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Phylogenetic and biochemical studies of *Thermus thermophilus* respiratory proteins relevant to aerobic heterotrophy at hydrothermal vents: the archaeal-type cytochrome *ba3* from strain HB8 is a sulphide resistant cytochrome *c* oxidase

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Introduction

Thermus thermophilus (Oshima & Imahori, 1974) is a heterotrophic, non-sporulating, non-motile, multi-plasmid bearing, high G + C, aerobic eubacterium that grows over the temperature range of ~60 to 85°C. Numerous strains of this genus have been isolated from terrestrial and shallow oceanic hot springs. Its recent discovery at ocean vent sites on the Mid-Atlantic Ridge and at the Guaymas Basin in the Gulf of California indicates aerobic microbial heterotrophy in and around these sites (Marteinsson et al., 1995). Conditions in the vicinity of the vents are characterized by temperatures ranging from ~2 to ~350°C, micromolar to above millimolar concentrations of sulphide, O_2 concentrations ranging from 0 to ~ 100 µM, toxic concentrations of numerous metal ions, and, one can surmise, extreme patchiness of reduced carbon compounds and temporal instability of those environments suitable for the growth of *Thermus*.

The terminal oxidases of an organism relying on respiration for its survival in the vent environment will be susceptible to sulphide poisoning. Although the atomic-level mechanism has not been well studied, sulphide is known to be a powerful inhibitor of cytochromes aa_3, where it appears to react at the a_3-Cu_B site, in a manner similar to carbon monoxide, thereby precluding oxygen reduction. The reaction of sulphide with bovine cytochrome aa_3 is not extremely rapid, as is the case with HCN, rather several minutes may be required depending on concentration and other conditions; and it is not known if sulphide inhibition can be reversed.

Our laboratory has been studying the respiratory proteins from *Thermus thermophilus* HB8 since the late 1970's, during which time we identified two cytochrome *c* oxidases. The cytochrome caa_3, was initially described in 1980 and has been subjected to extensive biochemical and biophysical characterization, including gene isolation and sequencing. It is derived from only two structural genes, one coding for a covalently linked subunit I and subunit III combination while the other gene codes for a protein consisting of a subunit II covalently linked with a cytochrome *c*. The cytochrome ba_3 was initially reported in 1988 and has also been subjected to considerable biophysical and biochemical characterization extending to gene isolation and sequencing. While our genetic work is not complete, two genes encoding for the two subunits of the as-isolated enzyme have been sequenced. In spite of being constructed from covalently linked proteins, cytochrome caa_3 appears to be closely related to other eubacterial cytochrome *c* oxidases. By contrast, cytochrome ba_3 is highly divergent in both sequence and chemical reactivity (see below), and the argument has been made that the genes encoding ba_3 were possibly acquired by *Thermus* by gene transfer (Keightley et al., 1995). A recent description of a cytochrome ba_3 from the archaebacterium, *Natronobacterium pharaonis* Tindall et al., 1984 tends to support this contention (M. Engelhard, pers. comm.). In an effort to identify conditions under which expression of the two *Thermus* oxidases is optimized, we discovered that synthesis of cytochrome caa_3 is constitutive with respect to oxygen, whereas the synthesis of cytochrome ba_3 is greatly enhanced under microaerophilic growth conditions (see Fee et al., 1993; Keightley et al., 1995 for review and additional references).

In this communication we report (a) that cytochrome ba_3 from the eubacterium *T. thermophilus* is a homologue with

the archaebacterial cytochrome ba_3 from *N. pharaonis,* and (b) that the cytochrome ba_3 from *T. thermophilus* is highly resistant to sulphide inhibition, while the cytochrome caa_3 is strongly inhibited.

Material and methods

Phylogenetic analyses of haem-copper oxidase genes was carried out using the programs of D.-F. Feng and R.F. Doolittle implemented on a Silicon Graphics Iris Indigo computer.

Cytochromes caa_3 and ba_3 were purified from *Thermus thermophilus* HB8 as previously described (Keightley et al., 1995). Sulphide inhibition of cytochromes caa_3 and ba_3 was examined by first incubating the enzymes at room temperature with 10 mM ascorbate, 0.3 mM tetramethylphenylenediamine (TMPD) ± 1 mM Na_2S in 0.1% Triton X-100 and 0.1 M N-tris[hydroxymethyl]-methyl-3-aminopropanesulfonic acid (TAPS) buffer at pH 8 for one hour prior to measuring oxygen uptake in a Gilson oxygraph. The buffer was identical, and the final concentrations in the oxygraph solution were 0.67 mM ascorbate and 3.5 mM TMPD and 0.44 µM ba_3 or 3.5 mM TMPD and 0.076 µM caa_3 and sulphide was 0 or 67 µM.

Results and discussion

In a recent phylogenetic analysis of haem-copper oxidase sequences, Castresana et al. (1994) presented a phylogenetic tree calculated using the neighbour-joining algorithm and a bootstrap method. The progressive alignment of Doolittle and Feng reproduced this tree (not shown) with one notable exception: no evidence was found for a homologous relationship between the *Sulfolobus acidocaldarius* Brock et al., 1972 SoxB sequence with that of the *Thermus thermophilus* CbaA gene. That this must be the case was already pointed out by Keightley et al. (1995) who noted that the percent identity between CbaB and SoxB was exceedingly low (< 20%) and each sequence showed a similar percent identity to all other haem-copper oxidase subunits I. To explore this relationship further, we incorporated the subunit I sequence of cytochrome ba_3 from the archaebacterium *Natronobacterium pharaonis* and constructed a phylogenetic tree using progressive

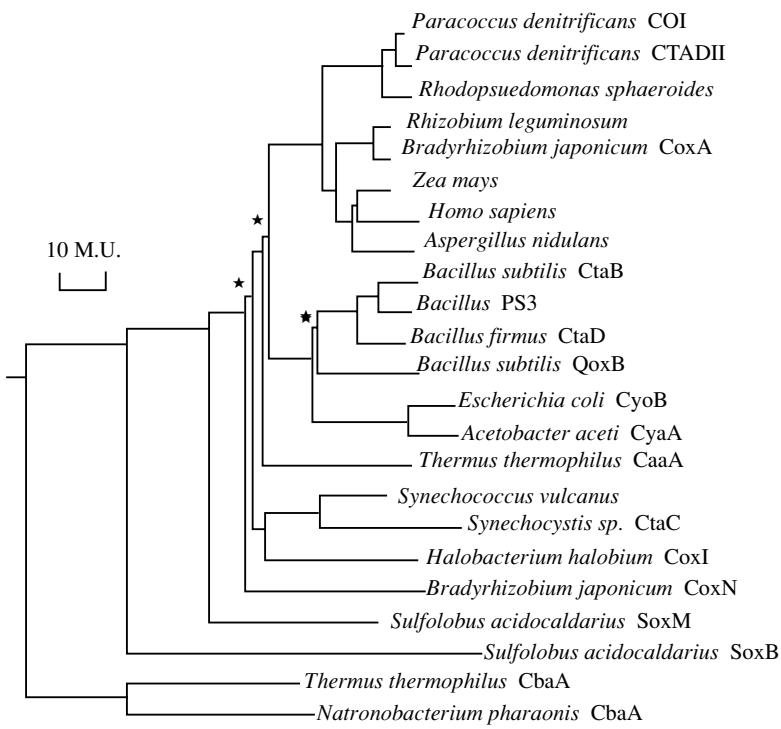

Figure 1. Unrooted phylogenetic tree of haem-Cu oxidase subunits I. The tree is based on the alignment of sequences that were N-terminally and C-terminally trimmed to avoid overlapping ends and included transmembrane elements II through XI. The progressive alignment programs of D.-F. Feng and R. F. Doolittle were used to generate the tree, and we gratefully acknowledge helpful discussions with Dr. Russell Doolittle. The matrix unit (M.U.) is defined as D = -lnS, where S is a measure of sequence similarity. A value of 1 was added to those branch lengths marked with a * in order to increase clarity of presentation. The *N. pharaonis* sequence was submitted to GenBank as Y10500 by Stephan Mattar and Martin Engelhard. Other sequences were obtained as described Castresana et al. 1994.

alignment. The unrooted tree is shown in Fig. 1, and with minor exceptions, it is similar to that reported by Castresana et al. (1994).

As long as the intersequence identities are greater than ~35 - 40%, both trees can be viewed as a group of several 'logical' clades derived from obviously common ancestral sequences. At and below this percent identity however, the tree displays a series of single branches leading first to *Bradyrhizobium japonicum* Jordan, 1982 CoxN then to *Sulfolobus* SoxM and SoxB and finally the two CbaA sequences (Fig. 1). In our opinion, these branch points must be viewed as ancestral sequences with considerable suspicion. Thus, until more intermediately branching sequences become available, we believe it is wiser to conclude that the five above mentioned haem-copper oxidase sequences are simply of unknown origin. However, the CbaA sequences from *T. thermophilus* and *N. pharaonis* share ~47% sequence identity and biochemical studies (M. Engelhard, pers. comm.) show that they are both cytochromes ba_3, making it reasonable to conclude that they are probably homologues. This raises an interesting question. How is it that *T. thermophilus,* a **eu**bacterium, and *N. pharaonis*, an **archa**ebacterium, come to share this homologous protein? While one may invoke a deeply rooted gene duplication, it could also arise from lateral gene transfer. The latter occurs, probably with high frequency, in natural populations of bacteria (see Sonea & Panisset, 1983 for discussion and speculation on the importance of gene transfer in forming microbial 'species'). *Thermus* cytochrome ba_3 may thus be of archaeal origin.

Divergent sequence implies the possibility of divergent biochemical behaviour. For example, we discovered many years ago that cyanide reacts with cytochrome ba_3 to reduce and to ligate the a_3 component, whereas it is well established that cyanide reacts with the oxidized a_3 component of cytochromes aa_3 to form the cyano complex (Ref. 58 in Fee et al., 1993). Here we describe additional divergent behaviour in the reaction of sulphide with *Thermus* cytochromes ba_3 and caa_3. Fig. 2 shows the effect of sulphide treatment on the TMPD oxidase activity of these enzymes. Each enzyme was treated for 1 hour with a reducing mixture of ascorbate and TMPD plus-or-minus Na_2S at 1 mM prior to being diluted into the oxygraph chamber. The kinetic traces of Panel A show that sulphide completely inhibits the enzyme activity of cytochrome caa_3 whereas the kinetic traces of Panel B show that cytochrome ba_3 suffers only ~50% inhibition after this treatment. In other experiments (data not shown) it was demonstrated that the use of cytochrome c_{552} as the electron donor, in place of TMPD, gave the same results. We are currently measuring the sulphide inhibition constants for the two enzymes. The K_I of bovine cytochrome aa_3 for sulphide is < 0.1 μM (P. Nicholls, pers. comm.), and we expect a similar value for *Thermus* cytochrome caa_3. By contrast, one can see already from Fig. 2 B that K_I for *Thermus* cytochrome ba_3 is ≥ 67 μM, which is the final concentration of sulphide in the oxygen electrode chamber.

In summary, we have analysed sequence information which indicates that the gene for cytochrome ba_3 is shared between archaebacteria and eubacteria, and we suggest that *Thermus* may have obtained this gene by lateral transfer. We also report, for the first time, that the cytochrome *c* oxidase activity of *Thermus* cytochrome ba_3 is extremely insensitive to sulphide inhibition while that of cytochrome caa_3 exhibits sensitivity toward sulphide that is typical of other cytochromes aa_3. There are several implications of these findings. First, the cytochrome *c* oxidase molecule can accrue amino acid replacements that, while retaining oxidase activity, greatly reduce inhibition by sulphide. Forthcoming structural information should reveal what changes are needed to attenuate sulphide affinity. Second, because of its resistance to sulphide, cytochrome ba_3 may be the terminal oxidase used by *Thermus* strains endemic to the vents (Marteinsson et al., 1995). This is testable using infrared spectroscopic techniques to quantify relative amounts of ba_3 and caa_3 in plasma membranes. Finally, to the extent that cytochromes ba_3 are generally insensitive to sulphide, it is reasonable to speculate that this enzyme may be utilized by other heterotrophs living at the hydrothermal vents.

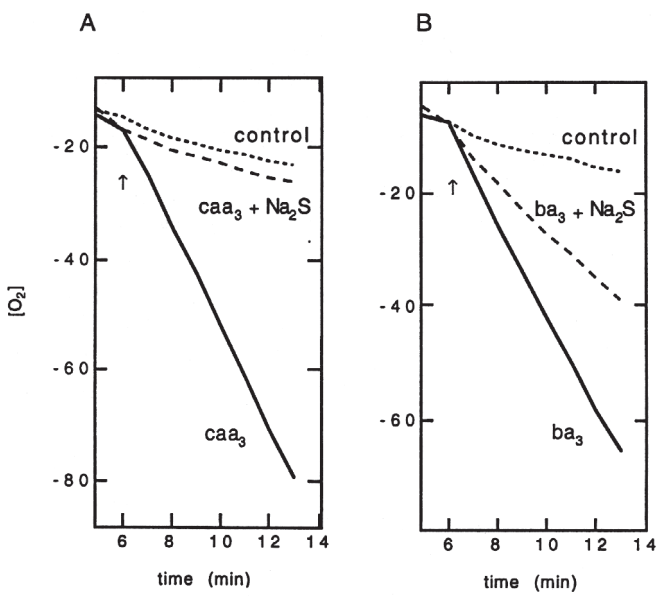

Figure 2. TMPD oxidase activity of cytochrome caa_3 (A) and cytochrome ba_3 (B) in the absence of enzyme (control) and in the presence of enzyme and absence (——) and presence (- - - -) of Na_2S. Data is presented as decreasing % relative oxygen concentration as a function of time. Final concentration of sulphide in the oxygen chamber was 0 or 67 μM. Further details of the experiment are presented in Material and methods.

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