Biochimica et Biophysica Acta, 1015 (1990) 264–268
Elsevier

BBABIO 43141

Mutational analysis of the function of the *a*-subunit of the F_0F_1-ATPase of *Escherichia coli*

Susan M. Howitt, Robert N. Lightowlers *, Frank Gibson and Graeme B. Cox

Division of Biochemistry and Molecular Biology, John Curtin School of Medical Research, Australian National University, Canberra (Australia)

(Received 25 July 1989)

Key words: *a*-Subunit; ATPase, F_0F_1-; Ion channel; Site-directed mutagenesis; (*E. coli*)

In a model proposed for the structure of the *a*-subunit of the *Escherichia coli* F_0F_1-ATPase (Howitt, S.M., Gibson, F. and Cox, G.B. (1988) Biochim. Biophys. Acta 936, 74–80), a cluster of charged residues, including one arginine and four aspartic acid residues, lie on the periplasmic side of the membrane. On the cytoplasmic side, three pairs of lysine residues and an arginine residue are present. Site-directed mutagenesis was used to investigate the roles of these residues. It was found that none was directly involved in the proton pore. However, the substitutions of Asp-124 or Asp-44 by asparagine or Arg-140 by glutamine had similar effects in that the membranes from such mutants from which the F_1-ATPase was removed were proton-impermeable. A combination of the Asp-44 mutation with either the Asp-124 or Arg-140 mutations in the same strain resulted in complete loss of oxidative phosphorylation. It was tentatively concluded that Asp-124 and Arg-140 form a salt bridge, as did Asp-44 with an unknown residue, and these salt bridges were concerned with the maintenance of correct *a*-subunit structure. Further support for this conclusion was obtained when second site revertants of a Glu-219 to histidine mutant were found to have either histidine or leucine replacing Arg-140. Thus, the lack of the Asp-124/Arg-140 salt bridge might enable repositioning of the helices of the *a*-subunit such that His-219 becomes a functional component of the proton pore.

Introduction

The F_0F_1-ATPase enzyme complex catalyses the terminal step in oxidative phosphorylation and photophosphorylation and is located in mitochondrial, chloroplast and bacterial membranes. In *Escherichia coli* [1] the enzyme comprises eight non-identical subunits, *a*, *b*, *c*, α, β, γ, δ and ε, encoded by the genes *uncB, F, E, A, D, G, H* and *C*, respectively. The *a*, *b* and *c* subunits are integral membrane proteins and form the F_0 portion of the complex which can function as a proton pore. The α, β, γ, δ and ε subunits are peripheral membrane proteins forming the F_1-ATPase portion of the complex which retains ATP hydrolysis activity when removed from the membrane.

The *a*, *b* and *c* subunits of the proton pore are present in a stoichiometry of 1 : 2 : 6–12 and secondary and tertiary structures of these subunits have been proposed [1–3]. Previous studies with mutants have identified several residues as components of the proton pore [4–7]. This has led to the suggestion that the proton pore consists of four amino acids: Arg-210, Glu-219 and His-245 of the *a*-subunit and Asp-61 of the *c*-subunit [8]. The possibility that Asp-44 was also involved had been raised, with the data obtained being consistent with either Asp-44 being required to pass protons from the bulk phase to the proton pore or being involved in stabilization of the *a*-subunit structure [8]. In the present paper the effect of altering eight additional charged residues of the *a*-subunit, proposed to be in the vicinity of the polar head group region of the membrane, have been described. One of these residues, Arg-140, was also found to be replaced in second-site revertants of a Glu-219 to histidine mutant *a*-subunit.

Experimental Procedures

Enzymes and chemicals. All chemicals and enzymes used were of the highest quality available. Oligonucleotides were synthesized by K. Newell, C.S.I.R.O., Division of Plant Industry, Canberra and G. Mayo, Division of Biochemistry and Molecular Biology, John Curtin School of Medical Research, A.N.U., Canberra. T4

* Present address: Institute of Molecular Biology, University of Oregon, Eugene, OR, U.S.A.

Correspondence: Dr. S.M. Howitt, Division of Biochemistry and Molecular Biology, John Curtin School of Medical Research, G.P.O. Box 334, Canberra, ACT 2601, Australia.

TABLE I

Strains of Escherichia coli and plasmids used

Chromosome nomenclature is that used by Bachman [9]; plasmid nomenclature is that used by Novick [10].

Bacterial strain or plasmid	Relevant genotype
AN727	*uncB402 argH pyrE entA recA* [11]
AN943	*uncE429 argH pyrE entA recA* [12]
AN2841	pAN496/*uncB402 argH pyrE entA recA*
AN2914	pAN562/*uncB402 argH pyrE entA recA*
AN2869	pAN520/*uncB402 argH pyrE entA recA*
AN2937	pAN574/*uncB402 argH pyrE entA recA*
AN2884	pAN532/*uncB402 argH pyrE entA recA*
AN2899	pAN547/*uncB402 argH pyrE entA recA*
AN2900	pAN546/*uncB402 argH pyrE entA recA*
AN2867	pAN518/*uncB402 argH pyrE entA recA*
AN2868	pAN519/*uncB402 argH pyrE entA recA*
AN2882	pAN530/*uncB402 argH pyrE entA recA*
AN2883	pAN531/*uncB402 argH pyrE entA recA*
AN3004	pAN597/*uncB402 argH pyrE entA recA*
AN2840	pAN495/*uncB402 argH pyrE entA recA*
AN2736	pAN174/*uncB402 argH pyrE entA recA*
AN3003	pAN596/*uncB402 argH pyrE entA recA*
pAN174	$Cm^r Tc^s$ [5]
pAN496	$Cm^r Tc^s$ *uncB599* $E^+ F^+$
pAN562	$Cm^r Tc^s$ *uncB619* $E^+ F^+$
pAN520	$Cm^r Tc^s$ *uncB605* $E^+ F^+$
pAN574	$Cm^r Tc^s$ *uncB621* $E^+ F^+$
pAN532	$Cm^r Tc^s$ *uncB610* $E^+ F^+$
pAN547	$Cm^r Tc^s$ *uncB623* $E^+ F^+$
pAN546	$Cm^r Tc^s$ *uncB622* $E^+ F^+$
pAN518	$Cm^r Tc^s$ *uncB603* $E^+ F^+$
pAN519	$Cm^r Tc^s$ *uncB604* $E^+ F^+$
pAN530	$Cm^r Tc^s$ *uncB608* $E^+ F^+$
pAN531	$Cm^r Tc^s$ *uncB609* $E^+ F^+$
pAN597	$Cm^r Tc^s$ *uncB627* $E^+ F^+$
pAN596	$Cm^r Tc^s$ *uncB626* $E^+ F^+$
pAN495	$Cm^r Tc^s$ *uncB*$^+$ $E^+ F^+$ [8]

polynucleotide kinase and T4 DNA ligase were obtained from Amersham (Australia) Pty Ltd as was [α-^{35}S]dATPαS.

Bacterial strains and plasmids. All of the bacterial strains used were derived from *E. coli* K-12 and are described, together with the plasmids used, in Table I.

Genetic techniques. The techniques used for genetic experiments were as outlined previously [13].

Preparation of plasmids. Plasmid DNA was isolated by the method of Selker et al. [14]. The isolation of single-stranded M13 phage DNA was done by using the method of Schreier and Cortese [15].

DNA sequencing. Nucleotide sequences were determined by the dideoxy chain-terminating method of Sanger et al. [16] using a Pharmacia T7 dideoxy nucleotide sequencing kit with [α-^{35}S]dATPαS.

Media and growth of organisms. The mineral salts minimal medium used and additions were as described previously [17]. Cells for the preparation of membranes were grown in 14 l fermenters as described previously [18]. The minimal salts medium in the fermenters was supplemented with 5% (v/v) Luria broth [19].

Turbidities of cultures were measured with a Klett-Summerson colorimeter. Growth yields were measured as turbidities after growth had ceased in medium containing limiting (5 mM) glucose.

Preparation of subcellular fractions. The preparation and treatment of subcellular fractions were as previously described [8].

Site-directed mutagenesis and construction of plasmids. Mutants were obtained as outlined in the Amersham handbook 'Oligonucleotide-directed in vitro mutagenesis system'. The oligonucleotides carrying the appropriate substitutions are shown in Table II. After confirmation of the presence of each mutation by DNA sequencing, a 2.2 kb *Hin*dIII-*Cla*I fragment carrying *uncB, uncE* and *uncF* was subcloned from M13mp18 replicative form into the vector pAN174 as previously described [8]. Plasmids containing the correct insert were then transformed into strain AN727 (*uncB402*). One transformant from each was purified and retained for further work. These strains are listed in Table I. For clarity, these strains are designated with reference to the amino-acid substitutions, i.e., AN2841 (D124 → N), AN2914 (R140 → Q), AN2869 (D44 → N, D124 → N), AN2937 (D44 → N, R140 → Q), AN2867 (D119 → A), AN2868 (D146 → N), AN2882 (K65 → Q, K66 → Q), AN2883 (K97 → Q, K99 → Q), AN2884 (E219 → H), AN2899 (E219 → H, R140 → L), AN2900 (E219 → H, R140 → H), AN3003 (D124 → N, R140 → Q) and AN3004 (E219 → Q, R140 → H).

Other methods. ATPase and atebrin fluorescence quenching activities were assayed as previously described [17]. Protein concentrations were determined using Folin's phenol reagent [20] with bovine serum albumin as standard.

TABLE II

Oligonucleotides used in the preparation of mutants

Underlined nucleotides denote differences from the normal sequence [1].

Oligonucleotide	Amino-acid substitution
5′-TCAATATTAACTCCATGTT-3′	Asp-44 → Asn
5′-GAACCTGATGGCTTTACTGCC-3′	Asp-119 → Ala
5′-TGCCTATCAACCTGCTGCC-3′	Asp-124 → Asn
5′-CCGTCTGCGAACGTGAACGTAACG-3′	Asp-146 → Asn
5′-CCTGCACTGCAAGTGGTTCCG-3′	Arg-140 → Gln
5′-GTATGCCGGCACCTGATTTTC-3′	Glu-219 → His
5′-ATGCCGGTCAGCTGATTTT-3′	Glu-219 → Gln
5′-AGCGTAGCCCAACAGGCGACCA-3′	Lys-65 → Gln, Lys-66 → Gln
5′-ATGGCCAAAGCCAGCTGATTGC-3′	Lys-97 → Gln, Lys-99 → Gln

Results

The effects of changing the a-subunit residues Asp-119, Asp-124, Asp-146, Arg-140, Lys-65, Lys-66, Lys-97 and Lys-99.

Strains AN2867 (D119 → A), AN2841 (D124 → N), AN2868 (D146 → N), AN2914 (R140 → Q), AN2882 (K65 → Q, K66 → Q) and AN2883 (K97 → Q, K99 → Q) were generated after site-directed mutagenesis using the oligonucleotides listed in Table II. Mutant strains of *E. coli* which have lost the ability to carry out oxidative phosphorylation are unable to grow on media in which succinate is the sole source of carbon. The above strains grew on succinate minimal medium and also had normal growth yields from limiting concentrations of glucose (Table III). Membranes were prepared from each of the above strains and ATPase activities and NADH-dependent and ATP-dependent atebrin fluorescence quenching activities determined (Table III). The atebrin fluorescence quenching activities were also determined on membrane preparations that had been stripped of the F_1-ATPase by dialysis against low ionic strength buffer in the absence of 4-aminobenzamidine. The mutant strains fell into two clear groups. In membranes from strains AN2867 (D119 → A), AN2868 (D146 → N), AN2882 (K65 → Q, K66 → Q) and AN2883 (K97 → Q, K99 → Q) all activities were similar to those obtained for membranes from the normal control, strain AN2840 (Table III). The second group, including strains AN2841 (D124 → N) and AN2914 (R140 → Q), had proton-impermeable stripped membranes, as indicated by the retention of NADH-dependent atebrin fluorescence quenching activity after dialysis. The effect of these mutations was therefore similar to that previously described for the Asp-44 to asparagine substitution [8] (also Table III). The ATPase activities varied between strains within this group, presumably due to differing effects on the level of assembly of the F_1-ATPase.

The effects of combining the changes Asp-44 to asparagine, Asp-124 to asparagine and Arg-140 to glutamine

The three charged residues which were found to have an effect on proton permeability were examined further by combining pairs of mutations. Strains AN2869 (D44 → N, D124 → N) and AN2937 (D44 → N, R140 → Q) had the same properties as the uncoupled control strain, AN2736 (Table III). In contrast, strain AN3003 (D124 → N, R140 → Q) had similar properties to both strains AN2841 (D124 → N) and AN2914 (R140 → Q) in that it was able to grow on succinate and had a normal growth yield but retained NADH-dependent fluorescence quenching in stripped membranes. The Asp-44 to

TABLE III

Properties of uncB mutant strains of E. coli

Bacterial strain	Plasmid-encoded amino-acid substitution	Growth on succinate [a]	Growth yield on 5 mM glucose (Klett units)	ATPase activity (μmol/min per mg protein)	% Fluorescence quenching		
					ATP-dependent	NADH-dependent	
						native	stripped
Coupled strains							
AN2840 [b]		+	236	0.8	82	86	35
AN2867	Asp-119 → Ala	+	231	0.8	82	86	39
AN2868	Asp-146 → Asn	+	229	0.7	74	88	31
AN2882	Lys-65 → Gln, Lys-66 → Gln	+	234	0.9	80	84	27
AN2883	Lys-97 → Gln, Lys-99 → Gln	+	240	0.8	76	82	35
AN2900	Glu-219 → His, Arg-140 → His	+	210	1.1	84	88	33
Coupled strains but with proton-impermeable stripped membranes							
AN2834 [d]	Asp-44 → Asn	+	232	0.5	58	82	84
AN2841	Asp-124 → Asn	+	231	1.1	82	88	74
AN3003	Asp-124 → Asn, Arg-140 → Gln	+	215	0.7	30	82	80
AN2914	Arg-140 → Gln	+	225	0.5	72	76	70
AN2899	Glu-219 → His, Arg-140 → Leu	+	208	1.1	28	86	86
Uncoupled strains							
AN2736 [c]		−	140	0.2	0	89	84
AN2869	Asp-44 → Asn, Asp-124 → Asn	−	141	0.3	10	85	86
AN2937	Asp-44 → Asn, Arg-140 → Gln	−	144	0.4	10	84	86
AN2884	Glu-219 → His	tr.	155	0.3	8	80	60
AN3004	Glu-219 → Gln, Arg-140 → His	−	151	0.6	14	80	80

[a] Colony size after 30 h incubation at 37 °C on solid succinate minimal medium. Growth was scored as normal colonies (+), slight growth (tr.) and no visible colonies (−).

[b] Coupled control strain.

[c] Uncoupled control strain.

[d] Ref. 8.

asparagine substitution was also combined with the Asp-119 to alanine change (results not shown) but the properties were indistinguishable from those of the strain carrying only the Asp-44 to asparagine substitution [8].

Isolation and characterisation of revertants of strain AN2884 (E219 → H)

Strain AN2884 (E219 → H) was generated after site-directed mutagenesis using the oligonucleotide indicated in Table II. The effects of this mutation have been described previously by Cain and Simoni [21] and our data are consistent with theirs except that our mutant had lower ATPase activity. This may have been due to inhibition of activity in our assay [7] or to a lower level of expression, and thus reduced assembly of the ATPase, from the vector used in our study [5]. The growth properties of strain AN2884 (E219 → H) indicated that this strain was effectively uncoupled (Table III). The properties of membranes from strain AN2884 (E219 → H) were consistent with this conclusion, with low ATPase activity, very low ATP-dependent atebrin fluorescence quenching activity and retention of NADH-dependent quenching in stripped membranes.

The interest in strain AN2884 (E219 → H) in the present context was in the nature of the revertants that were isolated following culture on succinate minimal medium. Plasmids were isolated from these revertants and used to transform strain AN727 (*uncB402*), selecting for growth on succinate minimal medium containing chloramphenicol. The plasmid isolation and transformation was then repeated to ensure that the final strains carried pure revertant plasmid. Four revertants were isolated and the entire *uncB* gene from each was sequenced after subcloning into phage M13mp18.

Three of the revertants showed a change of G to A at nucleotide 419, resulting in the replacement of Arg-140 by histidine. In the fourth, T replaced G in the same position, giving the sequence CTT, coding for leucine. The properties of one of the Arg-140 to histidine revertants, strain AN2900, and the Arg-140 to leucine revertant, strain AN2899, are summarized in Table III. Both had normal ATPase activity and a growth yield slightly lower than normal. The atebrin fluorescence quenching showed that strain AN2900 (E219 → H, R140 → H) was completely normal, while strain AN2899 (E219 → H, R140 → L) had only a small ATP-dependent fluorescence quench and retained NADH-dependent quenching after dialysis (Table III).

The replacement of Arg-140, particularly by histidine, reverses the effect of the Glu-219 to histidine substitution. One of the possible explanations for this effect was that, in the revertant strain, His-219 is now able to function as a component of the proton pore. A mutant strain was therefore constructed in which Arg-140 was replaced by histidine, and glutamine was the residue at position 219 rather than either glutamate or histidine. The strain carrying these two mutations, strain AN3004, was essentially similar to the uncoupled control strain (Table III).

Discussion

Seventeen charged residues in the *a*-subunit, postulated to be in or near the hydrophobic region of the membrane (Fig. 1), have now been altered by site-directed mutagenesis [4–8]. Only three of these, Arg-210, Glu-219 and His-245, are essential for ATP synthesis and it has been suggested that these residues are components of the proton pore [7,8]. Eleven are apparently not required for any aspect of ATPase function, since mutants in which these residues are replaced by polar residues are completely normal. These residues are Arg-61, Lys-65, Lys-66, Lys-97, Lys-99, Asp-119, Asp-146, Lys-167, Lys-169, Glu-196 and Lys-203. The remaining three residues, Asp-44, Asp-124 and Arg-140, may play a role in the stabilization of the *a*-subunit. Changes to these residues individually resulted in the loss of proton permeability when the F_1-ATPase was removed. Combinations of the Asp-44 to asparagine mutation with either of the other two had a more drastic effect. Strains AN2869 (D44 → N, D124 → N) and AN2914 (D44 → N, R140 → Q) were very similar in all respects to the

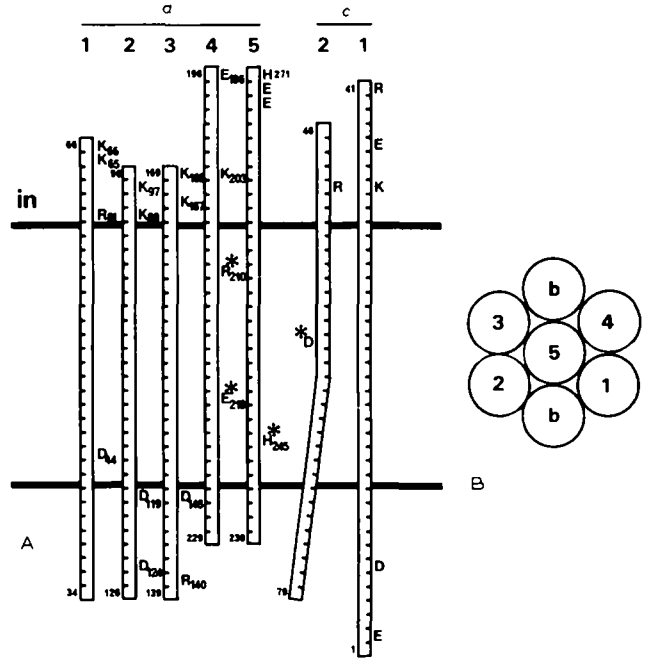

Fig. 1. (A) Proposed transmembrane helices of the *a*- and *c*-subunits from the *E. coli* F_0-ATPase. The N-terminal and C-terminal residues for each helix, and the residues altered by site-directed mutagenesis are numbered. D, aspartic acid; E, glutamic acid; H, histidine; K, lysine; R, arginine. The residues involved in the proton pore are indicated by a star (*). (B) Proposed packing of the transmembrane helices of the *a*- and *b*-subunits of the F_0-ATPase of *E. coli*. These helices have been proposed to be packed within a ring of *c*-subunits [6].

uncoupled control strain. In contrast, strain AN3003, in which the Asp-124 to asparagine and the Arg-140 to glutamine mutations were combined had a similar phenotype to the strains carrying either mutation alone. These results would suggest that Asp-124 and Arg-140 are functionally equivalent, one explanation being that Asp-124 and Arg-140 form a salt bridge which stabilizes the *a*-subunit structure.

This is consistent with our current structural model of the *a*-subunit, in which Asp-124 on helix 2 is adjacent to Arg-140 on helix 3 [8]. Such a salt bridge is apparently not absolutely required for correct assembly of the *a*-subunit structure, since replacements of Asp-124, Arg-140 or both do not result in complete loss of function. Only when Asp-44 is missing does this interaction become essential. Previous evidence suggested that Asp-44 was involved either in passing protons from the bulk phase to the proton pore or had a stabilizing effect on the F_0-ATPase. The results presented here make the latter more likely. Presumably, Asp-44 also participates in some stabilizing interaction with another residue.

The role of an Asp-124/Arg-140 charge pair in stabilizing the *a*-subunit may provide an explanation for the observation that in the revertants of strain AN2884 (E219 → H) Arg-140 had been replaced. Since Arg-140 could be replaced by either histidine or leucine, the loss of the arginine residue is clearly the main reason for the revertant phenotype. The most likely explanation of the phenotype of these revertants is that the absence of a stabilizing charge pair between Asp-124 and Arg-140 allows repositioning of the helices in such a way that His-219 can make the same interactions as the Glu-219 normally present. This is supported by the observation that strain AN3004, in which His-219 is replaced by glutamine in the presence of the Arg-140 to histidine change, is essentially uncoupled.

It therefore appears that it is only structural constraints which prevent His-219 replacing Glu-219 as a fully functional component of the proton pore. The fact that strain AN2900 (E219 → H, R140 → H) has properties closer to the wild type than strain AN2899 (E219 → H, R140 → L) may be because histidine can still form a stabilizing charge pair with Asp-124 while leucine cannot. The different geometry of the aspartic acid-histidine pair compared to the aspartic acid-arginine pair could explain the repositioning of His-219 in such a way as to regain function.

The results obtained with strains AN2884 (E219 → H), AN2900 (E219 → H, R140 → H) AN2899 (E219 → H, R140 → L) and AN3004 (E219 → Q, R140 → H) illustrate a possible mechanism for the gating of ion channels. In strain AN2884 (E219 → H), the channel is closed because His-219 cannot make the necessary interactions. However, breaking the charge pair of Asp-124/Arg140, by substituting histidine or leucine for Arg-140, repositions the helices so that His-219 can function and thus opens the channel. In gated ion channels, a similar structure, coupled with the breaking of a charge pair by interaction with a ligand, could provide the gating mechanism.

The results described here support our previous model of the *a*-subunit of the F_0F_1-ATPase (Fig. 1). None of the charged residues in helices 1, 2 and 3 is directly involved in the proton pore. The conclusion that Asp-124 and Arg-140 form a charge pair is consistent with helices 2 and 3 being adjacent, as predicted in the model. The revertants of strain AN2884 (E219 → H) have also provided evidence that this charge pair plays an important role in positioning the helices of the *a*-subunit of the F_0F_1-ATPase.

Acknowledgements

We thank P. Ewing, R. Taylor, D. Webb and S. Paxman for excellent technical assistance. We thank Sir Rutherford Robertson for stimulating discussions and thank the following for financial support: Sir John Proud, The Raymond E. Purves Foundation, The James N. Kirby Foundation, C.H. Warman, and the Bruce and Joy Reid Foundation.

References

1 Walker, J.E., Saraste, M. and Gay, N.J. (1984) Biochim. Biophys. Acta 768, 164–200.
2 Cox, G.B., Fimmel, A.L., Gibson, F. and Hatch, L. (1986) Biochim. Biophys. Acta 849, 62–69.
3 Senior, A.E. (1983) Biochim. Biophys. Acta 726, 81–95.
4 Cain, B.D. and Simoni, R.D. (1986) J. Biol. Chem. 261, 10043–10050.
5 Lightowlers, R.N., Howitt, S.M., Hatch, L., Gibson, F. and Cox, G.B. (1987) Biochim. Biophys. Acta 894, 399–406.
6 Lightowlers, R.N., Howitt, S.M., Hatch, L., Gibson, F. and Cox, G. (1988) Biochim. Biophys. Acta 933, 241–248.
7 Cain, B.D. and Simoni, R.D. (1989) J. Biol. Chem. 264, 3292–3300.
8 Howitt, S.M., Gibson, F. and Cox, G.B. (1988) Biochim. Biophys. Acta 936, 74–80.
9 Bachmann, B.J. (1983) Microbiol. Rev. 47, 180–230.
10 Novick, R.P., Clowes, S.N., Cohen, S.N., Curtiss, R., Datta, N. and Falkow, S. (1985) Microbiol. Rev. 40, 168–189.
11 Gibson, F., Cox, G.B., Downie, J.A. and Radik, J. (1977) Biochem. J. 162, 665–670.
12 Downie, J.A., Senior, A.E., Gibson, F. and Cox, G.B. (1979) J. Bacteriol. 137, 711–718.
13 Downie, J.A., Langman, L., Cox, G.B., Yanofsky, C. and Gibson, F. (1980) J. Bacteriol. 143, 8–17.
14 Selker, E., Brown, K. and Yanofsky, C. (1977) J. Bacteriol. 129, 388–394.
15 Schreier, P.H. and Cortese, R. (1979) J. Mol. Biol. 129, 169–172.
16 Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
17 Gibson, F., Cox, G.B., Downie, J.A. and Radik, J. (1977) Biochem. J. 164, 193–198.
18 Cox, G.B., Newton, N.A., Gibson, F., Snoswell, A.M. and Hamilton, J.A. (1970) Biochem. J. 117, 551–562.
19 Luria, S.E. and Burrous, J.W. (1957) J. Bacteriol. 74, 461–476.
20 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
21 Cain, B.D. and Simoni, R.D. (1988) J. Biol. Chem. 263, 6606–6612.