Respiratory Chains From Aerobic Thermophilic Prokaryotes

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Thermophiles are organisms that grow optimally above 50°C and up to ~120°C. These extreme conditions must have led to specific characteristics of the cellular components. In this paper we extensively analyze the types of respiratory complexes from thermophilic aerobic prokaryotes. The different membrane-bound complexes so far characterized are described, and the genomic data available for thermophilic archaea and bacteria are analyzed. It is observed that no specific characteristics can be associated to thermophilicity as the different types of complexes I–IV are present randomly in thermophilic aerobic organisms, as well as in mesophiles. Rather, the extensive genomic analyses indicate that the differences concerning the several complexes are related to the organism phylogeny, i.e., to evolution and lateral gene transfer events.

KEY WORDS: Complex I; succinate dehydrogenase; cytochrome *c* oxidase; oxygen reductase.

INTRODUCTION

The growth of an organism in a certain environment depends on a combination of chemical, biological, and physical factors, such as temperature, pH, and hydrostatic and osmotic pressures. Each organism grows only in certain ranges of each of those factors. Organisms that live at any of the "limits" of those factors are called extremophiles. Extremophiles not only tolerate the extreme conditions where they live, but require them for their survival. Very often, more than one type of extremophily is associated, such as very acidic pH and high temperatures (thermoacidophiles), or high salinity and high temperature (thermohalophiles). Organisms that have a maximal growth temperature above 50°C are called thermophiles (Edwards, 1990; Stetter, 1998). The presence of thermophilic organisms can be observed in both prokaryotic domains of life, i.e., in *archaea* and *bacteria*.

Proteins of thermophiles have to balance stability and flexibility in order to perform their functions. These proteins are considered to be more rigid than the corresponding mesophilic ones, what is true for mesophilic temperatures, but they have a similar degree of flexibility at the optimum growth temperatures of the respective organisms. Also, the specific activity of a thermoenzyme is in general comparable with that of the homologous mesophilic enzyme at each optimal temperature (Danson *et al.*, 1996; Jaenicke, 1991). Extrinsic factors, such as compatible solutes or glycosylation may contribute to protein stabilization. These factors may not be strictly required, since upon isolation and purification, many proteins from thermophiles retain their structure, function, and thermal stability. Furthermore, it is observed that recombinant thermophilic proteins expressed in mesophiles also retain their thermal stability (Vieille and Zeikus, 1996).

The stability of thermophilic proteins is achieved combining several mechanisms, involving electrostatic interactions, such as ion pairs, hydrogen bonds, and van der Waals forces, as well as hydration effects of nonpolar groups. Thus, thermostability of proteins can be correlated to an increase in the number of hydrogen bonds and ion pairs, and also an increase in the fractional polar surface, resulting in the addition of hydrogen bonds to water (Jaenicke, 1991; Vogt *et al.*, 1997). A decrease in the number of loops and turns is also observed, as well as stabilization of α helices. In summary, thermophilicity is an additive effect of multiple and subtle modifications.

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In this paper we describe the different enzymatic complexes involved in membrane-bound electron transfer chains of thermophilic and aerobic prokaryotes that have been isolated and characterized to date. Since the available biochemical data on these complexes is still very scarce, we carried out an extensive survey of respiratory complexes, predicted by the available complete genomes. This analysis aimed at answering a very simple question: Is there a specific characteristic associated with thermophilicity? The available genomes were analyzed, to detect the presence of respiratory chain components, being this data cross-linked to biochemical studies. When possible, amino acid sequence comparisons were performed to determine the similarities among proteins and to detect conserved motives. Comparisons with equivalent complexes from mesophiles were also performed to check for the exclusiveness of a specific characteristic in thermophilic organisms. Furthermore, the assignment on the genomic databases was confirmed by comparing again the target sequences against the databases.

A very important feature in this type of analyzes is the sampling considered. If the organisms chosen are from closely related phylogenetic groups, the conclusions of the analyzes are strongly biased, i.e., an observation that could be attributed to a thermophilic characteristic may in fact be the reflex of the phylogenetic relationship. Thus, a good sampling has to ensure representativity, not only in terms of living temperatures, but also considering the phylogenetic characteristics. In this study we considered all organisms that, to our knowledge, were described as being aerobic thermophiles, from which the genomes have been sequenced and/or biochemical characterization of the electron transfer chain has been performed. The organisms considered are (i) from the *archaea* domain: *Sulfolobus (S.) acidocaldarius, S. solfataricus, S. tokodaii, S. metallicus, Acidianus (A.) ambivalens, Pyrobaculum (P.) aerophilum, Aeropyrum (Ae.) pernix, Thermoplasma (Th.) acidophilum*, and *Th. volcanium*; and (ii) from the *bacteria* domain: *Aquifex (Aq.) aeolicus, Thermus (T.) thermophilus, Rhodothermus (R.) marinus, Geobacillus (G.) stearothermophilus, and Thermosynechococcus (Ts.) elongatus* (Table I).

Electron transfer chains couple electron transfer to proton translocation through the plasma membrane or the inner mitochondrial membrane, in prokaryotes or eukaryotes, respectively. A schematic representation of electron transfer chains in general is depicted in Fig. 1. These chains may contain several enzymes that accept electrons from the so-called electron donors or reducing equivalents (like NAD(P)H, succinate, $F_{420}H2$, glycerol-3-phosphate), and reduce quinones. At the end of the aerobic chains an oxygen reductase must be present. Other intermediate complexes, such as a quinol:electron carrier oxidoreductase may also be present. The best characterized electron transfer chains, those from mitochondria and related bacteria, are mainly composed of four complexes (named I–IV): NADH:quinone oxidoreductase, succinate:quinone oxidoreductase, quinol:cytochrome *c*

Table I. Distribution of Respiratory Complexes Among Aerobic Thermophilic Prokaryotes

Domain	Genus	NADH:quinone oxidoreductase	Succinate: quinone oxidoreductase	Quniol:cytochrome *c* oxidoreductase	Oxygen reductase	Electron carrier Quinone/metalloprotein	
	Sulfolobus	[a] NDH-2a NDH-2c	E	Dihaemic cyt *a*,[b] Rieske[b]	A1, B	Caldariella quinone *Sulfolobus* quinone tricyclic quinone	Sulfocyanine
Archaea	*Acidianus*	[c] NDH-2c	E	[c]	B	Caldariella quinone	[c]
	Pyrobaculum	[a] [c]	C	Dihaemic cyt,[b] Rieske[b]	Al,B	[a]	Cytochrome *c*
	Aeropyrum	[a] [c]	A	Dihaemic cyt[b] Rieske[b]	A1,B	[c]	Cytochrome *c*
	Thermoplasma	[a] NDH-2c	A	Dihaemic cyt,[b] Rieske[b]	bd	Themoplasma quinone	Sulfocyanine
Bacteria	*Aquifex*	NDH-1 [c]	E	bc_1	A2, B	[c]	Cytochrome *c*
	Thermus	NDH-1 [c]	c	Rieske	A2, B	Menaquinone 8	Cytochrome *c*
	Rhodothermus	NDH-1 [c]	B	bc	A2, C	Menaquinone 7	HiPIP Cytochrome *c*
	Geobacillus	[a] NDH-2a	[c]	b_6c_1	A1, B	[c]	Cytochrome *c*
	Thermosynechoccocus	[a] NDH-2a	E	b_6f	A2	[c]	[c]

[a] Unknown electron donor and/or electron donor interacting subunits, but homologues of Nqo4–Nqo14 encoding subunits are present in the genomes.
[b] Dihaemic cytochromes are part of the SoxABCD and SoxM complexes. The Rieske proteins mentioned are constituents of the SoxM complex.
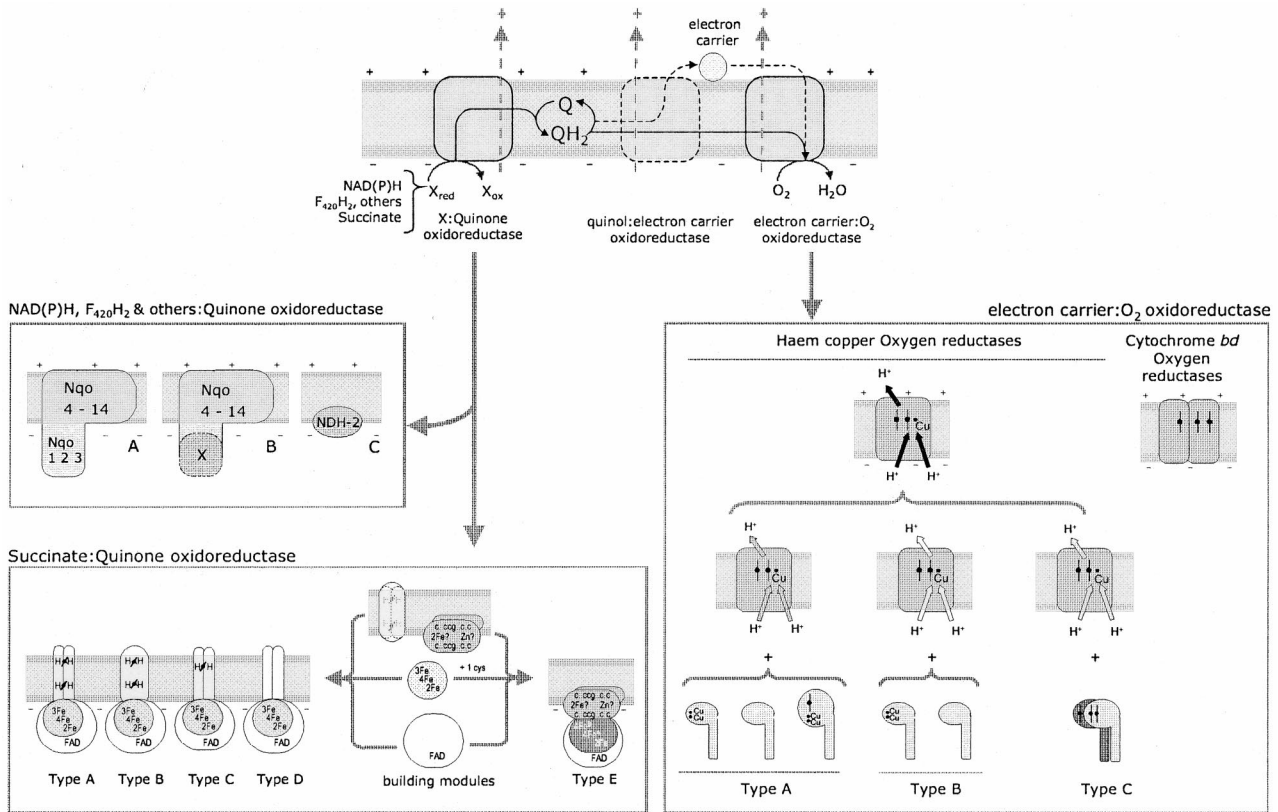[c] Unknown.

Fig. 1. General schematic representation of aerobic electron transfer chains. These chains receive electrons from different donors, such as NAD(P)H, succinate, $F_{420}H_2$, electron transfer protein, glycerol 3-phosphate, and others. The oxidation of these substrates is performed by oxidoreductases, which reduce quinones. The quinols, thus formed, may be directly oxidized by the oxygen reductases or trough intermediate complexes, which reduce metalloproteins (electron carriers, such as cytochrome c, HiPIP). NAD(P)H, $F_{420}H_2$, and others: quinone oxidoreductase. (A) Prokaryotic complex I, containing the subunits responsible for the oxidation of NADH (Nqo_1, Nqo_2, and Nqo_3). (B) Complex I-like enzymes, lacking Nqo_1, Nqo_2, and Nqo_3, thus with different electron donors (e.g., $F_{420}H_2$). (C) Type II NADH dehydrogenases. Succinate:quinone oxidoreductase. Classification of SQR according to the anchor (types A–D have a transmembrane anchor and different number of haems and type E has a monotopic anchor) and on the nature of the FeS clusters. 2Fe, $[2Fe–2S]^{2+/1+}$ cluster; 3Fe, $[3Fe–4S]^{1+/0}$ cluster; 4Fe, $[4Fe–4S]^{2+/1+}$ cluster; 4FeA and 4FeB, "canonical" and additional $[4Fe–4S]^{2+/1+}$ clusters, respectively. Haem B is represented by $[\phi]$. The "building modules" of the five types of enzymes are also represented: the flavoprotein is strictly conserved; the iron–sulfur subunit may contain an extra cysteyl that coordinates the $4Fe^B$, and the anchor subunits are not conserved. Electron carrier:O_2 oxidoreductase. The three families of haem–copper oxygen reductases, types A, B, and C, were established on the basis of their proton pathways, which are here schematically represented by arrows. A-type (*Pa. denitrificans*, unless otherwise indicated): D channel—GluI-278 (A1-type)/TyrI256 (*R. marinus*, A2-type), AspI-124, AsnI-199, AsnI-113, AsnI-131, TyrI-35, SerI-134, SerI-193; K channel—LysI-354, ThrI-351, SerI-291, and TyrI-280. B-type (*T. thermophilus*, ba_3): K channel (alternative)—ThrI-312, SerI-309, TyrI-244, and TyrI-237. C-type (*Bradyrhizobium japonicum*, cbb_3): K channel (alternative)—SerI-355, TyrI-295.

oxidoreductase, and cytochrome c: oxygen oxidoreductase. We restricted our analyzes to these four types of enzymes; after a brief introduction to each protein, the data for the thermophiles will be discussed. A summary of the obtained results is presented in Table I.

NADH:QUINONE OXIDOREDUCTASE

Three distinct types of membrane-bound enzymes are able to oxidize NADH, transferring the electrons to quinones: Type I—rotenone-sensitive NADH dehydrogenase, or complex I (NDH-1); Type II—the so-called alternative NADH dehydrogenase, or rotenone-insensitive NADH dehydrogenase (NDH-2); and Type III—the so-called Na^+-translocating NADH:quinone oxidoreductases.

Type I

Complex I is the largest complex of the respiratory chains and can be found in the three domains of life. It

is responsible for the transfer of electrons from NADH to quinones, through a number of prosthetic groups, coupling electron transfer to proton (in some cases sodium) translocation across the membrane (Hatefi, 1985). The electron microscopy data showed that the enzyme has an L-shaped structure, with two major domains: (i) a hydrophobic arm imbedded in the inner membrane and (ii) a peripheral arm protruding into the cytoplasm, containing the NADH oxidizing subunit, Nqo_1 (Nqo – NADH: $q$uinone $o$xidoreductase), with the FMN binding domain, and several subunits harboring iron–sulfur clusters (Ohnishi, 1998; Sazanov et al., 2003). A thin collar separates the two arms in Escherichia (E.) coli (Guenebaut et al., 1998) and in the bovine enzyme (Grigorieff, 1998). Recently, a horseshoe-shape was proposed for the active form of E. coli complex I, on the basis of cryomicroscopy studies, but this issue is still a matter of intense debate (Bottcher et al., 2002; Sazanov et al., 2003).

At least two distinct types of NDH-1-like complexes can be considered, regarding the electron donor and its interacting subunits. The NDH-1, whose electron donor is NADH, is widespread among bacteria and eukarya (Fig. 1(A)). The bacterial enzymes generally consist of 14 subunits, named Nqo_1–Nqo_{14}. The $F_{420}H_2$ dehydrogenases, which were described in the archaea Methanosarcina (M.) mazei (Baumer et al., 2000) and Archeoglobus fulgidus (Kunow et al., 1994), are composed of 11 out of the 14 subunits of NDH-1. This enzyme complex lacks the subunits responsible for the NADH dehydrogenase reaction (Nqo_1–Nqo_3), containing two other subunits, FpoO and FpoF, where the oxidation of their electron donor, $F_{420}H_2$, takes place. Other organisms contain genes encoding 11 subunits of complex I (Nqo_4–Nqo_{14}), but not those coding for Nqo_{1-3} or FpoF. This suggests that more subtypes will be found having different substrate-interacting subunits, and/or different electron donors, as more biochemical data become available (Fig. 1(B)).

Type II

Alternative NADH dehydrogenases (NDH-2) are able to oxidize NADH and/or NADPH and resistant to complex I specific inhibitors such as rotenone and piericidin A (Fig. 1(C)). NDH-2 usually contain noncovalently bound FAD (Yagi et al., 1993). The Trypanosoma brucei NDH-2 was the first example of an FMN containing NDH-2 (Fang and Beattie, 2002). Recent studies showed that some archaeal NDH-2 contain covalently bound FMN (see below) (Bandeiras et al., 2002, 2003). Consensus sequences forming an EF-hand secondary

structure motif for the binding of calcium have been reported in some type II NADH dehydrogenases primary structures, such as for Neurospora (N.) crassa external NDH-2 (Melo et al., 1999) and Solanum (S.) tuberosum NDB (Rasmusson et al., 1999).

On the basis of the above-mentioned biochemical observations and primary structure analyzes, we propose that the rotenone-insensitive NADH dehydrogenase/NDH-2 family can be divided into three distinct groups: (a) containing two dinucleotide-binding regions in a $\beta\alpha\beta$ fold (each displaying a conserved $G(X)GX_2G$ motif, which binds the ADP-moiety of the dinucleotide molecule) (Wierenga et al., 1986), and the noncovalently bound flavin; (b) containing two dinucleotide-binding motives plus an EF-hand motif to bind calcium and also noncovalently bound flavin; (c) with covalently bound flavin, containing one conserved dinucleotide-binding motif and a conserved histidine residue, the latter suggested to be involved in flavin covalent binding (Bandeiras et al., 2002).

The absence of the second dinucleotide-binding domain in group c strongly indicates that the first dinucleotide-binding motif is the substrate binding site for all NDH-2, corroborating Melo et al. hypothesis for NDE1 (Melo et al., 2001). Transmembrane helices are not commonly present among NDH-2 proteins, and the observation that hydrophobic and hydrophilic amino acids are located on opposite sides of some predicted α-helices suggests a membrane–protein interaction through the hydrophobic face of these amphipathic α-helices (Bandeiras et al., 2002). This feature is probably a common strategy in this family of enzymes.

Type III

The so-called Na^+-translocating NADH:quinone oxidoreductase catalyzes the oxidation of NADH, coupling Na^+ translocation across the membrane with electron transfer to quinones. The Vibrio (V.) cholerae (Barquera et al., 2002) and V. alginolyticus (Hayashi et al., 1994) enzymes are typical examples of such NADH:quinone oxidoreductases.

Thermophilic Enzymes

NDH-1

Significant similarities to complex I subunits were observed through all the genomes considered in this study. However, in some organisms the enzyme seems to have a different electron donor, as deduced by the lack of similarity to Nqo_1 (which contains the NADH and FMN binding domains), and in some cases, to Nqo_2 and Nqo_3.

A complete set of genes to assemble a complex I is observed in *T. thermophilus* HB-8 (Yano *et al.*, 1997) and *Aa. aeolicus* (Deckert *et al.*, 1998). In *T. thermophilus* there is a single operon containing all the 14 open reading frames encoding the 14 subunits of complex I. The enzyme comprises nine putative iron–sulfur binding motives, eight of which are generally found in bacterial complex I, and its mitochondrial counterpart (Nakamaru-Ogiso *et al.*, 2002). The 14 genes encoding *Aa. aeolicus* complex I are organized in several clusters disperse in the genome. The purified complex I presented NADH:decylubiquinone oxidoreductase activity, completely inhibited by rotenone (Peng *et al.*, 2003).

A complex I has also been isolated from the thermohalophilic bacterium *R. marinus*, as judged by rotenone-sensitive NADH oxidation activity. It was also reported that the electron transfer from NADH to menaquinone was coupled to the formation of a membrane potential (Fernandes *et al.*, 2002).

The thermophilic bacterium *G. stearothermophilus* genome is still under progress; however, already sequenced data are available (http://www.genome.ou.edu/bstearo.html). Amino acid sequences from all complex I subunits were searched in *G. stearothermophilus* database. Subunits Nqo_{12}, Nqo_4, and Nqo_6 displayed high scores, and Nqo_5, Nqo_7, Nqo_9, and Nqo_3 presented some similarity to *G. stearothermophilus* proteins. The homologues of Nqo_{12}, Nqo_4, Nqo_6, Nqo_9, and Nqo_5 are located in the same contig, what may suggest an operon organization. These observations allow us to speculate the presence of a complex I-like protein in this organism. However, since no similarity to Nqo_1 and Nqo_2 was found, the electron donor of the enzyme remains unknown. This may be also the case for the cyanobacterium *Ts. elongatus*, for which the genes encoding the flavoprotein domain subunits are not found in the genome (Nakamura *et al.*, 2002).

The genomes of thermophiles from the archaeal domain include open reading frames to encode most complex I subunits (Nqo_4–Nqo_{14}), organized in an operon as in *P. aerophilum* (Fitz-Gibbon *et al.*, 2002), *A pernix* (Kawarabayasi *et al.*, 1999), and in the genus *Thermoplasma* (Kawashima *et al.*, 2000; Ruepp *et al.*, 2000), or in more than one cluster as in the *Sulfolobus* species (Kawarabayasi *et al.*, 2001; She *et al.*, 2001). None of these organisms have homologues to the flavoprotein fraction of complex I, thus the electron donors for these enzymes are yet unknown. This unknown electron donor could be $F_{420}H_2$, since related proteins are reported in archaea (Baumer *et al.*, 1998).

The genomes of the organisms with an incomplete set of genes to encode a complex I-like enzyme were searched for similar sequences to the FpoF subunit (containing the motif to bind the $F_{420}H_2$) of the $F_{420}H_2$:methanophenazine oxidoreductase from *M. mazei* (Deppenmeier *et al.*, 2002). *Ts. elongatus* was the only organism whose genome presented a similar protein, though with a distinct localization from the complex I homologous subunits. However, all these organisms contain genes that may encode $F_{420}H_2$ related proteins in their genomes. On the basis of the above indications, the nature of the electron donors to these enzymes is not known. The hypothesis of being NADH or $F_{420}H_2$ cannot be excluded, but other electron donors are still possible, nevertheless different subunits should exist to bind the electron donors. Biochemical data are required to clarify this issue.

NDH-2

Rotenone-insensitive NADH dehydrogenases were described or found in the genomes of most organisms listed. No sequences encoding putative NDH-2 dehydrogenases were retrieved from protein sequence blast against *A. pernix*, *P. aerophilum*, and *Aa. aeolicus* genomes. Concerning the bacteria *R. marinus* and *T. thermophilus*, there are no genomic or biochemical data available.

The alternative NADH dehydrogenases found in the sampled thermophilic organisms belong to groups a and c, according to the conserved motives observed in their amino acid sequences. Group a comprises both archaeal and bacterial proteins, with two dinucleotide-binding motives, involved in noncovalently binding of NAD(P)H and flavins. Enzymes from this group can be found in *S. solfataricus*, *S. tokodaii*, *G. stearothermophilus*, and *Ts. elongatus.* Beyond the above-mentioned group a NDH-2, the *Sulfolobus* genus contains one enzyme belonging to group c. In this group, the absence of the second dinucleotide-binding region is consentaneous with the presence of a conserved histidine residue, and a covalently bound flavin suggested to be bound by the histidyl (Fig. 2). The covalent attachment between the protein backbone and the enzyme chromophore was suggested to increase the reduction potential of the flavin cofactor as observed for the *S. metallicus* and *A. ambivalens* enzymes (Bandeiras *et al.*, 2002, 2003). Genes encoding for this NDH-2 group are present in the *S. solfataricus*, *S. tokodaii*, *Th. acidophilum*, and *Th. volcanium* genomes (Fig. 2). The amino acid sequences of all archaeal NDH-2 and of representative homologues from bacteria and eukarya were aligned, and after manually adjustment, a dendrogram was constructed, using Clustal W (Fig. 3). The dendrogram fully supports the classification above proposed for these enzymes. Furthermore, the group c enzymes comprise exclusively thermophilic archaea.
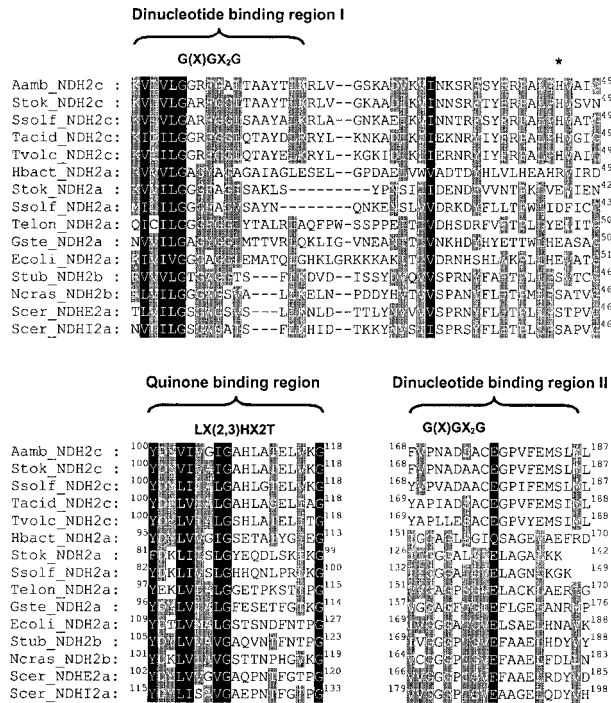
Fig. 2. Amino acid sequence alignment of type II NADH dehydrogenases. Comparison of amino acid sequences (NCBI accession number) from NDH-2 members of the three groups (a, b, c),: *A. ambivalens* (CAD33806); *S. tokodaii* (NP_378484); *S. solfataricus* (NP_343636); *Th. acidophilum* (NP_394588); *T. volcanium* (NP_111725); *Halobacterium* (NP_279851); *S. tokodaii* (NP_378575); *S. solfataricus* (NP_342489); *Ts. elongatus* (NP_681926); *G. stearothermophilus* (NP_391090); *Escherichia* (*E.*) *coli* (NP_415627); *Solanum* (*So.*) *tuberosum* (CAB52797); *N. crassa* (CAB41986); *Saccharomyces* (*Sa.*) *cerevisiae* (NP_013865, NP_013586). Conserved amino acid regions are highlighted. *Histidine residue proposed to be involved in flavin covalent binding. Multiple alignments were performed using Clustal W (1.6) (Thompson *et al.*, 1997) version and manually adjusted.

Envisaging the clarification of whether the presence of group c NDH-2 was due to the thermophily of these archaea, the genome of *Halobacterium sp.* (Ng *et al.*, 2000), the only aerobic mesophilic archaeon whose genome is available, was searched for type II NADH dehydrogenases, and the sequence obtained was included in our analysis. Since the latter aligned with group a, it can be speculated that the presence of group c may be a characteristic feature of the analyzed thermophilic archaeal respiratory chains.

Interaction between thermophilic archaeal NDH-2 and the quinone molecule is particularly emphasized by the presence of a quinone-binding motif of the $LX_{(2,3)}HX_2T$ type (Fisher and Rich, 2000) in the enzymes of group c. These residues form a common triad, containing a conserved histidyl hydrogen-bounded to one car-

bonyl of the quinone molecule. This presence is not so obvious in the other NDH-2 sequences (Fig. 2), although in the corresponding region we can observe similar residues able to bind quinones.

Secondary structure predictions of NDH-2 amino acid sequences were carried out. The results showed that among several putative α-helices, three of them are amphipathic and present at the same relative positions, suggesting a common membrane association within thermophilic NDH-2, which is extended to their mesophilic homologues.

Type III

Primary structures of the so-called Na^+-translocating NADH:quinone oxidoreductase were blasted against all the sampled organism genomes, but no similarities were found.

SUCCINATE:QUINONE OXIDOREDUCTASE

Complex II, Succinate:Quinone Oxidoreductase (SQR), catalyzes the oxidation of succinate to fumarate, donating electrons to quinones, and until now it was not shown to contribute to the establishment of the electrochemical membrane potential. The enzyme is composed by a cytoplasmatic and an anchor domain. The cytoplasmatic domain is built of two subunits, a flavoprotein (SdhA), harboring a covalently bound FAD, and an iron–sulfur protein (SdhB), containing one $[2Fe-2S]^{2+/1+}$ (S1), one $[4Fe-4S]^{2+/1+}$ (S2), and one $[3Fe-4S]^{1+/0}$ (S3) (or a second $[4Fe-4S]^{2+/1+}$) clusters. Depending on the anchor nature and on the FeS cluster composition, the enzymes can be divided into five types, A–E (Lancaster and Kroger, 2000; Lemos *et al.*, 2002) (Fig. 1). The anchor domain provides the binding site for the quinone and can be composed by transmembrane (types A–D) or by "putative" monotopic polypeptides (type E).

Thermophilic SQRs

A pernix, *Th. acidophilum*, and *Th. volcanium* contain genes that probably encode for type A enzymes; in the case of *Th. acidophilum*, whose enzyme was purified and characterized, it is already established the presence of two B-type haems. The available data for *P. aerophilum* suggests that it expresses a type C enzyme. *S. solfataricus*, *S. acidocaldarius*, *S. tokodaii*, and *A. ambivalens* contain genes encoding for type E enzymes.
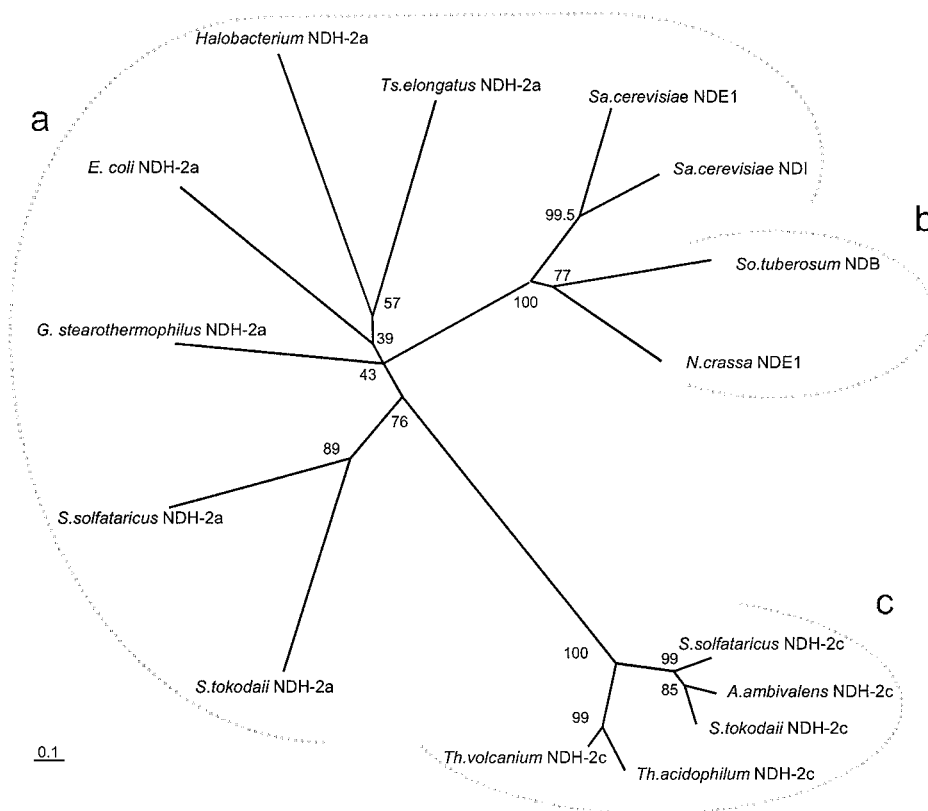
Fig. 3. Dendrogram for type II NADH dehydrogenases, based on the amino acid sequence alignment of Fig. 2. The dendrogram was performed using Clustal W Version 1.6 (Thompson *et al.*, 1997) excluding positions with gaps and correcting for multiple substitutions. Bootstrap values for the main nodes are indicated. Groups a, b, and c corroborate the classification proposed in the text.

Until now, these enzymes were only purified from thermophilic archaea, but genes encoding them are present in the genomes of *Campylobacter jejuni* and *Synechocystis sp* (both mesophilic bacteria).

Aa. aeolicus and *Ts. elongatus* are the only thermophilic bacteria that have available genomic data on SQRs, but none of these enzymes have been isolated yet. In both organisms, genes encoding for the different subunits are nonadjacent in the genomes. *Aa. aeolicus* possesses two different genes for subunit SdhB, with the extra cysteine residue attributed to be a ligand of the second $[4Fe–4S]^{2+/1+}$ present in type E enzymes. A protein with homology with both heterodissulfide reductase (Hdr) subunit B and SdhE is found in these genomes, being nonadjacent with any other Hdr subunit (see below). Thus, most probably these bacteria express type E enzymes.

Very few SQRs from thermophilic organisms have been isolated so far. As expected, the thermophilic enzymes have very high optimum activity temperatures, ranging from 75 to 81°C, which are obviously related to their optimum growth temperatures. On the other

hand, the kinetic parameters have similar values to other mesophilic bacterial, eukaryotic, and archaeal SQRs. Like some mesophilic SQRs (e.g., Azarkina and Konstantinov, 2002; and our unpublished results), the thermophilic enzymes catalyze the electron transfer from succinate to the artificial donor 2,6-dichlorophenolindophenol and are inhibited by the classical complex II inhibitors malonate and oxaloacetate, tetrachlorobenzoquinone being an extremely efficient inhibitor for *Th. acidopilum* and *S. acidocaldarius* SQRs (Anemuller *et al.*, 1995; Moll and Schafer, 1991).

R. marinus is the only thermophilic bacterium whose SQR has been purified and biochemically characterized (Fernandes *et al.*, 2001). It is a typical type B enzyme, nevertheless possesses as an atypical characteristic two conformations in the S3 center, revealed by EPR, but this could not be related yet to any other aspect of the enzyme or the organism. Also this center has an unusually high reduction potential of +130 mV (Pereira *et al.*, 1999b).

From the known thermophilic archaea SQRs, only four enzymes were purified and biochemically

characterized: the SQR from *S. acidocaldarius* (Janssen *et al.*, 1997; Moll and Schafer, 1991), *S. tokodaii* (former *Sulfolobus* strain7) (Iwasaki *et al.*, 1995, 2002), *A. ambivalens* (Gomes *et al.*, 1999; Lemos *et al.*, 2001), and *T. acidophilum* (Anemuller *et al.*, 1995; Bach *et al.*, 1993). The *Sulfolobales* enzymes are the only known examples, so far purified and characterized, of a type E enzyme. They are all composed of four subunits: SdhA (63–67 kDa), SdhB (31–37 kDa), and the two hydrophilic anchor subunits SdhE and SdhF (28–33 and 12–14 kDa, respectively). The EPR spectra of both the as-isolated enzyme (Iwasaki *et al.*, 1995, 2002; Janssen *et al.*, 1997; Moll and Schafer, 1991) as well as in the membrane-bound state (Gomes *et al.*, 1999) did not reveal the $[3Fe–4S]^{1+/0}$ center spectrum at $g = 2.02$ present in canonical SQRs. Upon addition of succinate, a rhombic-type signal with $g = 2.03$, 1.93, and 1.91, characteristic of reduced $[2Fe–2S]^{2+/1+}$ centers was detected (g values for *A. ambivalens*, similar to the other two enzymes). The presence of the tetranuclear center S2 could only be inferred by the microwave power saturation behavior of the S1 center in the succinate and dithionite reduced samples. In the *S. acidocaldarius* enzyme, a signal obtained for the reduced sample, in a temperature-difference spectrum (70 minus 25 K), was attributed to the extra tetranuclear center.

The anchor domain of type E enzymes is completely distinct from the canonical ones. With the exception of a small putative transmembrane helix close to the C terminus of the 33-kDa subunit, no other unequivocal transmembrane helices are predicted in the SdhC and SdhD proteins (because of this fact, we proposed to rename these polypeptides as SdhE and SdhF (Lemos *et al.*, 2002)). The 33-kDa (SdhE) subunit has a striking characteristic: it contains a duplicated cysteine residue-rich motif, $CX_{31-35}CCGX_{38-39}CX_2C$. A recent report by Iwasaki *et al.* (2002) showed that this subunit contains a novel type of a $[2Fe–2S]^{2+/1+}$ center, possibly bound to some of the extra cysteine residues.

Regarding the membrane attachment, since there were no predicted transmembrane helices, it was proposed an attachment through amphipathic helices; wheel projections of the predicted α-helices of *A. ambivalens* SdhE show an amphipathic nature of several helices (Lemos *et al.*, 2001, 2002). Thus, type E SQRs may have a monotopic anchor (Blobel, 1980), and since the protein is still embedded in the membrane, it explains its capability to interact with quinones. In fact, *A. ambivalens* SQR was isolated with caldariella quinone bound. Interaction with the quinone may occur through the newly discovered cluster in SdhE. It should be noticed also that known quinone binding motives are not present in SdhE or F.

QUINOL:CYTOCHROME *c* OXIDOREDUCTASE (COMPLEX III)

In mitochondria and Purple bacteria quinol: cytochrome *c* oxidoreductase activity is performed by the bc_1 complex. The minimal functional unit of this complex is composed by a dihaemic cytochrome *b*, a Rieske protein, and a cytochrome *c* (Hatefi, 1985; Trumpower, 1990a). This complex is proposed to translocate protons by a Q-cycle mechanism (Mitchell, 1975; Trumpower, 1990b). A very similar complex, the b_6f, catalyzes the quinol:plastocyanine/cytochrome c_6 oxidoreductase reaction in chloroplasts and Cyanobacteria (Cramer *et al.*, 1994). Cytochrome *b* is larger than cytochrome b_6, and cytochrome *f* differs from cytochrome c_1 in respect to its haem sixth ligand, which is the α amino group of the N terminus amino acid residue (Prince and George, 1995). In the *Bacillus* genus a b_6c_1 complex is responsible for the oxidation of menaquinol and reduction of cytochrome *c* (Yu *et al.*, 1995). A dihaemic cytochrome *c* is present in complex III from other gram-positive bacteria (Sone *et al.*, 2003). Copurification in the form of a supercomplex of complexes III and IV has been observed in several bacteria, such as *Paracoccus (Pa.) denitrificans* and mitochondria (Berry and Trumpower, 1985; Schagger and Pfeiffer, 2000).

Thermophilic Quinol:Cytochrome *c* Oxidoreductases

A b_6c_1 complex is expressed by *G. stearothermophilus* and its encoding genes have been sequenced (Sone *et al.*, 1996). For *T. thermophilus*, whose genome is still not fully sequenced, only a Rieske protein has been characterized (Fee *et al.*, 1984; Hunsicker-Wang *et al.*, 2003). *R. marinus* possesses a complex with quinol:cytochrome *c*/HiPIP (high potential iron–sulfur protein) oxidoreductase activity, with a minimum of three subunits (43, 27, and 18 kDa), containing five low-spin haem centers of the B- and C-types, in a ~1:4 ratio. A $[3Fe–4S]^{1+/0}$ center copurifies with this complex (Pereira *et al.*, 1999a). A bc_1 complex is expected to be present in *Aq. aeolicus*, since its genome contains genes coding for such a complex (Deckert *et al.*, 1998).

In *A. ambivalens* only a quinol:oxygen oxidoreductase is known, and thus the presence of a complex III may not be necessary. In fact there are no indications for the existence of such a complex in this organism. In the case of *S. acidocaldarius* two dihaemic cytochromes (SoxC and SoxG) similar to the bc_1 cytochrome *b* are present (Schafer, 1996; Schafer *et al.*, 2001). These are part of supercomplexes (so-called SoxABCD and SoxM,

respectively) together with the oxygen reductases (see next section), and the two operons coding for these complexes have been sequenced. In the SoxM complex, besides the dihaemic cytochrome (SoxG), a Rieske protein (SoxF) is also present (Schafer, 1996; Schafer *et al.*, 2001). There is no evidence for the presence of cytochromes c in this organism. A second Rieske protein (SoxL), which is not part of any of the mentioned supercomplexes, is also present in *S. acidocaldarius* (Schafer, 1996; Schafer *et al.*, 2001). The same observations can be extended to *S. solfataricus* and *S. tokodaii*, based in their genome sequences and protein characterization. Genes similar to those coding for SoxC/SoxG are also present in the genomes of *A pernix*, *P. aerophilum*, *Th. acidophilum*, and *Th. volcanium*. Rieske proteins are expected to be expressed in *A pernix* and *P. aerophilum*. Among the considered archaea, only *P. aerophilum* genome contains a gene coding for a protein with a haem C binding domain, which is not a cytochrome c_1.

In *S. metallicus* membranes, a new type of iron–sulfur cluster was detected, with redox properties similar to those of the Rieske proteins, which was suggested to be a functional substitute for these proteins (Gomes *et al.*, 1998).

In respect to complexes able to oxidize quinols and reducing metalloproteins, the available data for thermophilic prokaryotes is still very scarce. However, different complexes seem to be catalyzing this reaction, similarly to what may occur in several mesophiles.

OXYGEN REDUCTASES

Oxygen reductases are the last complexes of aerobic respiratory chains, catalyzing the reduction of dioxygen to water. Most of these enzymes belong to the superfamily of haem–copper oxygen reductases (Fig. 1), which are characterized by having in their subunit I a low-spin haem and a binuclear center harboring a high-spin haem and a copper ion, being able to couple oxygen reduction to proton translocation. These enzymes are able to oxidize peripheral or periplasmatic electron donors (such as cytochromes, HiPIPs, or copper proteins), or membrane-bound electron donors (quinols). The former have in their subunit II a mixed valence dinuclear copper center (CuA), which is absent in the latter. The cbb_3 oxidases are reported to be cytochrome oxidases, which instead of the subunit II of the other haem–copper oxidases, have one monohaemic and one dihaemic subunit.

To perform their function of reducing oxygen to water, and also to pump protons, these enzymes possess proton conducting channels. On the basis of the amino acid residues forming these channels, on amino acid sequence

comparisons, and on specific characteristics of subunit II, three families were established for haem–copper oxygen reductases, named A (which includes the subfamilies A1 and A2), B, and C (Pereira *et al.*, 2001). This classification is supported by the kinetic and ligand binding properties of the binuclear center (Pereira and Teixeira, in press).

Cytochrome *bd* is a quinol:oxygen oxidoreductase, present in many prokaryotic respiratory chains, that does not belong to the haem–copper oxygen reductases superfamily. Cytochrome *bd* is a two subunits protein complex, containing a low-spin B type haem and a catalytic center composed by two high-spin haems, one of the B and the other of the D type (Junemann, 1997). No structure of this type of oxygen reductases has yet been solved, and only a preliminary characterization of functional amino acid residues has been performed. Cytochromes *bd* do not pump proton, but are electrogenic.

In several eukaryotes, there is a quinol:oxygen oxidoreductase, the so-called alternative oxidase, which contains a di-iron center (Berthold *et al.*, 2000, 2002). The membrane attachment of this enzyme has been also proposed to occur through amphipathic helices (Joseph-Horne *et al.*, 2000).

Thermophilic Oxygen Reductases

Thermophilic aerobic prokaryotes have haem–copper oxygen reductases from all families and subfamilies, as well as cytochrome *bd* oxygen reductases.

The best characterized oxygen reductases from thermophiles are those that have been isolated from *S. acidocaldarius*, *A. ambivalens*, *T. thermophilus* (Giuffre *et al.*, 1999; Honnami and Oshima, 1984; Pinakoulaki *et al.*, 2002; Soulimane *et al.*, 2000), *R. marinus* (Pereira *et al.*, 1999c; Santana *et al.*, 2001), and *G. stearothermophilus* (Kusano *et al.*, 1996; Kusumoto *et al.*, 2000).

Two different oxygen reductases have been isolated from *S. acidocaldarius* (Schafer, 1996; Schafer *et al.*, 2001). One is encoded by the *soxABCD* operon and composed by a typical subunit I (SoxB) of haem–copper oxygen reductases, containing A_s type haems, a subunit II (SoxA) without the Cu_A site and a dihaemic cytochrome with As type haems (SoxC, mentioned before). The entire complex has quinol oxidase activity and is able to pump protons. Sequence analysis of subunits I and II showed that this oxygen reductase is a member of type B haem–copper reductases. SoxM, a second oxygen reductase isolated from *S. acidocaldarius*, is encoded by genes *soxM*, *H*, *G*, *F*, *E*, and *I*, organized in an operon (Lubben *et al.*, 1994). *soxM* codes for subunit I, which contains one

B- and one A_s-type haems. The subunit II, possessing a Cu_A center, is encoded by *soxH*. *soxG* and *soxF* encode a dihaemic cytochrome a similar to cytochrome *b* of the bc_1 complex, and for a Rieske protein, respectively. *soxE* encodes a copper protein, sulfocyanin, and the product of *soxI* is still unknown. Analyzing the amino acid residues constituents of the proton channels, this oxygen reductase complex is a member of the type A1 subfamily.

A proton pumping cytochrome aa_3 quinol oxidase has been isolated and characterized from *A. ambivalens* (Gomes *et al.*, 2001). The operon coding for this enzyme, *DoxBCEF*, has been sequenced (Purschke *et al.*, 1997) indicating that this is a type B oxygen reductase. *DoxB* encodes subunit I, containing A_s-type haems, while *DoxC* codes for subunit II, which does not possess the Cu_A center. The properties of the haem–copper binuclear center from this enzyme further corroborate its classification as a member of the B type family.

A caa_3 and a ba_3 oxygen reductases have been isolated from *T. thermophilus* and extensively characterized (Gerscher *et al.*, 1999; Giuffre *et al.*, 1999; Hon-nami and Oshima, 1984; Keightley *et al.*, 1995; Mather *et al.*, 1991; Soulimane *et al.*, 2000). Both are cytochrome *c* oxidases possessing in their subunits II the Cu_A center. A haem C is also present in subunit II of the caa_3 reductase. Both were shown to translocate protons. Sequence analyzes of their subunits I revealed that the caa_3 reductase is a member of type A2 subfamily while the ba_3 reductase belongs to type B family. As for the *A. ambivalens* oxygen reductase, the kinetic and ligand binding properties of the binuclear center of the ba_3 reductase also corroborate its classification (Aagaard *et al.*, 1999; Gilderson *et al.*, 2001; Giuffre *et al.*, 1997; Hellwig *et al.*, 2003).

R. marinus contains a caa_3 oxygen reductase similar to the one of *T. thermophilus*. This reductase has been extensively studied and was shown to be a member of type A2 subfamily (Pereira *et al.*, 1999c; Santana *et al.*, 2001). A type C oxygen reductase, a cbb_3, has also been isolated from *R. marinus* and part of a gene coding for subunit I has been sequenced, reinforcing the presence of such type of oxygen reductases in this thermophilic bacterium (Pereira *et al.*, 2000). A type A1 (Kusano *et al.*, 1996), biochemically characterized as a caa_3, and a type B, characterized as a $b(o/a)_3$ (Nikaido *et al.*, 1998; Sakamoto *et al.*, 1997), from *G. stearothermophilus*, as well as a cytochrome *bd* oxygen reductase (Kusumoto *et al.*, 2000; Sakamoto *et al.*, 1999), have been isolated and sequenced.

Oxygen reductases of types A1 and B may be expressed by *P. aerophilum* and *Ae. pernix*, based on the analysis of their genomes. Two oxygen reductases, an aa_3 and a ba_3, have been isolated from the membranes of *Ae. pernix* and were assigned to the genes expressing the type A1 and B reductases, respectively (Ishikawa *et al.*, 2002). Genes coding for cytochrome *bd* type oxidases have been sequenced from *Th. acidophilum*, *Th. volcanium*, and *Ts. elongatus*. The genome of *Th. elongatus* also contains genes encoding a type A2 oxygen reductase.

ELECTRON CARRIERS

Electron transfer between the different complexes of the respiratory chain is performed by quinones, which are lipophilic, and water-soluble or membrane-bound metalloproteins. Quinones can be divided into three major groups: the benzoquinones, the naphthoquinones (Collins and Jones, 1981), and benzothiophenquinones. Their different structures reflect different physical and functional properties, such as polarity, reduction potentials, as well as steric and interaction constritions.

Thermophilic Electron Carriers

Quinones are the membrane electron carriers from complexes I or II to complexes III or IV. Three different quinones have been isolated from *S. acidocaldarius* and *S. solfataricus*, all of them being benzothiophenquinones: caldariella quinone, sulfolobus quinone, and the tricyclic quinone (De Rosa *et al.*, 1977). The first two quinones are also present in *A. ambivalens* (Trincone *et al.*, 1989). *Th. acidophilum* contains thermoplasma quinone, which is closely related to menaquinone (Shimada *et al.*, 2001). *R. marinus* and *T. thermophilus* possess menaquinone with unsaturated isoprenoid chains, MK-7 in the former case and MK-8 in the latter (Tindall, 1991).

The electron transport between complex III and the oxygen reductase is usually performed by cytochrome *c*. Cytochromes of this type have been isolated from *T. thermophilus* (Yoshida *et al.*, 1984), and may be expressed in *G. stearothermophilus*, *Ts. elongatus*, *Aa. aeolicus*, and *P. aerophilum*, based on their genome analyzes. In *R. marinus*, an HiPIP was shown to be the electron carrier between the *bc* complex and the caa_3 oxygen reductase (Pereira *et al.*, 1999b). A cytochrome *c* isolated from this organism can also perform this role, although less efficiently. Sulfocyanin, a copper protein isolated from *S. acidocaldarius* and shown to be part of the SoxM complex, is proposed to participate in the electron transfer from the Rieske protein to Cu_A of subunit II of this oxygen reductase complex (Komorowski *et al.*, 2002). Genes coding for this copper protein are also present in the genomes of *S. sulfataricus* and *S. tokodaii*. A similar gene is present in *Th. acidophilum* genome.

CONCLUDING REMARKS

In summary, the electron transfer enzymatic complexes of the respiratory chains from aerobic thermophilic bacteria and archaea may be quite diverse, as made clear by the data collected in Table I. However, the same level of variability occurs in mesophilic organisms, such as the presence of different types of NADH:quinone oxidoreductases, succinate:quinone oxidoreductases, quinol:electron carrier oxidoreductases, and oxygen reductases. Also, there is no clear phylogenetic distinction between the respiratory complexes, *i.e.*, these complexes are randomly distributed among the bacteria and archaea domains. The only feature that is common to all thermophilic enzymes is that their optimum activity temperatures are high, in general close to those of the parental organism, as should be expected. Thus, it seems that at least in terms of the respiratory chains the adaptation to thermophilic conditions should be achieved by minimal structural changes, as those referred in the introductory section, to enhance their stability, rather than by the presence of specific thermophilic complexes.

Although a large amount of genomic data is already available, it is clear that the corresponding biochemical data is still very scarce. This is a mandatory task to confirm not only sequence predictions, but also to unravel alternative respiratory complexes, that may be anticipated to be present by physiological studies. The data described in this paper suggest that the variety found among thermophiles is just a consequence of the microbial diversity, which occurred throughout evolution.

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