*Biochimica et Biophysica Acta*, 986 (1989) 257–262
Elsevier



BBAMEM 74635

# The $F_1F_0$-ATPase of *Escherichia coli*. The substitution of glycine by valine at position 29 in the *c*-subunit affects function but not assembly

Anthony L. Fimmel and Ursula Morris

*Department of Biochemistry, Faculty of Science, Australian National University, Canberra (Australia)*

(Received 23 May 1989)

Key words: *c*-Subunit; Site-directed mutagenesis; ATPase, $F_1$-$F_0$-; (*E. coli*)

**Site-directed mutagenesis has been used to construct two mutations within the *uncE* gene, coding for the *c*-subunit of the $F_1F_0$-ATPase, resulting in the substitution of Gly-29 by Val and Gly-18 by Leu. The strain carrying the Gly-29 → Val substitution is unable to grow on succinate as sole carbon source and possesses an uncoupled growth yield, while the strain carrying the Gly-18 → Leu substitution possesses a wild-type phenotype. Membranes prepared from the strain carrying the Gly-29 → Val substitution possess low levels of ATPase activity and are proton-impermeable. The $F_1$-ATPase activity of this strain was found to be inhibited by approx. 75% when bound to the membrane. These results are discussed in relation to a previously proposed model for the $F_0$ (Cox, G.B., Fimmel, A.L., Gibson, F. and Hatch, L. (1986) Biochim. Biophys. Acta 849, 62–69).**

## Introduction

The membrane-bound energy-transducing $F_1F_0$-ATPase has a remarkably similar structure in mitochondria, chloroplasts and bacteria. The enzyme complex can be readily dissociated into two portions, the $F_1$-ATPase and the membrane-bound $F_0$ sector which forms a proton pore. The $F_0$ sector from all sources contains a 'proteolipid' of 70–82 amino-acid residues that is soluble in organic solvents. Many proteolipid subunits from various organisms have been sequenced and have been found to possess a number of features in common. Each of the proteins consists of two hydrophobic segments separated by a central polar region. Within the C-terminal hydrophobic segment there exists a conserved acidic residue, Asp-61 in *Escherichia coli*, which reacts with DCCD, resulting in inhibition of ATPase activity [1].

In *E. coli* the proteolipid or *c*-subunit is coded for by the *uncE* gene [2]. Analysis of DCCD-resistant mutants has indicated that the *c*-subunit is folded in a hairpin-like structure in the membrane, such that isoleucine at position 28 is close to the aspartic acid at position 61 [3]. Nuclear magnetic resonance studies of the *c*-subunit indicate that the molecule has extensive α-helical segments and support a hairpin structure for the molecule [4]. It appears likely that the proton pore of the $F_0$ sector consists of four amino acids, namely Arg-210, Glu-219 and His-245 of the *a*-subunit and Asp-61 of the *c*-subunit [5].

There exists within the *c*-subunit a helical arm of uniformly small amino acids consisting of Gly-18, Ala-21, Ala-25, Gly-29 and Gly-32. Strains carrying mutations at position 21 [6] and position 25 [7,8] which result in impaired $F_0$ function, have been isolated. A property common to all of the mutations in these strains is that they result in an increase in size of the amino acid substituted at these positions. In this paper we continue to investigate the role of amino-acid residues that lie on this helical arm of uniformly small amino acids.

## Materials and Methods

*Enzymes and chemicals.* All chemicals and enzymes used were of the highest quality available. Restriction endonucleases were obtained from Pharmacia. T4 DNA ligase, polynucleotide kinase, alkaline phosphatase, DNA polymerase (large fragment, Klenow enzyme), dideoxynucleotides and deoxynucleotides were obtained

Abbreviations: DCCD, dicyclohexylcarbodiimide; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

Correspondence: A.L. Fimmel, Department of Biochemistry, Faculty of Science, Australian National University, GPO Box 4, Canberra, ACT 2601, Australia.

TABLE I

*Strains of E. coli and plasmids used*

Chromosome nomenclature is that used by Bachmann [22]; plasmid nomenclature is that used by Novick et al. [23].

| Bacterial strain or plasmid | Relevant genotype | Source or reference |
|---|---|---|
| Bacterial strains | | |
| TG1 | *F'traD36 proAB lacI$^q$ZΔM15/ Δ(lac-pro) thi supE hsdD5* | Amersham |
| K37 | Hfr *supD* | N. Dixon |
| AN727 | *uncB402 recA* [a] | F. Gibson |
| AN943 | *uncE429 recA* [a] | F. Gibson |
| AN1440 | *uncF469 recA* [a] | F. Gibson |
| AF53 | pAF11/*uncE429 recA* [a] | this work |
| AF55 | pAN174/*uncE429 recA* [a] | Ref. 8 |
| AF89 | pAF19/*uncE429 recA* [a] | this work |
| AF105 | pAF23/*uncE429 recA* [a] | this work |
| Plasmids | | |
| pAF11 | Cm$^R$Tc$^S$ *uncB$^+$ E1002 F$^+$* | this work |
| pAF19 | Cm$^R$Tc$^S$ *uncB$^+$ E$^+$F$^+$* | this work |
| pAF23 | Cm$^R$Tc$^S$ *uncB$^+$ E1003 F$^+$* | this work |
| pAF27 | M13mp18 *uncB$^+$ E$^+$F$^+$H$^+$* | S. Howitt |
| pAN174 | Cm$^R$Tc$^S$ | F. Gibson |

[a] Other markers, *argH pyrE entA*.

from Boehringer-Mannheim, Australia. [α-$^{32}$P]dCTP and [γ-$^{32}$P]ATP were obtained from Amersham, Australia. Oligonucleotides were synthesized on an Applied Biosystems DNA Synthesizer.

*Bacterial strains and plasmids.* All the bacterial strains used were derived from *E. coli* K12 and are described, together with the plasmids used, in Table I.

*Media and growth of organisms.* 2 YT broth was prepared as described by Miller [9]. The mineral-salts minimal medium used and additions were as described previously [10]. Cells for the preparation of membranes were grown in 14-l fermenters essentially as described previously [11]. The media in the fermenter vessels were supplemented with 5% (v/v) Luria broth [12]. Turbidities of cultures were measured with a Klett–Summerson colorimeter. Growth yields were measured as turbidities after growth ceased in minimal media containing limiting (5 mM) glucose [7].

*Genetic techniques.* The techniques used for genetic experiments were as outlined previously [10,13,14].

*Preparation of plasmid and phage M13 DNA.* Plasmid DNA was prepared as described by Selker et al. [15]. The replicative form of phage M13 was prepared from a 250 ml 2YT broth culture inoculated with strain K37 and phage M13 at a multiplicity of infection of 1.0. Late-exponential phase cells were centrifuged and replicative form DNA was prepared as described for plasmid DNA.

The isolation of single-stranded phage M13 DNA was carried out as described by Messing [16].

*Site-directed mutagenesis.* Site-directed mutants were constructed using an Amersham in vitro oligonucleotide-directed mutagenesis kit. The oligonucleotide carrying the appropriate base substitution (see Table II) was annealed to the single stranded plasmid pAF27, extended using DNA polymerase and ligated using T4 DNA ligase. Strain TG1 was transformed with this preparation and the plaques so obtained were screened, using the appropriate γ-$^{32}$P-labelled oligonucleotide as probe, under conditions favouring hybridization of the probe to mutant plaques. Confirmation of the expected mutation was made by DNA sequence analysis. Single-site incorporation of each mutant oligonucleotide was confirmed by sequencing using that oligonucleotide as primer.

*DNA sequencing.* Nucleotide sequences were determined by the dideoxy chain-terminating method of Sanger et al. [17] using [α-$^{32}$P]dCTP.

*Preparation of cell membranes.* The preparation and treatment of membranes were as described previously [18].

*ATPase activity.* ATPase activity was measured by using the method described by Gibson et al. [19].

*Atebrin-fluorescence quenching.* Atebrin-fluorescence quenching was measured as described by Gibson et al. [19].

*Protein determination.* Protein concentrations were determined using Folin's phenol reagent [20].

## Results

### *Isolation of amino acid substitutions Gly-18 → Leu and Gly-29 → Val within the c-subunit*

Site-directed mutations resulting in amino acid substitutions Gly-18 → Leu and Gly-29 → Val were constructed using the appropriate oligonucleotide listed in Table II. The replicative form of the phage M13 carrying each mutation was digested with restriction endonucleases *Hin*dIII and *Cla*I, releasing a 2.2 kb fragment carrying the *uncB, uncE* and *uncF* genes. The digested replicative form was mixed with *Hin*dIII-*Cla*I digested

TABLE II

*Oligonucleotides used in site-directed mutagenesis and as sequencing primers for sequencing the uncE gene*

Underlined nucleotides denote differences from the wild-type sequence [21].

| Oligonucleotide | Amino-acid substitution |
|---|---|
| 5'-TGATGATGCTTCTGGCGGCA-3' | Gly-18 → Leu |
| 5'-ATCGGTATCGTCATCCTCGG-3' | Gly-29 → Val |
| 5'-AACACTACTACGTTTTAACT-3' [a] | |

[a] Sequencing primer used for sequencing from the *uncB-uncE* intergenic region.

vector pAN174 and ligated with T4 DNA ligase. A similar plasmid carrying the wild-type *uncB, uncE* and *uncF* genes was also constructed as a control. Strain AN727 (*uncB402*) was transformed with each ligation mix, and transformants were selected on rich medium containing chloramphenicol, and tested for ability to grow on succinate as the sole carbon source. Unexpectedly, transformants carrying the Gly-29 → Val mutation failed to grow on succinate minimal medium, whereas transformants carrying the Gly-18 → Leu mutation grew normally on this medium.

Plasmids were prepared from a small number of transformants carrying each mutation and analysed by digestion with restriction endonuclease *Hin*dIII and *Cla*I. A restriction pattern identical to that of control plasmid pAF19 was observed in each case, confirming the presence of the 2.2 kb fragment carrying each site-directed mutation. One such plasmid carrying the Gly-18 → Leu mutation was designated pAF11 and one carrying the Gly-29 → Val mutation was designated pAF23. The mutant allele resulting in the substitution of Gly-18 → Leu was designated *uncE1002*, while the allele resulting in the substitution Gly-29 → Val was designated *uncE1003*. It was concluded that the presence of the *uncE1003* allele on plasmid pAF23 (Gly-29 → Val) prevented the complementation of the *uncB402* allele in strain AN727 on succinate minimal medium containing chloramphenicol.

*Growth properties of strains carrying the Gly-18 → Leu and Gly-29 → Val mutations*

Strain AN943 (*uncE429*) was used to examine the properties of the mutations carried on plasmid pAF11 (Gly-18 → Leu) and plasmid pAF23 (Gly-29 → Val), since the chromosomally encoded *uncE429* gene product does not assemble into the membrane [24]. Plasmids pAF11 and pAF23 were transformed into strain AN943 (*uncE429*) and transformants were selected on rich medium containing chloramphenicol. The strains so constructed were designated strain AF53 (Gly-18 → Leu) and strain AF105 (Gly-29 → Val). Strain AF53 possesses growth properties similar to wild-type control strain AF89 (Table III), whereas strain AF105 is unable to grow on succinate as the sole carbon source and possesses a growth yield similar to the uncoupled control strain AF55 (pAN174/*uncE429*) (Table III).

The lack of complementation or dominance displayed by plasmid pAF23 (Gly-29 → Val) in complementation tests with strain AN727 (*uncB402*) was investigated. Plasmid pAF23 (Gly-29 → Val) was transformed into strain AN1440 (*uncF469*) and transformants were selected on nutrient medium containing chloramphenicol. Interestingly, these transformants failed to grow normally on minimal medium containing succinate as the sole carbon source, and one such transformant was found to possess an uncoupled growth yield of 162 Klett units.

*ATPase activities*

Cell membranes were prepared from mutant strains AF53 (Gly-18 → Leu) and AF105 (Gly-29 → Val), together with coupled control strain AF89 (pAF19/*uncE429*) and uncoupled control strain AF55 (pAN174/*uncE429*).

The ATPase activity of membranes from mutant strain AF105 (Gly-29 → Val) was similar to the uncoupled control strain AF55 (pAN174/*uncE429*) and were approx. 15% of the activity of membranes from the coupled control strain AF89 (pAF19/*uncE429*) (Table III). In contrast, the ATPase activity of strain AF53 (Gly-18 → Leu) was similar to that of coupled control strain AF89 (Table III).

When the membrane fractions were dialysed against low ionic strength buffer in the absence of *p*-aminobenzamidine and reassayed, the ATPase activity of strain AF105 (Gly-29 → Val) increased to 50% of the activity of the coupled control strain AF89 (pAF19/*uncE429*) (Table III). The ATPase activities of uncoupled control strain AF55 (pAN174/*uncE429*) and strain AF53 (Gly-18 → Leu) were unchanged by dialysis (Table III). The dialysis treatment causes the $F_1$-ATPase to be released from the membranes [25]. Two-dimensional gel electro-

TABLE III

*Properties of uncE mutant strains of E. coli*

| Bacterial strain | Plasmid-encoded amino-acid substitution | Growth on succinate | Growth yield on 5 mM glucose (Klett units) | ATPase activity (μmol/min per mg protein) | |
|---|---|---|---|---|---|
| | | | | predialysis | postdialysis |
| AF53 | Gly-18 → Leu | + | 215 | 0.58 | 0.57 |
| AF105 | Gly-29 → Val | − | 152 | 0.07 | 0.30 |
| AF55 [a] | | − | 158 | 0.03 | 0.05 |
| AF89 [b] | | + | 220 | 0.42 | 0.60 |

[a] Uncoupled control strain.
[b] Coupled control strain.

phoresis of membranes from strain AF105 (Gly-29 → Val) showed that the $F_1$-ATPase assembled normally on the membrane (Norris, U., unpublished data).

*Atebrin fluorescence-quenching*

Membrane preparations from the mutant and control strains were assayed for ATP-dependent and NADH-dependent atebrin fluorescence-quenching activities before and after the removal of the $F_1$-ATPase (Fig. 1). Membranes from the mutant strain AF105 (Gly-29 → Val) retained NADH-dependent atebrin fluorescence-quenching activity after removal of the $F_1$-ATPase while unstripped membranes from this strain lacked ATP-dependent atebrin fluorescence-quenching activity (Fig. 1). Membranes from the mutant strain AF53 (Gly-18 → Leu) possessed identical atebrin fluorescence-quenching activity to membranes from the coupled control strain AF89 (pAF19/*uncE429*) (Norris, U., unpublished data).

## Discussion

The mutant allele *uncE1003* codes for a *c*-subunit of the $F_1F_0$-ATPase in which Gly-29 is replaced by Val. Strain AF105, carrying the Gly-29 to Val substitution, possesses a low growth yield on limiting concentrations of glucose and is unable to grow on succinate as the sole carbon source. When carried on plasmid pAF23 (Gly-29 → Val), the mutation is dominant in complementation tests with strains carrying a mutation in either the *uncB* or *uncF* genes. Dominance has been observed before in complementation tests with the mutant *uncE1001* allele which codes for a *c*-subunit in which

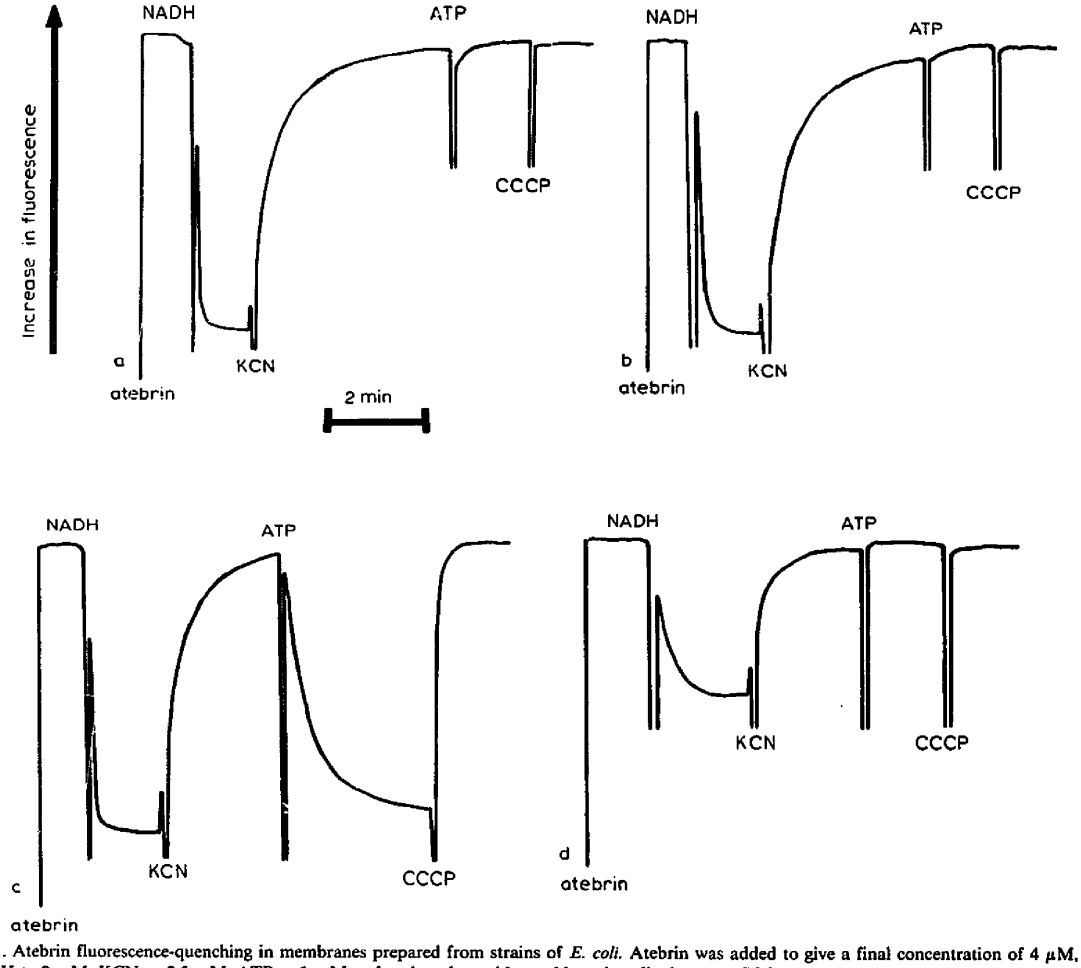


Fig. 1. Atebrin fluorescence-quenching in membranes prepared from strains of *E. coli*. Atebrin was added to give a final concentration of 4 μM, NADH to 2 mM, KCN to 2.5 mM, ATP to 1 mM and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) to 2 μM. (a) Membranes (0.4 mg of protein) from strain AF105 (Gly-29 → Val); (b) stripped membranes (0.7 mg of protein) from strain AF105; (c) membranes (0.7 mg of protein) from coupled control strain AF89; (d) stripped membranes (0.8 mg of protein) from coupled control strain AF89.

Ala-25 is replaced by Tyr [8]. These results indicate that the mutant *c*-subunit assembles into what would otherwise be a normal $F_1F_0$-ATPase complex and disrupts enzyme function. This occurs presumably as a result of the random assembly of both mutant and nonmutant *c*-subunits such that each $F_0$ has at least one mutant *c*-subunit.

Membranes prepared from strain AF105 (Gly-29 → Val) possessed low levels of ATPase activity, similar to the uncoupled control strain AF55 (pAN174/*uncE429*). Dialysis of membranes from strain AF105 (Gly-29 → Val) in the absence of *p*-aminobenzamidine resulted in the ATPase activity increasing to a level half that of the coupled control strain AF89 (pAF19/*uncE429*). This result indicates that the ATPase activity in strain AF105 (Gly-29 → Val) is inhibited by approx. 75% when the $F_1$-ATPase is bound to the membrane. Membranes from strain AF105 (Gly-29 → Val) lack ATP-dependent atebrin fluorescence-quenching activity and are proton impermeable even when the $F_1$-ATPase is removed. Two-dimensional gel electrophoresis indicates that the $F_1$-ATPase assembles normally in this strain.

In contrast, strains carrying the *uncE1002* allele, which codes for a *c*-subunit in which Gly-18 is replaced by Leu, possess a wild-type phenotype. Membranes prepared from strain AF53 (Gly-18 → Leu) possess both ATPase activity and ATP-dependent atebrin fluorescence-quenching activity similar to coupled control strain AF89 (pAF19/*uncE429*).

Other mutations in the *c*-subunit which result in the substitution of Ala-21 by Val [6] and Ala-25 by Thr [7] and by Tyr [8] have been described. If the α-helical hairpin structure of the *c*-subunit is correct, these amino-acid residues lie on a helical arm of uniformly small amino acids consisting of Gly-18, Ala-21, Ala-25, Gly-29 and Gly-32. All mutations so far described at positions 21, 25 and 29 affect oxidative phosphorylation and result in an increase in size of the amino acid substituted at these positions. Thus, an inverse relationship appears to exist between the size of the amino acids in these positions in the *c*-subunit and the level of ATPase activity. The observation that a 4-fold increase in ATPase activity can be released upon dialysis of membranes from strain AF105 (Gly-29 → Val) in the absence of *p*-aminobenzamidine suggests that steric hindrance may be responsible for the inhibition of ATPase activity in this strain. A similar level of inhibition of membrane-bound ATPase activity was observed in a strain carrying the Ala-25 to Tyr substitution [8]. These observations provide support for a rotational catalysis model for oxidative phosphorylation in which rotating *a*- and *b*-subunits interact with a ring of *c*-subunits [26]. According to this model, Ala-25 and Gly-29 lie on the same face of an α-helix and are directed towards the *a*- and *b*-subunits [27]. The increased size of amino acids at positions 25 and 29 may thus cause inhibition of both ATPase activity and proton translocation due to steric hindrance of rotational catalysis [27].

The Gly-18 to Leu substitution present in strain AF53 does not affect ATPase function. One possible explanation for this is that Gly-18 may be expected to reside at or below the bend in the opposite helix of the *c*-subunit at residue Pro-64. A proposed model for the structure of the $F_0$ [27] suggests that a bend in the helix at Pro-64 relieves steric constraints in the portion of the *c*-subunit below the bend, allowing greater flexibility in the substitution of amino acids in this region.

## Acknowledgements

We thank G. Cox and F. Gibson for their assistance and encouragement. We also thank F. Gibson, G. Cox and S. Howitt for the generous gift of bacterial and phage strains used in this work. We thank Mrs. P. Karp and Mr. R. Taylor for skilled technical assistance, Mr. G. Mayo for synthesis of oligonucleotides and N. Dixon for strain K37. This work was supported by a grant from the Faculties Research Fund, Australian National University, and by a grant from the Australian Research Grants Scheme.

## References

1 Sebald, W. and Hoppe, J. (1981) Curr. Top. Bioenerg. 12, 1–64.
2 Downie, J.A., Cox, G.B., Langman, L., Ash, G., Becker, M. and Gibson, F. (1981) J. Bacteriol. 145, 200–210.
3 Hoppe, J., Schairer, H.U. and Sebald, W. (1980) Eur. J. Biochem. 112, 17–24.
4 Moody, M.F., Jones, P.T., Carver, J.A., Boyd, J. and Campbell, I.D. (1987) J. Mol. Biol. 193, 759–774.
5 Howitt, S.M., Gibson, F. and Cox, G.B. (1988) Biochim. Biophys. Acta 936, 74–80.
6 Hoppe, J. and Sebald, W. (1984) Biochim. Biophys. Acta 768, 1–27.
7 Fimmel, A.L., Jans, D.A., Hatch, L., James, L.B., Gibson, F. and Cox, G.B. (1985) Biochim. Biophys. Acta 808, 252–258.
8 Fimmel, A.L. and Fordham, S.A. (1989) Biochim. Biophys. Acta 978, 299–304.
9 Miller, J.H. (1972) Experiments in Molecular Genetics, Cold Spring Harbor, New York.
10 Gibson, F., Cox, G.B., Downie, J.A. and Radik, J. (1977) Biochem. J. 164, 193–198.
11 Cox, G.B., Newton, N.A., Gibson, F., Snoswell, A.M. and Hamilton, J.A. (1970) Biochem. J. 117, 551–562.
12 Luria, S.E. and Burrous, J.W. (1957) J. Bacteriol. 74, 461–476.
13 Gibson, F., Downie, J.A., Cox, G.B. and Radik, J. (1978) J. Bacteriol. 134, 728–736.
14 Downie, J.A., Langman, L., Cox, G.B., Yanofsky, C. and Gibson, F. (1980) J. Bacteriol. 143, 8–17.
15 Selker, E., Brown, K. and Yanofsky, C. (1977) J. Bacteriol. 129, 388–394.
16 Messing, J. (1983) Methods Enzymol. 101, 20–78.
17 Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
18 Cox, G.B., Jans, D.A., Gibson, F., Langman, L., Senior, A.E. and Fimmel, A.L. (1983) Biochem. J. 216, 143–150.

19 Gibson, F., Cox, G.B., Downie, J.A. and Radik, J. (1977) Biochem. J. 162, 665–670.
20 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
21 Walker, J.E., Saraste, M. and Gay, N.J. (1984) Biochim. Biophys. Acta 768, 164–200.
22 Bachmann, B.J. (1983) Microbiol. Rev. 47, 180–230.
23 Novick, R.P., Clowes, R.C., Cohen, S.N., Curtiss, R., Datta, N. and Falkow, S. (1976) Bacteriol. Rev. 40, 168–189.
24 Jans, D.A., Fimmel, A.L., Langman, L., James, L.B., Downie, J.A., Senior, A.E., Ash, G.R., Gibson, F. and Cox, G.B. (1983) Biochem. J. 211, 717–726.
25 Cox, G.B., Downie, J.A., Fayle, D.R.H., Gibson, F. and Radik, J. (1978) J. Bacteriol. 133, 287–292.
26 Cox, G.B., Fimmel, A.L., Gibson, F. and Hatch, L. (1986) Biochim. Biophys. Acta 849, 62–69.
27 Cox, G.B., Jans, D.A., Fimmel, A.L., Gibson, F. and Hatch, L. (1984) Biochim. Biophys. Acta 768, 201–208.