Aust. J. Biol. Sci., 1977, **30**, 141–53

Effect of Dimethyl Sulphoxide on the Expression of Nitrogen Fixation in Bacteria

Mary L. Skotnicki and Barry G. Rolfe

Department of Genetics, Research School of Biological Sciences,
Australian National University, P.O. Box 475, Canberra, A.C.T. 2601.

Abstract

Storage in dimethyl sulphoxide (DMSO) of *Escherichia coli* K12 hybrids carrying *nif* $^+$ genes from *Klebsiella pneumoniae* can result in selection of a defective nitrogen-fixing phenotype. Similar results are obtained with *E. coli* K12 hybrids containing the nitrogen-fixing capacity from *Rhizobium trifolii*. DMSO appears to affect particular inner membrane proteins associated with energy metabolism in *E. coli* K12 and four chromosomal regions (*chlD*, *chlG*, *his* and *unc*) are associated with resistance to DMSO.

Introduction

Dimethyl sulphoxide (DMSO) is widely used as a cryoprotective agent (Ashwood-Smith 1967), but it has recently been shown to affect recombination (Ihrke and Kronstad 1975), mutagenesis (Anwar and Reddy 1975), DNA denaturation (Nakanishi *et al.* 1974*a*), the initiation of specific RNA transcripts (Nakanishi *et al.* 1974*b*), and specific membrane functions (Kunze 1974). During the course of our investigation of nitrogen fixation in different bacteria, we found that DMSO can have a marked effect on the expression of the nitrogen-fixing phenotype.

Nitrogen fixation in nature is classically a process in which atmospheric nitrogen is converted to ammonia by the enzyme nitrogenase (specified by *nif* genes) (Postgate 1974). The presence of nitrogenase in bacteria is commonly measured by their growth on nitrogen-free medium and by reduction of acetylene to ethylene (Postgate 1972). *Escherichia coli* and most strains of the closely related genus *Klebsiella* are phenotypically non-nitrogen fixers, and are generally regarded as being genetically devoid of *nif* functions (Shanmugam and Valentine 1975). However, various hybrids between *Klebsiella pneumoniae* strain M5a1 (a nitrogen fixer) and *E. coli* C and *E. coli* K12 have been made and shown to fix nitrogen, indicating successful transfer and expression of nif_{Kp} genes (*nif* genes from *K. pneumoniae*) in an *E. coli* background (Dixon and Postgate 1972; Cannon *et al.* 1974*a*).

Adequate supplies of energy and a low redox potential are necessary requirements for fixation of atmospheric nitrogen (Postgate 1974). In order to study the influence of mutations in pathways of energy metabolism and defects in anaerobic electron transport on the expression of nif_{Kp} genes in *E. coli* K12, a variety of hybrids was constructed. Recipient strains used were mutants uncoupled in oxidative phosphorylation (*unc*, Cox and Gibson 1974) and mutants defective in the nitrate reductase system [chlorate-resistant, *chlA* to *chlG* (Rolfe and Onodera 1972; Bachmann *et al.* 1976)]. This paper reports the effect of storing these hybrids in DMSO and the subsequent expression of nitrogen fixation in *E. coli* K12.

Materials and Methods

Bacterial Strains and Phages

The strains used are listed in Table 1. *E. coli* K12 strain KS649 is derived from strain KS60 (Shimada *et al.* 1972), which carries a heat-inducible λ prophage inserted into its chromosome near the *his* region; heat induction of this strain yielded several heat-resistant isolates. One of these was strain KS649, which was shown to contain an extensive deletion in its chromosome that included both the *his* genes and part of the λ genome, making it a λ cryptic strain. KS650, a strain similarly derived from KS60, has also lost part of the λ genome but is still *his*$^+$.

Table 1. Bacterial strains

Genetic symbols for chromosomal markers are those of Bachmann *et al.* (1976). *nif* denotes nitrogen fixation genes (Shagmugam and Valentine 1975), and Δ denotes deleted genes

Bacterium	Strain	Chromosomal markers	Reference
E. coli K12	AN249	*uncA*$^-$ *arg*$^-$ *entA*$^-$	Cox and Gibson (1974)
	AN283	*uncB*$^-$ *arg*$^-$ *entA*$^-$	
	AN285	*unc*405$^-$ *arg*$^-$ *entA*$^-$	Cox *et al.* (1974)
	C111	*chlD*^{A} *thi*$^-$ *bio*$^-$ *gal*$^-$ *leu*$^-$	Venables and Guest (1968)
	C113	*chlD*^{A} *thi*$^-$ *bio*$^-$ *gal*$^-$ *leu*$^-$	
	C122	*chlD*$^-$ *thi*$^-$ *bio*$^-$ *gal*$^-$ *leu*$^-$	
	C124	*chlA*$^-$ *thi*$^-$ *bio*$^-$ *gal*$^-$ *leu*$^-$	
	C123	*chlD*$^-$ *thi*$^-$ *bio*$^-$ *gal*$^-$ *leu*$^-$	Venables collection
	C183	*chlB*$^-$	
	C181	*chlA*$^-$	Venables (1972)
	C197	*chlE*$^-$	
	C198	*chlA*$^-$	
	C202	*chlE*$^-$	
	C218	*chlB*$^-$ *thi*$^-$ *gal*$^-$ *bio*$^-$ *thr*$^-$	
	DD27	(*gal chlD bio*)^A *pro*$^-$ *his*$^-$	Dykhuizen collection
	DD38	*chlG*$^-$ *bio*$^-$	Dykhuizen (1973)
	DD96	(*attλ chlA bis*)^A *his*$^-$	
	DD182	(*gal chlD bio chlA*)^A	Dykhuizen collection
	KS60	(*gal chlA*)^A	Shimada *et al.* (1972)
	KS649	(*gal chlA*)^A *his*$^-$ *gnd*$^-$ *som*$^-$	
	KS650	(*gal chlA*)^A	
	SB1801	(*his gnd RHA*-2A)^A *gal*$^-$ *chl*$^-$	Cannon *et al.* (1974*b*)
E. coli C	C-M7	*his*$^+{}_{Kp}$ *nif*$^+{}_{Kp}$ *rfb*$^+{}_{Kp}$ *arg*$^-$	Dixon and Postgate (1972)
K. pneumoniae	M5al	*nif*$^+$ prototroph	Mahl *et al.* (1965)
	Δ17	M5al (*gnd his nif shu*)^A	Shanmugam *et al.* (1974)
R. trifolii	T1K	T1 carrying R1-19 *drd* plasmid	Dunican and Tierney (1973)

Strain C-M7, a hybrid isolate from a conjugation experiment between *K. pneumoniae* M5a1 and *E. coli* C (Dixon and Postgate 1972), showed no tendency to produce *nif*$^-$ segregants when subcultured anaerobically on nitrogen-free medium and was thus classified as stable. Subsequent studies suggested that a segment of *Klebsiella* DNA, which included the *nif* genes, was probably integrated into the *E. coli* chromosome.

Rhizobium trifolii derivative T1K was constructed by transformation of strain T1 with drug-resistant plasmid R1-19*drd* DNA, selecting for transfer of kanamycin resistance (Dunican and Tierney 1973).

Bacteriophages used were λ, λ*c*I, λ*c*I857 (a heat sensitive *c*I repressor mutant of λ), λ*vir* (Rolfe *et al.* 1973), and the generalized transducing phage P1*cml clr*100 (Miller 1972) which has a heat-sensitive repressor and carries the resistance marker to chloramphenicol. Transducing phage lysates are made by incubating phage P1*cml clr*100 lysogens, previously grown at 30°C, at 40°C until the culture lyses.

Media

Saline phosphate buffer and growth media have been described elsewhere (Miller 1972; Rolfe *at al.* 1973). Nitrogen-free medium (NFM) was that of Cannon *et al.* (1974*a*). NFM plates were incubated at 30°C under 99% N_2–1% CO_2 by the method of Hill (1973). Growth in liquid NFM was measured by following turbidity in Pankhurst tubes (Campbell and Evans 1969; Postgate 1972). NFM, including agar, was devoid of detectable nitrogen (Microanalytical Service, John Curtin School of Medical Research, A.N.U.). When necessary, NFM was supplemented with appropriate amino acids at 25 μg/ml and vitamins at 10^{-3} μM.

Transduction by Generalized Transducing Phage P1

The hybrid strain C-M7 is resistant (non-adsorbing) to phages λ and P1 (Cannon *et al.* 1974*a*). However, we have found that C-M7 can be transduced to chloramphenicol resistance (12·5 μg/ml) using the heat-inducible transducing phage P1*cml clr*100. Chloramphenicol-resistant colonies appeared at the low frequency of about 10^{-8}; a suspension of one such colony was grown at 30°C until mid-log phase, followed by heat induction at 40°C to yield a phage lysate. This lysate was used to transduce the recipient *E. coli* K12 strain KS649 to *his*$^+$ *nif*$^+$ on nitrogen- and histidine-free medium under a nitrogen atmosphere.

Transductants were recovered at a frequency of approximately 6×10^{-6} and were purified by restreaking on similar selective medium before they were further tested. The transductants (1) had a stable His$^+$ phenotype after more than 10 subculturings and restreakings on minimal media, (2) were sensitive to chloramphenicol and phage P1, and were able to grow equally well at 30 and 40°C; and (3) when tested for sensitivity to phage λ, about a quarter of the *his*$^+$ *nif*$^+$ transductants were also co-transduced to phage λ resistance. All λ-sensitive transductants were tested for the presence of cryptic λ phage of the recipient host KS649 by complementation analysis using the phage amber mutant *susJ* (Shimada *et al.* 1972). The majority (98%) of transductants tested proved to be sensitive and able to complement phage λ *susJ*, and one example of the λ *susJ* complementing transductants (RB101) was shown to have a classical nitrogen-fixing capacity by growth on NFM, reduction of acetylene to ethylene, and repression of nitrogenase by ammonium ions [5 mM $(NH_4)_2SO_4$].

This characterization of the transductants indicated that (1) the *his*$^+$ *nif*$^+{}_{Kp}$ region was transferred by the generalized transducing phage P1, (2) the transductants do not retain any markers of the transducing phage, and (3) the resistance to phage λ in the donor strain C-M7 is probably due to the *Klebsiella* rough genes (*rfb*) integrated into the *E. coli* C chromosome (Cannon *et al.* 1974), which were then co-transduced into *E. coli* K12 strain KS649 with the *his*$^+$ *nif*$^+$ loci. About 60% of the λ-sensitive *his*$^+$ *nif*$^+$ transductants underwent spontaneous partial agglutination in broth culture, which may also indicate the transduction of some rough (*rfb*) markers from strain C-M7. Finally (4) this characterization of the transductants indicated that a recombination between the phage P1-transferred *his*$^+$ *nif*$^+{}_{Kp}$ region and the λ *crp* region of KS649, presumably within the *b*2 region of the λ *crp* phage, leaves a portion (at least the J genes of λ) of the cryptic λ genes still present near the *his* locus.

Matings

Rhizobium trifolii T1K and *E. coli* K12 strain KS650 were grown to mid-log phase in Luria (L) broth at 30°C. The donor strain, T1K, was irradiated with u.v. light (80% kill) and mixed with the recipient KS650 in the ratio 1 part T1K : 9 parts KS650 : 9 parts fresh broth. The mixture was incubated statically at 30°C for 4½ h. The cells were washed twice in saline phosphate buffer, plated on NFM and incubated under a nitrogen atmosphere for 5 days at 30°C before picking off presumptive nitrogen-fixing clones which arose at a frequency of about 10^{-7}. These hybrid clones were streaked twice on selective media (NFM under nitrogen) before single colonies were inoculated into L broth and grown overnight for further characterization. Two nitrogen-fixing *E. coli* hybrids isolated from this experiment and studied in more detail were RB95 and RB96. The criteria for classifying the nitrogen-fixing hybrids as *E. coli* and not *R. trifolii* are shown in Table 2.

In other conjugation experiments, *E. coli* K12 strain SB1801, containing the FN68 plasmid (F′ *nif*$^+{}_{Kp}$, *his*$^+$, *Cb*^{R}), was used as the donor. In addition to the *nif*$^+{}_{Kp}$ genes, this plasmid contains a stably linked carbenicillin resistance marker (*Cb*^{R}) (Cannon *et al.* 1976). Hybrids were made with *E. coli* K12 strains known to have mutations in pathways of energy metabolism; all the recipient strains (*chl*$^-$ and *unc*$^-$ mutants) were mal$^+$. Transfer was after the method of Bernstein *et al.* (1972), and mating mixtures for the various crosses were incubated at 30°C for 2 h. Hybrids carrying

mal$^+$ *Cb*^{R} were selected on EMB–maltose–carbenicillin (300 μg/ml) plates at 30°C. Hybrid isolates were purified by restreaking on the selection medium, and they were then tested for nitrogen fixation by growth on NFM and by acetylene reduction.

Acetylene Reduction Assay

Rates of formation of ethylene from acetylene were measured by the method of Tubb and Postgate (1973). Rates were always measured on cultures grown in NFM both with and without 100 μg/ml vitamin-free Casamino acids to ensure optimal growth without repression of nitrogenase activity.

Storage in DMSO

Hybrid strains freshly grown in L broth containing 0·5% glucose (Miller 1972) were stored in the same broth containing 7% DMSO (Sigma Grade 1, Lot 102C-2570) at −20°C. Samples of these frozen cultures were diluted into fresh broth and regrown when required (Monk *et al.* 1971).

Table 2. Characteristics of donor and recipient strains

Metabolic properties were assayed by commercial tests (API system for identification of Enterobacteriaceae, Hughes and Hughes Ltd, Brentwood, Essex), and only those showing differences between donor and recipient strains are listed

	Bacterial strain			
	R. trifolii T1K	*E. coli* K12 KS650	Hybrids RB95	RB96
Excessive production of polysaccharides	+	−	−	−
Urease activity	+	−	−	−
Sensitivity to phages λ, φ80, T_4, P1	−	+	+	+
Indole formation	−	+	+	+
Fermentation of:				
Glucose	−	+	+	+
Melibiose	−	+	+	+
Growth on NFM under N_2–CO_2	−	−	+	+
Resistance to drugs:				
Kanamycin (20 μg/ml)	+	−	+	+
Chloramphenicol (12·5 μg/ml)	+	−	+	+
Ampicillin (50 μg/ml)	+	−	+	+
Streptomycin (250 μg/ml)	+	−	+	+

Results

E. coli *K12* (*F'* nif$^+_{Kp}$) *Hybrid Strains and their Properties*

A variety of hybrid strains of *E. coli* K12 carrying the *nif*$^+_{Kp}$ genes on plasmid FN68 was constructed and stored in DMSO. These hybrids, after storage in DMSO, were regrown in fresh broth and then tested for their original properties and for the presence of the FN68 plasmid by resistance to carbenicillin and by their ability to grow on solid NFM, in liquid NFM (in Pankhurst tubes), and to reduce acetylene to ethylene.

As a control to test for the presence of plasmid-containing cells, samples of Pankhurst tube cultures were routinely checked by plating on solid NFM and L broth glucose plates. Colony numbers were similar on both media, and colony sizes on NFM always reflected the particular phenotypic stage of hybrids. Occasionally colonies from these NFM plates were restreaked onto fresh NFM plates and regrowth was always to the same extent as observed on the NFM plate from which the inoculum was isolated. Well-isolated single colonies from both the NFM and L broth glucose

plates were tested for the presence of the FN68 plasmid by resuspending colonies in buffer and directly checking their drug-resistance and nitrogen-fixing properties. These tests eliminated any possibility of plasmid loss or cross-feeding as an explanation of the DMSO effect.

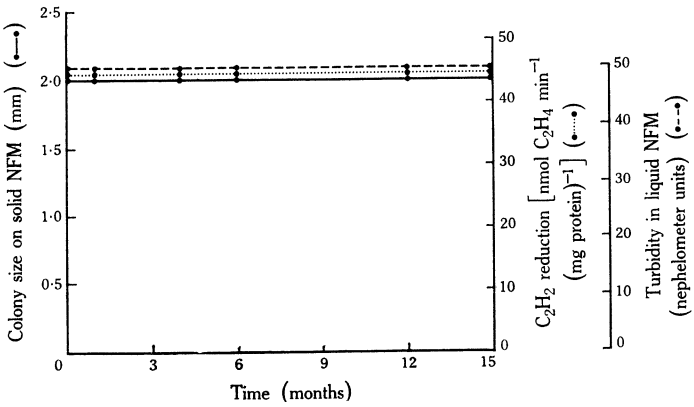


Fig. 1. Expression of *nif* $^{+}_{Kp}$ genes in *E. coli* K12 hybrid strains over a period of 15 months storage in DMSO was tested by growth on solid NFM under 99% N_2–1% CO_2 (Hill 1973) after 7 days (●——●), by growth in liquid NFM in Pankhurst tubes (Campbell and Evans 1969; Postgate 1972) (●– – –●), and by acetylene reduction to ethylene (Tubb and Postgate 1973) (●·····●). All incubations were at 30°C. Maintenance of stable expression of *nif* $^{+}_{Kp}$ genes on plasmid FN68 in *E. coli* K12 hybrids (class I); example shown is *uncB*$^{-}$ hybrid AN283 (FN68).

On several occasions frozen cultures were directly plated onto NFM and L broth glucose plates. Again well-isolated single colonies were directly tested for their nitrogen-fixing capacities. Changes in phenotypic expression observed by this method were similar to those found for cultures grown in broth before testing. DMSO-stored hybrid strains were regularly checked in this manner over a period of 15 months, and the results are shown in Figs 1, 2*a* and 2*b*. The responses of the hybrid strains can be divided into two classes (I and II). Fig. 1 shows that class I hybrids stably express all their nitrogen-fixing properties at a level similar to that prior to storage. Moreover, when these hybrids (stored in DMSO) were assayed for viability after 15 months, the class I strains still had a high viability of 10^{-2}–10^{-4} of original stored cells (Table 3). Examples of this class I response were found with both *unc*$^{-}$ (F′ *nif* $^{+}_{Kp}$) and *chl*$^{-}$ (F′ *nif* $^{+}_{Kp}$) hybrid strains. Similarly, over the same 15-month storage period the chromosomally integrated *E. coli* C hybrid C-M7 and *K. pneumoniae* strain M5a1 were stable with respect to their nitrogen-fixing properties.

In marked contrast, the various class II hybrids carrying the FN68 plasmid show a similar pattern of loss of the Nif^{+} phenotype although the actual onset of this decline varies with the hybrid strain, and ranges from 2 to 6 months (Figs 2*a* and 2*b*). The decline in these hybrids is first characterized by good growth on solid and liquid NFM but a complete inability to reduce acetylene to ethylene. This step is closely followed by a drop of about 60% in the extent of growth on solid and liquid NFM. The final step in the evolution of a defective nitrogen-fixing phenotype is characterized by very poor or no growth in liquid NFM and formation of small

colonies on solid NFM. Class II responses were found with both *unc*$^-$ (*nif*$^+_{Kp}$) and *chl*$^-$ (*nif*$^+_{Kp}$) hybrid strains; the survival of such strains in DMSO was greatly reduced as the viability after 15 months was only 10^{-6}–10^{-8} of original stored cells.

Class II hybrids progressively lost their nitrogen-fixing capacity even though the *Cb*^{R} plasmid was still present and could be mobilized into other *E. coli* K12 recipient cells previously stored in glycerol to give *Cb*^{R} *nif*$^+$ hybrids.

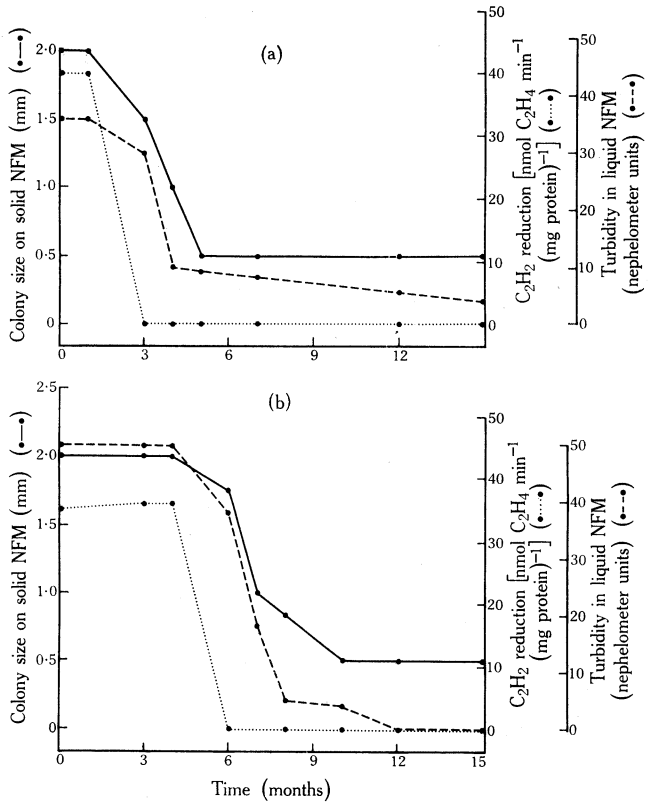


Fig. 2. Gradual decline of expression of *nif*$^+_{Kp}$ genes observed in class II hybrids. The decline is shown first by loss of acetylene reduction, then poor or no growth in liquid NFM, resulting finally in hybrids which can only form small colonies on solid NFM. See Fig. 1 legend for methods. (*a*) Very fast decline in *nif*$^+_{Kp}$ gene expression exhibited by the *chlA*$^-$ hybrid C181 (FN68). (*b*) Slower decline of expression by the *chlD*^{4} hybrid DD27 (FN68).

As this result was found for all class II hybrids tested, the Nif$^-$ phenotype of class II strains was studied in more detail by using the SB1801 (FN68) hybrid stored in DMSO for 15 months (phenotypically Nif$^-$) as a donor strain in conjugation experiments (Table 4). *Cb*^{R} *nif*$^+$ hybrids were formed at a frequency of 10^{-2} with any recipient strain, irrespective of the chlorate-resistant mutation present in the recipient. These nif$^+$ hybrids grew in liquid NFM and reduced acetylene to ethylene (Table 4).

DMSO Effect on nif$^{+}{}_{Kp}$ *Genes Incorporated into* E. coli *Chromosome*

To exclude the possibility that the DMSO effect was solely on plasmid-borne *nif*$^{+}{}_{Kp}$ gene expression, strain KS649 was transduced to *nif*$^{+}{}_{Kp}$*his*$^{+}$ with phage P1 grown on the *E. coli* C (*nif*$^{+}{}_{Kp}$) hybrid strain C-M7. One example of the λ *susJ* complementing transductants, RB101, was shown to be a stable nitrogen-fixing hybrid. This transductant RB101 was stored in DMSO and regularly checked for

Table 3. Effect of DMSO on expression of *nif*$^{+}{}_{Kp}$ genes and viability of hybrids

Hybrids were constructed by conjugation (Bernstein *et al.* 1972) with donor strain SB1801 (FN68), selecting for transfer of carbenicillin resistance and then testing for cotransfer of *nif*$^{+}{}_{Kp}$ genes by growth on NFM and by acetylene reduction. In all clones tested, the *Cb*^{R} marker did not segregate from the *nif*$^{+}{}_{Kp}$ genes. Class I of *nif*$^{+}{}_{Kp}$ expression is recognizable by stable expression of these genes (see Fig. 1); class II hybrids show a stepwise decline of *nif*$^{+}{}_{Kp}$ gene expression (see Fig. 2). Viability after storage in DMSO for 7 and 15 months is expressed as a fraction of original stored cells, and was measured on L broth glucose and NFM plates. n.t., not tested

Bacterium	Hybrid strain	Relevant mutation	Class of *nif*$^{+}{}_{Kp}$ expression	Viability after: 7 months	15 months
E. coli K12	AN283 (FN68)	*uncB*$^{-}$	I	5×10^{-2}	10^{-4}
	AN285 (FN68)	*unc*405	I	n.t.	10^{-4}
	AN249 (FN68)	*uncA*$^{-}$	II	10^{-4}	10^{-6}
	C113 (FN68)	*chlD*$^{-}$	I	10^{-1}	5×10^{-3}
	DD38 (FN68)	*chlG*$^{-}$	I	n.t.	4×10^{-3}
	C124 (FN68)	*chlA*$^{-}$	II	n.t.	10^{-7}–10^{-8}
	C181 (FN68)	*chlA*$^{-}$	II	10^{-5}	10^{-7}–10^{-8}
	C198 (FN68)	*chlA*$^{-}$	II	n.t.	10^{-7}
	DD96 (FN68)	*chlA*$^{\Delta}$	II	n.t.	10^{-7}
	C183 (FN68)	*chlB*$^{-}$	II	n.t.	10^{-7}–10^{-8}
	C218 (FN68)	*chlB*$^{-}$	II	10^{-5}	10^{-8}
	C122 (FN68)	*chlD*$^{-}$	II	10^{-4}	10^{-8}
	C123 (FN68)	*chlD*$^{-}$	II	10^{-5}	10^{-7}–10^{-8}
	C128 (FN68)	*chlD*$^{-}$	II	n.t.	10^{-7}
	C111 (FN68)	*chlD*$^{\Delta}$	II	n.t.	10^{-8}
	DD27 (FN68)	*chlD*$^{\Delta}$	II		10^{-7}–10^{-8}
	C197 (FN68)	*chlE*$^{-}$	II	n.t.	10^{-7}–10^{-8}
	C202 (FN68)	*chlE*$^{-}$	II	n.t.	10^{-7}
	DD182 (FN68)	*chlA chlD*$^{\Delta}$	II	n.t.	10^{-7}
	RB101	*chlA chlD*$^{\Delta}$	II	10^{-4}	10^{-6}
E. coli C	C-M7		I	n.t.	5×10^{-3}
K. pneumoniae	M5a1		I	4×10^{-1}	10^{-2}

its nitrogen-fixing properties over a 15-month period. A modified class II response was observed in this hybrid (Fig. 3). In contrast with the plasmid-bearing hybrids, RB101 does not show the 'all or none' response for acetylene reduction, but rather a gradual decline in both acetylene reduction and growth in liquid NFM. Good growth on solid NFM remains for a longer peroid, but after 15 months storage in DMSO strain RB101 has a completely Nif^{-} phenotype. However, as the *nif*$^{+}{}_{Kp}$ genes could be transduced from the phenotypically Nif^{-} transductant RB101 back into the original recipient host KS649, to give a Nif^{+} phenotype, DMSO does not directly affect the transferred *nif*$^{+}{}_{Kp}$ operon in the background of *E. coli* K12.

DMSO Effect on Nitrogen-fixing Capacity from R. trifolii

The extent of the DMSO effect on nitrogen-fixing capacities other than that of *Klebsiella* was investigated in hybrids between *R. trifolii* and *E. coli* K12. Conjugation experiments between *R. trifolii* and KS650 yielded nitrogen-fixing hybrid clones at a low frequency on NFM under anaerobic conditions (Skotnicki and Rolfe, unpublished data). Two examples of these hybrid clones which were picked for more detailed

Table 4. Transfer of *nif*$^{+}_{Kp}$ genes from a DMSO-stored Nif^{-} donor to glycerol-stored recipient strains
Acetylene reduction and growth on solid and liquid NFM were measured as described for Fig. 1. Hybrids marked with an asterisk (*) were constructed from donor SB1801 (FN68) which had been stored in DMSO for 15 months before mating with recipient strains stored in glycerol over the same period

Strain	Storage in DMSO (months)	C_2H_2 reduction [nmol C_2H_4 min^{-1} (mg protein)$^{-1}$]	Growth in liquid NFM (nephelometer units)	Growth on solid NFM, 99% N_2–1% CO_2 (colony size, mm)
SB1801	0	0	0	0
SB1801 (FN68)	0	45	43	2·0
SB1801 (FN68)	15	0	0	0·5
C124	0	0	0	0
C124 (FN68)*	0	35	50	2·0
C181	0	0	0	0
C181 (FN68)*	0	45	32	2·0
DD96	0	0	0	0
DD96 (FN68)*	0	40	53	2·0
DD182	0	0	0	0
DD182 (FN68)*	0	48	34	2·0

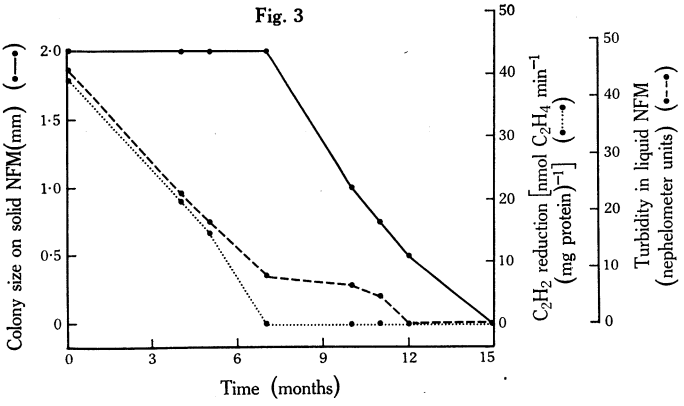
analysis were hybrids RB95 and RB96, which were largely *E. coli*-like in their properties. Hybrids RB95 and RB96 were stored in DMSO and their properties were checked regularly; the results are summarized in Fig. 4. The expression of the transferred nitrogen-fixing capacity from *R. trifolii* in this *E. coli* K12 background was very sensitive to storage in DMSO. In less than 3 months a clear class II response was found with both hybrids RB95 and RB96. However, colonies of RB96 isolated from NFM plates after 13 months storage in DMSO can be used as donor cells in conjugation experiments between RB96 and the *K. pneumoniae nif*$^{-}$ deletion mutant, Δ17, to transfer the Nif^{+} phenotype into the *Klebsiella* recipient host.

DMSO Effect on Recipient E. coli *K12 Cells*

Although the surviving cells of class II hybrids were still carbenicillin resistant and formed small colonies on solid NFM, they were unable to grow in liquid NFM and reduce acetylene. Since the cultures grown up from these survivors still have the FN68 plasmid, the simplest explanation is that storage in DMSO causes killing of the majority of hybrid cells with the complete Nif^{+} phenotype, leaving only cells which have a defective nitrogen-fixing phenotype.

One test of this hypothesis is to take a parental recipient strain stored in DMSO for 15 months and construct a *nif*$^{+}_{Kp}$ hybrid similar to the equivalent one which had been stored in DMSO over the same period and compare the nitrogen-fixing properties

of the two hybrids (Table 5). Parental strains C124 and C124-1 (the same strain stored in DMSO for 15 months) do not fix nitrogen. The original hybrid C124 (FN68) had a complete nitrogen-fixing phenotype when first made, but gave a class II response on storage in DMSO, growing poorly on solid and liquid NFM. The new hybrid



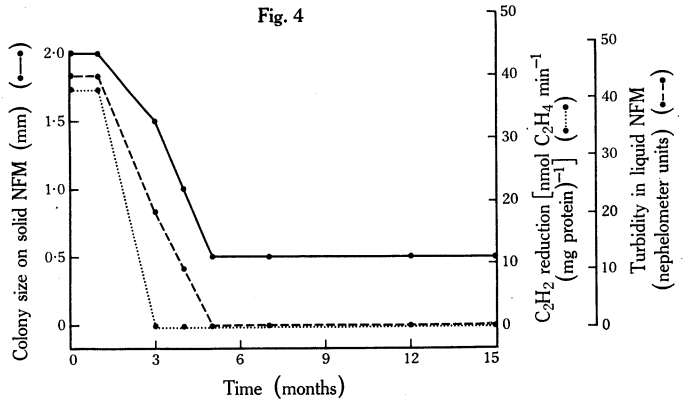


Fig. 3. Decline of expression of *nif* $^{+}{}_{Kp}$ genes in transductant strain RB101—an *E. coli* K12 hybrid between C-M7 and *E. coli* K12 strain KS649 with the *his nif*$_{Kp}$ region stably integrated into the chromosome. This strain was constructed by P1 transduction from host C-M7. At 15 months, the Nif^{+} phenotype could be restored by transducing the *his nif* region of the chromosome back into the original recipient KS649. See Fig. 1 legend for methods.

Fig. 4. Fast decline of expression of nitrogen fixation observed in *R. trifolii–E. coli* K12 hybrids RB95 and RB96. After 5 months storage in DMSO, both hybrids could grow only poorly on solid NFM, and no acetylene reduction or growth in liquid NFM was detectable. See Fig. 1 legend for methods.

C124-1 (FN68) was constructed in a conjugation experiment between glycerol-stored donor strain SB1801 (FN68) and recipient strain C124 which had been stored in DMSO for 15 months (C124-1). In these same conjugation experiments *nif* $^{+}{}_{Kp}$ Cb^{R} genes were transferred at a frequency of about 10^{-2} from donor SB1801 (FN68) to

a variety of recipient strains, including host C124-1, and there was no evidence of segregation of *nif*$^+$ genes from *Cb*^{R} in any *E. coli* K12 recipient. The newly constructed hybrid C124-1 (FN68), however, did not reduce acetylene and could grow only sparingly on solid and liquid NFM. Thus storage of *E. coli* K12 strains in DMSO can select for *E. coli* cells which are defective in the expression of *nif*$^+_{Kp}$ genes even before these genes are transferred to them (Table 5). Similar results were obtained with several *E. coli* K12 strains with mutations in different chlorate-resistant loci so that the effect is not dependent solely on the *chlA* locus.

Table 5. *nif*$^+_{Kp}$ expression in *E. coli* K12 stored in DMSO

C124 which had been stored in DMSO for 15 months (C124-1) was used as a recipient strain, with glycerol-stored donor SB1801 (FN68) in a conjugation experiment similar to that for C124 before DMSO storage. C124-1 (FN68) was tested for expression of *nif*$^+_{Kp}$ genes. Acetylene reduction and growth on solid and liquid medium was measured as described for Fig. 1

Strain	Storage in DMSO (months)	C_2H_2 reduction [nmol C_2H_4 (mg protein)$^{-1}$] min^{-1}	Growth in liquid NFM (nephelometer units)	Growth on solid NFM, 99% N_2–1% CO_2 (colony size, mm)
C124	0	0	0	0
C124 (FN68)	0	35	50	2·0
C124 (FN68)	15	0	4	0·5
C124-1	15	0	0	0
C124-1 (FN68)	0	0	8	0·5

Discussion

We have described the effect that storage of various hybrid strains in DMSO has on the subsequent expression of the nitrogen-fixing activity of these bacteria. The effect (1) is specific to DMSO storage, as the same hybrid strains stored in glycerol over a similar length of time showed no loss of nitrogen-fixing capacity; (2) is apparently specific to some *E. coli* K12 *nif*$^+_{Kp}$ hybrids and nitrogen-fixing hybrids between *R. trifolii* and *E. coli* K12, but is not limited to any one particular *E. coli* K12 background as the loss of nitrogen-fixing capacity occurs in a variety of *E. coli* K12 hybrids; and (3) is not limited to only *E. coli* K12 hybrids which carry the *nif*$^+_{Kp}$ genes on a plasmid, since a modified class II type response occurred when the *nif*$^+_{Kp}$ genes were integrated into the chromosome, as in transductant strain RB101. Furthermore, prolonged storage of *E. coli* K12 strains in DMSO can select for cells which are defective in their expression of *nif*$^+_{Kp}$ genes when these genes are subsequently transferred into them.

The original F′ *nif*$^+_{Kp}$ plasmid, FN39, carrying genes which correspond to the *E. coli* chromosomal region *metG gnd his shiA* was constructed by Skotnicki (nee Warren Wilson, unpublished data, and reported by Cannon *et al.* 1976) and found in early transfer experiments to segregate some plasmid-borne genes. The F′ *nif*$^+_{Kp}$ plasmid FN68 was constructed by translocation of carbenicillin resistance from R plasmid R68 to FN39 and it was found that the *Cb*^{R} *his*$^+$ *nif*$^+$ genes carried by this plasmid were now inherited stably in conjugation experiments with *E. coli* K12 strains. Although plasmid FN68 was found to give rise to Nif$^-$ derivatives when transferred to *Klebsiella pneumoniae*, *Salmonella typhimurium*, and *Erwinia herbicola*, on no occasion did this occur in matings with *E. coli* K12 (Cannon *et al.* 1976). The procedure used by Cannon *et al.* to measure nitrogenase activity was usually by acetylene reduction,

a method which precludes any possibility of observing partial loss of the Nif$^+$ phenotype as described in this paper.

Prolonged storage of many *E. coli.* K12 (FN68) hybrids in DMSO induces a Nif$^-$ phenotype in these hybrids when retested. This effect, however, is on expression of the *nif*$^+_{Kp}$ genes and not on dissociation of the FN68 plasmid, as FN68 can be readily transferred from phenotypically Nif$^-$ hybrids into other *E. coli* K12 backgrounds to give Cb^R Nif$^+$ hybrids.

However, the *uncB*$^-$ and *unc405*$^-$ hybrids, which are DMSO resistant, show no loss of the Nif$^+$ phenotype. Both *uncB*$^-$ and *unc*405$^-$ mutations are alterations within the inner membrane of *E. coli* K12, which contrasts with the *uncA*$^-$ mutation [defective in membrane-bound (Mg^{2+}–Ca^{2+}) ATPase; Cox and Gibson 1974] whose background is isogenic with that of the *uncB*$^-$ and *unc*405$^-$ strains, but whose hybrid [*uncA*$^-$ (F′ *nif*$^+_{Kp}$)] showed a class II response. This result, and the stability of some chlorate-resistant hybrids, implies that the DMSO effect is on particular inner membrane proteins associated with energy metabolism in *E. coli* K12 and is a potential probe of the energy coupling site needed for *nif*$^+$ expression in *E. coli* K12. In addition, the findings described here indicate at least four regions on the *E. coli* genome (Fig. 5) which are either directly or indirectly associated with resistance and

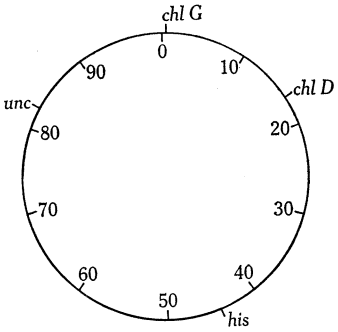


Fig. 5. Chromosomal map of *E. coli* K12 (Bachmann *et al.* 1976) showing four proposed regions (*chlD*, *chlG*, *his*, and *unc*) which are associated with resistance to DMSO.

sensitivity to DMSO. Furthermore, comparison of the class I response of strain C–M7 with the modified class II characteristics of transductant RB101, suggests that some marker(s) linked to the *his nif* region of the *K. pneumoniae–E. coli* C hybrid can influence the effect of DMSO on *nif*$^+_{Kp}$ expression in *E. coli* K12. Plasmid FN68 carries about 3 minutes of genome from *shiA* to *metG* (43·5 to 47 minutes) (Bachmann *et al.* 1976; M. L. Warren Wilson, personal communication) and fails to confer resistance to DMSO. This implies that a hybrid region in strain C-M7 confers the modified class II response of transductant RB101.

Development of the Nif$^-$ phenotype suggests a multi-step process giving a gradual loss of expression of the *nif*$^+_{Kp}$ operon in *E. coli* K12 similar to the evolution of penicillin resistance in bacteria by cell envelope alterations (Eriksson-Grennberg 1968).

Acknowledgments

One of us (M.L.S.) is the recipient of a Commonwealth Scholarship and Fellowship Plan Award and a Ph.D. Scholarship, Australian National University. The following people are thanked for cultures: G. B. Cox, R. A. Dixon, D. Dykhuizen, F. Gibson, M. Gottesman and W. A. Venables.

We thank Drs P. M. Gresshoff and K. Williams for helpful criticisms.

References

Anwar, S. Y., and Reddy, G. M. (1975). Effect of dimethyl sulphoxide in combination treatments with diethyl sulphate in the induction of chlorophyll mutations in *Oryza sativa* L. *Indian J. Exp. Biol.* **13**, 187–8.

Ashwood-Smith, M. J. (1967). Radioprotective and cryoprotective properties of dimethyl sulfoxide in cellular systems. *Ann. N.Y. Acad. Sci.* **141**, 45–62.

Bachmann, B. J., Low, K. B., and Taylor, A. L. (1976). Recalibrated linkage map of *Escherichia coli* K12. *Bacteriol. Rev.* **40**, 116–67.

Bernstein, A., Rolfe, B. G., and Onodera, K. (1972). Pleiotropic properties and genetic organisation of the *tol*A, B locus of *Escherichia coli* K12. *J. Bacteriol.* **112**, 74–83.

Campbell, N. E. R., and Evans, H. J. (1969). Use of Pankhurst tubes to assay acetylene reduction by facultative and anaerobic nitrogen-fixing bacteria. *Can. J. Microbiol.* **15**, 1342–3.

Cannon, F. C., Dixon, R. A., Postgate, J. R., and Primrose, S. B. (1974*a*). Chromosomal integration of *Klebsiella* nitrogen fixation genes in *Escherichia coli*. *J. Gen. Microbiol.* **80**, 227–39.

Cannon, F. C., Dixon, R. A., Postgate, J. R., and Primrose, S. B. (1974*b*). Plasmids formed in nitrogen-fixing *Escherichia coli–Klebsiella pneumoniae* hybrids. *J. Gen. Microbiol.* **80**, 241–51.

Cannon, F. C., Dixon, R. A., and Postgate, J. R. (1976). Derivation and properties of F-prime factors in *Escherichia coli* carrying nitrogen fixation genes from *Klebsiella pneumoniae*. *J. Gen. Microbiol.* **93**, 111–25.

Cox, G. B., and Gibson, F. (1974). Studies on electron transport and energy-linked reactions using mutants of *Escherichia coli*. *Biochim. Biophys. Acta* **346**, 1–25.

Cox, G. B., Gibson, F., and McCann, L. (1974). Oxidative phosphorylation in *Escherichia coli* K12. *Biochem. J.* **138**, 211–15.

Dixon, R. A., Cannon, F. C., and Kondorosi, A. (1975). Derivation of a P-type R factor carrying the nitrogen fixation genes from *Klebsiella pneumoniae*: *nif* expression in unrelated bacteria. *Proc. Soc. Microbiol. II* (*2*), 43.

Dixon, R. A., and Postgate, J. R. (1972). Genetic transfer of nitrogen fixation from *Klebsiella pneumoniae* to *Escherichia coli*. *Nature* (*London*) **237**, 102–3.

Dunican, L. K., and Tierney, A. B. (1973). Transformation of an R-factor from *Pseudomonas aeruginosa* into *Rhizobium trifolii*. *Molec. Gen. Genet.* **126**, 187–90.

Dykhuizen, D. (1973). Genetic analysis of the system that reduces biotin-d-sulfoxide in *Escherichia coli*. *J. Bacteriol.* **115**, 662–7.

Eriksson-Grennberg, K. G. (1968). Resistance of *Escherichia coli* to penicillins. II. An improved mapping of the *amp*A gene. *Genet. Res.* **12**, 147–56.

Hill, S. (1973). A simple method for exposing bacterial cultures on solid media to a defined gas mixture using nylon bags. *Lab. Pract.* **22**, 193.

Ihrke, C. A., and Kronstad, W. E. (1975). Genetic recombination in maize as affected by ethylenediaminetetraacetic acid and dimethyl sulfoxide. *Crop Sci.* **15**, 429–31.

Kunze, M. (1974). Fermentation of lactose by permeaseless *E. coli* in the presence of dimethylsulfoxide. *Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1*: *Orig. Reihe A* **229**, 352–5.

Mahl, M. C., Wilson, P. W., Fife, M. A., and Ewing, W. H. (1965). Nitrogen fixation by members of the tribe *Klebsielleae*. *J. Bacteriol.* **89**, 1482–7.

Miller, J. H. (1972). In 'Experiments in Molecular Genetics'. (Cold Spring Harbor Laboratory: New York.)

Monk, M., Peacey, M., and Gross, J. D. (1971). Repair of damage induced by ultraviolet light in DNA polymerase-defective *Escherichia coli* cells. *J. Mol. Biol.* **58**, 623–30.

Nakanishi, S., Adhya, S., Gottesman, M., and Pastan, I. (1974*a*). Activation of transcription at specific promoters by glycerol. *J. Biol. Chem.* **249**, 4050–6.

Nakanishi, S., Adhya, M., Gottesman, S., and Pastan, I. (1974*b*). Effects of dimethylsulfoxide on the *E. coli gal* operon and on bacteriophage lambda *in vivo*. *Cell* **3**, 39–46.

Postgate, J. R. (1972). The acetylene reduction test for nitrogen fixation. In 'Methods in Microbiology'. (Eds J. R. Norris and D. W. Ribbons.) Vol. 6B, pp. 343–56. (Academic Press: London.)

Postgate, J. R. (1974). In 'Biological Nitrogen Fixation'. (Ed. A. Quispel.) (North Holland: Amsterdam.)

Rolfe, B. G., and Onodera, K. (1972). Genes, enzymes and membrane proteins of the nitrate respiration system of *Escherichia coli*. *J. Membr. Biol.* **9**, 195–207.

Rolfe, B., Schell, J., Becker, A., Heip, J., Onodera, K., and Schell-Frederick, E. (1973). A colicin-tolerant mutant of *Escherichia coli* with reduced levels of cyclic AMP and a strong bias towards λ lysogeny. *Molec. Gen. Genet.* **120**, 1–16.

Shanmugam, K. T., Loo, A. S., and Valentine, R. C. (1974). Deletion mutants of nitrogen fixation in *Klebsiella pneumoniae*: mapping of a cluster of *nif* genes essential for nitrogenase activity. *Biochim. Biophys. Acta* **338**, 545–53.

Shanmugam, K. T., and Valentine, R. C. (1975). Molecular biology of nitrogen fixation. *Science* **187**, 919–24.

Shimada, K., Weisberg, R. A., and Gottesman, M. E. (1972). Prophage lambda at unusual chromosomal locations. I. Location of the secondary attachment sites and the properties of the lysogens. *J. Mol. Biol.* **63**, 483–503.

Tubb, R. S., and Postgate, J. R. (1973). Control of nitrogenase synthesis in *Klebsiella pneumoniae*. *J. Gen. Microbiol.* **79**, 103–17.

Venables, W. A. (1972). Genetic studies with nitrate reductase-less mutants of *Escherichia coli*. *Molec. Gen. Genet.* **114**, 223–31.

Venables, W. A., and Guest, J. R. (1968). Transduction of nitrate reductase loci of *Escherichia coli* by phages P1 and λ. *Molec. Gen. Genet.* **103**, 127–40.

Manuscript received 13 August 1976