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The nucleotide sequence and deduced amino acid sequence of the cytochrome c_L gene of *Methylobacterium extorquens* AM1, a novel class of *c*-type cytochrome

David N. NUNN* and Christopher ANTHONY
Biochemistry Department, University of Southampton, Southampton SO9 3TU, U.K.

The nucleotide seuqence and deduced amino acid sequence of the cytochrome c_L of *Methylobacterium extorquens* (*Pseudomonas* AM1; *Methylobacterium* AM1) shows that this cytochrome *c* is completely different, except for its haem-binding site, from all other cytochromes.

INTRODUCTION

Methylobacterium extorquens strain AM1 is a pink facultative methylotroph able to grow on methanol, methylamine and multicarbon compounds, but not on methane. It used to be called *Pseudomonas* AM1 or *Methylobacterium* AM1, but it has been recently renamed (Green *et al.*, 1988). Like all methylotrophic bacteria growing on methanol it contains at least two periplasmic *c*-type cytochromes (Anthony, 1982, 1986, 1988). Cytochrome c_H is a small, basic, cytochrome *c*, donating electrons to the oxidase (O'Keeffe & Anthony, 1980*a*; Anthony, 1986; Fukumori & Yamanaka, 1987); its amino acid sequence is typical of the Class I *c*-type cytochromes whose function is electron transfer between the bc_1 or *bf* complexes and terminal oxidases or photosynthetic reaction centres (Ambler, 1982; R. P. Ambler, personal communication).

Cytochrome c_L, by contrast, is a larger, acidic, cytochrome which is specifically involved in electron transfer from the quinoprotein methanol dehydrogenase (O'Keeffe & Anthony, 1980*a*; Beardmore-Gray *et al.*, 1983; Anthony, 1986; Nunn & Lidstrom, 1986*b*). That it is relatively large (about 19 kDa) but contains only one haem indicates a structure that does not conform to any of the types in the Class I cytochromes. Attempted amino acid analysis showed it to be blocked at the *N*-terminal; it has been more convenient, therefore, to deduce its protein sequence by way of its gene sequence.

The cytochrome c_L gene has recently been cloned from *Methylobacterium extorquens* and expressed in *Escherichia coli* (Nunn & Lidstrom, 1986*a*,*b*; Anderson & Lidstrom, 1988). The present paper describes the nucleotide sequence of this gene together with its deduced amino acid sequence, which confirms that this cytochrome is a novel type of *c*-type cytochrome.

MATERIALS AND METHODS

Materials

Radioactive nucleotides were obtained from Amersham International, Amersham, U.K. DNA modification enzymes were obtained from Bethesda Research Laboratories Inc., or from Pharmacia/PL Biochemicals Inc. Sequenase was obtained from United States Biochemical Corp. 'Phagemid' pT7T319U was obtained from Pharmacia/PL Biochemicals, Inc. Calf liver pyroglutamate aminopeptidase (8.5 units/mg) was obtained from Sigma Chemical Co.

Purification and manipulation of DNA

Plasmid DNA was purified by two rounds of density gradient centrifugation. Restrictions and ligations of DNA were performed as recommended by the manufacturers.

Construction of plasmids and generation of nested deletions

A 2.2 kb fragment of DNA from *Methylobacterium extorquens* AM1, consisting of a 1.0 kb *Xho–Xho* fragment plus a contiguous 1.2 kb *Xho–Sal*I fragment, has been shown recently to contain the structural gene for cytochrome c_L (*MoxG*) (Nunn & Lidstrom, 1986*a*,*b*; Anderson & Lidstrom, 1988). Each of these fragments was independently ligated into the *Sal*I site of previously digested pT7T319U DNA, and clones were selected containing the inserted fragments in both possible orientations. DNA was purified from each of the resulting four clones and, depending on the fragment, digested with either *Bam*HI (1.0 kb *Xho*I–*Xho*I fragment) plus *Sac*I, or *Sma*I (1.2 kb *Xho–Sal*I fragment) plus *Sac*I to generate 5′ and 3′ protruding ends. The technique of Henikoff (1984) was used to generate a nested set of deletions of each of the cloned fragments, essentially as described, except that mung bean nuclease was substituted for S1 nuclease. All deletion derivatives were transformed and maintained in *E. coli* strain TB1, a restriction minus derivative of *E. coli* JM83 (Messing, 1983; BRL Focus, 1984). Plasmids containing deletions of 150–200 bp were selected for DNA sequencing so that each overlapped the next largest deletion by at least 50 base pairs, and so that the sequence could be fully determined in both senses of the DNA.

DNA sequencing

Nucleotide sequences were determined using the dideoxy chain termination method of Sanger *et al.* (1977) with the modifications recommended by the manufacturers for the use of Sequenase. All the reactions were

* Present address: Microbiology Department, School of Medicine, University of Washington, Seattle, WA 98195, U.S.A.
Sequence data in this paper have been submitted to the EMBL Nucleotide Sequence Data Library under the accession number X07856.

run in duplicate using both dGTP and dITP to resolve any compression that might arise due to the high G+C (%) content of *Methylobacterium* DNA. Initial attempts to purify single stranded DNA templates from deletion derivatives described above yielded insufficient DNA for sequencing. Therefore, deletion derivatives of the cloned fragments in pT7T319U were digested with *Hin*dIII and *Eco*RI and the resulting fragments transferred to bacteriophage M13mp19 DNA. Single-stranded template DNA was then prepared from transfected isolates of *E. coli* strain JM105 as described by Messing (1983). DNA sequence data were compiled by computer and verified by re-reading gels for comparison with previously entered data.

Protein sequencing

The cytochrome c_L was purified by a modification of that described by O'Keeffe & Anthony (1980*a*) in which the final purification was achieved by anion exchange chromatography by f.p.l.c. on a Mono-Q column.

Preliminary work had demonstrated (as confirmed by the DNA sequence work) that the *N*-terminal of cytochrome c_L is blocked. In order to generate peptides for sequencing the protein was digested with hydroxylamine, which cleaves between asparagine and glycine residues (Croft, 1980). The reaction mixture, consisting of 6 M-guanidinium chloride and 2 M-hydroxylamine hydrochloride, was prepared by dissolving the solids in the minimum volume of 4.5 M-LiOH which was used to adjust the solution to pH 9.0. Lyophilized cytochrome c_L (2 mg) was dissolved in 400 μl of hydroxylamine reagent and incubated at 45 °C for 6 h. The reaction was terminated by titration to pH 2.5 with 90% (v/v) formic acid. The cytochrome was desalted by gel filtration on a column of Sephadex G-25 (PD10 column) equilibrated with 50 mM-acetic acid (the pH was measured as 3.0).

In an attempt to remove the blocking pyroglutamate, the cytochrome c_L (18 nmol) was treated with pyroglutamate aminopeptidase [0.2 mg dissolved in 1 ml of 0.1 M-sodium phosphate buffer (pH 8.0) containing EDTA (10 mM) and dithiothreitol (5 mM)]. The reaction mixture was incubated for 16 h at 20 °C and then desalted into 20 mM-sodium phosphate buffer (pH 7.0). The method is based on that of Podell & Abraham (1978).

Amino acid sequences were determined using an Applied Biosystems 470A 'gas phase' (pulsed liquid) protein sequencer coupled to a model 120 PTH derivative analyser.

RESULTS AND DISCUSSION

DNA sequence

The DNA sequence of the gene for cytochrome c_L (*MoxG*) is presented in Fig. 1 together with the deduced amino acid sequence. The numbering system is that used in describing the complete 2.2 kb fragment (containing the gene) whose sequence was submitted to the EMBL library (accession no. X07856). The G+C (%) ratio for the whole fragment is 65.3%, and that for the *MoxG* gene is 65.1%. This is the first DNA sequence from a pink facultative methylotroph that has been published, and, for comparison with possibly-related organisms that might be used for future molecular biology of methylotrophs and related bacteria, the codon usage is presented in Table 1. As in other bacterial genes rare codons such as AGA, AGG and ATA are avoided and there is a clear

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                783--TTCGTCGGAAAGCGTCCTAAACGTGTGCTGAGGACCAAGAACAAG
                          +       +
828   ACG ATG ATG AAC CGC GTA AAG ATC GGA ACC GCC CTT CTC GGC CTA
-25      MET MET Asn Arg Val Lys Ile Gly Thr Ala Leu Leu Gly Leu

873   ACG CTC GCA GGC ATT GCC CTG CCC GCG CTC GCC CAG CCG CAA TCC
-11   Thr Leu Ala Gly Ile Ala Leu Pro Ala Leu Ala Gln Pro Gln Ser

918   GGG CCG CAG ACC GGC GTC GTG TTC CGC AAC ACC GTG ACC GGC GAG
 5    Gly Pro Gln Thr Gly Val Val Phe Arg Asn Thr Val Thr Gly Glu

963   GCG CTC GAC GTG TCG CAA GGC AAG GAA GGC GGC CGC GAC ACG CCC
 20   Ala Leu Asp Val Ser Gln Gly Lys Glu Gly Gly Arg Asp Thr Pro

1008  GCC GTG AAG AAG TTC CTC GAG ACC GGC GAG AAC CTC TAC ATC GAC
 35   Ala Val Lys Lys Phe Leu Glu Thr Gly Glu Asn Leu Tyr Ile Asp

1053  GAC AAG TCC TGC CTG CGG AAT GGC GAG AGT CTG TTC GCG ACG TCC
 50   Asp Lys Ser Cys Leu Arg Asn Gly Glu Ser Leu Phe Ala Thr Ser
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1098  TGC TCG GGC TGC CAC GGC CAC CTC GCC GAG GGC AAG CTC GGG CCG
 65   Cys Ser Gly Cys His Gly His Leu Ala Glu Gly Lys Leu Gly Pro

1143  GGC CTG AAC GAC AAT TAC TGG ACC TAC CCG TCC AAC ACC ACG GAT
 80   Gly Leu Asn Asp Asn Tyr Trp Thr Tyr Pro Ser Asn Thr Thr Asp

1188  GTG GGC CTG TTC GCC ACG ATC TTC GGC GGC GCC AAC GGC ATG ATG
 95   Val Gly Leu Phe Ala Thr Ile Phe Gly Gly Ala Asn Gly MET MET
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1233  GGC CCG CAC AAC GAG AAT CTG ACG CCC GAC GAG ATG CTT CAG ACC
 110  Gly Pro His Asn Glu Asn Leu Thr Pro Asp Glu MET Leu Gln Thr
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1278  ATC GCC TGG ATT CGC CAC CTC TAT ACG GGG CCG AAG CAG GAC GCC
 125  Ile Ala Trp Ile Arg His Leu Tyr Thr Gly Pro Lys Gln Asp Ala

1323  GTC TGG CTC AAC GAC GAG CAG AAG AAG GCC TAC ACG CCC TAC AAG
 140  Val Trp Leu Asn Asp Glu Gln Lys Lys Ala Tyr Thr Pro Tyr Lys

1368  CAG GGC GAA GTC ATC CCG AAG GAC GCC AAG GGC CAG TGC AAG CCG
 155  Gln Gly Glu Val Ile Pro Lys Asp Ala Lys Gly Gln Cys Lys Pro

1413  CTG GAC GAG TGA TCC TTC
 170  Leu Asp Glu ----------
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Fig. 1. The nucleotide sequence and deduced amino acid sequence of the gene for cytochrome c_L (*MoxG*)

The *MoxG* gene coding region extends from 829 to 1422. The numbering is that used in describing the complete 2.2 kb fragment submitted to the EMBL library (accession no. X07856). The following are underlined: a ribosome-binding site (bases 819–823); the deduced signal peptide (amino acids −25 to −1) (crosses indicate positively charged residues); deduced haem-binding site (amino acids 65–69); *N*-terminal regions obtained by analysis of peptides generated by hydroxylamine (amino acids 57–61 and 107–111).

bias in this organism against the A/T ending in triplets: 91% of codons terminate in C or G. A similar high proportion of codons terminating in C or G (85–87%) has been noted in *Rhodobacter* (Davidson & Daldal, 1987) and *Paracoccus* (Harms *et al.*, 1987; Kurowski & Ludwig, 1987; Steinrucke *et al.*, 1987) (Table 1). Together with other data, this has led Steinrucke *et al.* (1988) to suggest that these two organisms are in the same (α-3) division of the eubacterial kingdom (Woese, 1987); this division may well include, therefore, the pink facultative methylotrophs such as *Methylobacterium extorquens*. This suggestion is further supported by observations on the signal peptides of the three genera (see below).

Protein sequence

The 2.2 kb fragment contains the structural gene for cytochrome c_L (Nunn & Lidstrom, 1986*a*; Anderson & Lidstrom, 1988), and the chosen reading frame is consistent with the size of the proteins coded by this fragment, based on its expression in *E. coli*, using a coupled *in vivo*

Table 1. Amino acid composition of cytochrome c_L from various methylotrophs

Column 1 is from the protein sequence derived from the cytochrome c_L gene of *M. extorquens* AM1. Other compositions are from hydrolysed cytochromes: 2, *M. extorquens* (Beardmore-Gray *et al.*, 1982); 3, *Methylophilus methylotrophus* (Beardmore-Gray *et al.*, 1982); 4, *Acetobacter methanolicus* (Elliott & Anthony, 1988).

Amino acid	Composition (residues/molecule)			
	1	2	3	4
Ala	10	14	23	18
Arg	4	4	4	5
Asn	10	—	—	—
Asp	11	—	—	—
*(Asx	21	24	17	20)
Cys	4	3	3	—
Gln	9	—	—	—
Glu	11	—	—	—
*(Glx	20	26	30	16)
Gly	21	23	28	17
His	4	7	9	8
Ile	5	6	8	6
Leu	14	15	12	15
Lys	12	13	12	11
Met	3	3	3	4
Phe	5	5	7	10
Pro	11	13	16	14
Ser	7	7	2	15
Thr	14	14	4	21
Trp	3	2	3	—
Tyr	6	6	7	3
Val	8	9	5	9
Total residues	172	194	193	197

* (Asx) is Asn plus Asp; (Glx) is Glu plus Gln.

T7 RNA polymerase/promoter gene expression system (Anderson & Lidstrom, 1988). In the 2.2 kb fragment there was only one open reading frame coding for a protein of the size of cytochrome c_L and this was the only one that contained a sequence coding for a typical haem binding site (Cys-Xaa-Xaa-Cys-His, underlined in Fig. 1).

The molecular mass of the deduced protein is 18735, which corresponds to the published value of about 19 kDa, based on SDS/polyacrylamide gel electrophoresis. The amino acid composition of cytochrome c_L calculated from the deduced sequence is consistent with that determined by analysis of hydrolysates of cytochrome c_L which is given in Table 2, together with the compositions of cytochromes c_L from an obligate methylotroph (*Methylophilus methylotrophus*) and an acidophilic methylotroph (*Acetobacter methanolicus*) for comparison.

The deduced amino acid sequence indicates an *N*-terminal glutamine. This deduction is consistent with our observation that the mature protein is blocked for amino acid sequence analysis, possibly due to the spontaneous formation of pyroglutamate from glutamine at the *N*-terminal, as found in many other bacterial *c*-type cytochromes. The possession of an *N*-terminal pyroglutamyl-

M. extorquens		
Cytochrome c_L	LAGIALPALA:QPQSGP	(*a*)
Methanol dehydrogenase (second subunit)	LSGLAAPALA:YDGTKC	(*b*)
R. sphaeroides		
Cytochrome c_2	AAFAALPALA:QEGDPE	(*c*)
R. capsulatus		
Cytochrome c_2	ALVLAAPAFA:QDAAKG	(*d*)
P. denitrificans		
Cytochrome oxidase (subunit II)	ATMTAVPALA:QDVLGD	(*e*)
Methanol dehydrogenase	AVLTTAPATA:NDQLVE	(*f*)
Cytochrome c_1	TVALAGGAVA:QDASTA	(*g*)

Fig. 2. Signal peptides of *M. extorquens* compared with those of related bacteria

The sequences presented are the *C*-terminal of the signal peptides joined (by colon) to *N*-terminal of the mature proteins. *a*, present work; *b*, D. N. Nunn & C. Anthony (unpublished work); *c*, Donohue *et al.* (1986); *d*, Daldal *et al.* (1986); *e*, Steinrucke *et al.* (1987), Raitio *et al.* (1987); *f*, Harms *et al.* (1987); *g*, Kurowski & Ludwig (1987).

prolyl- pair would be consistent with the failure of pyroglutamate aminopeptidase to catalyse the removal of the pyroglutamate residue and hence to release the *N*-terminal sequence for amino acid analysis because this aminopeptidase does not catalyse the hydrolysis of *N*-terminal peptide bonds between pyroglutamate and proline (Podell & Abraham, 1978).

Further evidence that the deduced amino acid sequence corresponds to that of cytochrome c_L is the result of treatment with hydroxylamine, which cleaves proteins between asparagine and glycine residues (Croft, 1980). That there are two such pairs in the deduced sequence of cytochrome c_L (Fig. 1) is consistent with the observation that two (unblocked) peptides were generated by treatment with hydroxylamine; their *N*-terminal sequences were as follows: Gly-Glu-Ser-Leu-Phe and Gly-Met-Met-Gly-Pro (indicated by a broken line in Fig. 1).

The deduced signal peptide is shown underlined in Fig. 1. It starts with a methionine, coded by ATG, which is 10 bp downstream from a typical ribosome-binding site (the Shine–Dalgarno sequence underlined in Fig. 1). Possession of a signal sequence is consistent with the known periplasmic location of this *c*-type cytochrome (Alefounder & Ferguson, 1981; Anthony, 1988; Ferguson, 1988). The signal peptide is typical of Gram-negative bacteria, having a pair of positively-charged residues, which are probably involved in binding to the negatively-charged outer phospholipids of the periplasmic membrane. These are followed by a stretch of hydrophobic residues, essential for transport of the cytochrome across the membrane, and the peptide terminates in the peptidase recognition and cleavage site. Fig. 2 shows the sequence of this part of the signal peptide for cytochrome c_L together with those for other periplasmic proteins from *Paracoccus denitrificans* and *Rhodobacter* species. The marked similarity of these sequences provides further support for the suggestion that *Methylobacterium* may be considered to be in the same division of the eubacterial kingdom as *Paracoccus* and *Rhodobacter* (see above).

Cytochrome c_L is soluble, it has a high midpoint redox

potential (256 mV at pH 7), it has absorbance maxima in the reduced state at about 550 nm and in the oxidized state at 695 nm, it is low spin, and it has histidine and methionine as the fifth and sixth ligands to the single haem (O'Keeffe & Anthony, 1980*a*,*b*; Beardmore-Gray *et al.*, 1982). In these respects it appears to be typical of cytochromes *c* belonging to Class I as proposed by Ambler (1982). That it is not typical of this class of cytochromes, however, has been previously indicated by its novel function, its large size (about 19 kDa), its reaction with carbon monoxide (albeit slow and incomplete), its rapid autoreduction at high pH and the response of its redox potential to changes in pH (O'Keeffe & Anthony, 1980*a*,*b*; Beardmore-Gray *et al.*, 1982).

Except for the haem-binding site, the sequence of cytochrome c_L shows no homology with any cytochromes in the EMBL databases; in particular, none of the conserved features of *c*-type cytochromes are seen in the sequence of cytochrome c_L. One key feature of special importance in a typical Class I cytochrome *c* is the sixth ligand methionine which is usually about 60 residues (or more) towards the *C*-terminal from the haem-binding histidine. In cytochrome c_L the three methionines are all within 50 residues of this histidine, and the sequences around the methionines bear no relation to those around the methionines of other *c*-type cytochromes. This is consistent with the previous observations of cytochrome c_L that have indicated that it may have an unusual environment for the haem (O'Keeffe & Anthony, 1980*a*,*b*; Beardmore-Gray *et al.*, 1982), and that the axial methionine ligand has a novel configuration, as directly observed by n.m.r. studies (preliminary results quoted by Santos & Turner, 1988).

The position of many aromatic and lysine residues in the polypeptide chains of *c*-type cytochromes are highly conserved. The lysine residues arranged around the haem pocket are of particular importance in binding cytochrome *c* to its electron donors and acceptors (terminal oxidase or photosynthetic reaction centres). That there is no obvious homologous arrangement of lysines in cytochrome c_L, as shown in the present work, is not surprising because this cytochrome is unlikely to involved in reaction with the oxidase (Anthony, 1986, 1988). Its function is to reaction with methanol dehydrogenase and with the small basic cytochrome c_H. This reacts with the oxidase and is similar in structure to other such Class I cytochromes; its lysines are likely, therefore, to be involved in binding to carboxylate residues on the oxidase and on the cytochrome c_L. It might be expected, therefore, that we will find conserved regions of carboxylate residues around the haem cleft of all cytochromes c_L in our future work on this novel class of cytochromes.

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REFERENCES

Alefounder, P. R. & Ferguson, S. J. (1981) Biochem. Biophys. Res. Commun. **98**, 778–784
Ambler, R. P. (1982) in From Cyclotrons to Cytochromes (Robinson, A. B. & Kaplan, N. O., eds.), pp. 263–280, Academic Press, London
Anderson, D. J. & Lidstrom, M. E. (1988) J. Bacteriol. **170**, 2254–2262
Anthony, C. (1982) The Biochemistry of Methylotrophs, Academic Press, London
Anthony, C. (1986) Adv. Microbial Physiol. **27**, 113–210
Anthony, C. (1988) in Bacterial Energy Transduction (Anthony, C., ed.), pp. 293–316, Academic Press, London
Beardmore-Gray, M., O'Keeffe, D. T. & Anthony, C. (1982) Biochem. J. **207**, 161–165
Beardmore-Gray, M., O'Keeffe, D. T. & Anthony, C. (1983) J. Gen. Microbiol. **129**, 923–933
BRL Focus (1984) **6**, no. 4, p. 7
Croft, L. R. (1980) Introduction to Sequence Analysis, John Wiley and Sons, Chichester
Davidson, E. & Daldal, F. (1987) J. Mol. Biol. **195**, 13–24
Daldal, F., Cheng, S., Applebaum, J., Davidson, E. & Prince, R. C. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 22012–22016
Donohue, T. J., McEwan, A. G. & Kaplan, S. (1986) J. Bacteriol. **168**, 962–972
Elliott, J. E. & Anthony, C. (1988) J. Gen. Microbiol. **134**, 369–371
Fukumori, Y. & Yamanaka, T. (1987) J. Biochem. (Tokyo) **101**, 441–445
Ferguson, S. J. (1988) in Bacterial Energy Transduction (Anthony, C., ed.). pp. 151–182, Academic Press, London
Green, P. N., Bousefield, I. J. & Hood, D. (1988) Int. J. Syst. Bacteriol. **38**, 124–127
Harms, N., de Vries, G. E., Maurer, K., Hoogendijk, J. & Stouthamer, A. H. (1987) J. Bacteriol. **169**, 3969–3975
Henikoff, S. (1984) Gene **28**, 351–359
Kurowski, B. & Ludwig, B. (1987) J. Biol. Chem. **262**, 13805–13811
Messing, J. (1983) Methods Enzymol. **101**, 10–89
Pettigrew, G. W. & Moore, G. R. (1987) Cytochromes *c*: Biological Aspects, Springer-Verlag, Berlin
Nunn, D. N. & Lidstrom, M. E. (1986*a*) J. Bacteriol. **166**, 581–590
Nunn, D. N. & Lidstrom, M. E. (1986*b*) J. Bacteriol. **166**, 591–597
O'Keeffe, D. T. & Anthony, C. (1980*a*) Biochem. J. **192**, 411–419
O'Keeffe, D. T. & Anthony, C. (1980*b*) Biochem. J. **192**, 481–484
Podell, D. N. & Abraham, G. N. (1978) Biochem. Biophys. Res. Commun. **81**, 176–185
Raitio, M., Jalli, T. & Saraste, M. (1987) EMBO J. **6**, 2825–2833
Sanger, F. S., Nicklen, S. & Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 5463–5467
Santos, H. & Turner, D. L. (1988) Biochim. Biophys. Acta **954**, 277–286
Steinrucke, P., Steffens, G. C. M., Panskus, G., Buse, G. & Ludwig, B. (1987) Eur. J. Biochem. **167**, 431–439
Steinrucke, P., Gerhus, E. & Ludwig, B. (1988) Proc. Eur. Bioenerg. Conf. 5th, 142
Woese, C. R. (1987) Microbiol. Rev. **51**, 221–271

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