BACTERIAL CYTOCHROMES *C* AND MOLECULAR EVOLUTION

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Abstract

Ambler, R. P. (Dept. of Molecular Biology, University of Edinburgh, Edinburgh EH9 3JR, Scotland) 1974. Bacterial Cytochromes c *and Molecular Evolution. Syst. Zool. 22: 554–565.*—Cytochromes *c* are very widely distributed in bacteria, and many organisms produce several different types of protein. The bacterial cytochromes *c* vary very greatly in size, in number of hemes, in spectrum, in oxidation reduction potential, and in function, and these differences are reflected in great differences in amino acid sequence.

In this paper, the present knowledge of the structure of bacterial cytochromes *c* is reviewed, and some new preliminary results presented. Sequence variation within classes of bacterial cytochromes *c* and relationships between cytochrome *c* classes are considered. The use of amino acid sequence results for the investigation of pre-Cambrian evolution is discussed, and pessimistic conclusions reached.

INTRODUCTION

The accurate study of the structure of proteins is an expensive and tedious process, and should not be undertaken without careful forethought. The determination of the amino acid sequence of yet another new protein requires to be justified by an appreciation of what questions it is likely to answer. The "evolutionary significance of proteins" is not a sufficient reason for the study of species variants of any protein that has been studied from one source. Up to now such work has mainly been done with proteins from higher organisms, of relatively recent divergence, and for most of which there is some evidence for their phylogeny. The extensive investigation of such amino acid sequences as the fibrinopeptides, mitochodrial cytochrome *c* and hemoglobin (Dayhoff, 1972) has answered the simpler questions one asks about the "evolutionary significance of proteins in higher organisms." The great attraction of the methods of molecular systematics are now the possibilities of applying them to problems for which paleontological methods are inapplicable, such as prokaryote phylogeny, the origin of organelles or the relationship of kingdoms to each other.

No serious discrepancies have been found yet between classically-based and sequence-based phylogenies for higher organisms, despite the very small proportion of the genome that is considered during the study of a single protein. This observed agreement is not proof that sequence methods will be able to be used reliably for phylogeny in the uncharted territory of much older and simpler organisms. We are profoundly ignorant about the relative importance of selection and chance in protein variation, and not enough is known about gene-transfer among simple organisms in natural environments. In the time that has elapsed since prokaryotes first evolved, events that are so rare as to be undemonstrable by experiment in our time could have had time to occur again and again. It seems possible that we may find there is no simple natural classification for prokaryotes based on their evolution, as there may have been so much gene-transfer between different genera that phylogenetic trees based on different genes (or even parts of genes) will be topologically distinct.

BACTERIAL CYTOCHROMES *C*

Cytochromes *c* are attractive proteins to use for phylogenetic study, since they are very widely distributed, and often present at high concentrations. They are colored which makes them easy to purify, and small which makes chemical characterization relatively simple. As they are critically

important parts of the respiratory systems, interacting with several other macromolecular systems, it is likely that any alterations to them have to be compatible with a large part of the phenotype of the organism.

Cytochromes of all the main classes have been found in bacteria, and their occurrence and properties have recently been extensively reviewed (Lemberg and Barrett, 1973). The very earliest studies (quoted in Keilin, 1966) showed that the absorption bands of prokaryotic cytochromes are often at different wavelengths to the bands in eukaryotes, and subsequent biochemical investigations have shown very great variations in the properties of cytochromes from different organisms. It is possible that they are polyphyletic, and in any case the erratic distribution of particular cytochromes is hard to interpret simply.

Protein chemical studies have largely been limited to the smaller soluble cytochromes. The amino acid sequence of one soluble *b*-type cytochrome has been determined (*Escherichia coli* cytochrome *b*-562; Itagaki and Hager, 1968), but most of the results have come from the various *c*-type cytochromes.

Mitochondrial cytochrome *c* has similar properties, structure and function whether it comes from a mammal or a lower plant. There is no such uniformity in the bacterial cytochromes *c*. They differ greatly in size, in number of prosthetic groups, in spectrum, in oxidation reduction potential, and in function, and these differences are reflected in great differences in amino acid sequence.

Bacterial cytochromes *c* are either designated by a numerical subscript (e.g., cytochrome c_2) or by their source and α-band wavelength (e.g., *Pseudomonas* cytochrome *c*-551). Cytochromes *c'* (Kennel et al., 1972) are proteins in which the heme iron is in a high-spin ligand field, although with the heme covalently attached to the protein in the normal cytochrome *c* way. The present system of nomenclature (Enzyme Nomenclature Recommendations, 1965) is confusing, and is being revised.

DISTRIBUTION AND STRUCTURE OF BACTERIAL CYTOCHROMES *c*

Many bacteria contain several types of cytochrome *c*, and since the proteins are easy to observe, and often easy to isolate, they have attracted a great deal of study. The many papers published on the subject are difficult to interpret since the many different proteins studied have been difficult to classify and to relate to each other. Up until now, they have been classified on the basis of their spectra, distribution, oxidation reduction potential, isoelectric point and size. These methods have proved adequate for the cytochromes of distinctive properties such as those containing high spin iron (cytochromes *c'*) or the very low oxidation reduction potential multiheme cytochromes c_3, but have been unable to discriminate between the many more typical cytochromes. Yamanaka and Okunuki (1964; Yamanaka, 1972) have attempted to study the evolution and relatedness of cytochromes *c* (both from prokaryotes and eukaryotes) by investigating their relative reactivities with different cytochrome oxidases, but the results are difficult to interpret. Despite the large amounts of cytochrome often synthesized, in few cases is the precise function of the protein known, and our ignorance is almost complete about the relative functions in cells that contain several distinct cytochromes of similar oxidation reduction potential. Several of the cytochromes are believed to act as penultimate components of the respiratory chains, passing on electrons to the cytochrome oxidase in aerobic respiration, or to the reductase in nitrate or sulfate respiration.

The determination of amino acid sequences of bacterial cytochromes *c* will both make a rational classification of these proteins possible and give information about the evolutionary history of the organisms that contain them. It is likely that in the next few years the number of classes of cytochromes *c* that exist will be delimited, and it will become possible to deduce phylogenetic trees that include

TABLE 1. PROPERTIES OF C-TYPE CYTOCHROMES FROM PROKARYOTES AND EUKARYOTES.

Complete or partial sequence information is available for cytochromes above the line. For cytochromes below the line, only amino acid compositions are known. References are given in the text.

Cytochrome	α-band max.	Source	Residues	Hemes	E'_0 (pH 7)	Isoelectric point
c_2	550	Athiorhodaceae	112	1	+280–+330	6–8
c_3	552	*Desulfovibrio*	102–110	4	—200	5–11
c_4	552	Pseudomonads *Azotobacter*	≃200	2	+300	≃5
c_5	556	Pseudomonads *Azotobacter*	87 ??	1	+320	≃5
c_7	551.5	"Chloropseudomonas"	68	3	—200	basic
c-551		*Azotobacter* denitrifying Pseudomonads	82	1	+280	≃4.5
c-555		"Chloropseudomonas" *Chlorobium*	86–99	1	+140	5–10
c-553		*Desulfovibrio*	82	1	—100–0	8.6
f	552–555	algal chloroplasts	83–87	1	+350	≃4
c	550–558	mitochondria	102–114	1	+255	11
c′	–	photosynthetic bacteria *Alcaligines* (one strain)	127	1	+130	6–10
c_1	553	mitochondria	400 ?	1	+225	acidic
c-550		*Spirillum itersonii*	94	1	+300	9.9
c-550		*Thiobacillus novellus*	118	1	+280	7.5
c-550		*Micrococcus denitrificans*	135	1	+250	acidic
c-550		*Bacillus licheniformis*	≃90	1	(?+250)	acidic

widely different organisms for each class of cytochrome.

The general properties of bacterial cytochromes for which there is already amino acid sequence information are summarized in Table 1, which also includes a few cytochromes for which sequence information is likely soon. Results of structural investigations are discussed individually in the following sections.

Cytochrome c_2

Cytochrome c_2 from *Rhodospirillum rubrum* was the first bacterial cytochrome purified (Vernon and Kamen, 1954), and the first to have its amino acid sequence investigated (Paleus and Tuppy, 1959). The complete amino acid sequence has now been determined (Dus et al., 1968), and appreciable similarity to the sequence of mitochondrial cytochrome *c* recognized. Physically similar proteins are known in all other purple non-sulfur photosynthetic bacteria (the Athiorhodaceae) that have been examined, but are not present in other photosynthetic bacteria (Meyer, 1970). Despite considerable effort, it has not yet been possible to prove whether the cytochrome functions in respiration or photosynthesis or in both. X-ray crystallographic analysis (Salemme et al., 1973) has shown that the general folding of the polypeptide chain is very similar to that of mitochondrial cytochrome *c*, and in particular that they both contain a short length of α-helix at the N-terminus.

Cytochrome c_3

This protein was discovered by Postgate (1956) in sulfate-reducing bacteria of the genus *Desulfovibrio*. Although of very similar molecular weight to mitochondrial

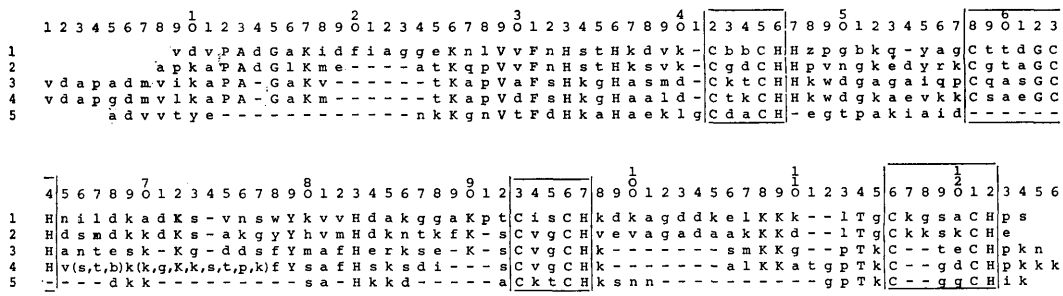

FIG. 1.—Amino Acid Sequences of Cytochrome c_3 and c_7 1, *Desulfovibrio gigas* NCIB 9332; 2, *D. vulgaris* NCIB 8303 (strain Hildenborough); 3, *D. desulfuricans* NCIB 8380 (strain El Agheila Z); 4, *D. salexigens* NCIB 8403 (strain British Guiana); 5, "Chloropseudomonas ethylica". Sequence no. 4 (*D. salexigens*) is tentative and incomplete (R. P. Ambler, M. Bruschi and J. Le Gall, unpublished observations). The one-letter notation used is that recommended by the IUPAC-IUB Commission on Biochemical Nomenclature (*Biochem. J.* (1969) 113:1–4). The heme-binding sites are boxed. Residues common to all sequences are shown in capitals. This choice is somewhat subjective, as deletions can be juggled to improve the apparent match.

cytochrome *c*, it has four heme groups attached to each polypeptide chain, and has an extremely low oxidation reduction potential. The genus *Desulfovibrio* is unusual in the wide range in the G + C content in the DNA of its constituent species (Postgate and Campbell, 1966), and this diversity is reflected in the great variation in the amino acid sequences of the cytochromes c_3 from different species (Fig. 1; Ambler, 1968; Ambler et al., 1969, 1971; R. P. Ambler, M. Bruschi and J. Le Gall, unpublished results).

Two soluble cytochromes *c* can be isolated in large amounts from the green photosynthetic bacterium "Chloropseudomonas ethylica" (Olson and Shaw, 1969). One of these (cytochrome *c*-551.5) has a high heme content and a very low oxidation reduction potential. Amino acid sequence determination (Ambler, 1971) has shown it to be a very small protein (68 residues) with three attached heme groups, making it the first representative of a new type of cytochrome *c* molecule for which the name cytochrome c_7 has been proposed. The sequence shows some similarity to those of the four-heme cytochromes c_3 (Fig. 1). Recent work (Gray et al., 1972) has shown that "Chloropseudomonas ethylica" is not a single organism, but a close association of a photosynthetic *Chlorobium* and a sulfate-reducer. The second *c*-type cytochrome (cytochrome *c*-555) from the system has also been investigated, with the results that are discussed in a following section.

Desulfovibrio cytochrome c-553

Desulfovibriones contain several cytochromes *c* besides cytochrome c_3. One of these is cytochrome *c*-553, which is a small monoheme protein with an oxidation reduction potential near zero. The sequence of the protein from *D. vulgaris* has been determined (Bruschi and Le Gall, 1972).

Cytochromes c_4, c_5 and c-551

These acidic proteins are found in *Azotobacter vinelandii* (Swank and Burris, 1969) and in some pseudomonads. The equivalence of the proteins from the different sources has only been recognized recently.

Cytochrome c_4 is a diheme protein with a molecular weight slightly greater than 20 000. The N-terminal sequences of the proteins from *A. vinelandii* and from three species of *Pseudomonas* have been examined (Ambler and Murray, 1973; Fig. 2). Kodama and Shidara (1969) showed that in *Ps. stutzeri* this cytochrome (which they called cytochrome *c*-552-II) was in the particulate fraction. Cytochromes c_4 have heme groups bound to two different

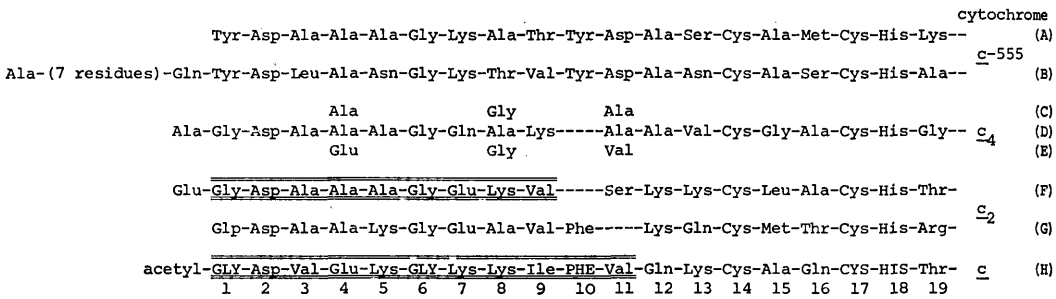

FIG. 2.—The N-terminal Segments of some Cytochromes c.

The sequences shown are: cytochromes *c*-555 from (A) *Chlorobium thiosulfatophilum,* (B) "Chloropseudomonas ethylica"; cytochromes c_4 from (C) *Azotobacter vinelandii,* (D) *Pseudomonas aeruginosa,* (E) *Pseudomonas stutzeri* and *mendocina;* cytochromes c_2 from (F) *Rhodospirillum rubrum,* (G) tentative sequence of *Rhodopseudomonas palustris* strain 2.1.6; (H) cytochrome *c* from horse. The residues marked xxx are believed to fold into an α-helix in the crystalline proteins. The "invariant" residues in mitochondrial cytochrome *c* are shown in capitals in sequence (H). References are given in the text. The abbreviation Glp represents pyrrolidone carboxylic acid.

sequences in the polypeptide chain, since two different cysteine/histidine peptides have been isolated from them after heme removal and tryptic digestion.

Cytochrome c_5 is a smaller monoheme protein. Different preparations have different molecular weights, and exposure to even mildly acid pH during preparation appears to produce heterogeneity and degradation (Swank and Burris, 1969). The most consistent preparations have been obtained from *Ps. mendocina,* and the sequence of an 87-residue cytochrome has been determined (Ambler and Taylor, 1973), although even this showed N-terminal heterogeneity. It seems possible that the cells contain an acid protease to which the cytochrome precursor is very susceptible. The size of the precursor is not known, and it is possible that cytochrome c_5 might really be a fragment of a large diheme cytochrome *c* such as the cytochrome *c* peroxidase (Lenhoff and Kaplan, 1956; Ellfolk and Soininen, 1970). Cytochromes c_5 have been identified but not fully purified in *A. vinelandii* and in

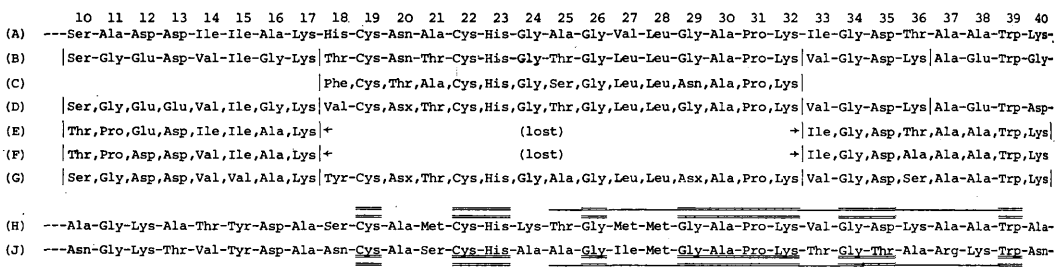

FIG. 3.—Comparison of the Sequences around the Heme Binding Sites of Cytochromes c_5 and Cytochromes *c*-555

The sequences shown are: cytochromes c_5 from (A) *Pseudomonas mendocina,* (B) *Ps. aeruginosa,* (C) *Ps. stutzeri,* (D) *Ps. denitrificans* NCIB 9496, (E) *Ps. fluorescens* biotype B, (F) *Ps. fluorescens* biotype D, (G) *Azotobacter vinelandii*; cytochromes *c*-555 from (H) *Chlorobium thiosulfatophilum,* (J) "Chloropseudomonas ethylica". For sequences (C)–(G) the only evidence is the amino acid composition and N-terminal analysis of the tryptic peptides shown. Residues in the cytochromes *c*-555 that are considered to match with cytochrome c_5 sequences are marked xxx, and residues that are considered to match very well are marked xxx.

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    1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3.4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5
1   E.D.P.E.V.L.F.K.N.K.G.C.V.A.C.H.A.I.D.T.K.M.V.G.P.A.Y.K.D.V.A.A.K.F.A.G.Q.A.G.A.E.A.E.L.A.Q.R.I.K.N.G.S.Q.G.V.
2   E.D.G.A.A.  F.  S.  P.  A.A.    T.I.D.S.  M.      A.L.K.E.  A.    N.A.G.V.K.D.A.D.K.T.L.A.G.H.    N.  T.Q.  V.
3   Q.D.G.E.A.  F.  S.  P.  A.A.    S.I.D.A.  L.      A.F.K.E.  A.    Y.A.G.Q.D.G.A.A.D.L.L.A.G.H.    N.  S.Q.  V.
4   A.S.G.E.E.  F.  S.  P.  G.A.    S.V.Q.A.  L.      A.L.K.D.  A.    N.A.G.V.D.G.A.A.D.V.L.A.G.H.    N.  S.T.  V.
5   S.T.G.E.E.  F.  A.  A.  V.A.    S.V.D.K.  L.      A.F.H.D.  A.    Y.G.A.Q.G.D.G.V.A.H.I.T.N.S.    T.  S.K.  N.
6   -.-.d.e.a.  f.  s.  p.  i.a.    s.v.d.a.  l.      s.l.k.e.  a.    h.a.g.g.v.e.a.e.e.l.l.a.g.h.    n.  s.s.  v.
7   E.T.G.E.E.  Y.  T.  G.  T.V.    A.I.D.S.  L.      S.F.K.E.  T.    Y.A.G.Q.A.G.I.A.D.T.L.A.A.K.    A.  G.S.  N.

    6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2
1   .W.G.P.I.P.M.P.P.N.A.V.S.D.D.E.A.Q.T.L.A.K.W.V.L.S.Q.K.   Pseudomonas aeruginosa NCTC 10332 (neotype)
2   .   P.I.      Q.  T.D.A.    L.T.    Q.  V.L.S.L.          fluorescens C-18 (ATCC 17588)
3   .   P.I.      P.  T.E.E.    K.I     E.  I.L.S.Q.          stutzeri 221 (ATCC 17400)
4   .   A.M.      P.  T.E.E.    K.T.    E.  V.L.T.L.          mendocina CH-110
5   .   P.I.      A.  S.P.E.    K.T.    E.  I.V.T.L.          denitrificans NCIB 9496 (ATCC 13867)
6   .   p.i.      a.  s.e.e.    q.t.    e.  v.l.t.l.                        NCIB 10465 (ATCC 19244)
7   .   Q.I.      P.  S.E.A.    K.T.    E.  V.L.T.H.      Azotobacter vinelandii O (NCIB 8789, ATCC 12837)
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FIG. 4.—Amino Acid Sequences of Cytochrome *c*-551 from Different *Pseudomonas* Species and from *Azotobacter vinelandii*. Sequence 6 (*Ps. denitrificans* NCIB 10465) is tentative and incomplete, with several segments aligned by homology with the other sequences, and so is shown in lower case. The one-letter notation used is that recommended by the IUPAC-IUB Commission on Biochemical Nomenclature (Biochem. J. (1969) 113:1–4). Blank positions indicate that the amino acid is identical to that in sequence 1.

several other pseudomonads, and the protein seems to have a wider distribution than cytochromes c_4 or *c*-551. Preliminary sequence investigations have shown all the cytochromes c_5 to be similar in sequence around the heme attachment site (Fig. 3), and all to contain a characteristic very acidic sequence containing two additional cyst(e)ine residues.

Pseudomonas cytochrome *c*-551 is an 82-residue monoheme protein that is also found in *A. vinelandii* (where it is probably identical to the cytochrome c_4-minor of Swank and Burris, 1969). *A. vinelandii* is the only organism in which it has yet been found which does not denitrify. The sequence of the protein from four different *Pseudomonas* species has been published (Ambler and Wynn, 1973), and the sequences from two further pseudomonads and from *A. vinelandii* are nearly complete (Fig. 4). The variation in the sequence of the protein between different isolates of two different species of *Pseudomonas* has also been investigated (Ambler, 1974).

Chlorobium cytochrome c-555

Green photosynthetic bacteria produce large amounts of a small monoheme cytochrome (Gibson, 1961). The amino acid sequences of the proteins from *C. thiosulfatophilum* and "Chloropseudomonas ethylica" (see above) have been determined (Van Beeumen and Ambler, 1973).

Cytochrome c′

These proteins (Kennel et al., 1972) are found in many photosynthetic bacteria, and in a single species of *Alcaligines*[1] (Suzuki and Iwasaki, 1962). They have a bewildering range of pseudonyms (e.g., RHP, cytochromoid, cryptocytochrome *c*). They differ from all other well characterized cytochromes *c* in that the heme iron is in a high spin ligand field. They are monoheme proteins containing about 130 amino acid residues. The sequence of the *Alcaligines* protein (Fig. 5) has been published (Ambler, 1973), and sequences of several cytochromes *c′* from photosynthetic bacteria will be reported soon (T. E. Meyer, R. P. Ambler and M. D. Kamen, in preparation).

[1] This organism (NCIB 11015) was formerly known as *Pseudomonas denitrificans*. The name-group *Ps. denitrificans* includes a very heterogeneous collection of organisms (see NCIB 9496 and 10465 in Fig. 4, and NCIB 8376, Ambler 1973a). Any strain that has been given this name should be worth looking at.

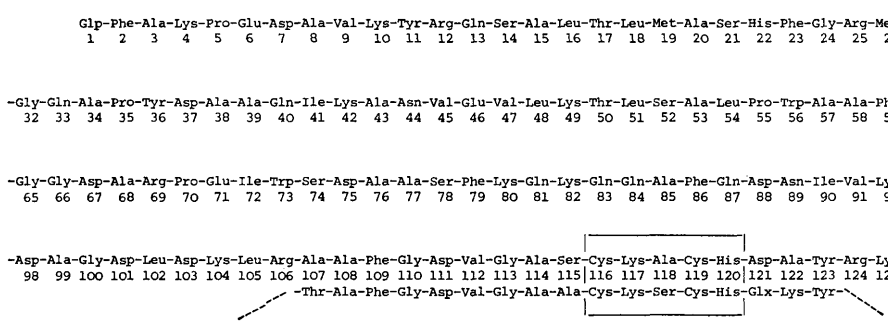

FIG. 5.—Amino Acid Sequence of Cytochrome *c'* from *Alcaligines*, strain NCIB 11015 (Ambler, 1973b). The heme binding sequence from the cytochrome *c'* from *Chromatium vinosum* (Kennel et al., 1972) is also shown. The heme binding sites are boxed.

The *Alcaligines* strain (NCIB 11015) produces an azurin (Suzuki and Iwasaki, 1962) which is clearly homologous to the azurins from pseudomonads and other *Alcaligines* (Ambler, 1972). Azurins are electron-transfer copper proteins. They have not been found in any photosynthetic bacteria.

Other bacterial cytochromes c

Many bacteria contain small monoheme cytochromes *c* that are generally similar in α-band maximum position and oxidation reduction potential to mitochondrial cytochrome *c*, cytochrome c_2 and *Pseudomonas* cytochrome *c*-551. These include *Spirillum itersonii* (Clark-Walker and Lascelles, 1970), various *Thiobacilli* (Yamanaka et al., 1971), *Micrococci* (Scholes et al., 1971) and *Bacilli*. Properties of some of these cytochromes are shown in Table 1. Many of the organisms, including *Bacillus licheniformis*, are denitrifiers. At present amino acid compositions but no sequence information are available for these proteins. When the latter becomes available, it is likely that it will be possible to allocate some of these proteins to known cytochrome classes.

Cytochromes of very low oxidation reduction potential are known from photosynthetic bacteria (e.g., *Rhodopseudomonas* cytochrome *c*-551.5; Meyer, 1970) and from blue-green algae (Holton and Myers, 1967). They appear to be small monoheme proteins, and so cannot be classed with cytochromes c_3.

Many organisms contain cytochromes with α-band maxima in the 553–555 nm region (Lemberg and Barrett, 1973). These cytochromes are generally minor components, and few have been fully characterized yet.

Several large and complex proteins that contain heme *c* as well as other prosthetic groups have been identified. This group includes *Ps. aeruginosa* cytochrome *cd* (= cytochrome oxidase, = nitrite reductase; Yamanaka and Okunuki, 1963; Nagata et al., 1970) and the flavocytochromes from *Chlorobium* and *Chromatium* (Bartsch et al., 1968). Cytochromes *c* peroxidase from bacteria are large diheme cytochromes *c* (Lenhoff and Kaplan, 1956; Ellfolk and Soininen, 1970), and may be related to the widely distributed "split-α" cytochromes (*Ps. aeruginosa;* Singh and Wharton, 1973: *Alcaligines faecalis;* Iwasaki and Matsubara, 1971).

SEQUENCE VARIATION WITHIN CLASSES OF BACTERIAL CYTOCHROMES *c*

The different groups of denitrifying pseudomonads have been very well characterized phenotypically (Stanier et al., 1966; Palleroni et al., 1970) and by DNA hybridization (Palleroni et al., 1972). The cytochromes *c* from many of these strains have been studied (Ambler and Wynn, 1973; Ambler, 1974; R. P. Ambler and E.

Taylor, unpublished results). The sequence differences between proteins from closely related species are in the range of 20–40%, and it is interesting to note that by this single genetic criterion, *A. vinelandii* is as good a *Pseudomonas* as any of the others (Fig. 4). These differences are very large by the standards of mitochondrial cytochrome *c*, being comparable to differences observed between phyla in the animal kingdom.

Strains of *Ps. aeruginosa* isolated from very different geographical and ecological situations have cytochromes *c*-551 that are in nine out of the ten examined cases identical (Ambler, 1974; the tenth protein has a single amino acid replacement). This finding can be interpreted as evidence that neutral mutations do not play a large role in the evolution of these proteins. The cytochromes *c*-551 within other species show more variation (*Ps. fluorescens* biotype C; Ambler, 1974: *Ps. stutzeri*; R. P. Ambler and E. Taylor, unpublished observations). These species are not as phenotypically homogeneous as *Ps. aeruginosa*, and the sequence differences show a tendency to conform to the phenotypic variation.

On the basis of the present preliminary information, cytochromes c_5 (Fig. 3) appear to be about as varied as the cytochromes *c*-551, while the cytochromes c_4 are less variable (Fig. 2).

The cytochromes c_3 from different *Desulfovibriones* show some similarity with each other (Fig. 1), particularly in the location of the four heme binding sites. To get any matching in the regions in between, many insertions and deletions must be made, and so it is not possible to quantitate the differences between the sequences. The four sequences shown in Fig. 1 are from representative strains of four out of the five species into which Postgate and Campbell (1966) divided the genus on phenotypic grounds. It will be interesting to see if proteins of intermediate sequence are produced by any of the numerous other strains that are available. Immunological methods have already been used to demonstrate the great variability of these proteins (Drucker et al., 1970), and ought to provide a very quick and satisfactory method for screening for such intermediates.

Only preliminary information is yet available for cytochromes c_2 from organisms other than *R. rubrum* (S. Murray and T. E. Meyer, personal communication). The proteins from three different strains of *Rps. palustris* differ from each other appreciably in amino acid composition, but the differences between each of them and the *R. rubrum* protein is about twice as great. Preliminary sequence studies suggest that the differences between the *R. rubrum* and the *Rps. palustris* cytochromes c_2 may be comparable to the differences in mitochondrial cytochromes *c* of organisms from different kingdoms.

The sequences of the two cytochromes *c*-555 from green photosynthetic bacteria (Van Beeumen and Ambler, 1973) show great similarities along long segments of the sequences, but other segments are quite different. The similarities are sufficiently great to indicate a close relationship between *Chlorobium thiosulfatophilum* and "Chloropseudomonas ethylica," so supporting the findings of Gray et al. (1972) on the nature of the latter "organism."

The sequences of cytochromes *c'* from photosynthetic bacteria (T. E. Meyer, R. P. Ambler and M. D. Kamen, in preparation) show clear homology with the sequence of the protein from *Alcaligines* (Fig. 5; Ambler, 1973), and very few insertions or deletions need to be postulated in any of these sequences to obtain a good match. All are monoheme proteins (Kennel et al., 1972), and in all the heme is bound near to the C-terminus of the polypeptide chain. The sequence of the heme binding site in *Chromatium vinosum* cytochrome *c'* (Fig. 5; Kennel et al., 1972) is very similar to that in *Alcaligines*, and quite different to that formerly postulated (Dus et al., 1962).

It will be seen that bacterial cytochromes from different but related sources show

much larger differences than are met with in eukaryotes. It is therefore essential that workers in this field should use well-defined strains of microorganisms and to ensure that these strains are deposited in culture collections.

RELATIONSHIPS BETWEEN CLASSES OF BACTERIAL CYTOCHROMES *c*

Many reviewers tacitly assume a common evolutionary origin for prokaryotic and eukaryotic cytochromes *c*. This is probably due to the clear homology that can be seen between the mitochondrial cytochromes *c* of even the most diverse eukaryotes, and a lack of appreciation of the very great biochemical diversity of prokaryotes. Some of the bacterial proteins (e.g., cytochromes *c'* and c_3) differ so greatly in structure and properties from mitochondrial cytochrome *c* that convergence is a much more likely explanation for the common -Cys-X-Y-Cys-His- heme binding site than divergence. If it is accepted that such sequences have evolved on three independent occasions, then the possibility that some of the more "normal" bacterial cytochromes have evolved separately must be considered.

The amino acid sequence and tertiary structure of *R. rubrum* cytochrome c_2 appear to show so much similarity to mitochondrial cytochrome *c* that homology between the two proteins must be accepted. Several attempts have been made to relate the sequence of *Pseudomonas* cytochrome *c*-551 to mitochondrial cytochrome *c* (McLachlan, 1971; Dayhoff, 1972), but the evidence is not overwhelming. It will be interesting to see if more advanced statistical methods can be devised using the sets of cytochrome *c*-551 sequences now available to obtain stronger evidence for or against homology, and tertiary structure studies may also soon help to resolve the problem. None of the more recently determined bacterial cytochrome *c* sequences show strong resemblances to mitochondrial cytochrome *c* (though see *Chlorobium* cytochrome *c*-555 in Fig. 2).

When similarities between different

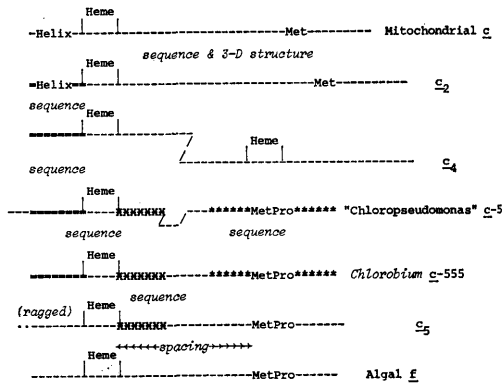

FIG. 6.—"Homologies" between Bacterial *c*-type Cytochromes. Summary of structural similarities between bacterial, chloroplast and mitochondrial cytochromes *c*. Details of the sequence similarities are given in Figs. 2 and 3, and in the text. ----, xxxx, and **** indicate segments of sequence from different classes of protein which show notable similarity.

classes of bacterial cytochromes are looked for, some confusing results are obtained (Figs. 2, 4 and 7). Segments of cytochromes from different classes appear to show a match, while no similarity at all can be seen between other parts of the same sequences. Segments of two different cytochromes present in the same organism (cytochromes c_4 and c_5) match with different parts of a single protein from another organism (*C. thiosulfatophilum* cytochrome *c*-555). The apparent matching of N-terminal regions of various cytochromes (Fig. 2) is particularly interesting since tertiary structure studies have shown that in the *R. rubrum* cytochrome c_2, residues 2–10 form a stretch of α-helix (Salemme et al., 1973) in a position relative to the heme very similar to the N-terminal α-helix in mitochondrial cytochromes *c* (see Dayhoff, 1972), even though the sequence similarity in this region between mitochondrial cytochrome *c* and cytochrome c_2 is not very great. The matching between the segments immediately after the heme attachment sites of cytochromes c_5 and *Chlorobium* cytochrome *c*-555 (Fig. 4) is remarkably good, though here again no similarities have been seen in the rest of the molecules.

In addition to mitochondrial cytochrome *c*, eukaryotes also contain mitochondrial cytochrome c_1 and chloroplast cytochrome *f* ($= c_6$) (see Lemberg and Barrett, 1973). The sequences of some bacterial cytochromes *c* may relate to these proteins better than to the main cytochrome *c*. Nothing except the amino acid composition of a large heme peptide has been published for cytochrome c_1 (Wada et al., 1968), but the sequence of at least three algal cytochromes *f* is known (*Monochrysis lutheri;* Laycock, 1972: *Euglena gracilis* and *Porphyra tenera;* G. W. Pettigrew, R. P. Ambler, T. E. Meyer and R. G. Bartsch, unpublished observations). The three cytochromes *f* are like *Pseudomonas* cytochrome *c*-551, *Chlorobium* cytochrome *c*-555 and cytochrome c_5 in having a -Met-Pro- sequence in a comparable position to the -Met-Pro- shown to be the sixth iron ligand methionine in *Ps. aeruginosa* cytochrome *c*-551 (Fanger et al., 1967), and it seems likely that further study (perhaps of cytochromes from blue green algae) will show a clear link between cytochrome *f* and some of the bacterial proteins.

Both the erratic distribution of bacterial cytochromes (e.g., the occurrence of cytochrome *c'* in *Alcaligines* and purple photosynthetic bacteria but nowhere else, or the close similarity of the *Azotobacter* and *Pseudomonas* cytochromes) and the appearance of matching segments in otherwise different sequences (Fig. 6) suggest that the course of prokaryote evolution has not been the simple dendritic divergence that is normally considered to have taken place in higher organisms. Gene transfer between distantly related organisms, such as the acquisition of a block of electron-transfer proteins by a proto-*Azotobacter* from a *Pseudomonas* ancestor could result in phylogenetic trees for different parts of the genome having quite different topologies. Unequal crossing over may mean that even different segments of the same protein may have evolved in response to different conditions. If the study of further bacterial protein sequences proves such hypotheses, it would mean that there will not be any simple natural classification for microorganisms, and that molecular methods based on now existing organisms may not be able to elucidate even the most general features of pre-Cambrian evolution.

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