The Evolutionary Stability of Cytochrome *c*-551 in *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* Biotype C

By R. P. AMBLER

Department of Molecular Biology, University of Edinburgh,
Edinburgh EH9 3JR, U.K.

(Received 10 July 1973)

Cytochrome *c*-551 was prepared from nine different strains of *Pseudomonas aeruginosa* and six of *Pseudomonas fluorescens* biotype C, and their amino acid sequences were compared with the sequences previously determined for the cytochromes of type strains of each species. The standard of sequence examination was such that all single amino acid substitutions, deletions or insertions ought to have been detected. Balanced double changes in sites in the same part of the sequence might have escaped detection. The standard of some of the quantitative amino acid analyses was not as high as would be required for the investigation of completely unknown sequences. Eight of the *Ps. aeruginosa* sequences could not be distinguished from the type sequence, whereas the ninth had a single amino acid substitution. The sequences from *Ps. fluorescens* biotype C were more varied, differing in from zero to four substitutions from the type sequence, with the most diverse sequences differing in seven positions. The results for *Ps. aeruginosa* are interpreted as evidence that neutral mutations are not responsible for much molecular evolution. The superficially paradoxical differences in the results for the two species are discussed.

Homologous proteins from different eukaryotic species differ in amino acid sequence, and for a particular protein, the amount of difference appears to be related to the evolutionary distance between the species (Dayhoff, 1972). Within species there is extensive protein polymorphism (Lewontin & Hubby, 1966; Harris, 1966). The functional significance of inter- and intra-species protein variation is in dispute. For a given organism in a given environment, the effect of a point mutation can be beneficial, harmful, or so slight as to have no selective effect. Kimura (1968) and King & Jukes (1969) have argued that a considerable proportion of evolutionarily accepted mutations are selectively neutral, and that much of the difference that is seen between, for example, the mitochondrial cytochromes *c* of eukaryotes (Dayhoff, 1972) is functionally irrelevant. In contrast, selectionists argue that neutral mutations are rare, and that most protein-sequence differences that are observed can result in phenotypic differences on which natural selection can act.

The workings of eukaryotic and prokaryotic cells are very similar at the level of protein function, so if it could be shown that selection can have a particular effect on protein variation in the simpler organisms, it is extremely likely that such an effect would be general.

Different patterns of intraspecies protein variation in prokaryotes are predicted by the neutralist and the selectionist theories. If many of the amino acid interchanges that can occur in a particular protein have no effect on the functioning of the organism, one would predict that natural populations would show very marked polymorphism, with amino acid alterations occurring at many different sites, and with little concordance between the variations at the different sites. One might also expect that many of the alterations would be between amino acids with similar side chains, such as valine and isoleucine or serine and threonine, although 'similarity' in this context is subjective. If most of the changes that occur can affect the functioning of the organism, one would expect less polymorphism, and concordance both between sets of amino acid alterations within the molecule and between particular alterations and other phenotypic characters.

Relatively little is known about inter- and intra-species protein variation in prokaryotes, and the situation is complicated by the lack of clear criteria for defining species. Immunological (Kauffmann, 1972) and chemical (McDonough, 1965) methods have shown very considerable variation in the flagellar protein of otherwise similar *Salmonella* organisms. Enzymological surveys have recognized a clear dimorphism in the penicillinase from different

Table 1. *Strains of Pseudomonas aeruginosa and Pseudomonas fluorescens used in the investigation*

Abbreviations: N.C.P.P.B. National Collection of Plant Pathogenic Bacteria, Harpenden, Herts., U.K.; N.C.T.C., National Collection of Type Cultures, Colindale, London NW9 5HT, U.K.; N.C.I.B., National Collection of Industrial Bacteria, Aberdeen AB9 8DG, U.K.; W.U.R.D.D., Western Utilization Research and Development Division, U.S. Department of Agriculture, Albany, Calif., U.S.A.

(a) *Ps. aeruginosa strains*

Strain no.	Obtained from	Culture collection		Accession from	Synonyms	Isolated by	Date isolated	Remarks (including source where known)
		Accession date						
54	R. Y. Stanier		W. C. Haynes	A.T.C.C. 7700 N.C.T.C. 7244				Isolated as <i>Ps. vendrelli</i> ; Puerto Rico, shallow well
129	J. E. Perez	1961	A. C. Monllor	N.C.P.P.B. 1224		A. C. Monllor	1961	Isolated as <i>Ps. polycolor</i> ; Puerto Rico, on <i>Aglaonema commutatum</i>
292	N.C.P.P.B.	1951	E. Hellmers	PP2		W.H.	1925	Isolated as <i>Ps. polycolor</i> ; Philippines, on <i>Nicotiana tabacum</i>
950	N.C.T.C.	1921	Parke Davis Co. U.S.A.	A.T.C.C. 19305 0.45 N.C.I.B. 950 B.U.C.S.A.V. 282		Burkholder		Source unknown
1224	N.C.P.P.B.	1962	J. E. Perez	PR129B		A. C. Monllor	1961	(see strain 129 above)
1896	N.C.P.P.B.	1966	C. F. Robbins	PP122		C. F. Robbins	1958	Isolated as <i>Ps. polycolor</i> ; Brazil, on <i>Allium</i> sp.
2000	N.C.T.C.	1924	-, Eyre	Eyre-I A.T.C.C. 14149		-, Eyre	1923	London, human pus
2195	N.C.P.P.B.	1968	D. Gandy	B-231		D. Gandy	1968	Sussex, U.K., on cultivated mushroom, interstipe tissue, suspected mummy disease
6009	S. Brenner	1958	N. O. Kaplan	P6009/1				Received as <i>Ps. fluorescens</i> (Lenhoff & Kaplan, 1956); <i>Ps. aeruginosa</i> cytochrome c-551 type sequence; C. Lamanna → N. O. Kaplan → S. Brenner, source unknown
8704	N.C.I.B.	1955	J. Senez	A.T.C.C. 10145				Marseille, France, from soil (hydrocarbon enrichment)
10332	N.C.T.C.	1966	O. Lysenko	N.C.I.B. 8295 C.C.E.B. 481				Neotype; source unknown; Merck, Sharpe and Dohme → F. Kavanagh → A.T.C.C. (in 1952)

(b) *Ps. fluorescens biotype C strains*

18	R. Y. Stanier		W.U.R.D.D.	P-7 A.T.C.C. 17400				From hen's egg; psychrophilic; Ralston group I*; <i>Ps. fluorescens</i> biotype C cytochrome c-551 type sequence
50	R. Y. Stanier			P3T-3 A.T.C.C. 17427		R. Y. Stanier		Berkeley, Calif; L-tryptophan enrichment of water sample; Ralston group I*
181	R. Y. Stanier		O. Jessen	PJ 70 A.T.C.C. 948 A.T.C.C. 11251 C.C.E.B. 295 N.R.R.L. B-10		M. Levine (strain 1013)	1957	Jessen biotype 52, group IV; Ralston group IA; Iowa, USA, from creamery waste → M. Levine → A.T.C.C. (as 948) → O. Lysenko → O. Jessen → R. Y. Stanier
191	R. Y. Stanier		O. Jessen	PJ 236 Rhodes 22/1 A.T.C.C. 17559		M. E. Rhodes	1951	Reading, U.K., raw T.T. milk; Jessen biotype 49, group IV; Ralston group IC
204	R. Y. Stanier		O. Jessen	PJ 682		O. Jessen		Øresund, Denmark, polluted sea water;
217	R. Y. Stanier		O. Jessen	A.T.C.C. 17571 PJ 905		O. Jessen		Jessen biotype 49, group IV; Ralston group ID
8376	N.C.I.B.	1955	S. D. Rubbo	A.T.C.C. 17584 PJ 132				Sukbertoppen, Greenland, polluted sea water; Jessen biotype 49, group IV; Ralston group IA received as <i>Ps. denitrificans</i> , source unknown; Jessen biotype 49, group IV

* Unassigned by Ralston to any subgroup.

isolates of *Bacillus licheniformis* (Pollock, 1965). Amino acid sequence results are still sparse. Smith *et al.* (1968) have shown that the subtilisins from two different species of *Bacillus* are clearly homologous, yet differ considerably (by 27% of the residues) in their sequences. Ambler & Wynn (1973) have shown that the sequences of the cytochromes *c*-551 from four different species of *Pseudomonas* differ from each other in 25–35% of the sequence. Long *N*-terminal segments of the tryptophan synthetase α -subunit of various enterobacteria and of *Pseudomonas putida* have been compared (Crawford & Yanofsky, 1971; Li & Yanofsky, 1972). Sequences of homologous rubredoxins (Benson *et al.*, 1971) and penicillinases (Ambler & Meadway, 1969) from distantly related organisms have been determined.

In the present investigation, the variation in the amino acid sequence of the cytochrome *c*-551 from several different isolates of two different species of *Pseudomonas* has been examined. The organisms concerned have been very thoroughly characterized in their phenotypic properties (Stanier *et al.*, 1966) and by DNA hybridization (Palleroni *et al.*, 1972), and are abundant in the wild. Several factors make this system suitable for study. Much of the discussion in the drift-versus-selection controversy has been about the variation in mitochondrial cytochrome *c*, which has a very similar function to cytochrome *c*-551, so the variation in the two sets of proteins may be governed by similar considerations. The cytochrome *c*-551 is easy to purify, and large-scale growth of the organisms is simple. The critical advantage is the small size of the protein (only 82 residues), which makes screening studies of the present sort feasible. Satisfactory proof of the identity of two sequences is far more difficult than the detection of a single mutational alteration, and requires almost as much evidence as the deduction of the sequence *ab initio*. Even with the present system, this proof of identity is not rigorous, but the standard of evidence is defined, so that the validity of the results can be judged. The different strains of bacteria chosen for investigation came from a wide range of geographical and ecological habitats, and have been maintained in laboratory culture for periods from a very few to 50 years. The strains of each species must be separated by a considerable (but inestimable) number of generations from their putative common ancestor, allowing opportunity for selective or non-selective divergence. The extent of this divergence has been discovered, and is discussed in terms of the selectionist and neutralist theories.

Experimental

The organisms used are listed in Table 1. The materials and methods used were as described by Ambler & Wynn (1973).

Results

Preparation and properties of cytochromes c-551

The strains used varied in their vigour of growth on the standard medium, and cytochrome yield (per weight of dry cells) also varied, but the standard method of purification (Ambler & Wynn, 1973) proved satisfactory in each case. The cytochrome *c*-551 from each of the *Ps. aeruginosa* strains was sufficiently pure by the end of step 5, but the full procedure was necessary to get pure cytochrome *c*-551 from the *Ps. fluorescens* biotype C strains. The proteins were examined by starch-gel electrophoresis at pH4.0 and pH8.5. The proteins from all the *Ps. aeruginosa* strains were homogeneous, and all except those from strains 129 and 1224 (which migrated considerably less rapidly) had similar mobilities towards the cathode. The proteins from the *Ps. fluorescens* biotype C strains were not uniform in electrophoretic behaviour, but the mobility differences were small.

Determination of amino acid sequences

Experimental approach. The amino acid sequences of cytochromes *c*-551 from representative strains of *Ps. aeruginosa* (strain 6009; Ambler, 1963) and *Ps. fluorescens* biotype C (strain 18; Ambler & Wynn, 1973) were known. These studies had shown that chymotrypsin hydrolysed both proteins into simple mixtures of easily fractionated peptides of moderate size, whereas tryptic digestion resulted in the formation of some unduly large peptides. In the present investigation, cytochrome *c*-551 (0.5–2 μ mol) from each of the new strains shown in Table 1 was treated to remove the haem, and then digested with chymotrypsin. The digests were fractionated by gel filtration followed by high-voltage paper electrophoresis and (for a few difficult peptides) paper chromatography. Samples of the purified peptides were analysed quantitatively for amino acid composition, and for *N*-terminal residues by the dansyl method. A few simple very small peptides were only analysed qualitatively for amino acid composition, and for a few others the *N*-terminal analysis was omitted. The cysteine-containing peptides were oxidized while on paper with performic acid vapour (Brown & Hartley, 1966) at an intermediate stage in their purification.

The patterns of peptides produced from the cytochromes from each strain were in all cases very similar to the patterns from their type sequences. Most peptides from each strain could not be distinguished from the corresponding peptides from the type sequences by electrophoretic mobility, amino acid composition or *N*-terminal residue (Tables 2 and 3). A few differences were apparent from

Table 2. *Peptides isolated from chymotryptic digests of cytochromes c-551 from different strains of Pseudomonas aeruginosa*

Peptides marked (1) had amino acid compositions different from that of the corresponding peptide in the *Ps. aeruginosa* cytochrome *c-551* type sequence (Fig. 1, and Ambler, 1963). Details are given in Table 4. The other peptides were examined by amino acid analysis, the quality of the composition results being indicated by symbols *a*, *b*, *c* or *z*. *a* indicates a satisfactory and *b* an adequate quantitative analysis, and *z* a satisfactory qualitative analysis (used for very small peptides only). *c* indicates cases where the analytical or other results were unsatisfactory, and each case is discussed in the text. The criteria for grading analyses are given in the text. *N*-Terminal residues were identified for all peptides except those marked *. Two naming systems are given for the peptides; † is similar to that used by Ambler & Wynn, 1973, and ‡ is the system used during the original type-sequence determination (Ambler, 1963). Position refers to the location of the peptide in the complete sequence (Fig. 1).

Peptide		Position	Strain									
†	‡		54	129	292	950	1224	1896	2000	2195	8704	10332
C414	C10	1-7	<i>a</i>	(1)	<i>a</i> *	<i>a</i> *	(1)	<i>a</i>	<i>a</i> *	<i>a</i> *	<i>a</i>	<i>a</i> *
C30/5		8-16	<i>b</i>	<i>a</i>	<i>b</i> *	<i>b</i> *	<i>a</i>	<i>a</i>	<i>c</i> *	<i>a</i> *	<i>b</i>	<i>b</i> *
C37a		17-22	<i>c</i>	<i>a</i>				<i>a</i>			<i>b</i>	
C37b		17-27			<i>c</i> *	<i>a</i> *	<i>a</i>		<i>b</i> *	<i>b</i> *		<i>b</i> *
C44		21-27	<i>b</i>	<i>a</i>				<i>a</i>			<i>b</i>	
C34	C5	28-34	<i>a</i>	<i>a</i>	<i>a</i> *	<i>a</i> *	<i>a</i>	<i>a</i>	<i>a</i> *	<i>a</i> *	<i>a</i>	<i>a</i> *
C311	C9	35-44	<i>a</i>	<i>a</i>	<i>a</i> *	<i>a</i> *	<i>a</i>	<i>a</i>	<i>a</i> *	<i>a</i> *	<i>a</i>	<i>a</i> *
C32/5	C6	45-56	<i>b</i> *	<i>a</i>	<i>a</i> *	<i>a</i> *	<i>a</i>	<i>a</i>	<i>a</i> *	<i>b</i> *	<i>b</i> *	<i>a</i> *
C210	C8	57-74	<i>a</i>	<i>a</i>	<i>a</i> *	<i>a</i> *	<i>a</i>	<i>a</i>	<i>a</i> *	<i>a</i> *	<i>a</i>	<i>a</i> *
C53	C3a	75-77	<i>a</i>	<i>a</i>	<i>z</i> *	<i>z</i> *	<i>z</i>	<i>a</i>	<i>z</i> *	<i>z</i> *	<i>a</i>	<i>z</i> *
C37d	C7a	78-79	<i>a</i>	<i>a</i>				<i>a</i>			<i>b</i>	
C33	C4	78-82			<i>b</i> *	<i>b</i> *	<i>a</i>		<i>b</i> *	<i>c</i> *		<i>b</i> *
C42	C3b	80-82	<i>a</i>	<i>a</i>				<i>a</i>			<i>a</i> *	

Table 3. *Peptides isolated from chymotryptic digests of cytochromes c-551 from different strains of Pseudomonas fluorescens biotype C*

Peptides marked (2), (3) etc. had amino acid compositions or other properties different from that of the corresponding peptide in the *Ps. fluorescens* biotype C cytochrome *c-551* type sequence (Fig. 1, and Ambler, 1963). Details are given in Tables 4 and 5. The other peptides were examined by amino acid analysis, the quality of the composition results being indicated by symbols *a*, *b*, *c* or *z*. *a* indicates a satisfactory and *b* an adequate quantitative analysis, and *z* a satisfactory qualitative analysis (used for very small peptides only). *c* indicates cases where the analytical or other results were unsatisfactory, and each case is discussed in the text. The criteria for grading analyses are given in the text. *N*-Terminal residues were identified for all peptides except those marked *.

Peptide	Position	Strain					
		50	181	191	204	217	8376
C413	1-7	<i>a</i>	(2)	(2)	(2)	(2)	<i>a</i>
C31/5	8-16	<i>a</i>	<i>a</i>	<i>a</i>	<i>b</i>	<i>b</i>	<i>a</i>
C37a	17-22	(3)	<i>a</i>	<i>b</i>	<i>a</i>	<i>a</i>	<i>b</i>
C15a	17-34	(3,4)	<i>a</i>	<i>a</i>	<i>b</i>	<i>c</i>	<i>c</i>
C24b	23-34	(4)	<i>b</i>	<i>a</i>	<i>b</i>	<i>b</i>	<i>b</i>
C26	35-44	<i>b</i> *	<i>a</i>	<i>b</i>	<i>a</i>	<i>a</i>	<i>b</i>
C34a/5	45-56	(7)*	(5)	(6)	<i>a</i>	(5)	<i>a</i>
C210	57-72	<i>a</i>	(8,9)	(10)	(9)	(8,9)	<i>b</i>
C47b	73-74				<i>z</i>	<i>z</i>	
C57	73-77	<i>z</i>	<i>z</i>	<i>a</i>		<i>z</i>	<i>z</i>
C67	75-77	<i>z</i>	<i>z</i>	<i>z</i>	<i>z</i>	<i>z</i>	<i>z</i>
C47a	78-79	(see text)	<i>z</i>	<i>z</i>	<i>z</i>	<i>z</i>	<i>z</i>
C42	80-82	<i>a</i> *	<i>z</i>	<i>a</i>	<i>z</i>	<i>z</i>	<i>z</i>

electrophoretic or chromatographic mobilities, and others were detected by the amino acid analyses. The peptides containing differences were further characterized, and evidence was obtained for the nature and position of the amino acid substitutions (Tables 4 and 5; Fig. 2).

Evidence for identity of peptides with those from type sequences

The primary evidence was quantitative identity of amino acid composition. The final amino acid analysis results were not all of as high a standard as

Table 4. Properties of peptides from cytochromes c-551 from *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* biotype C strains that differ from the type sequence

Each difference between a new sequence and its type sequence is numbered (1), (2) etc., and the altered residues are shown on Fig. 1. Peptides were named by using the same system as described by Ambler & Wynn (1973), and the same names are used for peptides from strains of *Ps. fluorescens* biotype C as were used for those from the corresponding region of the type sequence, but put in parentheses if differing significantly in electrophoretic mobility. Position refers to the location of the peptide in the sequence (Fig. 1). Electrophoretic mobilities were measured at pH 6.5, relative to lysine (+ve) for basic peptides, and to aspartic acid (–ve) for acidic peptides, and corrected for endosmosis. The purification procedure used for each peptide is shown, the steps being given in order of use. The symbols used are: G, gel-filtration through Sephadex G-25; high-voltage paper electrophoresis, 6, at pH 6.5, 3, at pH 3.5; B, paper chromatography in butan-2-ol–acetic acid–water–pyridine, 15:3:12:10 (by vol.); ox, oxidation with performic acid vapour before elution from paper. Impurity amino acids present at proportions above 0.1 mol/mol are shown in parentheses, and amino acid values outside the α limits (see the text) are shown in **bold type**. N-Termini were determined by the dansyl method. Tryptophan was not determined quantitatively.

Diff- erence	Strain	Peptide	Position	Mobility (pH 6.5)	Purifi- cation	Amino acid content (mol/mol of peptide)					
						Gly	Ala	Val	Leu	Ile	Ser
(1)	129	(C414)	1–7	–0.59	G6		1.0	1.0	1.0		
(1)	1224	(C414)	1–7	–0.59	G6		1.0	1.0	1.0		
(2)	181	C413	1–7	–0.68	G6	1.0	1.9		1.0		
(2)	191	C413	1–7	–0.68	G6	1.0	2.0		1.0		
(2)	204	C413	1–7	–0.68	G6	1.0	2.0		1.1		
(2)	217	C413	1–7	–0.68	G6	1.1	2.1		1.0		
(3)	50	C37a	17–22	0	G6B3ox	(0.22)		1.0			1.1
(3,4)	50	C15a	17–34	+0.18	G6	1.1	3.0	3.0	1.0		0.9
(4)	50	C24b	23–34	+0.27	G6	0.9	3.2	1.9	1.0		(0.12)
(5)	181	C34a/5	45–56	+0.38/+0.16	G6	2.0	1.0			0.9	1.0
(5)	217	C34a/5	45–56	+0.38/+0.16	G6	2.0	1.1	(0.11)	(0.11)	1.0	1.0
(6)	191	(C34a/5)	45–56	+0.18/0	G6	2.0	1.1	(0.14)	(0.20)	1.0	(0.20)
(6)	191	(T31a)	43–49	+0.32	G63	(0.20)	1.3		0.9	0.9	(0.29)
(6)	191	(T25)	39–49	~0	G6B3		1.9		1.1	1.1	(0.11)
(7)	50	(C34a/5)	45–56	+0.51/+0.31	G6	2.9	1.1			1.1	(0.11)

Amino acid content (mol/mol of peptide)											N- Terminus	(Peptide)
Thr	Asp	Glu	Phe	Trp	Met	Pro	Lys	His	Arg	Total		
		2.0	1.0			0.9				7	Glx	(C414)
		2.1	1.0			1.0				7	Glx	(C414)
	2.1		1.0							7	Asx	C413
	2.0		1.0							7	Asx	C413
	1.9		1.0							7	Asx	C413
	1.8		1.0							7	Asx	C413
0.8	1.2	(0.16)			1.0		1.0			6	Thr	C37a
0.9	3.1				0.9	1.0	3.0			18	Thr	C15a
	2.0					0.9	2.2			12	Val	C24b
1.0	1.9	1.1		Present			1.1	1.0		12	Ala	C34a/5
1.0	1.9	1.0		Present			1.1	1.0		12	Ala	C34a/5
0.9	3.0	1.0		Present			1.3	1.1		12	Ala	(C34a/5)
0.8	1.2	(0.11)					0.9	1.0		7	Thr	(T31a)
1.0	3.0						1.9	1.0		11	Asx	(T25)
0.9	1.9	1.0		Present			1.1		1.0	12	(not done)	(C34a/5)

Table 5. *Properties of peptides from 57-72 region of cytochromes c-551 from Pseudomonas fluorescens biotype C*

Details are given in the legend to Table 4. The peptides are shown in Fig. 2.

Difference	Strain	Peptide	Position	Mobility (pH 6.5)	Purification	Amino acid content (mol/mol of peptide)					
						Gly	Ala	Val	Leu	Ile	Ser
(8,9)	181	C210	57-72	-0.35	G6	1.0	3.1	1.0	1.0	1.0	(0.11)
—	181	C210X1	57-61	+0.63	6	0.8				1.1	
(8,9)	181	C210X4	62-72	-0.48	6	(0.21)	3.1	0.9	1.2		(0.15)
—	181	C210H1c	57-62	0	63	0.5				1.1	
(8,9)	181	C210H1b	63-65	0	63		0.9				
—	181	C210H3	66-70	-0.75	6	(0.21)	1.1	0.9			(0.21)
(8,9)	217	C210	57-72	-0.35	G6	1.0	3.0	1.0	1.0	1.0	(0.15)
—	217	C210X1	57-61	+0.63	6	0.8				1.0	
(8,9)	217	C210X4	62-72	-0.48	6		3.1	1.0	1.0		
—	217	C210H3	66-70	-0.75	6	(0.23)	1.2	0.9			(0.14)
(9)	204	C210	57-72	-0.35	G63	0.9	2.1	1.0	1.0	1.0	(0.14)
(9)	204	C210H1d	57-65	0	63	0.7				1.0	(0.24)
—	204	C210H3	66-70	-0.75	6	(0.18)	1.1	0.9			(0.14)
(10)	191	(C210)	57-72	-0.16	G6	1.0	2.1	1.0	1.0	1.1	(0.13)
(10)	191	(C210X3)	62-72	-0.23	6	(0.16)	2.0	1.0	1.0		(0.14)
—	50	C210	57-72	-0.32	G6	0.9	2.1	1.0	1.0	1.0	
—	50	C210X3	62-72	-0.45	6		2.1	0.9	1.0		
—	8376	C210	57-72	-0.32	G6	1.1	2.1	1.1	1.0	1.0	(0.16)
—	8376	C210H1e	57-65	0	63	0.9				1.1	(0.17)
—	8376	C210H3	66-70	-0.75	6	(0.18)	1.2	0.9			(0.11)

Amino acid content (mol/mol of peptide)

Thr	Asp	Glu	Met	Hse	Pro	Lys	Total	N-Terminus	(Peptide)
0.9	2.1	1.1	1.0		3.8		16	Gly	C210
				0.9	2.2		5	Gly	C210X1
0.8	2.0	1.1			2.0		11	Pro	C210X4
	(0.15)		0.8		2.9		6	(not done)	C210H1c
	1.1				1.1		3	Ala	C210H1b
0.9	1.0	1.0					5	Val	C210H3
1.0	2.0	1.1	0.9		3.7		16	Gly	C210
				0.8	2.0		5	Gly	C210X1
0.9	2.0	1.1			1.9		11	Pro	C210X4
0.8	1.1	1.0					5	Val	C210H3
1.0	2.1	1.1	0.9		5.1		16	Gly	C210
	1.0	(0.12)	0.9		4.1		9	(not done)	C210H1d
0.9	1.1	1.1					5	Val	C210H3
1.0	2.1	2.1	0.9		3.8	(0.24)	16	Gly	(C210)
1.0	2.0	2.0			1.8		11	Pro	(C210X3)
0.9	2.1	2.1	0.8		4.0		16	Gly	C210
0.9	2.1	2.2			1.9		11	Pro	C210X3
1.0	2.2	2.1	1.0		3.6		16	Gly	C210
	1.1	1.0	0.9		3.8		9	(not done)	C210H1e
0.8	1.1	1.1					5	Val	C210H3

would be required for the elucidation of a completely unknown sequence, as less material was used, and the peptides were not so extensively purified. For some of the strains, the starting protein may not have been completely pure, which could also affect the purity of peptides. The actual analysis results for peptides believed to be identical with those from their type sequences are not given, but Tables 2 and 3 show an assessment of the standard of these analyses. Results are classed in three categories,

a, *b* and *c*. Class *a* analyses were satisfactory by the criteria used for the type sequence determinations (Ambler & Wynn, 1973). No impurity amino acid was present in amounts as great as 0.2 mol/mol, and the relative amounts of amino acids (calculated on the basis that the average value was integral) that were present fell within the limits 0.8–1.2, 1.8–2.2, 2.7–3.3 or 3.7–4.3, except that for tyrosine 0.6 (= 1) was considered acceptable. Class *b* analyses were those in which impurity amino acids did not exceed

0.35 mol/mol, and molar ratios fell within the limits 0.7–1.3, 1.7–2.3, 2.7–3.4 or 3.6–4.4, or those in which, although a basic amino acid was present, it was not determined. Class c analyses were even worse, and the results are considered in individual detail below.

Evidence for amino acid differences

The paragraph numbers refer to the difference numbers as shown in Figs. 1 and 2 and Tables 2–5.

(1) Asp-2 → Ala-2 in strains 129 and 1224 (the only variations found in *Ps. aeruginosa* cytochromes c-551). The peptide corresponding to peptide C414 (peptide C10; Ambler, 1963) showed electrophoretic-mobility and amino acid-composition differences (Table 4). The *N*-terminal sequence (by the Dns-phenyl isothiocyanate method) was Glx-Ala-.

(2) Glu-1 → Asp-1 in strains 181, 191, 204 and 217. The peptides corresponding to peptide C413 (Table 3) showed differences in amino acid composition and *N*-terminus. The electrophoretic-mobility differences at pH 6.5 were small, but those at pH 3.5 were large, which is consistent with the replacing amino acid being aspartic acid, not asparagine. The *N*-terminus of the whole cytochrome c-551 from strain 191 was found to be Asx by the dansyl method.

(3) Ile-18 → Val-18 in strain 50. The peptides corresponding to peptides C37a and C15a showed differences in amino acid analysis (Table 4). The peptide corresponding to peptide C37a had a lower R_F value than that of the type-sequence peptide in the butan-2-ol-acetic acid-water-pyridine (15:3:12:10, by vol.) paper-chromatography system. The *N*-terminal sequence of the C37a peptide (by the Dns-phenyl isothiocyanate method) was Thr-Val-.

(4) Glu-29 → Asp-29 in strain 50. The peptides corresponding to peptides C15a and C24b showed differences in amino acid composition (Table 4). Samples of the C24b peptides from strains 50, 181, 191, 204 and 217 were each digested with trypsin, and the products separated by high-voltage paper electrophoresis at pH 3.5. The most basic component (Val-Gly-Pro-Ala-Leu-Lys) and the most acidic component (Asn) were common to all five digests. In the digests from strains 181, 191, 204 and 217, the intermediate fragment had the same electrophoretic mobility as peptide T37a from the type strain (sequence Glu-Val-Ala-Ala-Lys; Ambler & Wynn, 1973), but from strain 50 the fragment had a much slower mobility, the same as that of peptide T37 from *Ps. mendocina* cytochrome c-551 (sequence Asp-Val-Ala-Ala-Lys; Ambler & Wynn, 1973).

(5) Gly-46 → Ser-46 in strains 181 and 217. The peptides corresponding to peptides C34a/5 showed differences in amino acid composition (Table 4). Samples of the C34a/5 peptides from strains 181,

204, 217 and 8376 were digested with trypsin. The only differences detected between the strains were in histidine-containing fractions, strains 181 and 217 being indistinguishable from each other, but different from strains 204 and 8376. The *N*-terminal sequences of the C34a/5 peptides from strain 181 was Ala-Ser-His-, from strain 204 Ala-Gly-His-, and from strain 217 also Ala-Ser-His-, all by the Dns-phenyl isothiocyanate method. With the strain 181 C34a/5 peptide some Dns-Asp was noted contaminating the Dns-His at the second cycle, which was unexpected in view of the good analytical values obtained for this peptide (Table 4).

(6) Gly-46 → Asp-46 in strain 191. The peptide corresponding to peptides C34a/5 showed differences in amino acid composition and in electrophoretic mobility (Table 4), but there was not sufficient material left to locate the difference. Another 1 μ mol portion of the cytochrome was treated to remove the haem, digested with trypsin, and the peptides were fractionated and analysed for amino acid composition, *N*-terminal groups and electrophoretic mobility. Peptides that appeared identical with the type-sequence peptides T31/7, T27b, T37b, T34, T27a, T32a, T413 and T-I (Ambler & Wynn, 1973) were isolated. The peptide corresponding to peptide T411 showed difference (1) (see above). The peptides corresponding to the type-sequence peptides T25 and T31a differed in properties, as shown in Table 4, but the peptide corresponding to T31a was obtained in very low yield. The sequence of the peptide corresponding to T25 (residues 39–49) was found by the Dns-phenyl isothiocyanate method to be Asx-(n.s.)-(n.s.)-(n.s.)-(n.s.)-(n.s.)-Ala-Asx-His- (n.s. indicates that no sample was taken for *N*-terminal analysis at this step, owing to shortage of material). The electrophoretic mobilities of all these difference peptides of strain 191 indicated that the replacing residue was aspartic acid, not asparagine.

(7) His-47 → Arg-47 in strain 50. The peptides corresponding to peptides C34a/5 showed differences in amino acid composition and electrophoretic mobility (Table 4). The *N*-terminal sequence of the peptide was Ala-(fail)-Arg- by the Dns-phenyl isothiocyanate method; at the first cycle, *N*-terminal analysis failed to detect either the expected glycine or any other amino acid.

(8) Pro-63 → Ala-63 in strains 181 and 217.

(9) Gln-65 → Pro-65 in strains 181, 204 and 217.

(10) Glu-70 → Gln-70 in strain 191.

All these differences were detected in the region covered by the difficult (because of its very high proline content) peptide C210, the analyses for which are shown in Table 5. The sequences of the C210 peptides were investigated by further degrading the peptides with cyanogen bromide or with thermolysin. The peptides characterized are shown in Fig. 2 and Table 5.

Strain	Ala ¹
<i>Ps.</i> 129/1224	
<i>aeruginosa</i>	6009 Glu-Asp-Pro-Glu-Val-Leu-Phe-Lys-Asn-Lys-Gly-Cys-Val-Ala-Cys-His-Ala-Ile-Asp-Thr-Lys-Met-Val-Gly-Pro-Ala-Tyr-Lys-
	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28
<i>Ps.</i> <i>fluorescens</i>	18 Glu-Asp-Gly-Ala-Ala-Leu-Phe-Lys-Ser-Lys-Pro-Cys-Ala-Ala-Cys-His-Thr-Ile-Asp-Ser-Lys-Met-Val-Gly-Pro-Ala-Leu-Lys-
<i>biotype C</i>	50 Glu Val ³
	181 Asp ² Ile
	191 Asp ² Ile
	204 Asp ² Ile
	217 Asp ² Ile
	8376 Glu Ile
<i>Ps.</i> <i>aeruginosa</i>	6009 -Asp-Val-Ala-Ala-Lys-Phe-Ala-Gly-Gln-Ala-Gly-Ala-Glu-Leu-Ala-Gln-Arg-Ile-Lys-Asn-Gly-Ser-Gln-Gly-Val-Trp-
	29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56
<i>Ps.</i> <i>fluorescens</i>	18 -Glu-Val-Ala-Ala-Lys-Asn-Ala-Gly-Val-Lys-Asp-Ala-Asp-Lys-Thr-Leu-Ala-Gly-His-Ile-Lys-Asn-Gly-Thr-Gln-Gly-Asn-Trp-
<i>biotype C</i>	50 Asp ⁴ Gly Arg ⁷
	181 Glu Ser ² His
	191 Glu Asp ⁶ His
	204 Glu Gly His
	217 Glu Ser ² His
	8376 Glu Gly His
<i>Ps.</i> <i>aeruginosa</i>	6009 -Gly-Pro-Ile-Pro-Met-Pro-Pro-Asn-Ala-Val-Ser-Asp-Asp-Glu-Ala-Gln-Thr-Leu-Ala-Lys-Tyr-Val-Leu-Ser-Gln-Lys
	57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82
<i>Ps.</i> <i>fluorescens</i>	18 -Gly-Pro-Ile-Pro-Met-Pro-Pro-Asn-Gln-Val-Thr-Asp-Ala-Glu-Ala-Leu-Thr-Leu-Ala-Gln-Thr-Val-Leu-Ser-Leu-Lys
<i>biotype C</i>	50 Pro Gln Glu (see text)
	181 Ala ⁸ Glu
	191 Pro Gln Gln ¹⁰
	204 Pro (Pro ⁹) Glu
	217 Ala ⁸ Pro ⁹ Glu
	8376 Pro Gln Glu

Fig. 1. Sequence variants of cytochrome c-551 from strains of *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* biotype C

The sequences of the type protein from *Ps. aeruginosa* (strain 6009; Ambler, 1963) and *Ps. fluorescens* biotype C (strain 18; Ambler & Wynn, 1973) are shown complete. Eight out of the nine *Ps. aeruginosa* cytochromes could not be distinguished in sequence from strain 6009, but the ninth (strain 129 = strain 1224) had the replacement shown at residue 2. The different *Ps. fluorescens* biotype C cytochromes were more varied, and the residue believed present in each cytochrome at the eight sites where differences were detected are shown. Residues different from those of the type sequence are shown in *italics*. The superscript numbers refer to the individual differences as discussed in the text and in Tables 4 and 5. The evidence for the location of the difference residue shown in parentheses depends on sequence homology within a small peptide (see the text).

Fig. 2. Amino acid sequences of peptides C210 (residues 57-72) of cytochromes *c*-551 from different strains of *Ps. fluorescens* biotype *C*.

Difference amino acids are shown in *italics*, the superscript numbers referring to the individual differences as discussed in the text and in Table 5. Full lines indicate peptides that were analysed quantitatively (Table 5). Peptides substandard to level *b* are marked *, and to level *c* ** (see the text and Table 5). Broken lines indicate peptides that were only analysed qualitatively. → indicates end groups and subsequent residues revealed by phenyl isothiocyanate degradation, and detected by the dansyl method. -→ indicates such residues for which the dansyl amino acid was not positively identified. Peptides examined by carboxypeptidase A digestion (see the text) are marked †.

The *N*-termini of peptides C21OH1b from strains 181 and 217 were identified as alanine, establishing difference (8) in these two strains. Both differences (8) and (9) were established in strain 217 by the degradation experiments on peptide C21OX4 (Fig. 2). The evidence for difference (9) in strain 181 is indirect. The sequence of peptide C21OH1b was shown to be Ala(Asn,Pro) by electrophoretic mobility, amino acid composition and *N*-terminal group. The peptide was formed by thermolysin hydrolysis, so the sequence should be Ala-Asn-Pro, as thermolysin apparently will not split peptide bonds one residue *N*-terminal to a proline residue (Ambler & Meadway,

1968). There is no evidence for the location of difference (9) in strain 204, except that it must be between residues 57 and 65. However, any location except at position 65 would require there to be two alterations in this segment, and an alteration to Ile-59 or Met-61 would affect thermolysin cleavage of the C210 peptide. Therefore although the sequence of peptide C21OH1d could be Gly-Pro-Ile-Pro-Met-Pro-Pro-Pro-Asn, the sequence shown is more likely.

The peptide from strain 191 corresponding to peptide C210 had a less acidic electrophoretic mobility at pH 6.5, although the amino acid composition

was the same as that from the type sequence. When the peptide was treated with carboxypeptidase A, Gln, Ala and Leu were released, whereas from peptide C210 from strain 50, Ala, Leu and a trace of Glu were released, establishing difference (10).

Inadequacies in experimental results

Ps. aeruginosa sequences. *N*-Terminal analyses were not performed on the peptides from strains 292, 950, 2000, 2195 and 10332. Four peptides from different strains gave class c analyses (Table 2). These results are considered in detail below.

(a) Strain 2000 peptide C30/5. The peptide was recovered in such low yield that the analysis was well below the satisfactory amount for the instrument used, being only about 2 nmol/column. Although contaminants at proportions of 0.25 or more might not have been seen, the peptide appeared to be clean and the analysis reasonably stoichiometric.

(b) Strain 54 peptide C37a. Aspartic acid was not detected (owing to an analyser operating error, namely that the recorder was not switched on in time) and the peptide was badly contaminated with the 'paper impurity' amino acids glycine (0.4) and serine (0.4). Glutamic acid was absent, and as the electrophoretic mobility of the peptide was the same as that of peptides C37a from the other strains, the presence of aspartic acid is reasonably certain.

(c) Strain 292 peptide C37b. The peptide was recovered in low yield, and the analysis showed considerable contamination with the 'paper impurity' amino acids. The analysis results (on 7 nmol/column) were: Gly 1.4 (1), Ala 1.9 (2), Val 0.8 (1), Ile 1.1 (1), Thr 0.9 (1), Asp 1.3 (1), Met (as sulphone) 0.8 (1), Tyr 0.5 (1), Pro 0.9 (1), Lys (lost through instrument failure) (1), contaminated with Ser 0.35 (0) and Glu 0.25 (0). The predicted values are shown in parentheses. The peptide had the same electrophoretic mobility as the corresponding peptides from five other strains, which were analysed satisfactorily.

(d) Strain 2195 peptide C33. The peptide had the expected electrophoretic mobilities, and a qualitative amino acid analysis showed Val, Leu, Ile or Ser, Glu, Lys, with slight contaminating traces of Asp and Ala. In contrast, the quantitative analysis showed large quantities of several other amino acids, and was uninterpretable. This result is ascribed to the accidental use of a dirty hydrolysis tube.

Ps. fluorescens biotype C sequences. Two peptides from different strains gave class c analyses, and a dipeptide from another strain was not found, and had probably been lost in the peptide fractionation process.

(a) Strain 217 peptide C15a. The analysis showed: Gly 1.5 (1), Ala 3.1 (3), Val 2.2 (2), Leu 1.0 (1), Ile 1.1 (1), Ser 1.4 (1), Thr 0.9 (1), Asp 1.8 (2), Glu 1.1 (1), Met (as sulphone) 1.1 (1), Pro 1.1 (1), Lys 3.1 (3).

The theoretical values are shown in parentheses. The high values of glycine and serine are ascribed to contamination with 'paper impurities'.

(b) Strain 8376 peptide C15a. Although it ran with the expected electrophoretic mobilities, the peptide was recovered in such low yield that the analysis was uninterpretable owing to the high degree of contamination with 'paper impurities'. The same region of sequence was also isolated in peptides C37a and C24b, which were obtained in a sufficiently pure form to give good analyses.

(c) Strain 50 peptide C47a was not found. In other strains Val-Leu was found at positions 78–79, but the peptide was probably lost from the strain-50 digest because of over-running of a paper chromatogram, the peptide being expected to have a high R_F value. There is no evidence whatsoever that the sequence of residues 78–79 in strain 50 is not, for example, Ile-Phe. As peptide C42 was detected normally, the residue preceding it must be of a kind after which chymotrypsin normally splits. A deletion of residues 78 and 79 is also formally possible.

All the principal difference peptides, indicated by numbers in Tables 2 and 3, gave adequate analyses (class a or b), as shown in detail in Tables 4 and 5. One of the secondary peptides, derived from strain 181 peptide C210 by thermolysin, gave a class c analysis (peptide C210H1c, Table 5 and Fig. 2). Only 0.5 mol of the *N*-terminal glycine residue/mol was recovered after acid hydrolysis. It has been noticed on several occasions that the *N*-terminal residues of paper-purified peptides are recovered in lower yield than are internal residues, though this is an extreme example.

Discussion

Detection of amino acid differences

The amino acid sequences of the cytochromes *c*-551 from nine further strains of *Ps. aeruginosa* and six of *Ps. fluorescens* biotype C have been investigated (Table 1). The amino acid compositions and electrophoretic mobilities of the chymotryptic peptides from each protein have been compared with the corresponding peptides from the type sequences for each organism (Tables 2 and 3; Ambler, 1963; Ambler & Wynn, 1973). The peptides were not all purified to the extent that would have been necessary for the investigation of a completely unknown sequence, but the amino acid analyses should have been sufficiently good to detect all single amino acid substitutions, insertions or deletions. In particular, 'conservative' amino acid substitutions such as valine to isoleucine or serine to threonine are unlikely to have been missed. Not all cases of double substitutions ($A \rightarrow B$ at one site, compensated by $B \rightarrow A$ at another) would have been detected, as

some of the peptides analysed were fairly large. Thus the chance of a double substitution occurring in the 18-residue *Ps. aeruginosa* peptide C210 (Table 2 and Fig. 1) is appreciable.

These experiments showed that there were very few differences between any of the new proteins and their type sequences (Fig. 1). The few peptides that did differ in amino acid composition or electrophoretic mobility were further investigated, and in almost all cases the alteration was positively located.

Variations in Ps. aeruginosa cytochrome c-551

Ps. aeruginosa is a ubiquitous bacterium, and the strains of it chosen for investigation (Table 1) were selected to cover as wide a range in date, place and habitat of original isolation as possible. These strains included soil and water organisms as well as plant and human pathogens. Despite its very wide distribution, *Ps. aeruginosa* appears to be an 'ideal' bacterial species (Palleroni *et al.*, 1972), with all isolates having very similar phenotypic properties (Jessen, 1965; Stanier *et al.*, 1966) and DNA sequence.

Eight of the nine strains examined had cytochromes *c*-551 that could not be distinguished from the type sequence. The ninth strain, 1224, a plant pathogen from Puerto Rico, had a single amino acid substitution (Asp → Ala) at position 2 in the polypeptide chain (Fig. 1). As it was possible that this mutation had taken place since isolation from the wild, in the 10 years that the organism had been in the National Collection of Plant Pathogenic Bacteria and exposed to different selection pressures, another strain (129) was obtained, derived from the same isolate, but kept independently in Puerto Rico. The cytochrome *c*-551 from this organism also contained the single difference at residue 2.

Variation in Ps. fluorescens biotype C cytochrome c-551

There was more variation in the *Ps. fluorescens* biotype C cytochromes *c*-551 than in those from *Ps. aeruginosa* (Fig. 1). One of the new proteins appeared to be identical with the type sequence, that from strain 8376. The source of this organism is not known, but it is not likely to be the special source of the type strain (Table 1), and strain 8376 was sufficiently unlike typical *Ps. fluorescens* strains to have been put originally in another name group, *Pseudomonas denitrificans*. The cytochromes from two of the other strains (181 and 217) could not be distinguished from each other, although they were different from each of the other strains, and came from very different habitats (creamery waste and arctic sea water). The numbers of amino acid differences between strains were small, two to four differences between the new strains and the type sequence, and a maximum of seven differences between the most diverse proteins.

Nature of observed variation

The sequences of the cytochromes *c*-551 from four distinct species of *Pseudomonas* are known (Ambler & Wynn, 1973). They differ from each other in from 22 to 39% of their amino acids, with about half of the residues being the same in all four species. Ten amino acid interchanges have been found in the present investigation, in nine different sites. Of these, two are at positions 63 and 70 (Fig. 1), which are invariant in the four published sequences, whereas four of the others (at positions 1 and 2, and the two changes at position 46, Fig. 1) were to amino acids not previously found at these sites. The remaining four changes (at positions 18, 29, 47 and 65; Fig. 1) introduced amino acids known at these sites in other species. Only four of the ten interchanges substituted an uncharged side chain for another uncharged side chain, but the electrophoretic characterization of the different intact cytochromes was not done sufficiently thoroughly to be usable as a check to see if the predicted cases did show electrophoretic differences.

'Selection or drift'

Milkman (1972) suggested that experiments comparable with those reported here could help to assess the importance of stochastic processes in molecular evolution. He said 'If there are not scores of allozymes determined at each of many loci in *E. coli* (or some other suitable bacterium or virus), then Non-Darwinian evolution cannot be very important in higher organisms, because we shall have to conclude that very few differences are neutral'. The apparently extreme evolutionary stability of the cytochrome *c*-551 gene in *Ps. aeruginosa* suggests that in this organism very few amino acid replacements in the protein are possible that will not adversely affect its function. The large differences in sequence between the cytochromes *c*-551 of the different *Pseudomonas* species (Ambler & Wynn, 1973) show that great variations in the protein structure are possible without its gross function being altered, so that there cannot be constraints on its variation like those that must apply to histone IV (DeLange *et al.*, 1969).

Little is known about the geographical dispersion of bacteria, or about their average generation time in the wild. Nevertheless, it would seem that there must be a very large number (at present quite inestimable) of generations separating from their common ancestor an organism, for example, causing a plant disease in the Philippines and one causing a human infection in England 50 years ago. Chance connexions between the most diverse locations on Earth are possible, but it is quite inconceivable that they should exist between all the

Ps. aeruginosa strains examined in the present investigation.

At first sight the results observed for the variation in the cytochrome *c*-551 from the *Ps. fluorescens* biotype C strains are what might be expected if neutral mutations are responsible for much of molecular evolution. This is paradoxical, for should it be possible for selection to maintain a virtually invariant sequence in a protein in *Ps. aeruginosa*, the same mechanism ought to be able to act in the same way on the corresponding protein in a closely related organism. A possible way out of this difficulty is shown by the recent findings of E. Ralston & N. Palleroni (personal communication). They have shown that *Ps. fluorescens* biotype C can be divided up on phenotypic grounds by cluster analysis into several groups and subgroups. Strains 181 and 217 (which apparently have identical cytochrome *c*-551 sequences) are classed in the same subgroup, whereas the other strains, which have been examined in both ways, are all in different subgroups. Although the sample studied here is too small to be significant, these findings do suggest that the sequence variations may correlate with phenotypic differences. If further investigations of cytochromes *c*-551 from more *Ps. fluorescens* biotype C strains do show such a correlation, and concordance is found between the sets of amino acid alterations within different molecules, the paradox will be resolved, and the *Ps. fluorescens* biotype C sequence results will agree with those from *Ps. aeruginosa* in indicating that the role of neutral mutations in molecular evolution is small.

I thank Dr. D. R. Thatcher for discussions, Mrs. Elizabeth Taylor for technical assistance, and Mr. S. Hughes and Mr. T. Bruce for help with the large-scale bacterial cultures. I am grateful to Dr. R. Y. Stanier, Dr. J. E. Perez, Dr. J. M. Shewan (N.C.I.B.), Mr. R. A. Lelliott (N.C.P.P.B.) and Mr. L. R. Hill (N.C.T.C.) for bacterial strains. The work was supported by the Medical Research Council (Research Group on Bacterial Enzyme Variation).

References

- Ambler, R. P. (1963) *Biochem. J.* **89**, 349–378
 Ambler, R. P. & Meadway, R. J. (1968) *Biochem. J.* **108**, 893–895
 Ambler, R. P. & Meadway, R. J. (1969) *Nature (London)* **222**, 24–26
 Ambler, R. P. & Wynn, M. (1973) *Biochem. J.* **131**, 485–498
 Benson, A., Tomoda, K., Chang, J., Matsueda, G., Lode, E. T., Coon, M. J. & Yasunobu, K. T. (1971) *Biochem. Biophys. Res. Commun.* **42**, 640–646
 Brown, J. R. & Hartley, B. S. (1966) *Biochem. J.* **101**, 214–228
 Crawford, I. P. & Yanofsky, C. (1971) *J. Bacteriol.* **108**, 248–253
 Dayhoff, M. O. (1972) *Atlas of Protein Sequence and Structure*, vol. 4, National Biomedical Research Foundation, Washington, D.C.
 DeLange, R. J., Fambrough, D. M., Smith, E. L. & Bonner, J. (1969) *J. Biol. Chem.* **244**, 5669–5679
 Harris, H. (1966) *Proc. Roy. Soc. Ser. B* **164**, 298–310
 Jessen, O. (1965) *Pseudomonas aeruginosa and other Green Fluorescent Pseudomonads: a Taxonomic Study*, Munksgaard, Copenhagen.
 Kauffmann, F. (1972) *Serological Diagnosis of Salmonella Species*, Munksgaard, Copenhagen
 Kimura, M. (1968) *Nature (London)* **217**, 624–626
 King, J. L. & Jukes, T. H. (1969) *Science* **164**, 788–798
 Lenhoff, H. M. & Kaplan, N. O. (1956) *J. Biol. Chem.* **220**, 967–982
 Lewontin, R. C. & Hubby, J. L. (1966) *Genetics* **54**, 595–609
 Li, S.-L. & Yanofsky, C. (1972) *J. Biol. Chem.* **247**, 1031–1037
 McDonough, M. W. (1965) *J. Mol. Biol.* **12**, 342–355
 Milkman, R. (1972) in *Evolution of Genetic Systems, Brookhaven Symposia in Biology No. 23* (Smith, H. H., ed.), pp. 217–229, Gordon and Breach, New York
 Palleroni, N. J., Ballard, R. W., Ralston, E. & Doudoroff, M. (1972) *J. Bacteriol.* **110**, 1–11
 Pollock, M. R. (1965) *Biochem. J.* **94**, 666–675
 Smith, E. L., DeLange, R. J., Evans, W. H., Landon, M. & Markland, F. S. (1968) *J. Biol. Chem.* **243**, 2184–2191
 Stanier, R. Y., Palleroni, N. J. & Doudoroff, M. (1966) *J. Gen. Microbiol.* **43**, 159–272