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Hypothesis

Branched Mitochondrial Electron Transport in the Animalia: Presence of Alternative Oxidase in Several Animal Phyla

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Summary

The mitochondrion of most eukaryotes has multiple electron transport components that increase the points of entry and/or exit of electrons, thus giving a branched nature to the respiratory chain. In plants and many other organisms, a prominent example is alternative oxidase, a non-energy conserving branch in the respiratory chain and an additional terminal oxidase for the exit of electrons. Our genome database searches have now revealed the presence of alternative oxidase in four animal species from three different phyla (Mollusca, Nematoda and Chordata), consistent with frequent reports of cyanide-resistant respiration in the Animalia. In *Ciona intestinalis* and *Crassostrea gigas*, alternative oxidase is expressed in several different tissues. Phylogenetic analysis is consistent with the animal proteins having originated by vertical inheritance. We hypothesize that alternative oxidase is likely widespread in the Animalia and discuss some of the potential role(s) for such a branched respiratory chain.

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Keywords Alternative oxidase; animal mitochondria; branched mitochondrial electron transport chain; *Ciona intestinalis*; *Crassostrea gigas*; cyanide-resistant respiration; *Meloidogyne hapla*.

INTRODUCTION

The standard textbook description of mitochondrial electron transport outlines that reducing equivalents (NADH, $FADH_2$) are oxidized by NADH dehydrogenase (Complex I) and succinate dehydrogenase (Complex II), and that electrons are subsequently passed to the ubiquinone pool, followed by Complex III, cytochrome (cyt) c and Complex IV. Complex IV (cyt oxidase) is the terminal oxidase that passes the electrons to O_2. Further, this electron transfer process is coupled at three sites (Complex I, III, IV) to proton translocation from the matrix of the mitochondrion to the inner membrane space and the resulting electrochemical gradient is used to drive the synthesis of ATP by the process of oxidative phosphorylation. This description fits well the basic design of mammalian electron transport but, as recently reviewed (*1*), this description is incomplete for most eukaryotic mitochondria (as well as prokaryotic respiratory systems), which instead rely on additional electron transport components that increase the points of entry and/or exit of electrons, thus giving a branched nature to the electron transfer process.

Alternative oxidase (AOX) is a mitochondrial inner membrane protein which functions as a component of electron transport in all plants (*2, 3*). It catalyzes the oxidation of ubiquinol and reduction of O_2 to H_2O, thus representing both a branch in the respiratory chain and an additional terminal oxidase for the exit of electrons. Significantly, electron flow from ubiquinone to O_2 via AOX is not coupled to proton translocation. Hence, AOX represents a non-energy conserving branch of electron transport, bypassing the last two sites of proton translocation (Complex III and IV) associated with the cyt pathway. Besides its ubiquitous presence in plants, AOX is also widespread in fungi (*4*) and protists (*5–7*). Its origin in eukaryotes may be the endosymbiotic event that gave rise to mitochondria since it has also now been identified in the α-proteobacterium *Novosphingobium aromaticivorans* (*8*).

Until the mid-1980s, AOX was best described as a CN-resistant component of respiratory O_2 consumption in plants and some other organisms (*9*). Since then, a molecular characterization of AOX has occurred in many plants (*10*) and fungi (*11*), in the green alga *Chlamydomonas reinhardtii* (*12*), and in several parasitic protists including *Trypanosoma brucei brucei* (*6*), *Phytomonas* sp. (*5*) and *Cryptosporidium parvum* (*7*).

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Abbreviations: AOX, alternative oxidase; cyt, cytochrome; ROS, reactive oxygen species; SHAM, salicylhydroxamic acid.

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Given the wasteful nature of AOX in terms of energy conservation, effort has been put toward understanding the role of this pathway in respiration. In general, AOX may represent a means to integrate the coupled processes of carbon metabolism and electron transport, thus preventing redox and carbon imbalances and promoting metabolic homeostasis (*13*). One hypothesis put forth is that AOX may influence the generation of reactive oxygen species (ROS) by the respiratory chain (*14*). Mitochondrial electron transport chains are a significant source of ROS and the rate of ROS generation is dependent upon the reduction state of key respiratory components. These mitochondrial ROS may have important signaling functions within cells, while their excessive generation may cause oxidative damage to the mitochondrion (*15, 16*). By preventing the over-reduction of the respiratory chain, AOX could act to reduce ROS generation. In support of this hypothesis, transgenic plant cells lacking AOX display higher rates of mitochondrial ROS generation (*17*), particularly under growth conditions that may promote a more reduced chain (*18*).

Besides modulating ROS generation, AOX may also represent a means to maintain respiration if the cyt pathway is impaired. In support of this hypothesis, loss of the cyt pathway (by chemical or genetic means) results in an induction of AOX expression in plants, fungi and protists (*2, 5, 19, 20*). This is significant because numerous organisms are exposed to potent inhibitors of cyt oxidase such as CN, NO and sulfide. These inhibitors may accumulate in tissues as a result of their abundance in particular environments or simply as products of normal physiological and biochemical processes (*3, 21–24*).

Interestingly, a component of respiratory O_2 consumption that is resistant to CN has also been noted in several invertebrates, particularly in marine worms such as *Arenicola marina* (*25*), *Sipunculus nudus* (*26*), *Nereis pelagica* (*27*) and *Marenzelleria viridis* (*28*), as well as the bivalve *Arctica islandica* (*27*) and the mussel *Geukensia demissa* (*29*). In most of these cases, it is further shown that the CN-resistant O_2 consumption is less tightly coupled to ATP production and sensitive to salicylhydroxamic acid (SHAM). Interestingly, these are both characteristic features of AOX respiration. However, the molecular nature of CN-resistant respiration in these animals has not been elucidated and, in particular, no molecular evidence for AOX in animals has ever been reported.

EXPERIMENTAL PROCEDURES

In silico Analyses

AOX homologues were identified by tBLASTn searches of databases at NCBI (http://www.ncbi.nlm.nih.gov), TIGR (http://www.tigr.org/tdb/), and the Department of Energy Joint Genome Institute (http://www.jgi.doe.gov/) using the *Dictyostelium discoideum* AOX (BAB82989). The *Ciona intestinalis* sequence identified was then used to identify other animal AOX sequences.

Sequence Alignment and Phylogenetic Analyses

Deduced amino acid sequences were aligned by Clustal X Version 1.81 using default gap penalties (*30*). Sequences complete enough to include all four of the iron-binding motifs (*31, 32*) were used for the generation of phylogenies using MEGA version 2.1 (*33*). The neighbor-joining method was used with the p-distance model, but phylogenies generated using the number of distances, gamma, or poisson models yielded identical topologies.

RNA Isolation and Analysis

Live Pacific oysters (*Crassostrea gigas*) were purchased locally, shucked and dissected to isolate gill, heart, mantle and adductor muscle tissues. These tissues (along with hemolymph collected from the pericardial cavity) were frozen in liquid N_2 and RNA was isolated using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). DNA was eliminated from RNA samples prior to RT-PCR using amplification-grade DNase I (Invitrogen). Oligonucleotide primers (forward, 5′-GAGTCAATGAAGAACCCTCC-3′ reverse, 5′-CTCATCTGCTCGAATTGC-3′) were designed to amplify a 663 bp AOX fragment. RT-PCR was performed using the Access RT-PCR System (Promega, Madison, WI, USA). Reaction products were then analyzed by agarose gel electrophoresis. Northern analyses were done as before (*32*) using the 663 bp cDNA product (see above) as probe.

RESULTS AND DISCUSSION

AOX in the Animalia

We have identified AOX genes in species from three different animal phyla: Mollusca, Nematoda and Chordata. AOX is present in the genome of the oyster *C. gigas*, the plant pathogenic root-knot nematode *Meloidogyne hapla*, and the sea squirts *C. intestinalis* and *C. savignyi* (Table 1). Interestingly, our database searches also revealed for the first time AOX genes in two other important taxonomic groups: a diatom (*Thalassiosira pseudonana*) and a red alga (*Cyanidioschyzon merolae*) (Table 1).

RT-PCR showed that AOX is expressed in all of the oyster tissues tested and Northern analyses detected an AOX transcript of approximately 1.5 kb (Fig. 1). Further, analysis of an EST databank of *C. intestinalis* (*34*; http://ghost.zool.kyoto-u.ac.jp/indexr1.html) indicates that AOX is expressed in several different cell types and at different developmental stages. EST counts gave the following expression profile: eggs (3/29,444), blood cells (8/28,596), cleavage stage embryos (2/26,796), tailbud embryos (3/31,209), and young adults (1/29,138).

We compared the AOX protein sequences of oyster and *C. intestinalis* with those from a wide taxonomic range of

Table 1
AOX genes from a wide range of taxonomic groups including animals. Unless otherwise indicated, each is present in the GenBank database.

Kingdom	Phylum or division	Species	Accession number or identifier
Animalia	Mollusca	*Crassostrea gigas*	BQ426710[a]
	Nematoda	*Meloidogyne hapla*	BM901810[a]
	Chordata	*Ciona intestinalis*	TC17302[a,b]
		Ciona savignyi	AACT01009170,[a] AACT01012415[a]
Fungi	Ascomycota	*Ajellomyces capsulatus*	AF133237
		Aspergillus niger	AB016540
		Blumeria graminis	AF327336
	Basidiomycota	*Cryptococcus neoformans*	AF502293
Plantae	Anthophyta	*Arabidopsis thaliana*	NM_113135, NM_113134, NM_125817
		Populus tremula	CAB64356
		Catharanthus roseus	AB055060
		Zea mays	AAL27795
		Triticum aestivum	BAB88646
		Oryza sativa	AB004813
		Cucumis sativus	AAP35170
		Glycine max	U87906
Protista	Chlorophyta	*Chlamydomonas reinhardtii*	AF047832, AF314255
		Acetabularia acetabulum	CF258325
	Rhodophyta	*Cyanidioschyzon merolae*	AP006491[a]
	Bacillariophyta	*Thalassiosira pseudonana*	DOE, scaffold_278; nucleotides 4692–5382 and 1321–2018[a,c]
	Acrasiomycota	*Dictyostelium discoideum*	BAB82989
	Apicomplexa	*Cryptosporidium parvum*	AY312954
	Euglenozoa	*Trypanosoma brucei brucei*	AB070617
	Oomycota	*Pythium aphanidermatum*	CAE11918
Eubacteria	Proteobacteria	*Novosphigobium aromaticivorans*	ZP_00095227

[a]AOX sequences not previously reported in the literature; [b]at the TIGR database; [c] at the Department of Energy Joint Genome Institute Database.

organisms that includes plants, fungi, green algae, a red alga, a diatom, a slime mold, parasitic protists and an α-proteobacterium (Fig. 2; Table 1). It was previously shown that AOX proteins from diverse taxonomic groups nonetheless all share key conserved amino acid residues in the central region of the protein (*32*). These include the six iron-binding residues distinctive of these di-iron carboxylate proteins (*31*), other residues within the four iron-binding motifs, and several other amino acids. Importantly, all of these residues are also completely conserved in the animal proteins (Fig. 2). The other animal sequences found (from *M. hapla* and *C. savignyi)* are incomplete in this region and were thus not included in the alignment. However, the partial sequences from these animals do possess the expected conserved features (including two of the four iron-binding motifs) and thus almost certainly also encode AOX proteins.

Figure 3 shows an unrooted protein phylogeny of the AOX proteins generated using distance methods. The plant AOXs cluster together and are most closely related to those of the red alga. The animal AOXs of *C. gigas* and *C. intestinalis* group most closely with each other and are more distantly related to the AOXs of fungi. Since only a limited number of branches are robustly supported and since some aspects of the topology disagree with established eukaryotic phylogenetic relationships (e.g. the separation of green algae and land plants) it is not possible to make strong phylogenetic/evolutionary interpretations. Given the limited data presently available (such as the lone α-proteobacterial sequence), it is possible that an

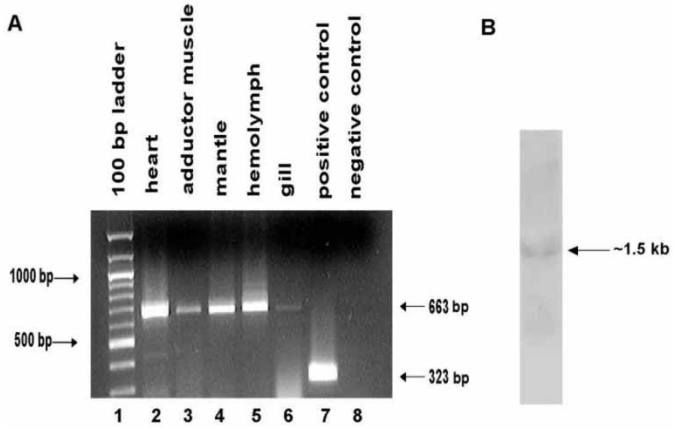

Figure 1. Expression of AOX in the oyster *C. gigas*. (A) RT-PCR results. Lane 1: 100 bp DNA ladder; Lanes 2–6: Expected 663 bp RT-PCR product generated from different oyster tissue RNA; Lane 7: RT-PCR kit control with expected 323 bp product; Lane 8: reaction containing gill RNA but lacking reverse transcriptase. (B) Northern blot analysis showing the expression of an $\sim$ 1.5 kb AOX transcript.

insufficient number of phylogenetically informative characters is present in the alignment.

It does not seem conceivable that, in each case, these animal AOX sequences represent contamination of source material, such as by symbionts or parasites. First, DNA (RNA) for each of the sequencing projects was derived from specific source cells, be it sperm, eggs, or hemocytes (*35–37*). Second, a sea squirt EST project reveals AOX expression in several different cell types and developmental stages (see above). Third, while the *C. gigas* sequence is from an oyster challenged by strains of *Vibrio* bacteria, we could not find AOX in the complete genomes of several *Vibrio* strains available at NCBI. Oysters can also be infected by parasitic protists such as *Perkinsus marinus* (*38*). Several parasitic protists have AOX but these sequences are not closely related with the animal AOX sequences in our phylogeny (Fig. 3). Fourth, we could demonstrate expression of AOX in live oysters expected to be free of parasitic organisms because they were obtained from a shellfish harvest area enforced by the Canadian Shellfish Sanitation Program (Fig. 1). Fifth, the multiple sequence alignment and phylogenetic analyses show that the oyster and sea squirt sequences group most closely to one another and are not closely embedded within any of the other taxonomic groups (Fig. 3).

What is the Origin and Distribution of Animal AOXs?

Molecular phylogenies support the hypotheses that animals evolved from single-celled protists, and that animals and fungi have a common ancestry (*39*). Several extant protozoan and fungal groups possess AOX, supporting our assumption that animal AOXs were derived by vertical inheritance. The current understanding of Metazoan evolution is that fungi diverged first, followed by the mesomycetozoa, and finally the choanoflagellates and animals (*39*). No complete genome data is yet available for any members of the mesomycetozoa or choanoflagellates to examine whether AOX is retained in these groups. Its presence in the two *Ciona* species suggests that AOX was maintained in the animal lineage at least until the divergence of urochordates from the rest of the Chordata. We hypothesize that animals lacking AOX (as is likely the case in the subphylum Vertebrata) represent a lineage that has lost the gene over evolutionary time.

Another possible explanation for the presence of AOX in four animal species would be multiple horizontal gene transfer events. For example, a cellulose synthase gene in *C. intestinalis* and the possible presence of algal photosynthetic genes in a molluscan sea slug are suggested to represent horizontal gene transfer events (*40, 41*). Despite such examples, it seems unlikely that animals from three different phyla would be subject to horizontal gene transfer events resulting in animal AOX genes so similar to one another (Fig. 3). This would suggest that, in each case, the same or very similar organisms were the source of transferred DNA.

New genomic data continues to uncover AOX sequence in new taxonomic groups, most recently being uncovered in α-proteobacteria (*8*) and now in red algae, diatoms, and animals (Fig. 2; Table 1). Within some taxa, previous study has already implicated the existence of an AOX-like terminal oxidase based on such biochemical features as CN-resistant and SHAM-sensitive respiration. However, it appears that the well-described CN-resistant respiration of the parasitic protist *Plasmodium falciparum* is not due to AOX since we and others (*7, 42*) are unable to find AOX in its sequenced genome. This fact certainly points out that one must be cautious until both biochemical and molecular data are available.

While AOX appears to be ubiquitous in the Plantae, its distribution in the Protista and Fungi is clearly more sporadic. For example, within the Trypanosomatidae, *T. brucei brucei* and *Phytomonas* sp. have AOX, but *Leishmania* sp. do not (*5, 6*). The green alga *C. reinhardtii* has AOX, but its close relative *Polytomella* sp. does not (*43*). While AOX is widespread in yeast, it is absent in *Saccharomyces cerevisiae* (*4*). The available molecular and biochemical evidence suggests that AOX will also be widely but sporadically distributed in the Animalia. For example, while the nematode *M. hapla* has AOX, *Caenorhabditis elegans* and several other nematode genomes or ESTs available for searching clearly do not.

In addition to the three animal phyla in which we have uncovered AOX genes, previous biochemical and physiological studies also suggest the existence of such a pathway in the phyla Annelida (*N. pelagica* and *A. marina*; *25, 27*), Sipuncula (*S. nudus*; *26*) and possibly Arthropoda (*Spodoptera eridania*; *44*).

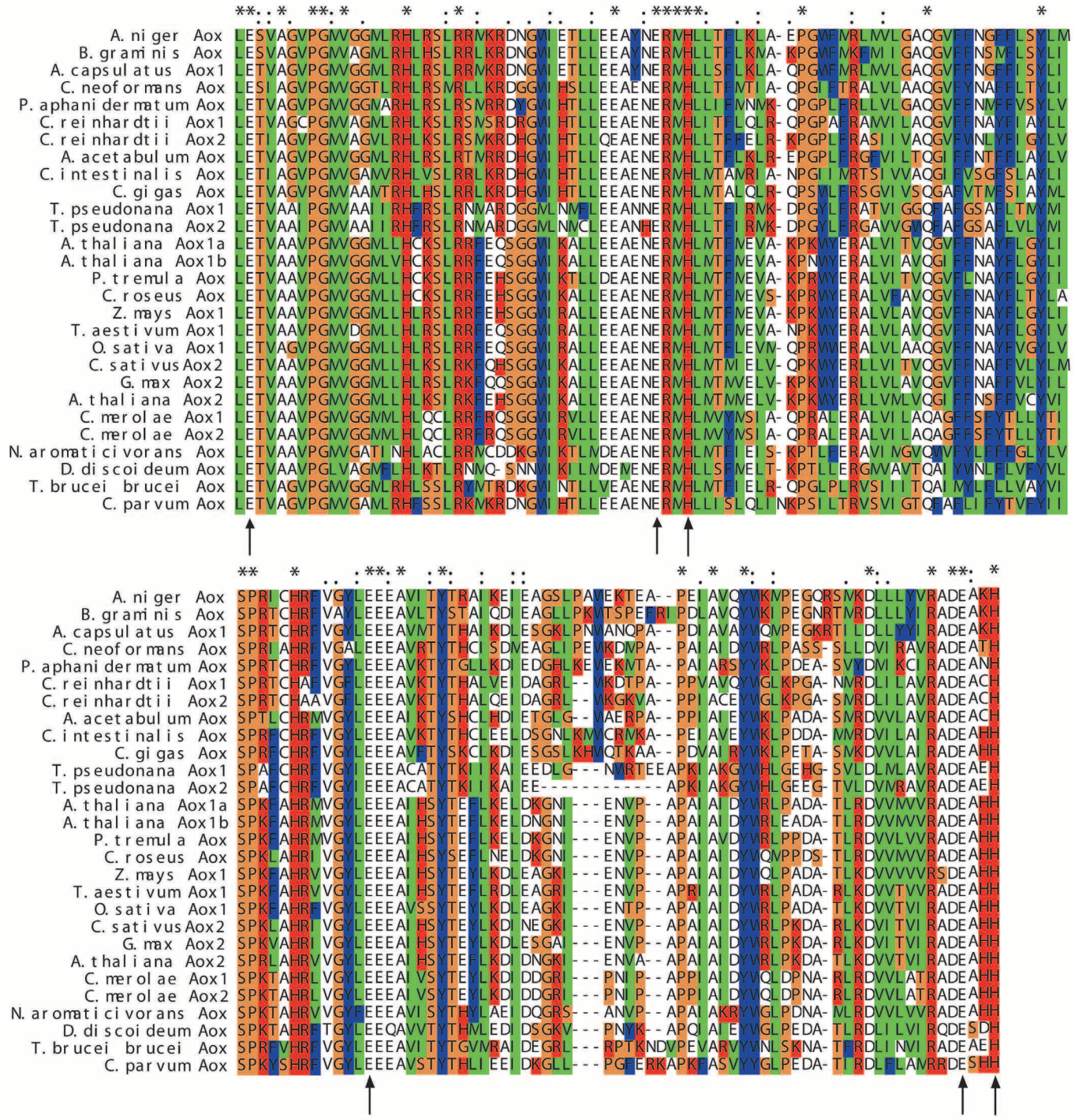

Figure 2. A multiple sequence alignment of 28 AOX proteins from 23 species representative of a wide range of prokaryotic and eukaryotic taxa. Black arrows indicate the putative iron-binding residues. The colors and other symbols above the alignment follow the default conventions defined by the Clustal X program, regarding conservation of amino acids between sequences (*30*). The colour conventions for amino acids are: orange, GPST; red, HKR; blue, FWY; and green, ILMV. The * symbol indicates positions which have a single, fully conserved residue, : indicates that a strong group is fully conserved, and . indicates that one of the weaker groups is fully conserved. See the Clustal X program for a full list of the strong and weak groups (*30*).

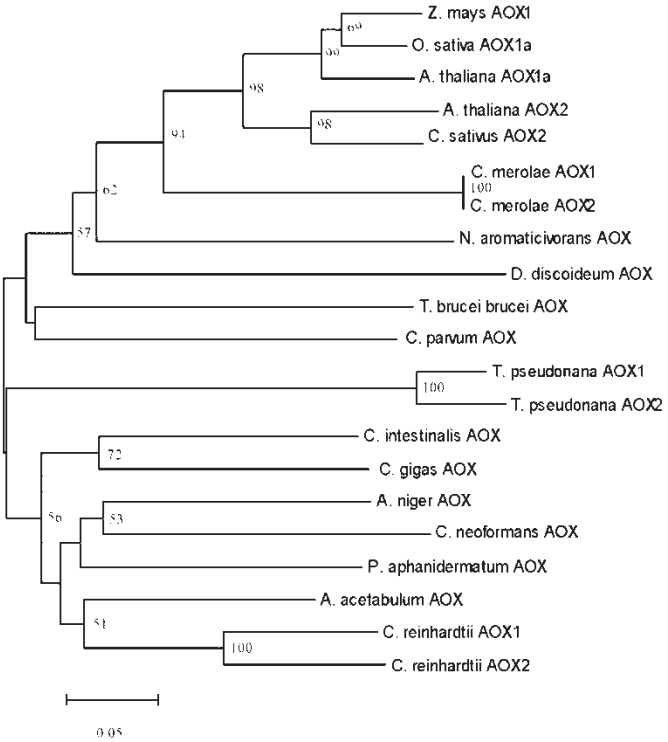

Figure 3. Phylogeny of eukaryotic and prokaryotic AOX proteins, generated by the neighbour-joining method using the p-distance model. Bootstrap values are based on 1000 trials. Branch lengths are indicated by the scale at the bottom left.

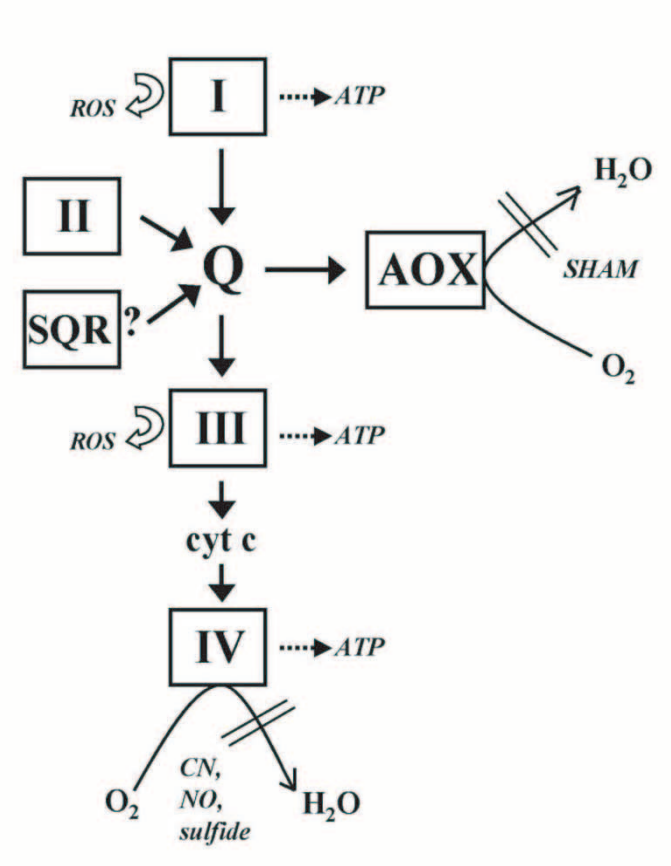

Figure 4. The branched mitochondrial electron transport chain hypothesized to exist in some animals. Note that electron transport chain complexes I, III and IV generate proton motive force used to produce ATP via ATP synthase (not shown) and that complexes I and III are major sites of ROS generation. Electron transport from ubiquinone (Q) to O_2 via AOX is not coupled to proton translocation. Potential naturally occurring inhibitors of cyt oxidase (Complex IV) are shown along with a commonly used artificial inhibitor of AOX. Also shown is the potential sulfide-quinone oxidoreductase (SQR) pathway responsible for sulfide oxidation. See text for details.

What is the Functional Role of an Animal AOX?

Given the animal species for which we have uncovered AOX genes, as well as the species for which previous work implicates its presence, several hypotheses can be put forth about its functional role in animal respiration. The first hypothesis describes a general role that may be applicable to many AOX-expressing animals while the remaining hypotheses may be more specific to particular groups.

1. Adaptation to Stress. In plants, AOX has become closely associated with stress conditions induced by both biotic and abiotic factors (*45*). In general, such conditions tend to disrupt metabolic homeostasis and the importance of AOX may be to promote homeostasis by providing additional flexibility in metabolism. This flexibility could potentially allow the mitochondrion to (a) modulate the rate of ATP production; (b) maintain electron flux to oxygen when other downstream electron transport chain components or ADP are limiting; or (c) modulate the reduction state of respiratory components, thereby controlling the rate of generation of ROS (Fig. 4). Such flexibility may be important for preventing stress-induced cell death, since transgenic plant cells lacking AOX show increased susceptibility to several different programmed cell death-inducing compounds (*46*). It is perhaps revealing that marine intertidal species (including oyster and sea squirt) are likely to experience large fluctuations in parameters such as oxygen concentration, temperature and nutrient availability (*23, 47, 48*).

2. Sulfide-resistant respiration. Sulfide is a potent inhibitor of cyt oxidase (*49*). Marine intertidal environments can experience high concentrations of sulfide and the invertebrates in these zones display elevated sulfide resistance resulting from a number of physiological and metabolic adaptations (*23*). Of particular interest here is the mitochondrial oxidation of sulfide. Mitochondria from marine invertebrates readily oxidize sulfide to thiosulfate and pass the electrons to oxygen via the respiratory chain (*23*). The enzyme responsible for this

activity is likely sulfide:quinone oxidoreductase (Fig. 4). Genes encoding this protein have been uncovered in a wide range of eukaryotes including animals (*50*), in keeping with the wide range of eukaryotic mitochondria that have now been shown capable of sulfide oxidation (*23*). A sulfide-quinone oxidoreductase gene is indeed present in the *C. intestinalis* genome (TC38167).

In marine invertebrates experiencing moderate tissue sulfide levels, electrons from sulfide oxidation could be passed to oxygen via the usual cyt pathway and coupled to ATP production. However, at higher sulfide concentrations (when cyt oxidase is inhibited by sulfide), it has been hypothesized that electrons are passed to oxygen by another terminal oxidase. This would allow aerobic metabolism to continue at high sulfide concentrations and allow for the continued detoxification of sulfide, thus promoting survival of the animal (*23*). The identity of this oxidase is unknown but is reminiscent of AOX, being reported as CN-resistant, SHAM-sensitive and less tightly coupled to ATP production (*23*, Fig. 4).

3. Pathogenesis. Parasites and other pathogens have developed numerous strategies allowing survival within their host. Interestingly, many parasitic protists and pathogenic fungi have AOX respiration (*5, 7, 51–53*). In the case of *T. brucei brucei* (the cause of sleeping sickness in humans) AOX is the dominant respiratory pathway in the long slender form of the parasite that resides in the host's bloodstream (*51*). The fungus *Cryptococcus neoformans* is a major human pathogen, the AOX gene of which is induced by mammalian body temperatures and contributes to the virulence of this organism (*53*). Many plant pathogenic fungi also maintain AOX respiration (*52*). Here, we have reported an AOX gene in the nematode *M. hapla*. Many nematodes are also parasites of animals or plants (*54*). Further, inhibitor studies on isolated mitochondria from several parasitic nematodes have suggested the existence of an alternative respiratory pathway branching from the cyt pathway upstream of Complex III, which would be consistent with the presence of AOX (*55–57*).

Both plants and animals generate NO as part of their defense response against pathogens (*58*). Further, the defense response of many plants involves the release of CN (*21*). NO and CN are each potent cyt oxidase inhibitors (Fig. 4) and both are implicated in defense against nematodes (*59–61*). Since AOX is resistant to both CN and NO (*22*), its presence in some nematodes could maintain respiration if cyt oxidase were disabled by host defenses, thus contributing to the virulence of these organisms.

4. Oxyconformity. Two strategies characterize the response of animal respiration to changes in environmental oxygen concentration. Oxyregulating species maintain a constant rate of oxygen consumption over a wide range of ambient oxygen concentrations while oxyconformers increase their oxygen consumption rate as ambient oxygen concentrations increase. Marine intertidal zones have highly fluctuating ambient oxygen concentrations and several marine invertebrates in these environments display strict oxyconformity at the tissue and mitochondrial level. Examples include the worms *S. nudus* and *N. pelagica*, as well as the bivalve *A. islandica* (*26, 27*). Further, it has been hypothesized that these species utilize a second terminal oxidase to increase oxygen consumption at high ambient oxygen concentrations. This oxidase activity appears to be SHAM-sensitive, to be non-energy conserving and to display a lower affinity for oxygen than cyt oxidase. The lower affinity could ensure the oxidase becomes more active as oxygen levels rise and the non-energy-conserving nature of the path would allow respiration to increase without an increase in ATP turnover. Interestingly, K_m oxygen values for plant AOX are reported to be 10 to 100 fold higher than that of cyt oxidase (*62, 63*). Hence, characteristics of the oxidase responsible for oxyconformity in marine invertebrates are consistent with the presence of AOX (Fig. 4).

Other Implications of an Animal AOX

There is abundant interest in the biology of the animal species for which we have uncovered AOX genes. Pacific oyster represents 97% of worldwide oyster production for human consumption (*37*). The root-knot nematode *M. hapla* is a major crop pest responsible for massive agricultural losses worldwide (*64*). *Ciona* is a model animal for studies in evolution and development since the larval stage of the life cycle exhibits a notochord and a nerve cord (*65*). It may thus possess a good estimate of the complement of genes present in the animal lineage prior to the separation of urochordates from other chordates (*66*). Despite this interest, we were unable to find any physiological or biochemical studies that address the complement of respiratory terminal oxidases for these species. Conversely, many other marine intertidal species and nematodes have been extensively studied at the physiological and biochemical level and display characteristics reminiscent of AOX (see above). In these cases, however, no genome sequence or other molecular data is available. Filling these gaps of knowledge will undoubtedly contribute to our understanding of respiration in these animals.

The discovery of AOX in a nematode has potential implications for human and veterinary medicine. As discussed earlier, several parasitic nematodes have already been hypothesized to contain an AOX-like respiratory oxidase that, if indeed present, would represent a novel target in efforts to develop new drug therapies. Such efforts are already underway for the AOX-containing parasitic protists *T. brucei brucei* and *C. parvum* (*42, 67*).

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REFERENCES

1. Berry, S. (2003) Endosymbiosis and the design of eukaryotic electron transport. *Biochim. Biophys. Acta* **1606**, 57–72.
2. Vanlerberghe, G. C., and McIntosh, L. (1997) Alternative oxidase: from gene to function. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **48**, 703–734.
3. Millenaar, F. F., and Lambers, H. (2003) The alternative oxidase: *in vivo* regulation and function. *Plant Biol.* **5**, 2–15.
4. Veiga, A., Arrabaca, J. D., and Loureiro-Dias, M. C. (2003) Cyanide-resistant respiration, a very frequent metabolic pathway in yeasts. *FEMS Yeast Res.* **3**, 239–245.
5. van Hellemond, J. J., Simons, B., Millenaar, F. F., and Tielens, A. G. M. (1998) A gene encoding the plant-like alternative oxidase is present in *Phytomonas* but absent in *Leishmania* spp. *J. Euk. Microbiol.* **45**, 426–430.
6. Ajayi, W. U., Chaudhuri, M., and Hill, G. C. (2002) Site-directed mutagenesis reveals the essentiality of the conserved residues in the putative diiron active site of the trypanosome alternative oxidase. *J. Biol. Chem.* **277**, 8187–8193.
7. Suzuki, T., Hashimoto, T., Yabu, Y., Kido, Y., Sakamoto, K., Nihei, C., Hato, M., Suzuki, S., Amano, Y., Nagai, K., *et al.* (2004) Direct evidence for cyanide-insensitive quinol oxidase (alternative oxidase) in apicomplexan parasite *Cryptosporidium parvum*: phylogenetic and therapeutic implications. *Biochem. Biophys. Res. Commun.* **313**, 1044–1052.
8. Stenmark, P., and Nordlund, P. (2003) A prokaryotic alternative oxidase present in the bacterium *Novosphingobium aromaticivorans*. *FEBS Lett.* **552**, 189–192.
9. Henry, M.-F., and Nyns, E.-J. (1975) Cyanide-insenstive respiration. An alternative mitochondrial pathway. *Sub-Cell. Biochem.* **4**, 1–65.
10. Considine, M. J., Holtzapffel, R. C., Day, D. A., Whelan, J., and Millar, A. H. (2002) Molecular distinction between alternative oxidase from monocots and dicots. *Plant Physiol.* **129**, 949–953.
11. Joseph-Horne, T., Hollomon, D. W., and Wood, P. M. (2001) Fungal respiration: a fusion of standard and alternative components. *Biochim. Biophys. Acta* **1504**, 179–195.
12. Baurain, D., Dinant, M., Coosemans, N., and Matagne, R. F. (2003) Regulation of the alternative oxidase *Aox1* gene in *Chlamydomonas reinhardtii*. Role of the nitrogen source on the expression of a reporter gene under the control of the *Aox1* promoter. *Plant Physiol.* **131**, 1418–1430.
13. Vanlerberghe, G. C., and Ordog, S. H. (2002) Alternative oxidase: Integrating carbon metabolism and electron transport in plant respiration. In *Photosynthetic Nitrogen Assimilation and Associated Carbon and Respiratory Metabolism*. (Foyer, C. H. and Noctor, G., eds). Kluwer Academic Publishers, The Netherlands.
14. Purvis, A. C., and Shewfelt, R. L. (1993) Does the alternative pathway ameliorate chilling injury in sensitive plant tissues? *Physiol. Plant.* **88**, 712–718.
15. Finkel, T., and Holbrook, N. J. (2000) Oxidants, oxidative stress and the biology of aging. *Nature* **408**, 239–247.
16. Dröge, W. (2002) Free radicals in the physiological control of cell function. *Physiol. Rev.* **82**, 47–95.
17. Maxwell, D. P., Wang, Y., and McIntosh, L. (1999) The alternative oxidase lowers mitochondrial reactive oxygen production in plant cells. *Proc. Natl. Acad. Sci. USA* **96**, 8271–8276.
18. Parsons, H. L., Yip, J. Y. H., and Vanlerberghe, G. C. (1999) Increased respiratory restriction during phosphate-limited growth in transgenic tobacco cells lacking alternative oxidase. *Plant Physiol.* **121**, 1309–1320.
19. Dufour, E., Boulay, J., Rincheval, V., and Sainsard-Chanet, A. (2000) A causal link between respiration and senescence in *Podospora anserina*. *Proc. Natl. Acad. Sci. USA* **97**, 4138–4143.
20. Funes, S., Nargang, F. E., Neupert, W., and Herrmann, J. M. (2004) The Oxa2 protein of *Neurospora crassa* plays a critical role in the biogenesis of cytochrome oxidase and defines a ubiquitous subbranch of the Oxa1/YidC/Alb3 protein family. *Mol. Biol. Cell* **15**, 1853–1861.
21. Poulton, J. E. (1990) Cyanogenesis in plants. *Plant Physiol.* **94**, 401–405.
22. Millar, A. H., and Day, D. A. (1996) Nitric oxide inhibits the cytochrome oxidase but not the alternative oxidase of plant mitochondria. *FEBS Lett.* **398**, 155–158.
23. Greishaber, M. K., and Völkel, S. (1998) Animal adaptations for tolerance and exploitation of poisonous sulfide. *Annu. Rev. Physiol.* **60**, 33–53.
24. Cooper, C. E. (2003) Competitive, reversible, physiological? Inhibition of mitochondrial cytochrome oxidase by nitric oxide. *IUBMB Life* **55**, 591–597.
25. Völkel, S., and Grieshaber, M. K. (1997) Sulphide oxidation and oxidative phosphorylation in the mitochondria of the lugworm *Arenicola marina*. *J. Exp. Biol.* **200**, 83–92.
26. Buchner, T., Abele, D., and Pörtner, H. O. (2001) Oxyconformity in the intertidal worm *Sipunculus nudus*: the mitochondrial background and energetic consequences. *Comp. Biochem. Physiol. B* **129**, 109–120.
27. Tschischka, K., Abele, D., and Pörtner, H. O. (2000) Mitochondrial oxyconformity and cold adaptation in the polychaete *Nereis pelagica* and the bivalve *Arctica islandica* from the Baltic and white seas. *J. Exp. Biol.* **203**, 3355–3368.
28. Hahlbeck, E., Arndt, C., and Schiedek, D. (2000) Sulphide detoxification in *Hediste diversicolor* and *Marenzelleria viridis*, two dominant polychaete worms within the shallow coastal waters of the southern Baltic Sea. *Comp. Biochem. Physiol. B.* **125**, 457–471.
29. Parrino, V., Kraus, D. W., and Doeller, J. E. (2000) ATP production from the oxidation of sulfide in gill mitochondria of the ribbed mussel *Geukensia demissa*. *J. Exp. Biol.* **203**, 2209–2218.
30. Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucl. Acids Res.* **24**, 4876–4882.
31. Berthold, D. A., and Stenmark, P. (2003) Membrane-bound diiron carboxylate proteins. *Annu. Rev. Plant Biol.* **54**, 497–517.
32. McDonald, A. E., Amirsadeghi, S., and Vanlerberghe, G. C. (2003) Prokaryotic orthologues of mitochondrial alternative oxidase and plastid terminal oxidase. *Plant Mol. Biol.* **53**, 865–876.
33. Kumar, S., Tamura, K., Jakobsen, I. B., and Nei, M. (2001) MEGA2: Molecular Evolutionary Genetics Analysis Software, Arizona State University, USA.
34. Satou, Y., Kawashima, T., Kohara, Y., and Satoh, N. (2003) Large scale EST analyses in *Ciona intestinalis*. Its application as Northern blot analyses. *Dev. Genes Evol.* **213**, 314–318.
35. McCarter, J., and 34 others (1999) The Washington Univ. Nematode EST Project.
36. Dehal, P., and 86 others (2002) The draft genome of *Ciona intestinalis*: insights into chordate and vertebrate origins. *Science* **298**, 2157–2167.

37. Gueguen, Y., Cadoret, J. P., Flament, D., Barreau-Roumiguiere, C., Girardot, A. L., Garnier, J., Hoareau, A., Bachere, E., and Escoubas, J. M. (2003) Immune gene discovery by expressed sequence tags generated from hemocytes of the bacteria-challenged oyster, *Crassostrea gigas*. *Gene* **16**, 139–145.
38. Elandalloussi, L. M., Leite, R. M., Afonso, R., Nunes, P. A., Robledo, J. A., Vasta, G. R., and Cancela, M. L. (2004) Development of a PCR-ELISA assay for diagnosis of *Perkinsus marinus* and *Perkinsus atlanticus* infections in bivalve mollusks. *Mol. Cell. Probes* **18**, 89–96.
39. Lang, B. F., O'Kelly, C., Nerad, T., Gray, M. W., and Burger, G. (2002) The closest unicellular relatives of animals. *Curr. Biol.* **12**, 1773–1778.
40. Hanten, J. J., and Pierce, S. K. (2001) Synthesis of several light-harvesting complex I polypeptides is blocked by cycloheximide in symbiotic chloroplasts in the sea slug, *Elysia chlorotica* (Gould): a case for horizontal gene transfer between alga and animal? *Biol. Bull.* **201**, 34–44.
41. Nakashima, K., Yamada, L., Satou, Y., Azuma, J., and Satoh, N. (2004) The evolutionary origin of animal cellulose synthase. *Dev. Genes Evol.* **214**, 81–88.
42. Roberts, C. W., Roberts, F., Henriquez, F. L., Akiyoshi, D., Samuel, B. U., Richards, T. A., Milhous, W., Kyle, D., McIntosh, L., and Hill, G. C. (2004) Evidence for mitochondrial-derived alternative oxidase in the apicomplexan parasite *Cryptosporidium parvum*: a potential anti-microbial agent target. *Int. J. Parasitol.* **34**, 297–308.
43. Reyes-Prieto, A., El-Hafidi, M., Moreno-Sanchez, R., and Gonzalez-Halphen, D. (2002) Characterization of oxidative phosphorylation in the colorless chlorophyte *Polytomella* sp. Its mitochondrial respiratory chain lacks a plant-like alternative oxidase. *Biochim. Biophys. Acta* **1554**, 170–179.
44. Heisler, C. R., Hodnick, W. F., and Ahmad, S. (1988) Evidence for target site insensitivity to cyanide in *Spodoptera eridania* larvae. *Comp. Biochem. Physiol.* **91C**, 469–472.
45. Simons, B. H., and Lambers, H. (1999) The alternative oxidase: is it a respiratory pathway allowing a plant to cope with stress? In *Plant Responses to Environmental Stresses: From Phytohormones to Genome Reorganization*. (Lerner, H. R., ed), pp. 265–286, Marcel Dekker Inc., New York, N.Y.
46. Robson, C. A., and Vanlerberghe, G. C. (2002) Transgenic plant cells lacking mitochondrial alternative oxidase have increased susceptibility to mitochondria-dependent and -independent pathways of programmed cell death. *Plant Physiol.* **129**, 1908–1920.
47. Wohlgemuth, S. E., Taylor, A. C., and Grieshaber, M. K. (2000) Ventilatory and metabolic responses to hypoxia and sulphide in the lugworm *Arenicola marina* (L.). *J. Exp. Biol.* **203**, 3177–3188.
48. Abele, D., Heise, K., Pörtner, H. O., and Puntarulo, S. (2002) Temperature-dependence of mitochondrial function and production of reactive oxygen species in the intertidal mud clam *Mya arenaria*. *J. Exp. Biol.* **205**, 1831–1841.
49. Nicholls, P. (1975) The effect of sulfide on cytochrome aa_3. Isosteric and allosteric shifts of the reduced α-peak. *Biochim. Biophys. Acta* **396**, 24–35.
50. Theissen, U., Hoffmeister, M., Grieshaber, M., and Martin, W. (2003) Single eubacterial origin of eukaryotic sulfide:quinone oxidoreductase, a mitochondrial enzyme conserved from the early evolution of eukaryotes during anoxic and sulfidic times. *Mol. Biol. Evol.* **20**, 1564–1574.
51. Tielens, A. G. M., and van Hellemond, J. J. (1998) Differences in energy metabolism between trypanosomatidae. *Parasitol. Today* **14**, 265–271.
52. Joseph-Horne, T., and Hollomon, D. W. (2000) Functional diversity within the mitochondrial electron transport chain of plant pathogenic fungi. *Pest Manag. Sci.* **56**, 24–30.
53. Akhter, S., Mcdade, H. C., Gorlach, J. M., Heinrich, G., Cox, G. M., and Perfect, J. R. (2003) Role of alternative oxidase gene in pathogenesis of *Cryptococcus neoformans*. *Infect. Immun.* **71**, 5794–5802.
54. Jasmer, D. P., Goverse, A., and Smant, G. (2003) Parasitic nematode interactions with mammals and plants. *Annu. Rev. Phytopathol.* **41**, 245–270.
55. Mendis, A. H. W., and Townson, S. (1985) Evidence for the occurrence of respiratory electron transport in adult *Brugia pahangi* and *Dipetalonema viteae*. *Mol. Biochem. Parasitol.* **14**, 337–354.
56. Armson, A., Grubb, W. B., and Mendis, A. H. W. (1995) *Strongyloides ratti*: mitochondrial enzyme activities of the classical electron transport pathway in the infective (L3) larvae. *Int. J. Parasitol.* **25**, 257–260.
57. Kita, K., Hirawake, H., and Takamiya, S. (1997) Cytochromes in the respiratory chain of Helminth mitochondria. *Int. J. Parasitol.* **27**, 617–630.
58. Wendehenne, D., Pugin, A., Klessig, D. F., and Durner, J. (2001) Nitric oxide: comparative synthesis and signaling in animal and plant cells. *Trend. Plant Sci.* **6**, 177–183.
59. Selkirk, M. E., Smith, V. P., Thomas, G. R., and Gounaris, K. (1998) Resistance of filarial nematode parasites to oxidative stress. *Int. J. Parasitol.* **28**, 1315–1332.
60. Brunet, L. R. (2001) Nitric oxide in parasitic infections. *Int. Immunopharmacol.* **1**, 1457–1467.
61. Widmer, T. L., and Abawi, G. S. (2002) Relationship between levels of cyanide in sudangrass hybrids incorporated into soil and suppression of *Meloidogyne hapla*. *J. Nematol.* **34**, 16–22.
62. Millar, A. H., Bergensen, F. J., and Day, D. A. (1994) Oxygen affinity of terminal oxidases in soybean mitochondria. *Plant Physiol. Biochem.* **32**, 847–852.
63. Ribas-Carbo, M., Berry, J. A., Azcon-Bieto, J., and Siedow, J. N. (1994) The reaction of the plant mitochondrial cyanide-resistant alternative oxidase with oxygen. *Biochim. Biophys. Acta* **1188**, 205–212.
64. Trudgill, D. L., and Blok, V. C. (2001) Apomictic, polyphagous root-knot nematodes: exceptionally successful and damaging biotrophic root pathogens. *Annu. Rev. Phytopathol.* **39**, 53–77.
65. Corbo, J. C., Di Gregorio, A., and Levine, M. (2001) The ascidian as a model organism in development and evolutionary biology. *Cell* **106**, 535–538.
66. Leveugle, M., Prat, K., Popovici, C., Birnbaum, D., and Coulier, F. (2004) Phylogenetic analysis of *Ciona intestinalis* gene superfamilies supports the hypothesis of successive gene expansions. *J. Mol. Evol.* **58**, 168–181.
67. Nihei, C., Fukai, Y., and Kita, K. (2002) Trypanosome alternative oxidase as a target of chemotherapy. *Biochim. Biophys. Acta* **1587**, 234–239.