrome bo_3 UOX [13], which is encoded by *cyoABCD* in the *cyoABCDE* operon. *cyoE* encodes a protoheme IX-farnesyl transferase that functions as a heme O synthase (HOS) [14]. Homologs of *E. coli cyoE* are found in other bacteria and eukaryotes, such as *Bacillus subtilis ctaB*, *Paracoccus denitrificans Orf1*, and *Saccharomyces cerevisiae COX10* (see [12] for a review). *B. subtilis ctaA*, located just upstream of *ctaB*, and *COX15* in *S. cerevisiae* function as heme A synthase (HAS) (see [12] for a review). An additional assembly factor, Surf1, is involved in COX biogenesis in humans [15,16], but its role remains unclear [16]. *Surf1* homologs exist in many prokaryotes and eukaryotes [17]. In *P. denitrificans*, Surf1 homologs Surf1c and Surf1q function specifically in the assembly of cytochrome aa_3 COX and cytochrome ba_3 UOX, respectively [18].

To investigate the evolution of AAB terminal oxidase, we cloned the putative genes *ctaB* and *ctaA* from *A. pasteurianus* IFO 3284 and demonstrated their function as HOS and HAS, respectively. We also examined the phylogenetic relationship of the UOX and COX gene clusters in AAB and other Proteobacteria, and showed that the UOX of AAB is phylogenetically unique and close to the β,γ-Proteobacterial group, but the COX gene cluster clusters with the α-Proteobacterial group.

2. Materials and methods

2.1. Materials

Restriction enzymes and *E. coli* DH5α were kindly supplied by Toyobo. *E. coli* DH10B was purchased from Invitrogen. The pGem-T Easy Vector System was purchased from Promega. All other materials were of analytical grade and were obtained from commercial sources.

2.2. Bacterial strains and culture conditions

A. pasteurianus IFO 3284 (formerly *A. aceti* IFO 3284 [5]), *Gluconacetobacter diazotrophicus* PAl 5, *Gluconacetobacter xylinus* IFO 3288 and *Acidiphilium cryptum* NBRC 14242 were used in this study (Table 1). The strains were maintained on an agar slant, which was prepared by adding 1.7% agar and 0.5% $CaCO_3$ to a potato medium consisting of 0.5% glucose, 2% glycerol, 1% yeast extract, 1% polypeptone and 100 ml/l potato extract. The cell growth of *A. pasteurianus* and *Ga. diazotrophicus* was performed in a YPG medium (pH 6.5) consisting of 1% glycerol, 0.5% polypeptone and 0.5% yeast extract, and that of *Ga. xylinus* and *A. cryptum* was done in a YPG medium containing 2% ethanol and in a NBRC234 medium (pH 3.0) consisting of 0.1% glucose, 0.01% peptone, 0.1% yeast extract, 0.005% $MgSO_4$, 0.005% K_2HPO_4, 0.001% KCl and 0.2% $(NH_4)_2SO_4$, respectively. *E. coli* ST2592 (Δ*cyd*::Km^r, Δ*cyo*::Cm^r, Δ*rec*::Tc^r) [19] was maintained on an LB (Luria-Bertani) agar plate containing 1% glucose and antibiotics anaerobically. It was cultivated overnight in 10 ml of LB medium (pH 7.5) containing 1% glucose and the respective antibiotics in a sealed tube, and then the seed culture was transferred to 300 ml of LB medium containing 1% glycerol, 0.5% sodium nitrate and the respective antibiotics. The culture was

Table 1
Bacterial strains and plasmids used in this study.

Strains or plasmids	Relevant properties	References
Strains		
Acetobacter pasteurianus IFO 3284	Wild-type strain (originally called *Acetobacter aceti*, but now reclassified as *Acetobacter pasteurianus* based on 16S rRNA analysis)	NBRC 3284
Gluconacetobacter diazotrophicus PAl 5	Wild-type strain	ATCC49037
Gluconacetobacter xylinus IFO 3288	Wild-type strain	NBRC 3288
Acidiphilium cryptum JF-5	Wild-type strain	NBRC 14242
E. coli strains		
DH5a	F$^-$, *endA1*, *hsdR17*, *recA1*, *supE44*, *thi*-1, f80*lacZ*ΔM15	Toyobo
ST2592	*cyo*, *cyd* double-deletion strain (Δ*cyd*::Km^r, Δ*cyo*::Cm^r, *rec*::Tc^r) constructed from W3110	Minagawa, J. [19]
DH10B	F$^-$, *mcrA*, Δ(*mrr-hsdRMS-mcrBC*), *f*80d/*lacZ*, Δ*lac X*74, *deoR*, *rec*A1, *ara*D139, Δ(*ara-leu*)7697, *galU*, *galK*, l-*rpsL*, *endA*1, *nupG*	Invitrogen
Plasmids		
PUCC13	Ap^r; 2.6 kb Pst I DNA fragment of *A. pasteurianus* IFO 3284 strain containing *ctaB* inserted in pUC18 (expressed from lac promoter)	This work
pBAD24	Ap^r; *araC*	Guzman L.M. [51]
pBADctaA	Ap^r; PCR *ctaA* product from the genome of *A. pasteurianus* IFO 3284 strain inserted into EcoRI and Pst I sites of pBAD24	This work

saturated with argon and grown with rotary shaking at 100 rpm up to 70 Klett units. This cultivation procedure was also used to prepare competent cells of ST2592 and to prepare the cytoplasmic membranes. *E. coli* DH5α and DH10B were cultivated on LB medium with rotary shaking at 200 rpm overnight at 37 °C.

2.3. Preparation of membranes

The cells were harvested from the cultures described above by centrifugation at 6500 ×*g* for 10 min. The cells were washed with 50 mM potassium phosphate buffer (pH 5.0 for *A. cryptum*; pH 6.5 for other AAB; pH 7.5 for *E. coli*) at 10 ml/g wet cells, and centrifuged at 6500 ×*g* for 10 min. The washed cells were resuspended in the same buffer at a concentration of 4 ml/g wet cells. The cell suspension was passed through a French press at 16,000 psi twice and centrifuged at 3000 ×*g* for 10 min to remove cell debris. The supernatant was then centrifuged at 120,000 ×*g* for 90 min to remove the cytosol. The resultant pellet was resuspended in the same buffer using a Teflon homogenizer, and used as a membrane fraction. Protein content was determined by the modified Lowry method [20].

2.4. Cloning of ctaB and ctaA from A. pasteurianus IFO 3284

Cloning of these DNA fragments was carried out before obtaining the genome sequence data of *A. pasteurianus* IFO 3284 described below, and thus is simply summarized here. PCR amplification was performed on genomic DNA of *A. pasteurianus* IFO 3284 with several primers listed in Table S1. Oligo-nucleotide primers, CtaB-1 and CtaB-3, were designed on the conserved regions of the respective amino acid sequences, GALNMWY and WTPPHF, of Proteobacteria for sub-cloning the HOS (372 bp) gene. Other primers, CtaA-12 and CtaA-13, were also designed on the basis of the conserved amino acid sequences of GG(F)I(V) GWWM and NTWPLMN(D), respectively, to obtain the partial *ctaA* 296 bp PCR product.

For *ctaB* cloning, genomic DNA of the IFO 3284 strain was digested with several restriction enzymes, and Southern hybridization was performed using the *ctaB* 372 bp PCR product described above. The positive Pst I 2.5 kb DNA fragment was selected and ligated into pUC18 for further cloning. Resultant plasmid DNA was transformed to *E. coli* DH5α, and then colony hybridization was performed with a probe prepared from the *ctaB* PCR fragment to obtain a positive clone. Plasmid extracted from the positive colony was sequenced using several sequence primers (Table S1) and also using M4-RV and M4 (pUC18-derived primers), and was used for *ctaB* expression as described elsewhere. For *ctaA* cloning, PCR *in vitro* cloning (LA PCR *in vitro* Cloning Kit, TaKaRa) was performed with genomic DNA of the IFO 3284 strain, which was digested with a restriction enzyme (Sph I or Eco RI) and ligated to the respective specific cassette (TaKaRa), according to the supplier's protocol. Primer sets, CtaA-1 and CtaA-2, were designed from the partial *ctaA* 296 bp PCR product for cloning the upstream region, and also CtaA-3 and CtaA-4 primers for cloning the downstream region. Using DNA fragments having cassette DNA as a template, the first PCR was carried out with primer CtaA-1 or primer CtaA-3, and primer C1 (for the cassette). The first PCR products were 100 times diluted and used as a template for the second PCR, performed with primer CtaA-2 or primer CtaA-4, and the cassette primer C2. The upstream ~700 bp fragment was obtained from the Sph I fragment, while the downstream ~700 bp fragment was from the Eco RI fragment. These PCR products were cloned into T vector and sequenced.

The sequences of *ctaB* and *ctaA* thus obtained were confirmed to be the same by comparing with the draft genome sequencing data.

2.5. DNA sequencing of the DNA fragments

The nucleotide sequence of a cloned fragment was determined by the dye terminator method (Sanger's method). Both strands of the sequences were determined mainly using specific pUC18-derived primers, FITC primers M4 and M4-RV, which are useful for the pGEM-T easy vector, and the oligonucleotide corresponding to the part of the cloned fragment for the walking method was also used. DNA sequencing was performed with an ABI PRISM 310 Genetic Analyzer. The obtained nucleotide sequence was analyzed using the Genetyx-Mac program (Software Development Co., Ltd.).

2.6. Expression of cloned ctaB and ctaA in E. coli

A 2.5 kb Pst I DNA fragment containing *ctaB* was inserted into pUC18 to make pUCC13 (Table 1), which was transformed into *E. coli* DH5a or *E. coli* ST2592. Then, the strains harboring pUCC13 or pUC18 (vector control) were grown aerobically or anaerobically by nitrate respiration, respectively, as described above. After reaching 50 Klett units, isopropyl-β-D-thiogalacto-pyranoside (IPTG) was added to a final concentration of 100 μM and harvested after 2-h cultivation at 30 °C.

To examine the function of CtaA, a plasmid harboring the *ctaA* gene of *Acetobacter* sp. IFO 3284 S strain, pBADctaA, was constructed. The *ctaA* gene was amplified with two primers, CtaA-5′-EcoRI and CtaA-3′-PstI, designed around starting and terminating codons of *ctaA* of this S strain (Table S1). The amplified *ctaA* gene was digested with EcoR I and Pst I, and ligated into pBAD24 (Table 1) to produce pBADctaA. The pBADctaA was transformed into *E. coli* DH10B, and then the

transformant was cultivated in 100 ml of LB medium, with or without 0.1% L-(+)-arabinose, at 37 °C for 12 h.

2.7. Other analytical procedures

The hemes of the cytoplasmic membrane were extracted into cold acid acetone as described by Weinstein and Beale [21]. These hemes in acetone were then transferred into ether by the addition of aliquots of cold water. After separating the phase, the heme-containing ether layer was removed, washed with cold water and evaporated. The hemes were then dissolved in HPLC solvent (71% ethanol, 17% acetic acid, 12% distilled water), and analyzed by reversed-phase HPLC using a Zorvax ODS column C18 (25 cm × 4.6 mm with 5-μm particles) at a flow rate of 1.0 or 1.2 ml/min in the HPLC elution solvent and monitored using a SPD-M 10A UV–vis photodiode array detector (Shimadzu). Heme *b* content was determined by measuring the reduced-minus-oxidized difference spectra of pyridine hemochrome using a millimolar absorption coefficient of 20.7 [6].

The *in vitro* heme O synthesis assay was performed according to the established method [14], except for farnesyl pyrophosphate and the buffer. Farnesyl pyrophosphate ammonium salt (Sigma) and potassium phosphate buffer (pH 7.0) were used in this study, instead of farnesyl pyrophosphate bromide salt and Tris–HCl buffer.

Low-temperature redox spectra were measured with each 400 μl of the membrane suspension put into both sample and reference cuvettes. A few grains of sodium hydrosulfite and 4 μl of 100 mM potassium ferricyanide were added to the sample and reference cuvettes, respectively. Both the cuvettes were then frozen in liquid nitrogen, and reduced-minus-oxidized difference spectra were measured from 700 nm to 500 nm with a Hitachi 557 dual wavelength spectrophotometer.

SDS-PAGE was performed with a slab gel composed of stacking and separating gels of 5% and 12.5% acrylamide, respectively.

2.8. Sequence retrieval and data construction for motif search analysis

The amino acid sequences from 359 complete microbial genomes (one genome per genus) were obtained from the KEGG FTP site at ftp://ftp.genome.jp/pub/kegg/genes/organisms/ [22], and used for comparative analyses. Pfam version 24.0 was downloaded from the Pfam FTP site at ftp://ftp.sanger.ac.uk/pub/databases/Pfam/ and used for the motif search analysis [23].

2.9. Identification of the terminal oxidase gene clusters with surf1 homolog

SURF1 motif in the dataset of all proteins was detected using the HMMER version 3.0 package with E-value $\leq 10^{-4}$ [24]. The detected ORFs were listed. In addition, COX and UOX in the dataset of all proteins were also detected. Genes assigned with KEGG ortholog numbers K02256, K02257, K02258, K02259, K02260, K02261, K02262, K02263, K02264, K02265, K02266, K02267, K02268, K02269, K02271, K02272, K02273, K02274, K02275, K02276, K02277, K02297, K02298, K02299, K02300, K02301, K02826, K02827, K02828 and K02829 were collected and listed. Next, these two lists were merged and sorted with ORF number (Table S2).

2.10. Phylogenetic analysis

To construct the phylogenetic tree, sequence alignment was carried out using ClustalW [25]. We used the MEGA version 5.05 package to generate the phylogenetic tree to study the phylogenetic relationships with the maximum-likelihood (ML) approach and 100 bootstrap replicates [26,27].

3. Results and discussion

3.1. Genes related to UOX synthesis in A. pasteurianus IFO 3284

Bacterial terminal oxidase gene clusters, at least in the A-type heme–copper oxidases, include HOS gene and/or HAS gene in addition to the oxidase structural genes (Fig. 2A and B) [28–30]. Since no HOS and HAS genes were found near the structural gene of UOX of *A. aceti* 1023 [10] and *A. pasteurianus* IFO 3283 [11], both genes were cloned from the genome of IFO 3284. The gene product for putative HOS (CtaB) shares 73% identity to *Gluconobacter oxydans* GOX1864 (putative HOS), 53% identity with *Agrobacterium tumefaciens* CtaB, 51% with *P. denitrificans* CtaB, 41% with *Pseudomonas aeruginosa* PA0113 (putative HOS) and 27% with *E. coli* CyoE, and the ORF found upstream of *ctaB* shares low identity with *G. oxydans* GOX1863 (CtaD homolog) (23%) and with COX subunit I (CtaD) of *P. denitrificans* (22%) (Fig. 2E). However, the gene product for putative HAS (CtaA) (Fig. 2E) shares low but significant identity with the COX assembly protein of *A. tumefaciens* and *P. denitrificans* (42 and 35% amino acid identity). *ctaB*, *ctaD*, and *ctaA* genes of IFO 3284 (Fig. 1E) are identical to those of *A. pasteurianus* IFO 3283-01 [11]. *A. pasteurianus* has a single UOX gene cluster, *cyaBACD*, with no HOS or HAS genes nearby (Fig. 2E).

3.2. Functional analysis of Acetobacter CtaB and CtaA

To determine the functionality of IFO 3284 CtaB, the gene was expressed in *E. coli* DH5α and in *E. coli* ST2592 (Δ*cyo*Δ*cyd*) [19]. Membranes were prepared from DH5α harboring pUCC13 or pUC18 (vector control), and the heme composition was analyzed by reversed-phase HPLC (Fig. 3A). Membranes from *E. coli* DH5α (pUCC13) exhibited two heme peaks with heights 2.5- and 5.5-fold higher than in the vector control. The elution peaks exhibited spectra characteristic of heme B (399 nm) and heme O (395 nm) (see Fig. 4D). Although we cannot explain why pUCC13 produced a much higher level of heme B than the control, heme O content relative to heme B (0.28 *vs.* 0.13) was greater in *E. coli* (pUCC13), suggesting that CtaB is involved in heme O synthesis. Clearer evidence was obtained from ST2592 membranes, which do not accumulate heme O [19]; in this strain, a new peak corresponding to heme O appeared in the presence of pUCC13, and was 1.74-fold more prevalent than heme B (Fig. 3B). Membranes prepared from the ST2592 (pUCC13) accumulated heme O during incubation at 30 °C in a reaction mixture containing hemin, farnesyl diphosphate, magnesium sulfate, and dithionite (Fig. 3C); these accumulation was dependent on each of these reactants (data not shown). Thus, CtaB of *A. pasteurianus* is a protoheme IX farnesyltransferase that functions as an HOS.

To examine the function of CtaA, a plasmid harboring *ctaA* (pBADctaA) was transformed into *E. coli* DH10B. The transformant showed extremely poor growth in the presence of arabinose, and the cell pellets exhibited a deep red color due to the extreme increase in cytochrome *b* of which the absorption at 554 nm peak could be seen in the low-temperature redox spectra (data not shown). SDS-PAGE showed an increase in a 32 kDa band in DH10B cultivated with arabinose (Fig. 4A), indicating high expression of CtaA, although the mass was lower than the calculated mass (40 kDa) based on the amino acid sequence. HPLC analysis of membrane-derived heme revealed a large amount of heme B and a small amount of heme O in *E. coli* (pBADctaA) in the absence of arabinose (Fig. 4B); in the presence of arabinose, *E. coli* (pBADctaA) produced an additional small peak corresponding to heme A (Fig. 4C), which could be distinguished from heme O by its absorbance (395 nm for heme O, 399 nm for heme B, and 405 nm for heme A [8]) (Fig. 4D). The addition of arabinose increased the heme B content from 0.63 to 2.16 nmol/mg; the area of the heme B peak on HPLC increased ~3.4-fold (see Fig. 4B and C), consistent with the increase in the 554 nm peak in the redox spectra. The increased heme B content suggests an increase in CtaA, which is a heme B-containing protein

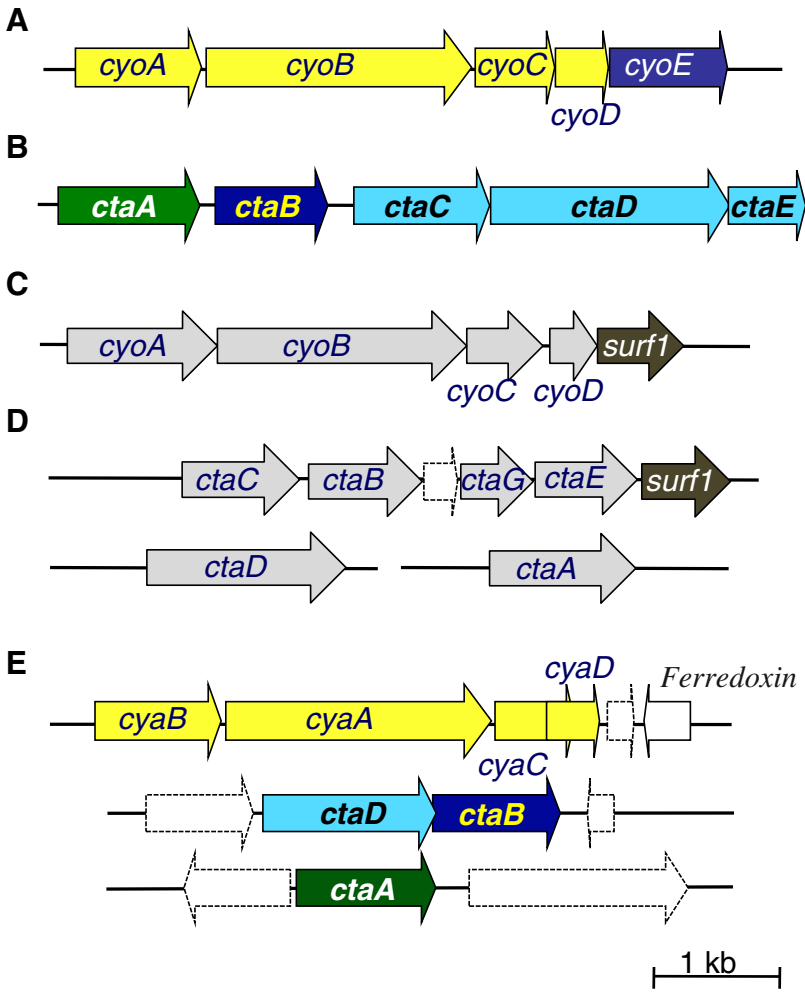

Fig. 2. Schematic representations of the gene organizations for several different UOX and COX gene clusters. (A) *E. coli* UOX genes: *cyoABCDE* (CyoE is HOS). (B) *B. subtilis* COX genes: *ctaABCDEF* (CtaA and CtaB are HAS and HOS, respectively). (C) *P. denitrificans* UOX genes: *cyoABCD* with *surf1* (*surf1q*). (D) *P. denitrificans* COX genes: *ctaCBGE* with *surf1* (*surf1c*), *ctaD*, and *ctaA*. (E) *A. pasteurianus* IFO 3284 UOX genes and COX genes: *cyaBACD*, *ctaBD* and *ctaA*.

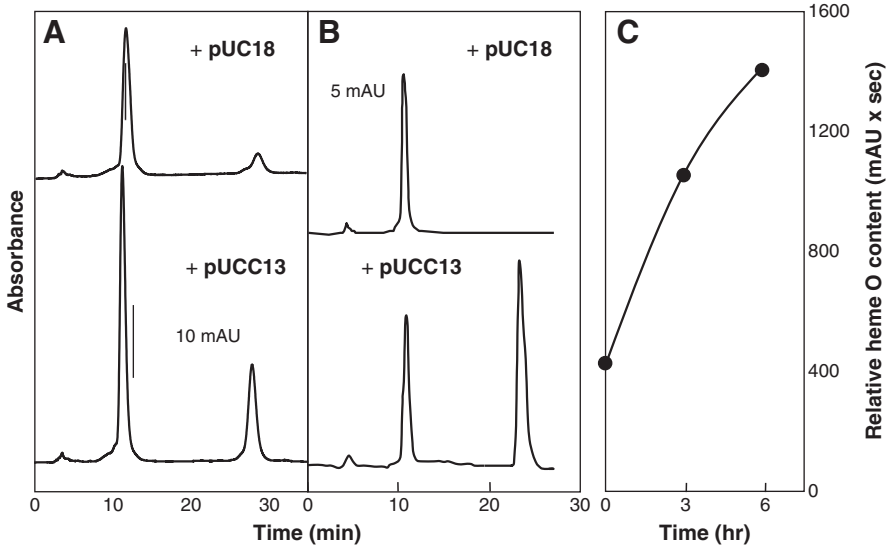

Fig. 3. Reversed-phase HPLC analysis of hemes extracted from the membranes of *E. coli* DH5a or ST2592 expressing *A. pasteurianus* CtaB. (A) Hemes were extracted from 2 mg (protein) of the membrane of DH5a harboring pUC18 and pUCC13, and analyzed by reversed-phase HPLC, and monitored by absorbance at 398 nm. (B) Hemes were extracted from 800 μg (protein) of the membrane of ST2592 (pUC18) and ST2592 (pUCC13). (C) Membranes of ST2592 (pUCC13) were used for reaction. The reaction mixture consisted of 800 μg of the membrane protein, farnesyl-diphosphate, hemin and magnesium sulfate. The reaction was started by the addition of dithionite, and incubated at 30 °C up to 6 h. Hemes were extracted and analyzed by reversed-phase HPLC, and monitored by absorbance at 398 nm. The content of heme O synthesized was determined on the basis of the respective heme O peak area (mAU × s).

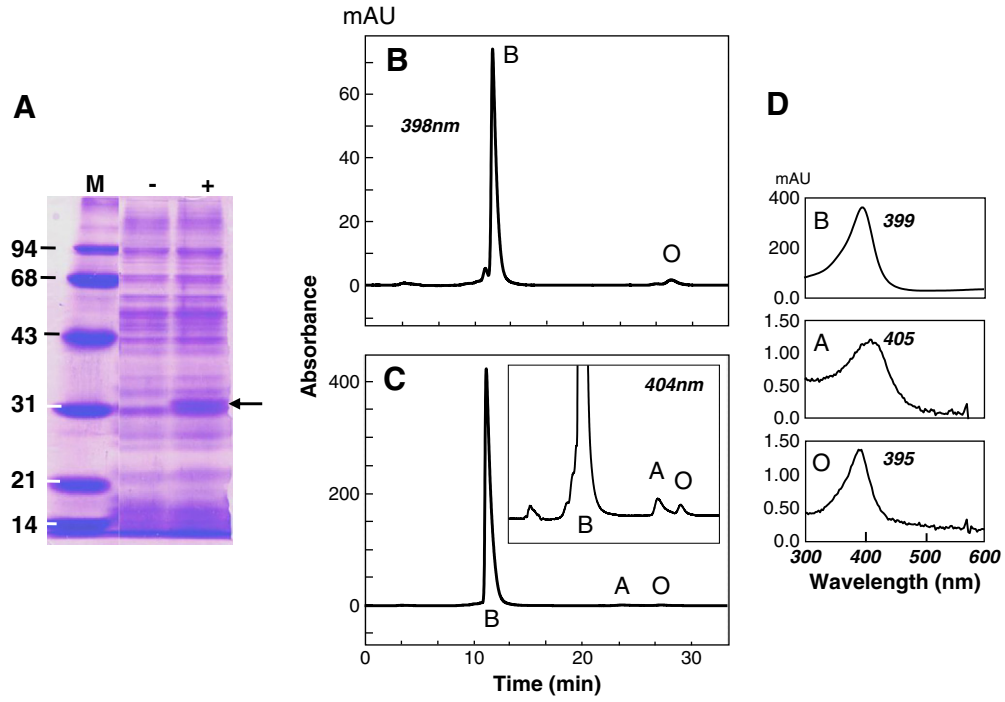

Fig. 4. Expression of *A. pasteurianus* CtaA from pBADctaA in *E. coli* DH10B. *E. coli* DH10B harboring pBADctaA was cultivated in the presence of 0.1% L-arabinose at the beginning of culture. Membranes were prepared from *E. coli* DH10B (pBADctaA) grown in the absence or presence of 0.1% arabinose. (A) SDS-PAGE was performed with membrane fractions (30 μg of protein each) prepared from the cells grown without (−) and with arabinose (+). Lane M: marker proteins, phosphorylase b (94 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.1 kDa) and lysozyme (14.4 kDa). (B, C) HPLC analysis of heme components extracted from the membranes prepared from the cells grown without (B) and with arabinose (C). Absorbance was monitored at 398 nm in panels B and C, but at 404 nm in the inset of panel C. (D) Absorption spectra (photo-diode array) of peaks B, A and O in panel C.

Table 2
Gene repertoires of *cya* and *cta* gene clusters in acetic acid bacterial genomes.

Gene clusters	Gene name	Species					
		A. cryptum	*G. bethesdensis*	*Ga. xylinus*	*Ga. diazotrophicus*	*A. pasteurianus*	*G. oxydans*
Ubiquinol oxidase	CyaB	–	GbCGDNIH1_1276	GLX_26640	GDI_2035	APA01_00900	GOX1911
	CyaA	–	GbCGDNIH1_1275	GLX_26650	GDI_2034	APA01_00910	GOX1912
	CyaC	–	GbCGDNIH1_1274	GLX_26660	GDI_2033	APA01_00920	GOX1913
	CyaD	–	GbCGDNIH1_1273	GLX_26670	GDI_2032	APA01_00930	GOX1914
Cytochrome *c* oxidase	CtaA	Acry_1224, Acry_1225	GbCGDNIH1_2184	GLX_10080	GDI_1341	APA01_00780	–
	CtaE	Acry_2164	GbCGDNIH1_2250	–	–	–	–
	CtaB	Acry_2165	GbCGDNIH1_2257	GLX_26210	GDI_2100	APA01_16820	GOX1864
	CtaD	Acry_2166	GbCGDNIH1_2258	GLX_26200	GDI_2101	APA01_16830	GOX1863
	CtaC	Acry_2167	GbCGDNIH1_2259	–	–	–	–
Ubiquinol oxidase (different type)	CyoC	Acry_1094	–	–	–	–	–
	CyoB	Acry_1095	–	–	–	–	–
	CyoA	Acry_1096	–	–	–	–	–
Cytochrome *c* oxidase (different type)	CtaE	–	–	–	GDI_2595	–	–
	CtaD	–	–	–	GDI_2596	–	–
	CtaC	–	–	–	GDI_2597	–	–

A. cryptum: *Acidiphilium cryptum* JF-5.
A. pasteurianus: *Acetobacter pasteurianus* IFO 3283-01 [11].
G. oxydans: *Gluconobacter oxydans* 621H [52].
G. bethesdensis: *Granulibacter bethesdensis* CGDNIH1 [53].
Ga. diazotrophicus: *Gluconacetobacter diazotrophicus* PAl 5 [43].
Ga. xylinus: *Gluconacetobacter xylinus*) [44].

[31]. More importantly, the increase in CtaA was accompanied by a decrease in heme O content relative to heme B (the peak area) of 0.049 to 0.007, and the peak area of heme O decreased by 50% (Fig. 4). The heme A content increased and the heme A:heme O ratio was 0.47:0.53 (inset, Fig. 4C). Thus, CtaA expression increased the heme B content and heme A content relative to heme O also increased. However, the addition of cyanide to the membrane suspension did not affect the spectra; heme A produced in CtaA-expressing membranes was not incorporated into UOX, suggesting that cytochrome bo_3 might not be converted to cytochrome ba_3. If heme A, not heme O, is functionally inserted into UOX, a sharp peak around 590 nm should be seen as a result of high-spin heme A reacting with cyanide [6]. This was not observed even when *Bacillus ctaA* was expressed in *E. coli* [31], in which heme A was also produced in trace amounts but no spectral change indicating the presence of a heme A-containing oxidase was obtained as in the case of *A. pasteurianus ctaA*; thus, it was expected that heme A is tightly bound to CtaA as an unreleased reaction product together with the prosthetic group, heme B [32–34]. This study clearly indicates that CtaA in *A. pasteurianus* functions as an HAS but seems to require some other protein(s) such as Surf1 to integrate heme A into an oxidase protein in *E. coli*.

3.3. Comparison of the cya operon and cta genes in the acetic acid bacteria

A. pasteurianus seems to produce a cytochrome ba_3 UOX by a combination with the oxidase proteins from the *cyaBACD* operon and the prosthetic groups, Cu_B, heme B, and heme A; heme A is generated by the products of *ctaB* and *ctaA*. In AAB, cytochrome ba_3 and/or bo_3 UOXs have been purified from *Acetobacter* and *Gluconobacter* [8,35]. To characterize the distribution of the terminal oxidases in AAB, we analyzed the phylogenetic relationships of UOX synthetic genes in six *Acetobacteraceae*. All tested genomes with the exception of *A. cryptum* have a *cyaBACD* operon in the same phylogenetic clade (Table 2), but *A. cryptum* has a partial gene cluster, *cyoCBA* (synonymous with *cyaCAB*), which is phylogenetically different from the other AAB *cya* operons (see Fig. S4). A complete set of COX genes (*ctaABCDE*) was found only in *A. cryptum* and *Granulibacter bethesdensis*, both of which diverged earlier than the other so-called AAB species, *Gluconacetobacter*, *Acetobacter* and *Gluconobacter* within the *Acetobacteraceae* (Fig. 5). Of these AAB species, *A. pasteurianus*, *Ga. xylinus* and *Ga. diazotrophicus* carry *ctaBD* and *ctaA*, while *G. oxydans* has *ctaBD* but not *ctaA*, which is consistent with the production of cytochrome bo_3 with heme O instead of cytochrome ba_3 with heme A.

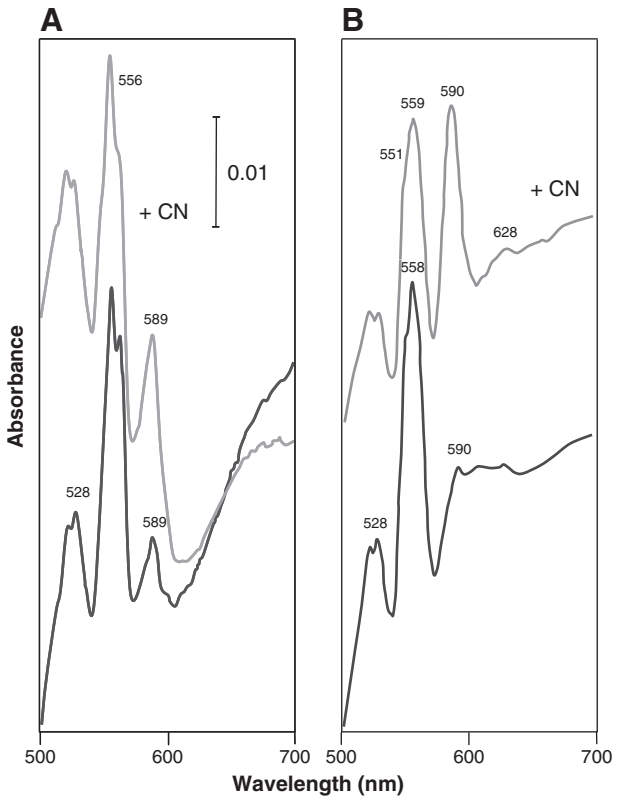

Fig. 6. Low-temperature redox spectra of the membranes prepared from the cells of *Gluconacetobacter diazotrophicus* (A) and *Gluconacetobacter xylinus* (B). Membranes prepared from the cells grown in YPG and YPG containing 2% ethanol for *Ga. diazotrophicus* and *Ga. xylinus*, respectively, were suspended in 50 mM potassium phosphate buffer (pH 6.5), and then the low-temperature redox spectra were measured with or without 1 mM cyanide (CN), as described in Materials and methods.

To determine whether the *Gluconacetobacter* species also produces a functional cytochrome ba_3 UOX, we prepared membranes from *Ga. diazotrophicus* PAl 5 and *Ga. xylinus* IFO 3288, and examined the UOX

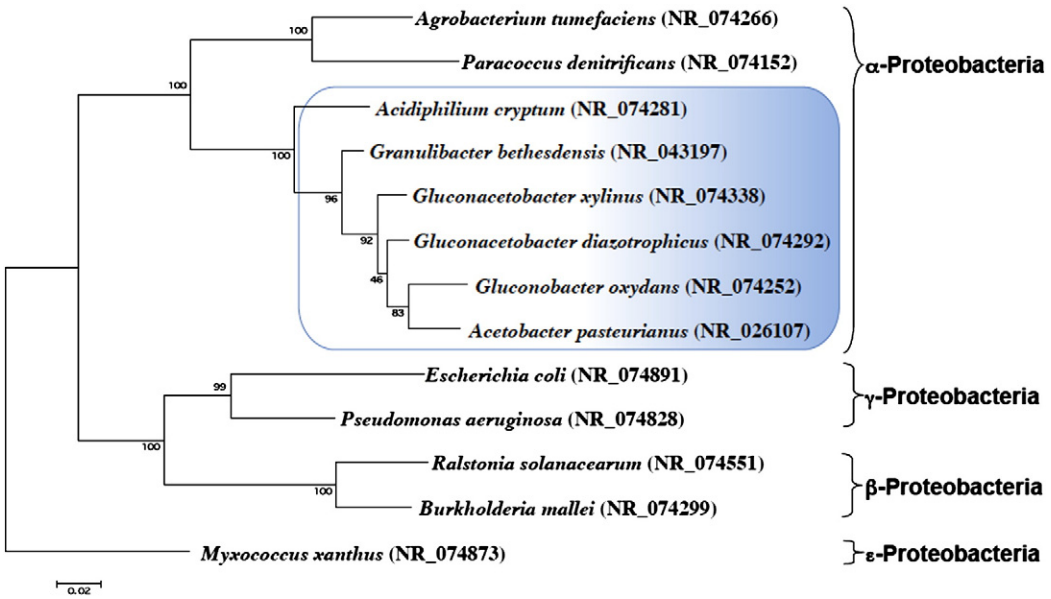

Fig. 5. Maximum-likelihood phylogenetic tree of 16S rRNA sequence of Proteobacteria including six *Acetobacteraceae* species. Of these 6 *Acetobacteraceae* species, *Gluconacetobacter*, *Acetobacter* and *Gluconobacter* are called AAB.

Table 3
Amino acid residues mutated (bold and gray shade) in the essential residues involved in the metal ligands and H^+ channels in CtaD of AAB.

	Conserved residues[1])	Amino acid residues in CtaD of these species				
		A. cryptum	*A. pasteurianus*	*G. oxydans*	*Ga. diazotrophicus*	*Ga. xylinus*
Heme ligand residues	His94 (*a*)	His	His	His	His	His
	His411 (a_3)	His	**–**	**–**	**–**	**Asp**
	His413 (*a*)	His	His	His	His	His
CuB ligand residues	His276	His	**Gly**	**Leu**	**Gly**	**Gly**
	His325	His	**Lys**	**Asp**	His	His
	His326	His	**Ser**	**Gly**	**Asp**	**Asp**
Residues involved in D–channel	Tyr35	Tyr	Tyr	**Phe**	Tyr	Tyr
	Asn113	Asn	**Ser**	**Gln**	**Ser**	Asn
	Asp124	Asp	**Gly**	**Arg**	Asp	**Ala**
	Asn131	Asn	Asn	**Thr**	Asn	**Ala**
	Ser134	Ser	**Gly**	**Gly**	**Cys**	**Gly**
	Ser193	Ser	**The**	**Ile**	**Met**	**Met**
	Glu278	Glu	**Val**	Glu	Glu	Glu
Residues involved in K–channel	Tyr280	Tyr	**Leu**	**Ala**	**Gly**	**Ala**
	Ser291	Ser	**Phe**	**Ala**	**Cys**	Ser
	Thr351	Thr	**Ala**	**Ser**	**Met**	**Val**
	Lys354	Lys	**Leu**	**Leu**	**Leu**	**Val**

[a]*P. denitrificans* cytochrome aa_3 *ctaD* (Pden_1938) numbering.

activity and redox spectra of the membranes. Both exhibited high UOX activity (8.9 and 4.3 U/mg, respectively) and a typical spectrum due to cytochrome ba_3 in the presence of cyanide (Fig. 6) [7,8]. Heme A was detected in these membranes by HPLC. Since the *A. cryptum* and *G. bethesdensis* genomes have a complete set of *cta* genes, we examined COX activity in the membranes of *A. cryptum* and *Ga. diazotrophicus*, which has a partial COX operon, *ctaCDE* (GDI_2595, 2596, 2597). *A. cryptum* exhibited 4.1 nmol O_2/min/mg COX activity with horse heart cytochrome *c* as the substrate and 33.1 nmol O_2/min/mg with its own cytochrome *c*, while no activity was detected with *Ga. diazotrophicus* membranes.

Thus, considering the phylogenetic divergence of the *Acetobacteraceae* family, *A. cryptum* and *G. bethesdensis* seem to have retained a complete set of *cta* genes, but the later-diverged AAB species have *ctaBD* and *ctaA*, and are missing *ctaCE*. Unlike the functional *ctaB* and *ctaA*, *ctaD* may not be functional. The *ctaD* of AAB produced an unusually extended phylogenetic tree with extensive variations, despite the normal phylogenetic relationship between the *ctaD*s of *A. cryptum* and *G. bethesdensis*, ancestors of AAB (Fig. S1A). Therefore, we examined CtaD sequence alignments for the AAB strains and other heme–copper oxidase subunit I's for several A-type COXs, including *A. cryptum* and *G. bethesdensis*, two A-type UOXs, and four B-type COXs (Fig. S2). Almost all essential amino acids for heme and copper ligands at the binuclear center and for H^+ channels [1,3] are mutated in AAB (Table 3). One may consider that CtaD in AAB is close to the B-type enzymes, which do not have a D-channel and have an alternative K-channel that differs from those in A-type enzymes. However, (i) CtaD of ABB species seems to be phylogenetically close to CtaD (A-type enzymes), although they are phylogenetically distant from non-mutated A-type enzymes (Fig. S1). (ii) Although CtaD of AAB contains mutations in the D- and K-channels, some residues are conserved in some AAB enzymes (Table 3). However, the conserved residues (alternative K-channel) of B-type enzymes were not found in the AAB enzymes (Fig. S2).

Thus, CtaD seems to have lost its function in the AAB genera *Gluconobacter*, *Acetobacter* and *Gluconacetobacter*. Instead, these species retained the *cyaBACD* operon (except for *A. cryptum*). We suggest AAB species use UOX instead of the ancestral COX.

3.4. Phylogenetic relationship of AAB UOX synthesis genes

In order to determine the phylogenetic relationship of genes associated with UOX synthesis, we analyzed *cyoB* (*cyaA*), *ctaB*/*cyoE* and *ctaA* in comparison to other typical bacterial species, of which 16S rRNA phylogeny is shown in Fig. S3.

The phylogenetic analysis of *ctaB* (*cyoE*) showed that *ctaB* of AAB is located within the *ctaB* category of α-Proteobacteria, but far from *cyoE*, which forms a single clade in the tree (Fig. S1B) and diverged from *yjdK* of *Bacillus subtilis* (BSU12080), which complements the *ctaB* mutation [36]. The *ctaA* of AAB is also located in a clade of α-Proteobacterial *ctaA* (Fig. S1C). These results suggest that AAB acquired heme O and heme A synthesis (CtaB and CtaA) genes in AAB evolved phylogenetically.

The Proteobacterial UOX gene (*cyoB*/*cyaA*) seems to originate from the COX gene (*ctaD*) of an ancestor of *B. subtilis* (BSU14900) before they were transferred into an ancestor of α/β- and γ-Proteobacteria (Fig. 1). However, *cyaA* of *A. pasteurianus* (APA01_00910) did not cluster with the UOX gene of α-Proteobacteria but with those of γ-Proteobacteria. Therefore, we performed a phylogenetic analysis of 73 UOX subunit I's (Fig. S4). As shown in Fig. 7 (a simplified version of Fig. S4), they separated into two major clusters: one formed by α and

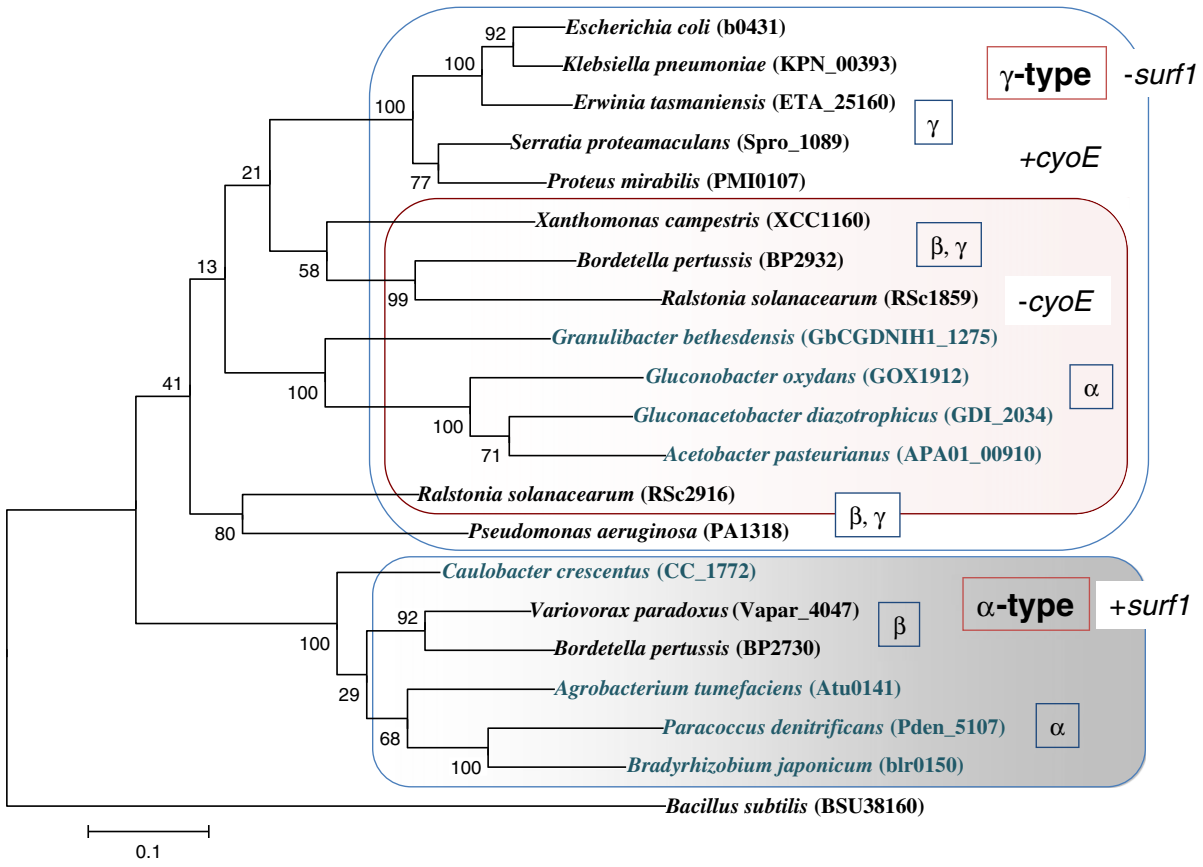

Fig. 7. Maximum-likelihood phylogenetic tree of UOX subunit I in α, β and γ-Proteobacteria. This tree was separated into two large clades; one is called γ-type and the other α-type; the former does not have the *surf1* gene (−*surf1*), but the latter has the gene (+*surf1*), respectively. Furthermore, the γ-type group (+*cyoE*) includes another group having that does not have any *cyoE* gene (−*cyoE*). Gene numbers correspond to organisms as follows: *A. pasteurianus* (APA01_00910), *A. tumefaciens* C58 (Atu0141), *Bradyrhizobium japonicum* (blr0150), *Caulobacter crescentus* CB15 (CC_1772), *G. bethesdensis* (GbCGDNIH1_1275), *Ga. diazotrophicus* PAl 5 (Brazil) (GDI_2034), *G. oxydans* (GOX1912), *P. denitrificans* (Pden_5107), *Bordetella pertussis* (BP2730, BP2932), *Ralstonia solanacearum* (RSc1859, RSc2916), *Variovorax paradoxus* (Vapar_4047), *E. coli* K-12 MG1655 (b0431), *Erwinia tasmaniensis* (ETA_25160), *Klebsiella pneumoniae* (KPN_00393), *P. aeruginosa* PAO1 (PA1318), *Proteus mirabilis* (PMI0107), *Serratia proteamaculans* (Spro_1089), and *Xanthomonas campestris* pv. campestris ATCC 33913 (XCC1160). *B. subtilis* (BSU38160) was used as an outgroup.

β-Proteobacteria (α-type UOX), and the other formed by a single clade of γ-Proteobacteria (Enterobacteria) and a mixed clade consisting of α-, β- and γ-Proteobacteria (γ-type UOX). When examining the UOX subunit I and UOX gene cluster, we noted that the gene clusters could be distinguished by the accessory protein related to heme synthesis or insertion, CyoE or Surf1. It is known that the α-Proteobacterium *P. denitrificans* has both COX and UOX gene clusters, both of which include *surf1* homologs (Fig. 2C & D). The functions of these Surf1 homologs have been experimentally validated to be involved in heme A insertion into subunit I of COX or UOX [18,38]. Most Surf1 homologs in the α-, β-, and γ-Proteobacteria (see Table S2) were detected in COX gene clusters, but small numbers of homologs were found only in α-type UOX. In contrast, the γ-type UOX did not carry *surf1*, but in some cases carried a *cyoE* gene in addition to *cyoABCD*. *cyoE* was found only in γ-Proteobacteria, including a large group of Enterobacteria and other groups such as *Acinetobacter* or *Pseudomonas* (Fig. S4). The most typical case is the UOX operon of *E. coli* (Fig. 2A), in which the CyoE functions as an HOS and produces heme O [37]. The *cyoE*-negative group was located within a mixed clade of α-, β- and γ-Proteobacteria, of which most of the α-Proteobacteria (5 in 7 species) were *Acetobacteraceae* (Figs. 7 & S4). Since the UOX gene clusters of *Acetobacteraceae* are more similar to those of the β/γ-Proteobacteria than the α-Proteobacteria, they may have been acquired by horizontal gene transfer.

P. denitrificans (α-Proteobacteria) carries an α-type UOX operon and *surf1*, and expresses cytochrome ba_3 UOX with heme A at the binuclear center [39]. In contrast, γ-Proteobacteria such as *E. coli* and *Azotobacter vinelandii* have a γ-type UOX operon with *cyoE* and express cytochrome bo_3 UOX, which binds heme O (Fig. 2A) [40–42]. Thus, it seems that the distribution of cytochrome bo_3/cytochrome ba_3 UOXs is consistent with two types of UOX (Table S2). However, *Ga. diazotrophicus*, *Ga. xylinus*, and *A. pasteurianus* express cytochrome ba_3 UOX in spite of these species having γ-type UOX and lacking a Surf1 homolog [7,43,44]. The subgroup of γ-type UOX (−*cyoE*) including *Acetobacteraceae* all have *ctaB* and *ctaA*, and thus produce heme O and heme A. The exception is *G. oxydans*, which has *ctaB* but not *ctaA* and thus express cytochrome bo_3. Thus, these subgroups including AAB could produce cytochrome ba_3 in spite of the absence of a *surf1* homolog, suggesting the existence of another heme A insertion mechanism. In contrast, bacteria that carry the γ-type UOX (+*cyoE*) lack the COX gene clusters, including *ctaB*, with the exception of five genomes (see Table S2). The results clearly show that *ctaB* in the COX gene cluster was replaced with *cyoE* from the *cyoABCDE* operon. *cyoE* is most closely related to *ctaB* of *Firmicutes*, suggesting that the ancestor of γ-Proteobacteria *cyoE* was acquired by horizontal gene transfer.

The respiratory chain of AAB having such a unique UOX is involved in a rapid oxidation of many sugars or sugar alcohols with several primary dehydrogenases located in the outer surface of the cytoplasmic membranes [5]. In *G. oxydans*, the respiratory chain has extraordinary high oxidase activity in membranes (0.8–1.1 μmol O_2/min/mg) [45] and intact cells (~2.0 μmol O_2/min/mg) [46]. These values are unique in comparison to other bacteria such as *Corynebacterium glutamicum*,

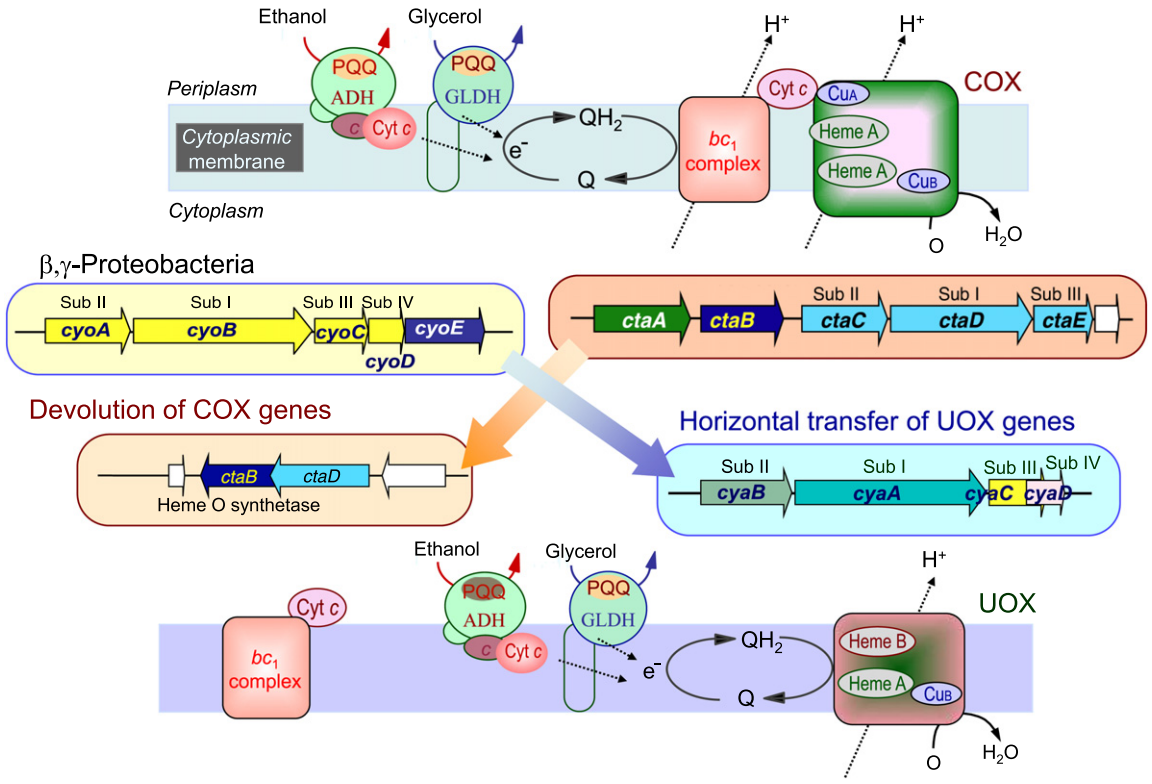

Fig. 8. Schematic model for evolution of the terminal oxidases in AAB. Ancestral species of AAB seem to have a respiratory chain consisting of the cytochrome bc_1 complex and COX similar to that of a typical aerobic respiratory chain of α-proteobacterial species. The respiratory chain has PQQ-containing quinoprotein dehydrogenases such as alcohol dehydrogenase (ADH) or glycerol dehydrogenase (GLDH) as the periplasmic primary dehydrogenase, and the enzymes donate electrons to ubiquinone (Q), and transfer electrons to COX via the bc_1 complex, and then to oxygen. During the evolutional process, AAB seems to have accepted UOX genes from some species of β,γ-Proteobacteria, and then to have created a truncated respiratory chain in which electron transfer to oxygen occurs via UOX directly accepting electrons from ubiquinol (QH_2). The truncated respiratory chain would generate less energy per single substrate oxidation but oxidizes the substrate much more rapidly. Such a rapid oxidation system would be beneficial in AAB, as described in the Conclusion. Thus, AAB started not to use the COX system for the respiratory chain, and lacks a large part of the COX genes, except for *ctaB* and *ctaA*, which are now used for heme O and heme A synthesis, respectively, for UOX biogenesis.

which has a typical cytochrome bc_1 and COX system with almost 10-fold lower oxidase activity in membranes (0.02–0.17 μmol O_2/min/mg) [47] and intact cells (0.138 μmol O_2/min/mg) [48]. This large difference in the respiratory activity between *G. oxydans* and *C. glutamicum* could be explained partly by the high expression of *G. oxydans* cytochrome bo_3 (2–3 fold higher than that of *C. glutamicum* cytochrome aa_3), and also by the high turnover number of the quinol oxidation rate of the purified cytochrome bo_3 (3–4 fold higher than that of *C. glutamicum* bc_1aa_3) (see Table S3). In addition, AAB such as *G. oxydans* [45,49] and *A. pasteurianus* [50] generate an extraordinarily low H^+/O ratio of 1–2 although they express a typical A-type UOX.

Thus, the uniquely high oxidation ability and low energy generation of AAB could be related to the truncated respiratory chain in AAB expressing UOX instead of COX.

4. Conclusions

Our results suggest that AAB have acquired UOX genes from β- and γ-Proteobacteria by horizontal transfer, while almost all the COX genes are missing, except for *ctaB* and *ctaA*, which now supply the heme moieties for UOX.

The truncated respiratory chain in AAB with UOX (Fig. 8) may generate less energy and a reduced proton-motive force, but has acquired a higher electron transfer ability. This change in the respiratory chain may enable AAB to perform incomplete oxidation, which contributes to the rapid oxidization of the many sugars or sugar alcohols present in fruit or flowers, the AAB habitat, and to accumulate high concentrations of acid products in their environment. Oxidative reaction products such as acetic acid are harmful to other microorganisms and thus contribute to the fitness of AAB in the competitive microbial world in fruit and flowers. This may be the driving force for AAB acceptance of UOX genes by horizontal gene transfer and the loss of COX gene function. This evolutionary adaptation enables AAB to survive in a fruitful habitat but not more harsh environments.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbabio.2014.05.355.

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