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Sequence variability in bacterial cytochromes *c*

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Cytochromes *c* are proteins that can be defined both phenotypically and by their possession of a characteristic sequence motif. Many sequences from bacterial sources are known, and new ones are being reported every year. An analysis can be made as to what fraction of new sequences are members of already known classes or subclasses, and how many map into previously uninhabited regions of sequence space.

Introduction

Sequence space is now uniformly occupied. This is a proposition that can now be considered using the information that is available about the amino-acid sequences of bacterial cytochromes *c*. These are a set of proteins that can both be defined phenotypically through the characteristic adsorption spectrum, and by their possession of the haem-attachment sequence motif (-Cys-X-Y-Cys-His-). While this sequence is present in other proteins, two-thirds of its occurrences in the data base are in cytochromes *c* of various kinds. In general, the soluble cytochromes *c* are located in the periplasm, as shown directly by extraction and indirectly through gene sequencing and the identification of appropriate signalling sequences.

There are now more than a hundred known and distinct sequences that possess these properties, and we can begin to ask questions about their relationships and distribution. In 1982, Ambler [1] recognised four sequence classes of cytochromes *c*. Class I includes the classical soluble cytochromes *c* of mitochondria and bacteria, with the haem-attachment site towards the N-terminus, and the sixth ligand provided by a methionine residue about forty residues further on towards the C-terminus. Distinct sub-classes can be recognized (Table I), and three-dimensional structures have been determined for several varieties, in each of which most of the elements of the 'cytochrome fold' [2,3] can be seen. Class II includes the high-spin cytochromes *c'* and various low-spin cytochromes (e.g., *Rhodopseudomonas palustris* cytochrome *c*-556; [4,5]. The haem-attachment site is close to the C-terminus, and in the low-spin proteins the sixth ligand seems likely to be a methionine residue close to the N-terminus. The three-dimensional structure is completely different, the proteins folding as a cluster of four α-helices [6]. Class III comprises the low redox potential multiple haem cytochromes c_3 and c_7, with only around thirty residues of amino acid per haem group, and Class IV was created to hold the complex proteins that have other prosthetic groups as well as haem *c*, such as the flavocytochromes *c* and the cytochromes *cd*. Does this classification still fit the great increase in information gained in the last 10 years?

Of the hundred-odd known sequences, about two-thirds clearly fit into Class I. All but perhaps six fit into the five subdivisions (Table I) proposed by Ambler [1]. About a quarter of the sequences are of Class II, while seven are of Class III, and about the same number do not fit readily into either Class I, II or III (Table II). Information is becoming available about the haem subunits of flavocytochromes *c* (e.g. Ref. 8), and it seems possible that these may be accommodated in Class I.

Cytochromes *c* occur in a wide variety of bacteria, and occur in high yield in many different Gram-negative organisms. They were also detected in Gram-positive bacteria as early as 1928 by Yaoi and Tamiya [9], but few from this source have been characterized [10–12]. Gram-positive bacteria lack a periplasm in which soluble cytochromes *c* can be retained if they are to function outside the cell membrane, and so their cytochromes appear to be bound on the outer surface of this membrane; they can be extracted in a soluble form only after partial degradation with proteinases [10,13].

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Cytochromes *c* function in the electron transport chains of bacteria with many different sorts of energy metabolism, including phototrophes, methylotrophes, denitrifiers, sulphate reducers and the nitrogen-fixing *Azotobacter*. Proteins of the same sequence class operate in different electron transport chains, while organisms which share the same metabolic strategy may use quite distinct electron transport components. The structure, function and evolution of cytochromes *c* have recently been the subject of an excellent two-volume monograph by Pettigrew and Moore [14,15].

In the present paper, the validity of the 1982 cytochrome *c* sequence classification will be discussed by considering some of the proteins that have been characterized since then, and mention will be made of cytochromes *c* whose sequences require class or subclasses not recognized in 1982.

TABLE I

Subdivision of sequence class I cytochromes c

Class IA. Long cytochrome c_2
Contain several extra loops when compared with class IB, although three-dimensional structure is generally similar. The insertions are in different places in the different members of this class.
Examples: Rhodospirillum rubrum cytochrome c_2 [57]; *Aquaspirillum itersonii* cytochrome *c*-550 [58].

Class IB. Mitochondrial cytochrome *c*
Examples: Rhodopseudomonas globiformis cytochrome c_2 [59]; *Nitrobacter agilis* [60].

Class IC. Split-α-band cytochromes *c*
Possess a widened or split α band of lowered absorptivity, which does not seem to correlate with dimerization. Sequence characteristics include -M------LS---I-----Y- (sixth ligand region) and aromatic and proline residues after the haem binding site.
Examples: monomeric, *Spirulina maxima* (cyanobacterial) cytochrome c_6 [61]; *Thiobacillus neapolitanus* cytochrome *c*-554 (547) [62]; pseudodimeric, halophilic *Paracoccus* cytochrome *c*-554(548) [63]; dihaem, *Azotobacter vinelandii* cytochrome c_4 [64].

Class ID. Cytochrome c_8
Sequence characteristics include several proline residues around the sixth ligand methionine and a tryptophan residue near the C-terminus (Fig. 3).
Examples: *Pseudomonas* cytochrome *c*-551 [53]; *Rhodospirillum tenue* cytochrome *c*-553 [56]; *Hydrogenobacter thermophilus* cytochrome *c*-552 [55]; *Methylophilus methylotrophus* cytochrome c_H (Ambler and Daniel, unpublished results).

Class IE. Cytochrome c_5
Sequence characteristics include extra cysteine residues that form a short disulphide loop [18,21], and an absence of aromatic residues near the C-terminus. Although generally isolated as a protein with the haem attachment site near the N-terminus (Fig. 2), this short from is processed from a sequence with about 30 more residues on the N-terminus (see text).
Examples: Pseudomonas mendocina cytochrome c_5 [18]; organism H-1-R cytochrome c_5 (Fig. 2).

TABLE II

Prokaryotic cytochromes c of known sequence that do not fit into the Ambler's 1982 classes [1]

The positions in the sequence at which the putative haem or iron binding residues occur are shown, or all cysteine, histidine and methionine residues if direct information is not available. The final italic number is the total number of residues

	Ref.
(1) Cytochrome *c*-552 *Pseudomonas perfectomarinus* C45, 48; H49; M56, 60, 108, 119; C134, 138; H139; M145, 190 (*about 247*)	[65]
(2) Flavocytochrome *c* (haem subunit) *Pseudomonas putida* C15, 18; H19; M50 (H17) (*78*)	[8]
(3) Flavocytochrome *c* (haem subunit) *Chlorobium limicola* C18, 21; H22; M60, 85 (*86*)	[66]
(4) Cytochrome c_L *Pseudomonas* AM1 C65, 68; H69; M108, 109, 121 (C53, 167) (*172*)	[32]
(5) Photosynthetic reaction centre (haem subunit; cytochrome *c*-558) *Rhodopseudomonas viridis* C87, 90, H91, M74; C132, 135, H136, M112; C244, 247, H248, M233; C305, 308, H309, H124; (S-glycero-C1) (*336*)	[67],[68]
(6) Cytochrome *c″* (CO-binding) *Methylophilus methylotrophus* (43 residues from N-terminus, no cysteine in this sequence)	[30]
(7) Cytochrome *c* peroxidase *Pseudomonas aeruginosa* C51, 54; H55; M95, 132, 173; C177, 180; H181; M254 (*302*)	[69]
(8) Cytochrome *c Rhodobacter capsulatus* C34, 37; H38; M184 (*259*) (for *Paracoccus denitrificans*: see [40])	[38],[39],[70]

Cytochromes c_5 and c_3

Cytochrome c_3, discovered by Postgate in 1956 [16], is a small tetrahaem cytochrome (class III) of very low redox potential that has until now been found only in sulphate-reducing bacteria. Cytochrome c_5 is a class IE monohaem cytochrome discovered first in the nitrogen fixer *Azotobacter vinelandii* by Tissieres [17], but also present in denitrifying pseudomonads [18]. We have investigated the cytochrome complement of organism H-1-R, a purple photosynthetic bacterium isolated as a contaminant from a culture of *Chromatium gracilis* strain Hol-1 (Meyer, T.E., personal communication). Two soluble cytochromes can be isolated in high yield from the organism, one of which proves to be a cytochrome c_5 (Fig. 1), while the other is a Class III tetrahaem cytochrome (Fig. 2), which may or may not prove to be homologous to the *Desulfovibrio* cytochromes c_3.

The three-dimensional structures of two cytochromes c_3 have been independently determined [19,20], and the sixth ligands have been identified (as particular histidine side-chains) for each of the four haem groups. The H-1-R protein also contains a plethora of histidine residues, but while two are located so that they could be homologous with the sixth ligands of haems 1 and 2 of

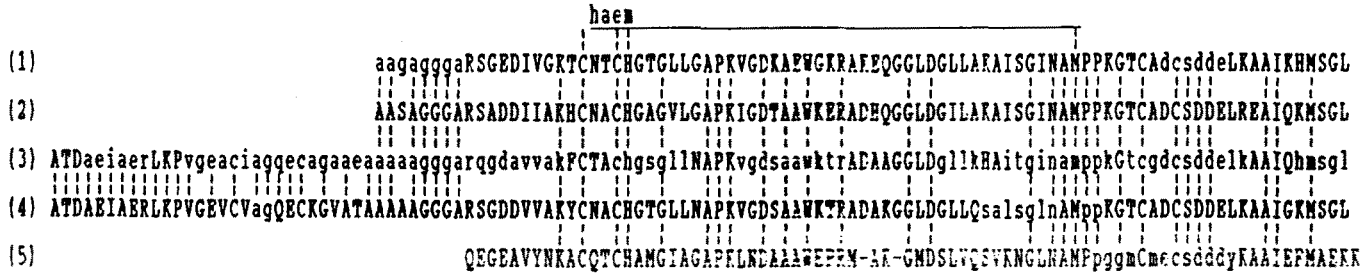

Fig. 1. Amino acid sequence of cytochromes c_5. (1) *Pseudomonas aeruginosa* P6009; (2) *Pseudomonas mendocina* Ch110 [15]; (3) *Pseudomonas stutzeri* Stanier-221; (4) *Azotobater vinelandii* strain O; (5) Organism H-1-R. The protein is also present in *Pseudomonas fluorescens* biotypes B, C, D and E, and in *Pseudomonas denitrificans* NCIB 9496. Residues aligned only from peptide compositions are shown in lower-case letters. Vertical lines join residues identical in all sequences compared. Sequences (apart from (2)) are unpublished results of R.P. Ambler.

cytochromes c_3, no suitably situated residues appear to be present for the other two haems.

The cytochrome c_5 of organism H-1-R has the features that are recognizable in these proteins from fluorescent pseudomonads [18] or *Azotobacter vinelandii* [21], including the extra disulphide-linked cysteine residues close after the sixth-ligand methionine. If cytochrome c_5 is extracted from cells under conditions in which proteolytic cleavage is minimized, the form isolated contains around 120 residues [22], whereas if some autolysis occurs during isolation, a smaller form containing about 100 residues [23] is also formed, and may predominate [18]. The extra residues are present in an extra N-terminal domain which in at least two representatives contain two further cysteine residues (Fig. 1). In this long form the haem attachment site is so far from the N-terminus that the proteins might be considered not to meet the Class I sequence criteria, except that three-dimensional structure studies [21] show that the short form certainly has the 'cytochrome fold'.

A similarly anomalous cytochrome *c* distribution was observed in *Chloropseudomonas ethylica* [24,25], where this green photosynthetic system was found to contain both a three-haem Class III cytochrome *c* and a cytochrome *c*-555 closely similar in sequence to that from *Chlorobium thiosulphatophilum*. The anomaly was explained when it was realized that *'Chloropseudomonas ethylica'* was really a consortium of a green photosynthetic *Chlorobium* and a novel sulphur-reducer, *Desulphurobacter acetoxidans* [26,27]. Attempts have been made to resolve H-1-R into more than one component microscopically, and by trying to isolate more than one type of 5S rRNA (Hreggvidsson, G., personal communication), but without success. However, one will only be convinced that H-1-R really is a novel organism when it has been well characterized by the classical techniques of bacteriology.

Cytochromes *c* in methylotrophes

Methylotrophes are rich sources of soluble cytochromes *c*, and several groups have purified multiple

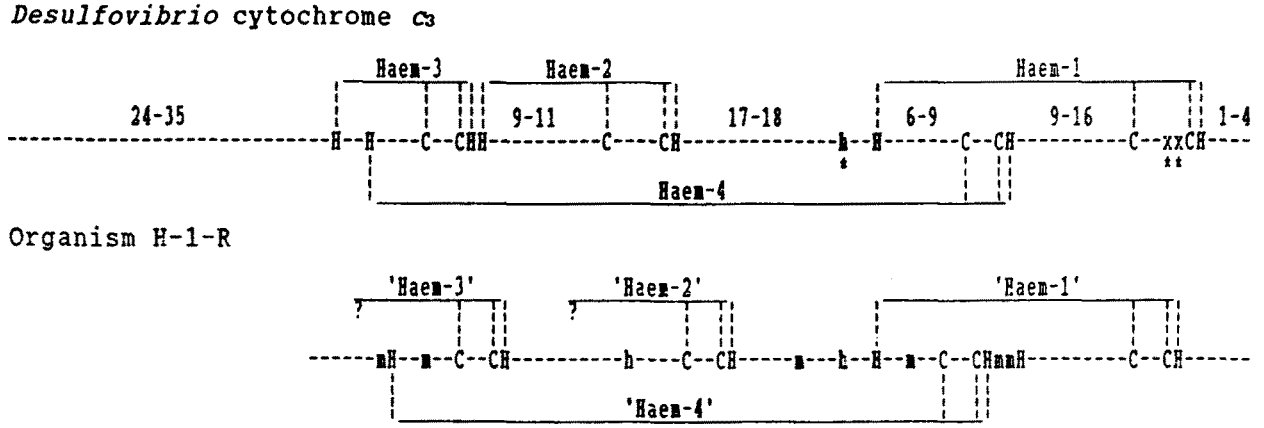

Fig. 2. Iron-coordination in four-haem cytochromes *c*. Only cysteine, histidine and methionine residues are shown, with those residues that function in haem binding and as iron ligands in the cytochromes c_3 of known tertiary structure shown in upper case, together with the putatively homologous residues in the H-1-R protein. The amino-acid sequences for cytochromes c_3 from six species of *Desulfovibrio* are known. Residues marked * are not present in all sequences. The numbers above the *Desulfovibrio* sequence give range of numbers of residues in different sequences. The haem groups are numbered as by Higuchi [19].

cytochromes *c* from different organisms, and have shown that they differ in physical properties and in function. For instance, in the methanotrophe *Methylococcus capsulatus*, the predominant soluble cytochrome *c* [28], while not close in sequence to any other known protein, probably fits into sequence class IC (Table I). Anthony and his associates purified two distinct proteins from *Methylophilus methylotrophus* which they call cytochromes c_L and C_H [29]. The cytochrome c_H proves (Ambler, R.P., unpublished results; quoted in Ref. 30) to be the same sequence class as the well-known *Pseudomonas* cytochrome *c*-551 (Fig. 1), class ID in Table I. This class is sufficiently characteristic and widely distributed to merit a specific name, and I propose that it should be called cytochrome c_8. Santos and Turner [30] have reported an N-terminal sequence from a 15 kDa monohaem protein of unusual spectral properties which they call cytochrome *c″*; there is no haem attachment site in the 44 sequenced residues. No blue copper proteins have yet been reported from this organism.

Methylobacterium extorquens (commonly known as *Pseudomonas* AM1 [31]) produces a 'cytochrome c_L', so called because it possesses an isoelectric point lower than that of the other main cytochrome. The gene for this protein has been cloned and sequenced [32], and codes for a monohaem cytochrome *c* quite different from anything previously reported (Table II). The 'cytochrome c_H' from this organism proves to have a sequence of the mitochondrial cytochrome *c* type (class IB, short cytochrome c_2, Table I; Ambler, unpublished results). The organism also produces two blue copper proteins, amicyanin and pseudoazurin [33], which probably have a role in the periplasm similar to that of cytochromes *c*.

Lidstrom and her associates [34] purified several cytochromes *c* from *Methylomonas* sp. strain A4, and have determined N-terminal sequences for three of them. A cytochrome *c*-552 shows sequence similarity to the *Methylobacterium extorquens* cytochrome c_L [32], but the small cytochrome $c\text{-}554_H$ does not appear to be either a cytochrome c_2 or a cytochrome c_8. A dihaem cytochrome *c*-551 has the sequence up to the first haem quite similar to those of the dihaem cytochromes c_4 from *Azotobacter* and *Pseudomonas* (Fig. 3), but the information available is not sufficient to assure this connexion.

Methylomonas J [35] has been shown to produce several distinct cytochromes *c* [36], and the organism uses two distinct azurins in electron transport [37], one of which was synthesized only during growth on methylamine. Unfortunately, this organism now appears to have been lost, and so it is not possible to study the

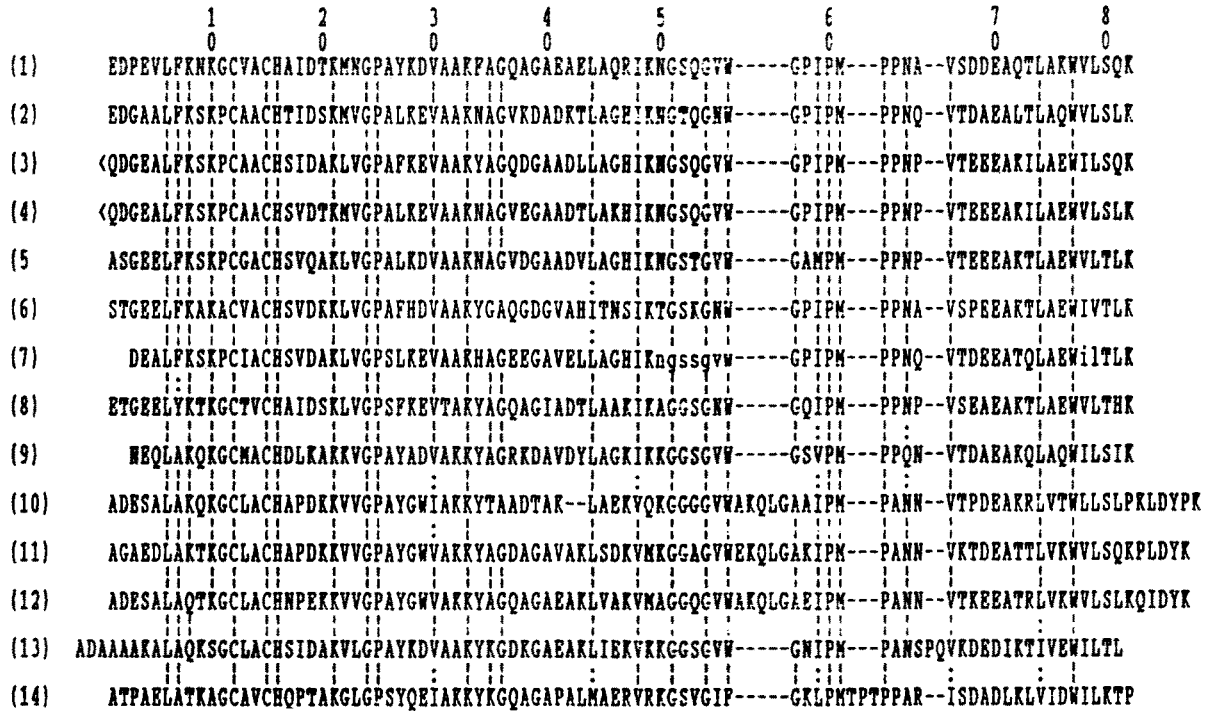

Fig. 3. Amino-acid sequences of cytochromes c_8. (1) *Pseudomonas aeruginosa* cytochrome *c*-551; (2) *Pseudomonas fluorescens* C-18; (3) *Pseudomonas stutzeri* 221; (4) *Pseudomonas stutzeri* 320; (5) *Pseudomonas mendocina* CH-110; (6) *Pseudomonas denitrificans* 9496; (7) *Pseudomonas denitrificans* 10465; (8) *Azotobacter vinelandii* O; (9) *Hydrogenobacter thermophilus*; (10 *Rhodocyclus purpureus*; (11) *Rhodospirillum tenue* 2761; (12) *Rhodospirillum tenue* 3761; (13) *Methylophilus methylotrophus*; (14) *Rhodopseudomonas gelatinosa*. Residues which are identical in the majority of the sequences shown are joined by vertical bars, with colons marking structurally similar residues in such positions. Residues shown in lower case letters are aligned only from peptide compositions. For sequences: (1) see Ref. 53, (3,5,6,7,8) see Ref. 54, (9) see Ref. 55, (11,14) see Ref. 56; (4,10,12,13) are from unpublished results of R.P. Ambler.

cytochromes *c* any further unless another isolate can be recovered.

These observations demonstrate the wide variety of different cytochromes *c* used in the electron transport chains associated with C-1 metabolism, and indicate the caution that must be used in trying to decide the functional equivalents of different components in different organisms.

How many more cytochrome *c* classes?

In Table II are shown the bacterial cytochromes *c* of known sequence which do not appear to fit in to Ambler's 1982 classes [1]. Most of these are proteins larger than the small soluble class I cytochromes, except for the haem subunits of flavocytochromes *c*, which may prove to be class I proteins. There are many membrane-associated cytochromes, including characterized ones similar to mitochondrial cytochrome c_1 from *Rhodobacter capsulatus* [38,39,70], and *Paracoccus denitrificans* [40].

In addition there are small class I proteins that do not closely fit the subclasses recognized in Table I. These include *Thermus thermophilus* cytochrome *c*-552 [41], *Ectothiorhodospira halophila* (Ambler, R.P. and Meyer, T.E., unpublished results) and *Desulfovibrio vulgaris* cytochrome *c*-553 [42,43]. In my opinion, the latter protein is not closely related to the cytochromes c_8, despite the claims and alignment of Nakano et al. [42], as nine insertion or deletion events had to be postulated, and the *Desulfovibrio* protein lacked most of the residues characteristic of the class ID cytochromes c_8, particularly in the C-terminal half of the sequence. This belief has just been confirmed by the reporting of the three-dimensional structure of the protein [44]. The N- and C-terminal segments are α-helices as in other Class I cytochromes *c*, but the distribution of secondary structure elements in the rest of the molecule is different, with the 6th ligand methionine being in a helical region. When aligned with the help of the three-dimensional structure, the sequences show only 28% identity to *Pseudomonas aeruginosa* cytochrome c_8.

Conclusions

Studies of interspecies differences in cytochromes *c* among bacteria show that such differences are large when compared with those among animal species. Thus the difference between *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* cytochromes c_8 is comparable to that between insects and mammals for mitochondrial cytochrome *c* [45], and differences of similar magnitude occur for the cytochromes c_2 among the species of the Rhodospirillaceae [46]. Within a bacterial species, cytochrome sequences are well conserved [47], although species within the same genus vary in the tightness with which their sequences cluster. Thus *Pseudomonas aeruginosa, Rhodospirillum rubrum* and *Rhodopseudomonas sphaeroides* are 'good' species, while isolates of *Pseudomonas fluorescens* biotype C or *Rhodopseudomonas palustris* show considerably more variation in their cytochrome sequences, although the sequences all clearly belong to the same cluster, and distant from other recognized species.

Further representative of the class I subclasses (Table II) continue to be found, and currently make up about half of the new cytochrome *c* sequences that are reported. This high proportion suggests that we have already recognized most of the main classes of small soluble cytochromes *c* that have a wide distribution.

However, about a quarter of the new sequences are not obviously related to any of those known previously, and at least two of them are dihaem cytochromes [48]. This relevant abundance of recent new discoveries is probably a result of the use of new methods; of cytochrome purification that extend to more hydrophobic proteins; of protein sequencing that obtains information from large proteins synthesized at low levels; and from the application of gene sequencing to bacterial cytochromes. Success in this latter approach has generally come through using probes designed from partial experimentally determined amino-acid sequences. Probing with heterologous sequences has not generally been very successful, because of the great interspecies differences, and the possibility of a DNA probe specific for a cytochrome *c* class or sub-class is still only a dream.

Woese [49] believes that by the sole criterion of 16S rRNA sequences he can divide the bacteria into groups, and can arrange these groups as branches of a phylogenetic tree interpretable as representing consistent divergence from a remote common ancestor. Hence, if a characteristic is present in representatives of more than one branch it should be a property that was present in the common ancestor.

Woese puts almost all the Gram-negative bacteria into a group he calls the purple bacteria, divided into four subgroups, (alpha, beta, gamma and delta) with further numerical subdivisions. Cytochrome *c* sequences are known from each of his purple groups, as well as from more diverse organisms, such as the Bacilli, cyanobacteria and the green sulphur bacteria, and the protein is believed to occur even in archaebacteria [50].

The distributions of cytochrome *c* sequence classes and subclasses span Woese's divisions. Thus the class II cytochromes *c* are known from groups alpha, beta and gamma, and cytochromes c_8 (Fig. 3) from groups beta-1, beta-2 and gamma-3. Major bacterial metabolic functions such as photosynthesis, denitrification, methylotrophy and the fixation of dinitrogen are also sporadically distributed among the branches of his tree. If Woese's hypothesis is correct, the 'primitive' trunk

organisms will have had the ability to carry out all these functions, and have possessed separate genes each recognisably ancestral to the genes coding for modern cytochrome classes. Such functions and such genes will need to have been lost from most lines of modern bacteria, and the last $3 \cdot 10^9$ years of bacterial history will have been a tale of regress, not progress. Sonea's alternative hypothesis, that bacterial genomes can continually be reinforced by lateral transfer of genes from anywhere in Gaia's gene-pool [51,52], is to me preferable, particularly when judged with Ockham's Razor.

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