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Cytochrome cbb₃ of *Thioalkalivibrio* is a Na⁺-pumping cytochrome oxidase

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Cytochrome c oxidases (Coxs) are the basic energy transducers in the respiratory chain of the majority of aerobic organisms. Coxs studied to date are redox-driven proton-pumping enzymes belonging to one of three subfamilies: A-, B-, and C-type oxidases. The C-type oxidases (cbb₃ cytochromes), which are widespread among pathogenic bacteria, are the least understood. In particular, the proton-pumping machinery of these Coxs has not yet been elucidated despite the availability of X-ray structure information. Here, we report the discovery of the first (to our knowledge) sodium-pumping Cox (Scox), a cbb₃ cytochrome from the extremely alkalophilic bacterium *Thioalkalivibrio versutus*. This finding offers clues to the previously unknown structure of the ion-pumping channel in the C-type Cox and provides insight into the functional properties of this enzyme.

**Significance**

The majority of aerobic living organisms use oxygen for respiration. The key enzyme, which directly reduces oxygen to water during respiration, is the terminal cytochrome c oxidase. It generates a large portion of the utilizable energy provided by the respiratory chain. Accumulation of biologically available energy by means of cytochrome c oxidases is believed to be due to the proton-motive force across the mitochondrial or bacterial membrane. Details of this energy conversion are still unclear. Here we report the discovery of a sodium-pumping cytochrome c oxidase that converts energy of respiration into sodium-motive force. This finding provides clues to understanding the mechanism of cytochrome c oxidase that is not available when applying knowledge of the proton-pumping versions of the enzyme.


The authors declare no conflict of interest.

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Data deposition: The nucleotide sequences of *Thioalkalivibrio* cbb₃ oxidase have been deposited in the EMBL database (accession no. HE575403.1).

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concentrations (17). *T. versutus* is a chemolithotroph that oxidizes sulfur compounds and employs Cox as a terminal component of its aerobic electron transport chain. Here we report that *T. versutus* uses a novel C-type Cox that (i) specifically requires Na⁺ for its activity and (ii) electrogenically exports Na⁺ from cells or right-side-out subcellular membrane vesicles, the process being coupled to oxidation of ascorbate by O₂.

**Results and Discussion**

Experiments were performed using *T. versutus* cells (exhausted of endogenous substrates) and the right-side-out subcellular vesicles derived from them. Thus, both cells and vesicles displayed no respiration without substrate addition. To start respiration at the level of Cox in respiratory chain, we used the exogenous substrates Wurster’s blue [\(N,N,N',N'-\text{tetramethyl-p-phenylenediamine} (\text{TMPD})\)], which is nonspecific for all terminal oxidases, or cytochrome *c*, which is specific for Cox, both in the presence of a reductant ascorbate. Addition of any of the substrates initiated rapid O₂ consumption (Fig. 1 *A* and *B*) and generation of an electric membrane potential (\(\Delta \psi\)) (Fig. 1 *D*). Similar to the Na⁺-motive NQR (14), these activities were specifically Na⁺-dependent, showing optimum at alkaline pH (8.5–9.5) (Fig. 1 *C* and *D*). In contrast, the well-known H⁺-motive Cox from *Rhodobacter sphaeroides* (7) and *Paracoccus denitrificans*, used as controls (Fig. S1 *A–C*), as well as from *Pseudomonas stutzeri* (18), showed no Na⁺ specificity (SI Text, section S1). Cytochrome *c*, unlike TMPD, was inefficient toward *T. versutus* Cox at high salt concentrations, similar to Cox from other species; therefore, TMPD was used later on. The Cox inhibitor cyanide fully arrested both Cox activities (Fig. 1 *D* and Fig. S1D).

Thus, Cox operating in *T. versutus* membranes is a Na⁺-dependent oxidase (Scox) that can be either a Na⁺-activated H⁺ pump or Na⁺-activated redox loop lacking in H⁺ pump.

To discriminate between these two possibilities, we tested H⁺ pumping by the oxidase. Unlike H⁺-pumping Cox, which acidify the external medium during respiration in the presence of the K⁺ ionophore valinomycin (Fig. S2A) (see also refs. 7 and 19), Scox did not mediate H⁺ extrusion at alkaline pH (Fig. 2A and Fig. S2B), as shown earlier (20). Instead, Scox evoked alkalinization of the medium in response to O₂ pulse when valinomycin was replaced by protonophore, and H⁺ could serve as counter ion instead of K⁺ (Fig. 2B and inset and Fig. S2C). Reversibility of this alkalinization in the time scale (upper green curve, Fig. 2, Inset) points that it cannot be attributed to H⁺ consumption during respiratory chemical reaction of water production because of irreversibility of the latter reaction. These effects could be explained by assuming that the oxidase is Na⁺-motive and electrogenic. In such case, \(\Delta \psi\) formed by Na⁺ efflux, in the presence of a protonophore, should be counterbalanced by H⁺ influx, leading to reversible alkalinization of the outer space. Fig. 2D shows these relationships. Consistent with this scheme, a respiration-driven, fully cyaniode-sensitive export of \(^{22}\text{Na}^+\) was found in bacterial cells and vesicles preloaded with this isotope (Figs. 2C and 3A and *B*). Export of \(^{22}\text{Na}^+\) could be stimulated by protonophores [carbonyl cyanide m-chlorophenylhydrazone (CCCP) and 2-heptyl-4-hydroxyquinoline N-oxide (HQNO), a protonophore operating at alkaline pH (Fig. S3 and SI Text, section S2)] as well as valinomycin. Like respiratory activity of *T. versutus* Scox, export of \(^{22}\text{Na}^+\) occurred at alkaline pH (8.0–10.0) (Fig. 2C). These data confirm that *T. versutus* Scox operates as a primary Na⁺ pump rather than an Na⁺/H⁺ antiporter or a K⁺ pump.

To identify the Na⁺-pumping Scox, we considered the three known types of Cox (21, 22): A, B, and C (Fig. 4A), which are represented by cytochromes aa₃/cco₃, ba₃, and cbh₂, respectively. Because *T. versutus* membranes contained heme B but not heme A (Fig. S4), A- and B-type Cox can be excluded in our case. Instead, we identified an operon of a cbh₂ oxidase (C-type Cox).
in the *T. versutus* genome (EMBL accession no. HE575403.1) and detected its expression product (Fig. S5 and SI Text, section S1; for the enzyme pattern, see Fig. 2D), which might operate in this bacterium as a Na⁺ pump. Consistent with our hypothesis, *P. denitrificans* AO1 vesicles, which lack all Coxs and displayed no active Na⁺ transport (Figs. 2C and 3C), became capable of

![Image](https://example.com/image1)

**Fig. 3.** Evidence of a primary Na⁺ pump in *T. versutus* (*Tv*). (A) Substrate-induced Na⁺ efflux from 22Na-loaded *Tv* cells, (B) *Tv* vesicles, (C) *P. denitrificans* AO1 vesicles without *Tv* cbb₃ (no Scox), (D) *P. denitrificans* AO1 vesicles with *Tv* cbb₃ (+Scox), and (E and F) WT *P. denitrificans* vesicles was assessed by radioactivity of 22Na retained in cells or vesicles after substrate addition. (G) Substrate-induced Δψ generation in vesicles from *Tv* and (H) WT *P. denitrificans* monitored by spectral changes of safranine O (10 μM). A pH 9.2 (A–D and G), 7.5 (E and H), and 9.6 (F and H) reaction medium for “Na⁺ transport measurements” was used (Materials and Methods). Additions (final concentrations): valinomycin, 0.5 μM or 2 μM (A and G); protonophores: HQNO, 5 μM (G), 25 μM (B–D and G) or 50 μM (A); monensin, 0.1 μM (B and D) or 38 μM (A); KCN, 1 mM (B–D) or 10 mM (A); proteases of cells, 2.7 mg/mL; vesicles, 0.7 mg/mL (B), 1 mg/mL (C–F), 50 μg/mL (Left) or 76 μg/mL (Right) (G), and 50 μg/mL (H). Arrows at “0” time show substrate addition: TMPD, 40 μM (A, B, G, and H), or 3 mM (C–F) in the presence of ascorbate, 3 mM (B, G, and H) or 10 mM (A and C–F).

![Image](https://example.com/image2)

**Fig. 4.** Phylogenetic relationships of representative Coxs based on catalytic subunit protein sequences (Table S1). (A) The brief phylogenetic tree with representative Coxs: A-type (blue), B-type (green), and C-type (pink). *Thioalkalivibrio* clade (yellow, see also B) with R(K)308L(M) substitution is inside C-type Coxs. The sequences given in ref. 10 (gray) cluster separately from C-type Coxs (Unknown). E323 is conserved only in the catalytic subunit of C-type Coxs (see C); residue numbering corresponds to *P. stutzeri* cbb₃ (here and elsewhere). (B) Inset derived from the detailed phylogenetic tree with representative Coxs (Fig. S6); *T. versutus* cbb₃ Scox is orange-contoured. Blue spheres indicate nodes with clade credibility values <90; other nodes have clade credibility values ≥90. (Scale bar: 0.5 substitutions per residue.) (C) Amino acid sequences of helices IX–XI of catalytic subunit of C-type Coxs aligned using WebLogo software (23). The upper panels of each pair under an individual α-helix contain representatives of the Na⁺-motive-like cbb₃ Coxs (yellow-colored in the C-type cluster); the lower panels contain all other representatives of the C-type cluster that are H⁺-motive-like (pink-colored in the C-type cluster). Red arrows show R(K)308L(M) substitution and conservative T312, G344, S348, and T389 in the Na⁺-motive-like representatives, E323 conserved in both groups, W386 conserved among H⁺-motive-like representatives. Red asterisks in the Na⁺-motive template indicate residues forming Na⁺ coordination shell (shown in Fig. S6).
oxidase-driven primary $^{22}$Na$^+$ pumping (Figs. 2C and 3D) similar to that of T. versutus vesicles (Figs. 2C and 3B) after expression of T. versutus cbb$_3$. We checked whether the observed $^{22}$Na$^+$ export from the energized cells and vesicles could be explained by a $\Delta$ψ-potentiated $^{22}$Na$^+$/Na$^+$ exchange or specified by a potential-dependent Na$^+$ channel. Because dissipation of $\Delta$ψ by yalinomycin, as well as by protonophore (Fig. 3G), increased $^{22}$Na$^+$ export in T. versutus vesicles (Fig. 3B), a passive $\Delta$ψ-potentiated $^{22}$Na$^+$/Na$^+$ leakage or "Na" transfer through a $\Delta$ψ-activated Na$^+$ channel can be excluded. Consistent with this conclusion is the absence of $\Delta$ψ-driven $^{22}$Na$^+$ export in $^{22}$Na$^+$-loaded WT P. dentrificans vesicles that lack T. versutus Scox (Fig. 3E, F, and H). The data also reveal that P. dentrificans ad$_3$ and cbb$_3$ Scox, expressed in the expressed growth conditions (Materials and Methods), do not pump Na$^+$. Thus, these experiments directly demonstrated that T. versutus cbb$_3$ is a primary Na$^+$ pump.

The cbb$_3$ oxidases studied previously were shown to operate as H$^+$ pumps (7, 19, 24, 25). However, an individual pathway of pumped H$^+$ analogous to the D channel in A-type Cox has not been identified (10, 24). Only a K-pathway analog, involved in delivering chemical H$^+$ to BNC to produce water in Cox of all C-type members. Additionally, W386 seemed to be fully conserved among the Na$^+$-motive-like (Thioalkalivibrio clade) and H$^+$-motive-like (all other C-type members). Additionally, W386 seemed to be fully conserved among the Na$^+$-motive-like but not in the Na$^+$-motive-like C-type members.

Another indication that the Na$^+$-pumping channel is located within helices IX–XI in the D-type Coxs is provided by the observation that there is a unique conserved, ionizable, membrane-embedded residue, E323, that is found in the middle of helix IX (Fig. 4C). This E323 is surrounded by several polar residues that form a Na$^+$-coordination shell and could function as Na$^+$ chelators (Fig. 5B). In the R. sphaeroides H$^+$-motive cbb$_3$, the corresponding glutamate (E383) was initially suggested to participate in H$^+$ pumping (24). However, its functional role in H$^+$ pumping was challenged later based on the absence of E323 in some putative C-type Coxs (10). To resolve discrepancies about the role of E323, we performed phylogenetic analyses of the C-type Coxs sequences used in ref. 10 where the study was limited to sequence-alignment level. We found that the previously assigned C-type Cox sequences that lack E323 do not actually cluster with other C-type enzymes in the phylogenetic tree and form a phylogenetically distinct clade (gray-colored, Fig. 4A and B). Taking into consideration such clustering, we divided all protein sequences of C-type Coxs into two groups for further consideration such as clustering, we divided all protein sequences of C-type Coxs into two groups for further consideration such as clustering, we divided all protein sequences of C-type Coxs into two groups for further consideration such as clustering, we divided all protein sequences of C-type Coxs into two groups for further}

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Fig. 6. Putative mechanism of ion translocation by the H+-motive (P. stutzeri) and Na+-motive (T. versutus) cbb₃ Coxs for a single electron transfer. (A) Free energy of Na⁺ binding by E323 in ccoN of P. stutzeri (left) and T. versutus cbb₃ (right), estimated by the free energy perturbation method within MD modeling of the complexes in 1 M Na⁺ solution (Table S2). (B) Combined models of the H⁺-pumping channel in the H⁺-motive cbb₃ by several hypotheses (circled numbers): 1 (10) + 2 (26) or 1 + 3 (25, 32) + 4 (24). (C) Functional model of the sodium pumping in the Na⁺-motive cbb₃ (this study) formed by α-helices IX-XI (green) of the catalytic subunit; α-helix (question-marked rectangle) in the Na⁺-motive Cox analogous to the auxiliary α-helix U (olive) in the H⁺-motive Cox is presumed. In the H⁺-motive and Na⁺-motive cbb₃ Coxs, a large gray rectangle depicts helices I-VIII and XII: “K channel” (blue) delivers chemical H⁺ to the active center to produce water during reduction of O₂. Notably, helices IX and X of the catalytic subunit of P. stutzeri cbb₃ (H⁺-motive pattern) as well as of T. versutus cbb₃ (Na⁺-motive pattern) contain features that disrupt regularity of the helices, conferring flexibility that might be necessary for ion transfer (Fig. 5E). Additionally, in P. stutzeri cbb₃ the only large cavity seems to be located within helices IX–XI of the main subunit (Fig. 5C) and the recently identified auxiliary subunit U (26) (for details, see SI Text, section S3). When water-filled, this cavity could link the critical residues R(K)308 and E323, providing a water-filled H⁺-translocation pathway (Fig. 5D). These facts allow us to assume that in the Na⁺-motive cbb₃ such cavity could serve as the Na⁺-pumping channel. Finally, we evaluated the Na⁺-binding capacity of the H⁺-motive and Na⁺-motive cbb₃ Coxs using molecular dynamics (MD). MD simulations predicted that the main subunit of T. versutus, but not that of P. stutzeri, can bind Na⁺ in the E323 site in a redox-linked manner (Fig. 6 A and B and Table S2). According to the prediction, one electron coming into the metal active center switches the enzyme from a Na⁺-nonbinding oxidized (ox) state to a Na⁺-binding reduced (red) state. Under the tentative model, the subsequent entry of H⁺ to the active center via the chemical reaction through the “K channel” results in Na⁺ extrusion (Fig. 6B). The predicted coupling stoichiometry is one electron for each pumped Na⁺, which is consistent with the experimental data (Fig. 2B and Fig. S2 B and C) according to which Scox operation fits best with the following equation:

\[ 4\text{cyt}^{2+} + 4H^{+}_{\text{chem}} + 4\text{Na}^+_{\text{pump}} + O_2 → 4\text{cyt}^{3+} + 4\text{Na}^+_{\text{out,pump}} + 2H_2O. \]

Evidently, the capacity of T. versutus Scox to bind Na⁺ is provided by the E323-involving Na⁺-coordination shell (see Fig. 5B), which is absent in P. stutzeri Cox due to S348(A) substitution.

The fact that the Na⁺-pumping function was revealed among C-type Coxs may shed light on evolution of Coxs. At present, the phylogenomics-based evolutionary scenario of Coxs remains obscure (1, 22, 34). Therefore, in our reasoning we take into consideration several independent markers of possible Cox evolution and generally accepted facts: (i) geological evidence on oxygen-free atmosphere on the Earth preceded an oxygen atmosphere (35), (ii) close relationship between nitric oxide reductases and C-type Coxs and their far distance from A- and B-type Coxs (1, 36, 37), and (iii) the conclusion that Na⁺ energetics appeared before H⁺ energetics (38). If we believe the mentioned facts and views are true, then C-type Coxs—which include Na⁺-motive and H⁺-motive representatives—might be the ancestors of H⁺-motive A- and B-type Coxs, as assumed earlier (37). C-type Coxs were shown to bind O₂ much more tightly than A-type Coxs (39). Owing to this property C-type Coxs operate under low O₂ conditions and would be the first energy-transducing enzymes capable of oxygen reduction when the early atmosphere of the Earth was formed, being gradually filled with O₂.

**Conclusion**

We provide the first direct demonstration to our knowledge that the T. versutus cytochrome cbb₃ (Cox) is a primary Na⁺ pump. This finding is collectively based on the observation of (i) specific Na⁺ dependence of Cox activity, (ii) protonophore- or valinomycin-stimulated Na⁺ pumping in T. versutus cells and vesicles, (iii) no H⁺ pumping in them in the presence of valinomycin and reversed stimulation of O₂ evolution by valinomycin in the absence of protonophore without valinomycin, (iv) expression of the T. versutus cbb₃ in P. denitrificans cells with the result that membranes of this bacterium are competent in protonophore/valinomycin-activated respiration-supported Na⁺ pumping, and (v) a Na⁺-binding coordination shell in the active center of Cox. These results raise the question of whether Na⁺ pumping is inherent in some other C-type Coxs. In this context, whether the Na⁺ transport in Vitreoscilla (40) is evoked by a primary Na⁺-pumping bo-type quinol oxidase or by a secondary pumping Na⁺/H⁺ antiporter is still unclear for the lack of ionophore tests after bo oxidase expression in Escherichia coli, which contains self-Na⁺/H⁺ antiporters (41) (for details, see SI Text, section S2). Our findings are consistent with the hypothesis of using Na⁺-motive energy transducers in organisms living under low ΔpH and high salinity (12) and might stimulate further progress in the study of energy-transduction mechanisms of Coxs.

**Materials and Methods**

Cultivation of bacterial strains, phylogenetic analysis, molecular dynamic simulations, and other methods are detailed in SI Materials and Methods.

**Bacterial Strains.** T. versutus AL2 and P. denitrificans strain AO1 [expresses no cytochrome c oxidase activity (42)] and WT strain (PD 1222) were batch-cultured aerobically as previously described (17, 43).

**Membrane Vesicle Isolation.** Right-side-out membrane vesicles were isolated from freshly grown cells in a medium containing 50 mM CAPSO-KOH (pH 9.9), 50 mM K₂SO₄, 0.1 M sucrose, 0.1 mM EGTA, and 0.35 M Na₂SO₄ by disruption in a French press cell according to a standard procedure.

**Expression of Coxs in P. denitrificans.** For heterologous expression of T. versutus cbb₃ Cox in P. denitrificans, the T. versutus AL2 cccOΔNQP operon was identified (HE575403.1) and amplified by PCR. The PCR fragment was cloned into the XbaI-HindIII-digested derivative of the broad host-range plasmid pBBR1MCS (42, 43) to produce the pBBR1/cccOΔNQP plasmid. For protein expression, the recombinant plasmid pBBR1/cccOΔNQP was transferred by conjugation into the Paracoccus recipient strain AO1. Exogenously expressed T. versutus cbb₃ Cox in strain AO1 reached a level comparable to that in T. versutus AL2 strain, as monitored using Western blotting. Expression of self-cbb₃ Cox in P. denitrificans WT strain grown aerobically (this study) reached the same high level as in semiaerobically grown cells (44), as quantified by real-time quantitative PCR. Each self-Cox level, aₕ and cbb₃, reached 70 pmol Cox/mg of membrane protein as determined by CO-reduced minus reduced spectra using extinction coefficients of 7 mM⁻¹ cm⁻¹.

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Electrical membrane potential generation in right-side-out membrane vesicles was monitored by the safranine method (49) or by tetraphenylphosphonium-selective electrode (50) at 25 °C.

H+ release in intact cells and membrane vesicles in O2-pulse was assessed by a standard method (51) in 1 mL of anoxic incubation mixture. Respiration of samples was initiated by addition of water (5–20 μL) saturated with air at 25 °C. The evolved changes in pH in the incubation mixture were estimated by titration with argon-saturated 0.5 mM H3O4.

Respiratory activity was assessed using a Clark-type electrode at 25 °C.

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