THE PATHWAYS OF ENERGY METABOLISM REQUIRED FOR BACTERIAL NITROGEN FIXATION

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STATEMENT

The results described in this thesis were obtained by myself in the Department of Genetics, Research School of Biological Sciences, Australian National University, except for the results described in Table 4.6 which were obtained by Dr. M. Reporter and Dr. B. Rolfe, and the electron micrograph in Figure 4.7, which was prepared by Ms. J. Hughes.

Mary L. Skotnicki

M.L. Skotnicki

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Summary

The central aim of this study was to determine which biochemical pathways provide the large amounts of energy required for bacterial nitrogen fixation. The phenotypic expression of nitrogen fixation genes from *Klebsiella pneumoniae* (nif^{+}_{Kp} genes) was examined in various well-defined mutants of *Escherichia coli* K12 defective in pathways of energy metabolism. The results obtained were then used as a model to investigate whether similar pathways were used to provide energy for nitrogen fixation in the symbiotic bacterium *Rhizobium trifolii*.

In *E. coli* K12 (F'nif^{+}_{Kp}) hybrids, electron transport-dependent phosphorylation is not necessary for anaerobic nitrogen fixation, and substrate level phosphorylation can provide sufficient ATP from glucose for nitrogenase activity. The fumarate reduction system is essential in these hybrids for passaging of electrons to nitrogenase. This system is probably also involved in maintaining the membrane in the energized state, thereby allowing nitrogen fixation to occur.

The nitrate reduction system, which can energize the membrane like the fumarate reduction system, is not necessary for nitrogenase activity in the *E. coli* K12 (F'nif^{+}_{Kp}) hybrids. However, two nitrate reductase

genes, *chlA* and *chlB*, are essential for nitrate inhibition of nitrogen fixation, Nitrate, rather than its product nitrite, inhibits nitrogenase activity, and this inhibition is most probably effected through a regulator factor coded for by *chlA* and *chlB*.

It was not possible to transfer the *nif* genes from *R. trifolii* to *E. coli* K12, in order to investigate the pathways needed for phenotypic expression of nif^{+}_{Rt} genes in the same way as done for the nif^{+}_{Kp} genes. Therefore, the pathways of energy metabolism used in *R. trifolii* were investigated by isolating and characterizing various mutants defective in energy conservation.

Five of the mutants isolated are uncoupled in oxidative phosphorylation (unc^{-}). These mutants have all the characteristics of *E. coli* K12 unc^{-} mutants defective in the F_1 portion of the ATPase complex, including a much-reduced ATPase activity.

The five *R. trifolii* unc^{-} mutants form effective, nitrogen-fixing nodules on three clover species. Bacteroids isolated from these nodules have a high level of ATPase activity, but when they are redifferentiated into the bacterial form they re-acquire their original bacterial characteristics, including very low ATPase activity.

Therefore, in *Rhizobium* bacteroids, a new system for transporting electrons, possibly including a new or altered ATPase, is probably necessary to ensure that sufficient ATP is generated for maintenance of the membrane in an energized state and for bacteroid nitrogen fixation.

List of Publications

1. Skotnicki, M.L. & Rolfe, B.G. Interaction between the oxidative phosphorylation genes of *Escherichia coli* K12 and the nitrogen fixation gene cluster of *Klebsiella pneumoniae*. *Biochem. Biophys. Res. Commun. 75*, 651-658 (1977).

2. Skotnicki, M.L. & Rolfe, B.G. Interaction between the nitrate respiratory system of *Escherichia coli* K12 and the nitrogen fixation genes of *Klebsiella pneumoniae*. *Biochem. Biophys. Res. Commun. 78*, 726-733 (1977).

3. Skotnicki, M.L. & Rolfe, B.G. Interaction between the fumarate reductase system of *Escherichia coli* and the nitrogen fixation genes of *Klebsiella pneumoniae*. *J. Bacteriol. 133*, 415-417 (1978).

4. Skotnicki, M.L. & Rolfe, B.G. Stepwise selection of defective nitrogen-fixing phenotypes in *Escherichia coli* K12 by dimethyl sulfoxide. *J. Bacteriol. 130*, 939-942 (1977).

5. Skotnicki, M.L. & Rolfe, B.G. Effect of dimethyl sulphoxide on the expression of nitrogen fixation in bacteria. *Aust. J. Biol. Sci. 30*, 141-153 (1977).

6. Skotnicki, M.L. & Rolfe, B.G. Transfer of nitrogen fixation genes from a bacterium with the characteristics of both *Rhizobium* and *Agrobacterium*. *J. Bacteriol. 133*, 518-525 (1978).

7. Skotnicki, M.L. & Rolfe, B.G. Differential stimulation and inhibition of growth of *Rhizobium trifolii* strain T1 and other *Rhizobium* species by various carbon sources. *Microbios* (in press, 1977).

8. Gresshoff, P.M., Skotnicki, M.L., Eadie, J.F. & Rolfe, B.G. Viability of *Rhizobium trifolii* bacteroids from clover root nodules. *Plant Sci. Lett. 10*, 299-304 (1977).

9. Gresshoff, P.M., Eadie, J.F., Skotnicki, M.L. & Rolfe, B.G. Defective regulation of nitrate metabolism in soybeans and its consequence on nodulation. *Proc. 2nd Intl. Tiss. Cult. Conf. N.Z., D.S.I.R.* [ed. K. Giles] (in press, 1977).

10. Skotnicki, M.L. & Rolfe, B.G. The genetic control of nitrogen fixation and nitrate reduction in *E. coli*. *Proc. Genet. Soc. Aust. 23*, 15 (1976).

11. Eadie, J.F., Skotnicki, M.L. & Rolfe, B.G. The metabolic state of nitrate reductase in *E. coli* K12 and its biological implications. *Proc. Genet. Soc. Aust. 23*, 15 (1976).

12. Rolfe, B.G., Skotnicki, M.L. & Eadie, J.F. Phage lambda lysogeny as a measure of the metabolic state of *E. coli* K12. *Proc. Genet. Soc. Aust. 23*, 15 (1976).

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TABLE OF ABBREVIATIONS USED

MEDIA

LBG - Luria broth with glucose

NA - Nutrient agar

NAG - Nutrient agar with glucose

EMB - Eosin-methylene blue medium

MM - Davis and Mingioli minimal medium

NFM - Nitrogen-free medium

TM - Trifolii medium

TMY - Trifolii medium with yeast extract

ANTIBIOTICS

Ap - Ampicillin

Cb - Carbenicillin

Km - Kanamycin

Nm - Neomycin

Sm - Streptomycin

Sp - Spectinomycin

ATP - Adenosine triphosphate

ADP - Adenosine diphosphate

ATPase - Adenosine triphosphatase

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CHAPTER 1

GENERAL INTRODUCTION

CHAPTER 1 - GENERAL INTRODUCTION

1.1 The importance of nitrogen fixation

All living matter known to us contains nitrogen; the ultimate source of this organic nitrogen is the atmosphere, where the strongly bonded gaseous molecule N_2 is the predominant gas (79.08% by volume of the gases). Since the average nitrogen content of fundamental rocks may be as low as 10-12 ppm [145, 206] almost all nitrogen used for primary production must come initially from the atmosphere. Thus, in the nitrogen cycle, fixation of atmospheric nitrogen plays an indispensable role in replenishing nitrogenous substances leached out of the soil or used for plant growth; a shortage of fixed nitrogen in the soil is the most common limiting factor for primary production. The few species of micro-organisms which are capable of using elementary N_2 are therefore very important for increasing the nitrogen content of the soil.

Since ancient times, agricultural production has been based on rotation of crops in which legumes figure prominently due to their ability to form symbiotic root nodules with nitrogen-fixing bacteria. Although recently there has been a decrease in the use of legumes, and an increase in the use of industrial fertilizers, a need to increase food production in overpopulated countries, and concern over limited resources of fossil fuels and possible contribution of fertilizers to water pollution has led to renewed interest in extending biological nitrogen fixation.

All available evidence indicates that nitrogenase, the complex enzyme responsible for fixation of atmospheric nitrogen, is limited to a number of prokaryotic organisms including bacteria and blue-green algae. Some of these micro-organisms are able to fix nitrogen in the free-living state, while others do so only in association with higher plants or animals [20, 29, 74, 167]. The most important examples of such associations are the rhizobia which form symbiotic root nodules on leguminous plants. In order that the *Rhizobium*-legume symbiosis may be extended to its maximum efficiency for agricultural production, it is first necessary to gain a better understanding of the biochemistry and physiology of this association.

Because of the complexity of the *Rhizobium*-legume association, the free-living nitrogen-fixing bacteria, *Klebsiella pneumoniae*, *Azotobacter vinelandii* and *Clostridium pasteurianum* have been more widely used to study the nitrogenase system. However, the more limited data on this system in *Rhizobium* [119] indicates that nitrogenase of these symbiotic bacteria is similar to that of free-living species.

1.2 The nitrogenase system

In spite of a wide variation in the types of nitrogen-fixing organisms, the central reaction catalysed by nitrogenase is the same in all organisms so far studied:

$$N_2 + 6e^- + 6H^+ + nATP \rightarrow 2NH_3 + nADP + nP_i$$

The basic biochemical requirements for fixation are:

(a) nitrogenase

(b) a supply of strong reductant usually as reduced ferrodoxin or flavodoxin

(c) ATP as an energy source

(d) in aerobic bacteria, protection of the nitrogen-fixing system from oxygen denaturation and competition for reductant.

Nitrogenase is a complex enzyme consisting of two proteins, the molybdenum-iron (Mo-Fe) protein of molecular weight 100,000-300,000 daltons [34, 54, 55, 73, 136, 218], and the iron (Fe) protein of molecular weight 50,000-70,000 daltons [73, 136, 152, 218]. Spectroscopic evidence, and the content of iron and acid-labile sulphide, suggest that the Fe protein contains one iron-sulphur cluster typical of bacterial ferredoxins [135, 156, 157, 237].

The isolation of highly purified Mo-Fe and Fe proteins has established that only these two proteins, together with adequate supplies of ATP and reductant, are essential for nitrogen fixation *in vitro*. However, the individual proteins do not catalyse the fixation of N_2; only the enzyme complex can reduce N_2 to the produce ammonia [136].

In all nitrogen-fixing systems studied, the only product is two molecules of ammonia formed by addition of 6 protons and electrons to N_2; no intermediates have been detected [35, 36, 77]. Recent evidence [157] indicates that the sequence of electron flow is from the

Fe protein to the Mo-Fe protein, which would require the N_2 reduction site to be on the Mo-Fe protein.

Nitrogenase can also reduce several other substrates, and its ability to reduce acetylene to ethylene [64, 184] has greatly facilitated the study of nitrogen fixation.

1.3 Ammonia assimilation and regulation

Ammonia, the primary product of nitrogen fixation, is incorporated mainly into glutamate, catalysed by glutamine synthetase (GS), which has a very low K_m for ammonia, and glutamate synthase (GOGAT) [121, 150]. Glutamate dehydrogenase (GDH) functions in assimilation of ammonia only when the level of ammonia in the cell is high [150], as with nitrate reduction (Figure 1.1).

In nodules produced by *Rhizobium*, it has been proposed that plant rather than bacteroid enzymes are important in the assimilation of ammonia produced by fixation of N_2 in the bacteroids [175, 176]. It seems likely that *Rhizobium* bacteroids excrete ammonia, as has been demonstrated for both free-living rhizobia in culture [154] and for bacteroids of *R. japonicum* [14, 21], and that this ammonia is subsequently assimilated by plant enzymes.

Ammonia also regulates the synthesis of nitrogenase and other enzymes involved in the assimilation of ammonia into glutamate and glutamine [150, 214]. Repression of nitrogenase synthesis by ammonia has been demonstrated for many different nitrogen-fixing organisms including

Figure 1.1

During nitrogen fixation the level of ammonia formed is low, and ammonia is assimilated *via* glutamine synthetase and glutamate synthase. When the level of ammonia is high, as with nitrate reduction, ammonia is assimilated *via* glutamate dehydrogenase. This enzyme has a higher K_m for ammonia than glutamine synthetase.

GS; glutamine synthetase

GOGAT; glutamate synthase

GDH; glutamate dehydrogenase

Nitrogen-fixing system

α-ketoglutarate

GDH

nitrogenase

high NH_3

glutamate → proteins

nitrite reductase

low NH_3

GS

GOGAT

ATP

glutamine + ADP

α-ketoglutarate

nitrate reductase

glutamate

Nitrate reducing system

K. pneumoniae [220] and *Rhizobium* species [25, 192]. Recently, a model has been proposed in which ammonia repression is mediated by the intracellular levels of glutamine synthetase [142, 143], and it has been suggested that this enzyme acts as a positive control element for nitrogenase synthesis in a manner similar to that proposed for histidase synthesis in *K. aerogenes* [168, 212, 219].

1.4 Protection from oxygen

Although nitrogen fixation occurs in a wide range of aerobic, facultatively anaerobic, and anaerobic bacteria, all nitrogenases examined *in vitro* are oxygen-labile and function only under anaerobic conditions [32, 73, 117, 231]. Aerobic nitrogen-fixing bacteria have developed various devices to protect their nitrogenase systems from oxygen including special respiratory protection [32, 99, 165], conformational protection [56, 155, 236], compartmentation [82, 207], and production of extracellular slime [94]. In *Rhizobium* nodules, leghaemoglobin of plant origin also plays a role in protection of nitrogenase from O_2 inactivation by facilitating the diffusion of O_2 to the bacteroids at very low O_2 tension [5].

1.5 Electron donors and ATP hydrolysis

Nitrogen fixation is a reductive process and a reductant of low potential is necessary for activity. In cell-free preparations, the most commonly used reductant

is sodium dithionite, which donates electrons directly to nitrogenase in the absence of other electron carriers [30]. In *Clostridium*, ferredoxin is the principal electron donor, and donates electrons directly to nitrogenase [148]; under iron-limiting conditions, another electron carrier, flavodoxin, becomes important in nitrogen fixation [126].

Iron-sulphur proteins active in electron transport to nitrogenase have also been isolated from soybean ndoule bacteroids [127, 234] from *Azotobacter* [195,233] and *Bacillus polymyxa* [196].

Many nitrogen-fixing organisms also contain the enzyme hydrogenase [124, 151], which catalyses the reaction:

$$\text{Ferredoxin (reduced)} + 2H^+ \rightleftharpoons \text{Ferredoxin (oxidized)} + H_2$$

This hydrogenase activity is completely independent of ATP, but nitrogenase can also exhibit an ATP-dependent H_2-evolution [30]. Thus, depending on the conditions in the cell, H_2 can either function as an electron donor for nitrogenase by coupling through hydrogenase, or else can be evolved by nitrogenase. Nitrogenase evolves H_2 in the presence or absence of N_2; under 1 atm N_2 about one third of the electrons transferred by nitrogenase are used for H^+ reductions [89, 104].

Although the free energy for reduction of molecular nitrogen is negative [3], additional energy supplied by hydrolysis of ATP to ADP and orthophosphate is required for *in vitro* nitrogenase activity [122]. Like various other ATP-hydrolysing enzymes, nitrogenase displays absolute specificity for ATP, and shows no activity with CTP, UTP,

GTP or ITP [73, 91]. Nitrogenase from all organisms tested requires ATP for activity; the actual substrate in the reaction is believed to be the Mg^{2+}-ATP complex since free ATP in the absence of Mg^{2+} does not show activity [136]. The stoichiometric relationship between ATP hydrolysis and electron transfer during nitrogen fixation has been studied closely [31, 89, 92, 107, 122, 137, 227], but is still a subject of some controversy. Considerable amounts of ATP are hydrolysed, but the ATP:$2e^-$ ratio (ratio of ATP molecules hydrolyzed to pairs of electrons transferred) varies with the ratio of Mo-Fe to Fe protein, temperature, pH, ADP concentration, reducible substrate and prior history of the preparation [33].

Studies of nitrogen-fixing *A. chroococcum* cells indicate consumption of 4-5 moles ATP per mole of N_2 fixed [57], but in contrast are values of 20 moles ATP for *Clostridium* [53] and about 30 for *Klebsiella* [165]. However, even higher ratios have been observed *in vitro* for some organisms [217]. Thus, nitrogen fixation is a very energy-demanding process.

1.6 Derivation of energy

As well as being involved in nitrogen fixation, ATP is required by the bacterial cell for a wide range of other metabolic functions. This ATP can be synthesized either by substrate level phosphorylation or by electron transport-dependent phosphorylation [87]. ATP is synthesized

oxidatively by the complex membrane-bound ATPase protein, which is coupled to the electron transport chain with oxygen as terminal electron acceptor (Figure 1.2). Under anaerobic conditions, this ATPase complex can synthesize ATP by coupling to the electron flow to the acceptors nitrate and fumarate [240]. For growth on a non-fermentable carbon source such as succinate, electron transport-dependent phosphorylation is the only mechanism which can generate sufficient intracellular ATP. A bacterial mutant which lacks either a functional ATPase or electron transport chain will therefore be unable to grow on succinate. It will, however, still be able to grow on a fermentable carbon source such as glucose, from which ATP can be produced by substrate level phosphorylation. Between 1 and 2 molecules of ATP can be produced in this way from each molecule of glucose fermented to pyruvate, depending on the pathway used (Figure 1.3). A further molecule of ATP can be generated from pyruvate (Figure 1.4), giving a maximum of 4 ATP/glucose molecules by substrate level phosphorylation, although the actual number is nearer to 3 [90, 98].

In addition, the bacterial cell must be able to establish and maintain a protonmotive force across the cell which "energizes" the membrane for uptake of various compounds. This requirement can be satisfied either by a functional proton-translocating respiratory chain or by ATP hydrolysis *via* the proton-translocating ATPase [87]. Thus, a mutant which lacks a functional

FIGURE 1.2

Scheme, summarizing oxidative phosphorylation in *E. coli* (after Haddock and Jones [88]). The scheme includes the various routes for aerobic electron transport, with dashed lines indicating alternative pathways for reducing equivalents. Protons are translocated across the membrane, and the electron transport chain can be coupled to the ATPase complex to produce ATP.

Fp: flavoprotein

Fe/S: iron-sulphur protein

Q_8: ubiquinone

F_0 & F_1: components of the ATPase complex

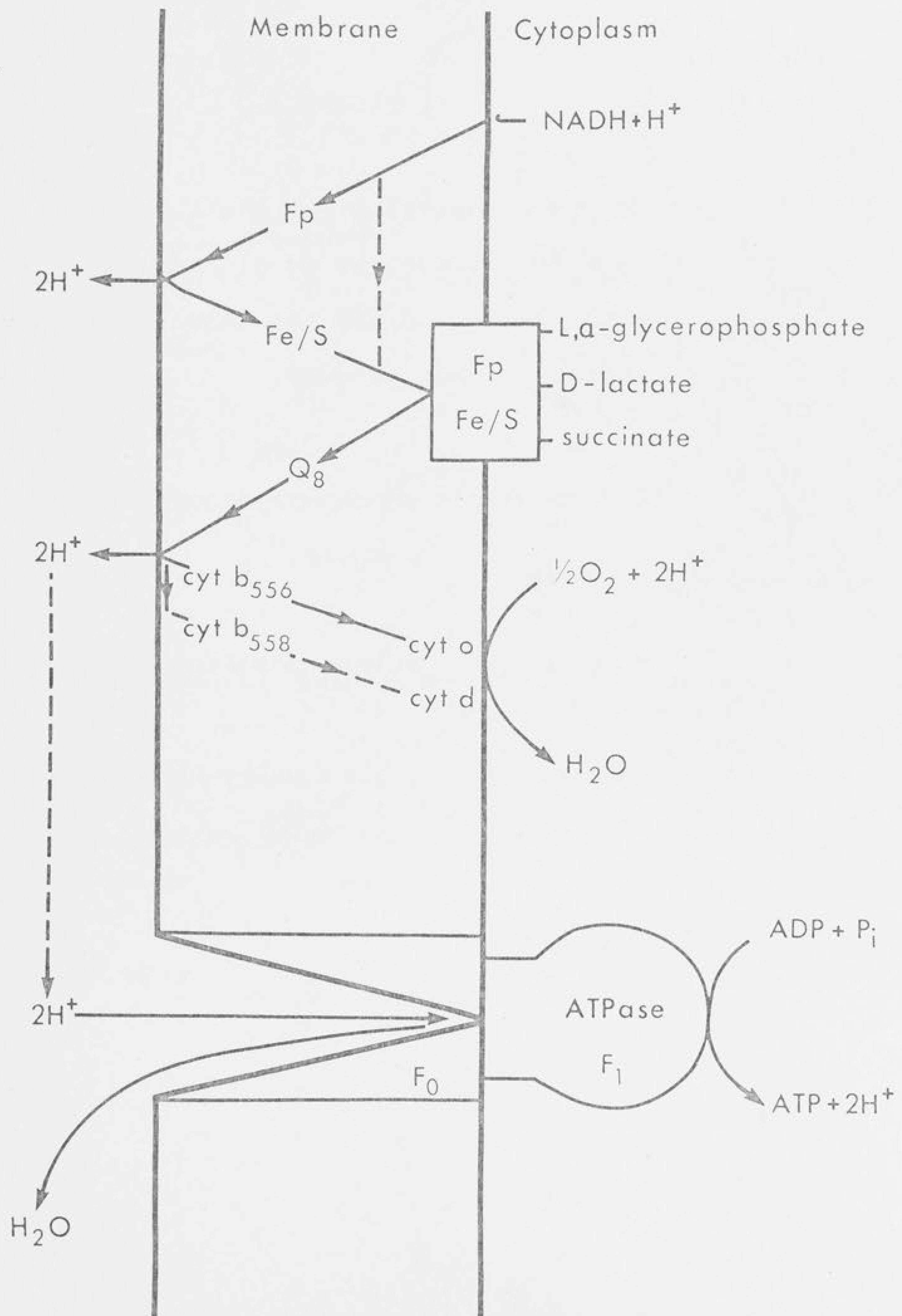

Membrane
Cytoplasm
NADH+H⁺
Fp
2H⁺
Fe/S
L,a-glycerophosphate
Fp
D-lactate
Fe/S
succinate
Q8
2H⁺
cyt b556
½O2 + 2H⁺
cyt b558
cyt o
cyt d
H2O
ADP + Pi
2H⁺
ATPase
F1
F0
ATP + 2H⁺
H2O

FIGURE 1.3

Scheme summarizing the possible pathways for catabolism of glucose to pyruvate, with numbers of ATP and $NADH_2$ molecules formed by each pathway.

EMP pathway: Embden-Meyerhof-Parnas pathway.

ED pathway: Entner-Douderoff pathway.

HMP pathway: Hexose monophosphate (pentose phosphate) pathway.

Net yield

	ATP	$NADH_2$
EMP:	2	2
ED:	1	2
HMP	1	4

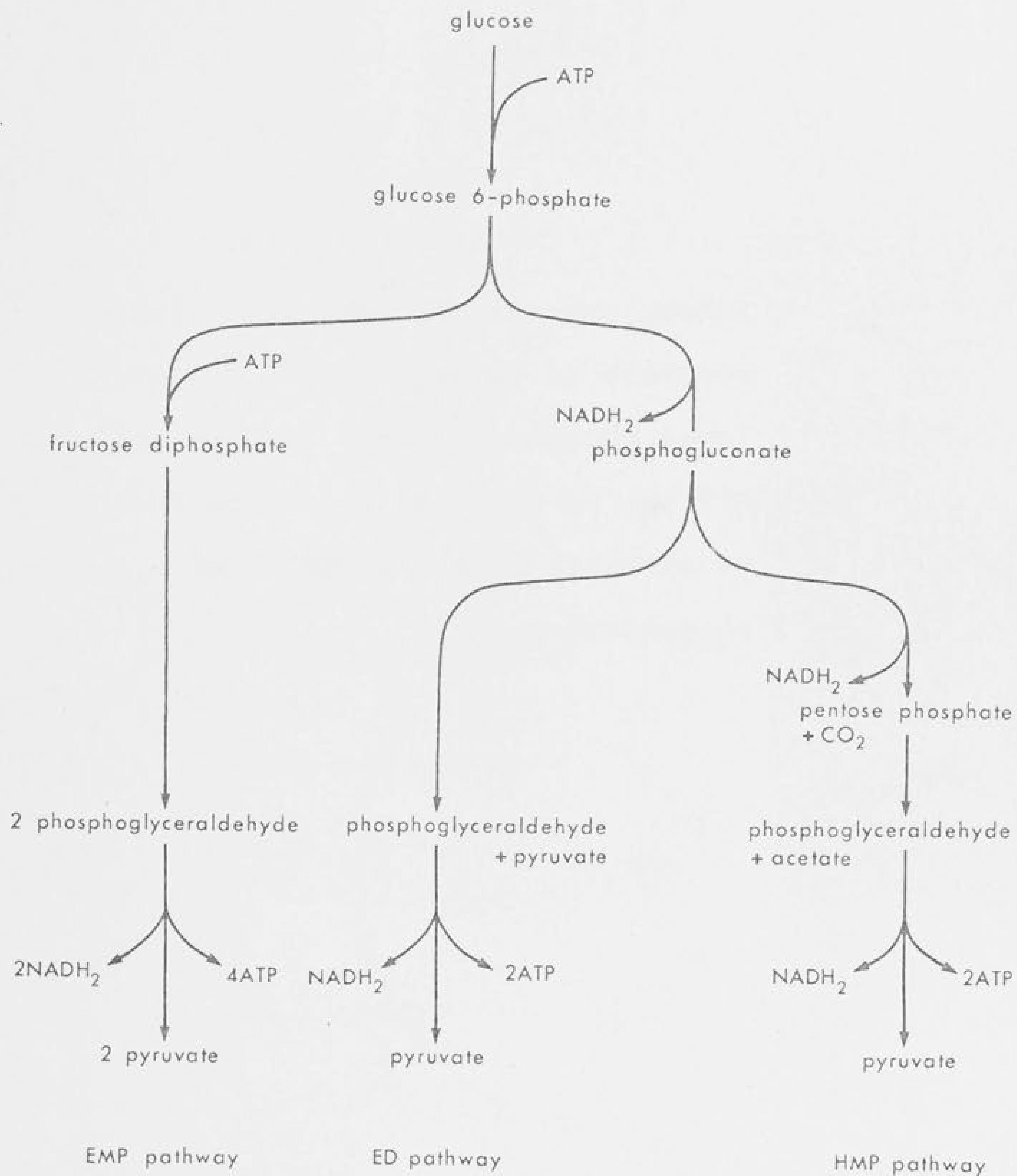
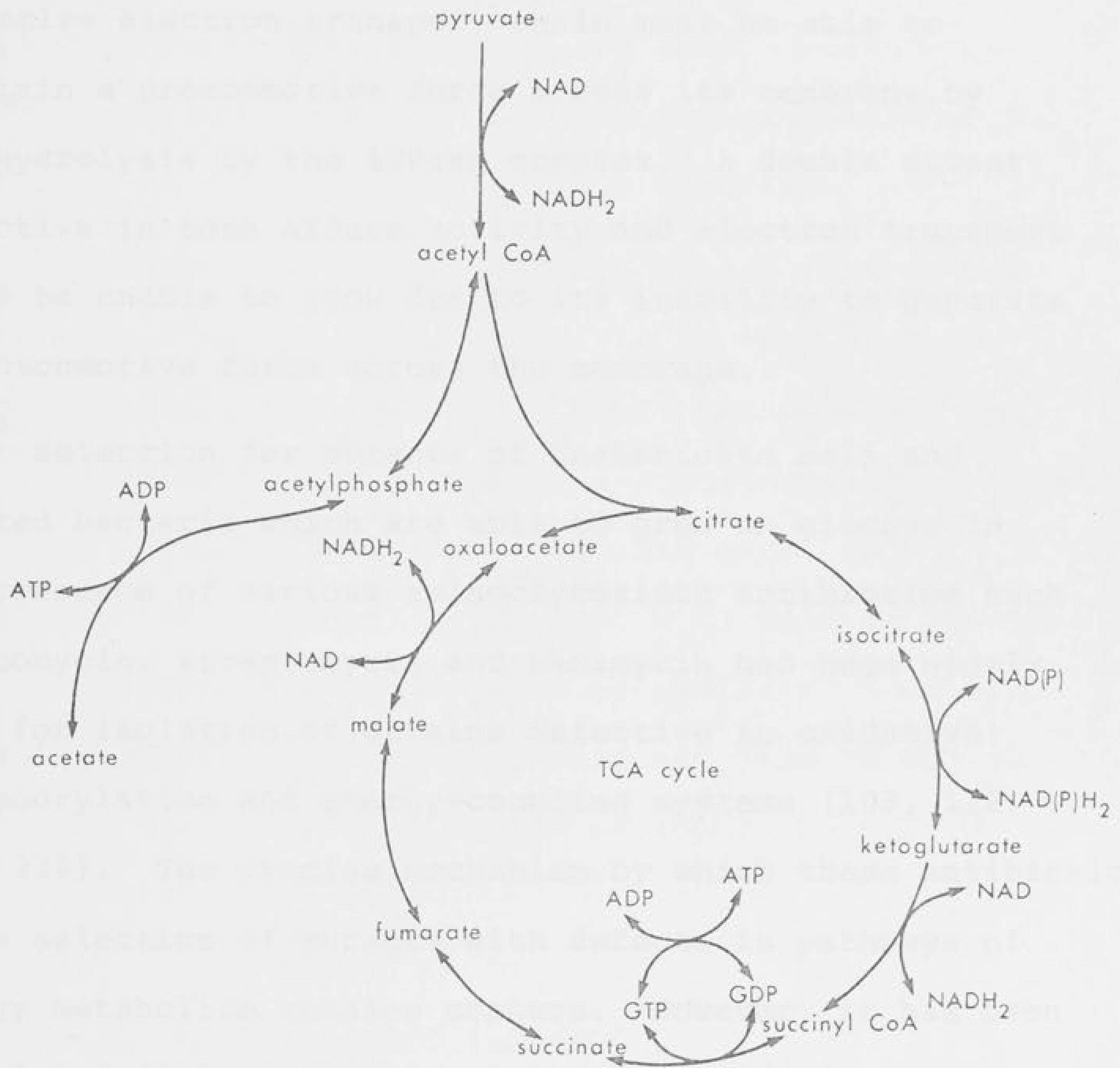

glucose
ATP
glucose 6-phosphate
ATP
fructose diphosphate
NADH$_2$
phosphogluconate
NADH$_2$
pentose phosphate
+ CO_2
2 phosphoglyceraldehyde
phosphoglyceraldehyde
+ pyruvate
phosphoglyceraldehyde
+ acetate
2NADH$_2$
4ATP
NADH$_2$
2ATP
NADH$_2$
2ATP
2 pyruvate
pyruvate
pyruvate
EMP pathway
ED pathway
HMP pathway

FIGURE 1.4

Scheme summarizing the catabolism of pyruvate to produce ATP by substrate level phosphorylation. One molecule of ATP can be produced from each molecule of pyruvate by substrate level phosphorylation.


pyruvate
NAD
$NADH_2$
acetyl CoA
ADP
acetylphosphate
ATP
acetate
citrate
$NADH_2$
oxaloacetate
NAD
malate
TCA cycle
isocitrate
NAD(P)
$NAD(P)H_2$
ketoglutarate
ATP
ADP
NAD
fumarate
GDP
GTP
$NADH_2$
succinyl CoA
succinate

ATPase will only be able to grow on a fermentable carbon source if its respiratory chain can still translocate protons and thereby maintain the required protonmotive force across the membrane. Similarly, a mutant with a defective electron transport chain must be able to maintain a protonmotive force across its membrane by ATP hydrolysis by the ATPase complex. A double mutant defective in both ATPase activity and electron transport would be unable to grow due to its inability to generate a protonmotive force across the membrane.

Selection for mutants of *Escherichia coli* and related bacteria which are ablt to grow on glucose in the presence of various aminoglycosidic antibiotics such as neomycin, streptomycin and kanamycin has been widely used for isolation of strains defective in oxidative phosphorylation and energy-coupling systems [103, 112, 179, 230]. The precise mechanism by which these antibiotics allow selection of mutants with defects in pathways of energy metabolism remains obscure. However, it has been postulated [112, 183] that respiratory-deficient cells incorporate less neomycin than ordinary cells, possibly due to an inability to generate sufficient energy to concentrate the antibiotic.

Under anaerobic conditions, the membrane may be energized for uptake of various compounds either by coupling to available ATP, the utilization of which requires a coupled ATPase [84], or by the electron transport chains which have nitrate and fumarate as their terminal

acceptors [130] (Figure 1.5).

Mutants of *E. coli* defective in these anaerobic electron transport pathways involving nitrate reductase, fumarate reductase, ubiquinone and menaquinone have been isolated and well-characterised, so that a wide range of strains defective in the various pathways of energy metabolism is now available in this organism.

Although *E. coli* is generally regarded as devoid of nitrogen fixation *(nif)* genes, it is very closely related to the genetically less well-characterised but nitrogen-fixing bacterium *Klebsiella pneumoniae*. Moreover, it is now possible to transfer the *nif* genes from *K. pneumoniae* (*nif*$_{Kp}$ genes) to *E. coli* with full expression [43, 67], thereby generating the possibility of testing which pathways of energy metabolism are used to provide the energy for nitrogen fixation by using the various *E. coli* mutants available.

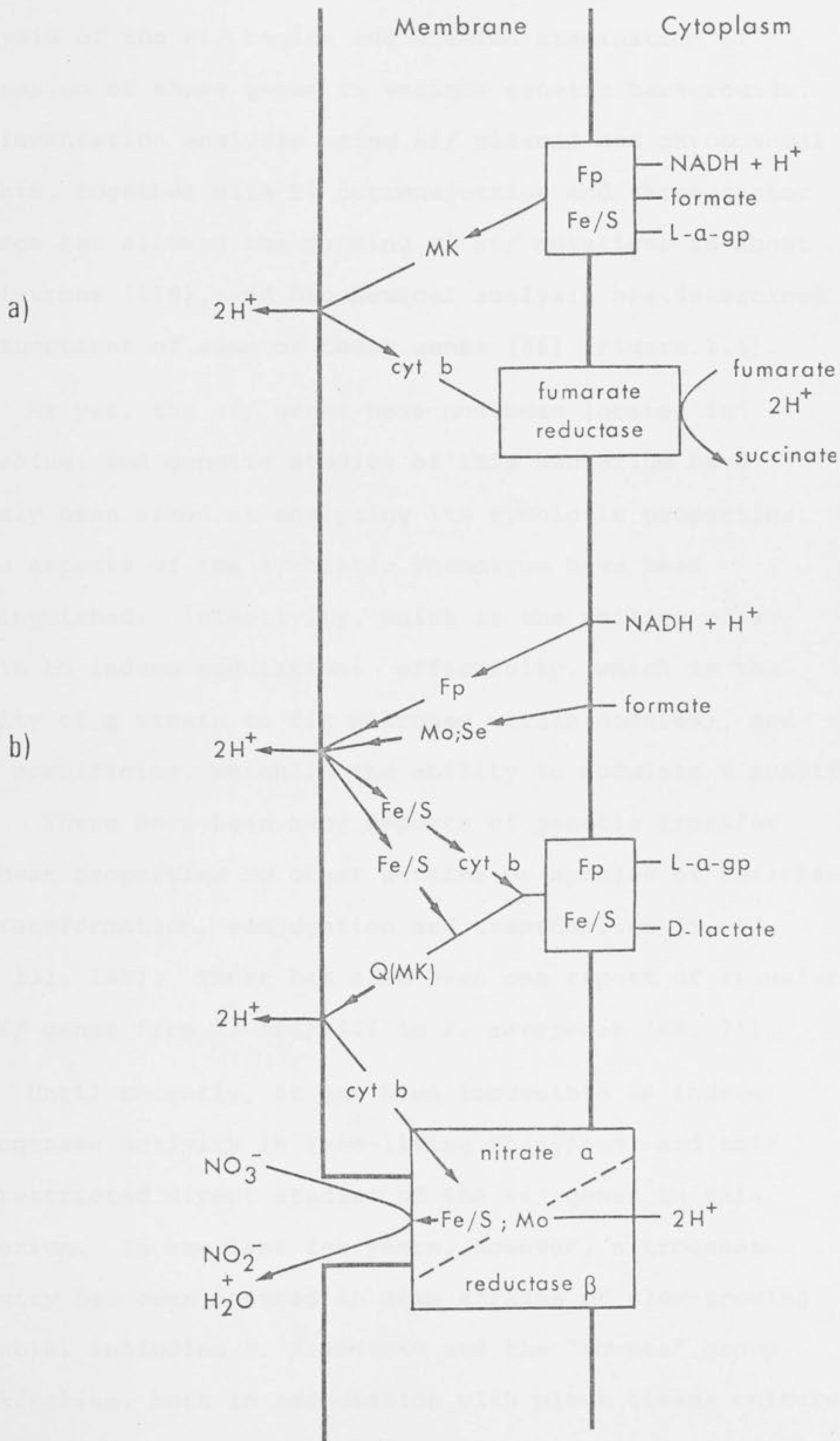
1.7 Genetics of nitrogen fixation

The *nif* genes of *K. pneumoniae* have been closely studied, and are located in a segment of the chromosome 15-20 average genes in length [46, 210, 211] proximal to the operator end of the operon for histidine biosynthesis [193]. The cluster of *nif* genes is currently being analysed using mutant strains unable to fix N_2. Initially, mutations were mapped by phage Pl transduction [208], but more recently construction of plasmids carrying the *K. pneumoniae nif* genes [43, 65] has facilitated a diploid

FIGURE 1.5

Scheme summarizing the proposed functional membrane organization in *E. coli* of the redox carriers responsible for anaerobic electron transport (a) with fumarate as terminal acceptor; (b) with nitrate as terminal electron acceptor (modified after Haddock and Jones [88]).

MK, menaquinone; Q, ubiquinone; cyt., cytochrome; Fe/S, iron-sulphur protein; Fp, flavoprotein; Mo, molybdenum-containing polypeptide; Se, selenium-containing polypeptide; α, β, sub-units of nitrate reductase; L-α-gp, L-α-glycerophosphate.



Membrane
Cytoplasm
a)
Fp
Fe/S
NADH + H$^+$
formate
L-α-gp
MK
2H$^+$
cyt b
fumarate reductase
fumarate
2H$^+$
succinate
b)
NADH + H$^+$
Fp
formate
Mo;Se
2H$^+$
Fe/S
Fe/S
cyt b
Fp
Fe/S
L-α-gp
D-lactate
Q(MK)
2H$^+$
cyt b
nitrate α
reductase β
NO_3^-
Fe/S ; Mo
2H$^+$
NO_2^- + H_2O

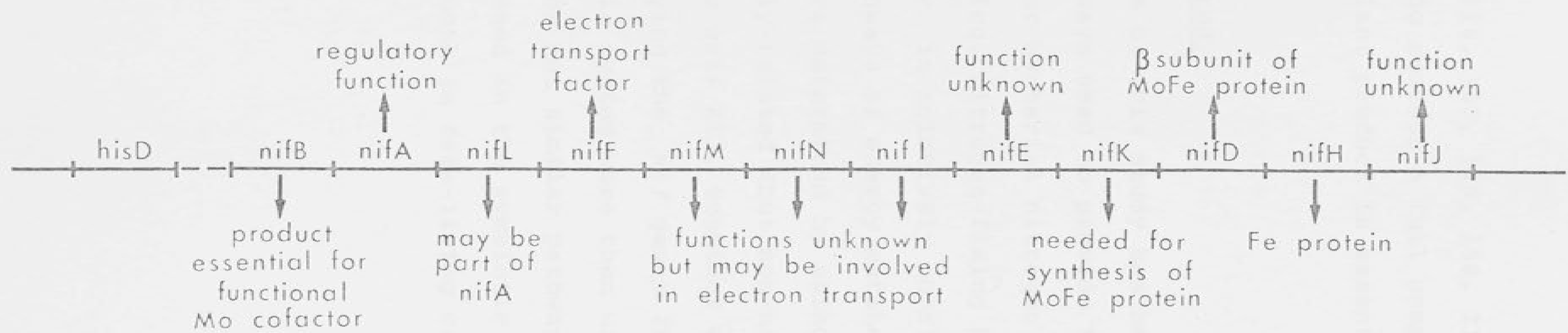
analysis of the *nif* region and enabled examination of expression of these genes in various genetic backgrounds. Complementation analysis using *nif* plasmid and chromosomal mutants, together with P1 cotransduction and three-factor crosses has allowed the mapping of *nif* mutations in about 12 cistrons [118], and biochemical analysis has determined the functions of some of these genes [66] (Figure 1.6).

As yet, the *nif* genes have not been located in *Rhizobium*, and genetic studies of this bacterium have largely been aimed at analysing its symbiotic properties. Three aspects of the symbiotic phenotype have been distinguished: infectivity, which is the ability of a strain to induce nodulation; effectivity, which is the ability of a strain to fix nitrogen within nodules; and host specificity, which is the ability to nodulate a specific host. There have been many reports of genetic transfer of these properties to other strains or species of *Rhizobium* by transformation, conjugation and transduction [9, 62, 108, 131, 188]. There has also been one report of transfer of *nif* genes from *R. trifolii* to *K. aerogenes* [69, 71].

Until recently, it has been impossible to induce nitrogenase activity in free-living *Rhizobium*, and this has restricted direct studies of the *nif* genes in this bacterium. In the last few years, however, nitrogenase activity has been induced in some strains of slow-growing rhizobia, including *R. japonicum* and the "cowpea" group of *Rhizobium*, both in association with plant tissue cultures [45, 79, 163, 164, 172, 191], and also in the absence of

FIGURE 1.6

Diagrammatic representation of the cluster of *nif* genes in *K. pneumoniae*, showing the functions of the various genes where known (Kennedy, personal communication [66, 118]).



electron
transport
factor
regulatory
function
function
unknown
β subunit of
MoFe protein
function
unknown
hisD
nifB
nifA
nifL
nifF
nifM
nifN
nif I
nifE
nifK
nifD
nifH
nifJ
product
essential for
functional
Mo cofactor
may be
part of
nifA
functions unknown
but may be involved
in electron transport
needed for
synthesis of
MoFe protein
Fe protein

any plant material [116, 132, 146, 158, 216]. This has shown that rhizobia do possess a full complement of *nif* genes, and that no plant product is essential for nitrogen fixation to occur.

1.8 Aims of this study

The central aim of this study has been to identify the biochemical pathways used to provide the large amounts of energy required for bacterial nitrogen fixation. Because the free-living, nitrogen-fixing bacterium *Klebsiella pneumoniae* is relatively poorly characterised genetically, the pathways of energy metabolism used for nitrogen fixation were determined by using well-defined mutants of the closely-related (but non-nitrogen-fixing) bacterium *Escherichia coli* K12, together with a transferable F-prime plasmid carrying the *nif* genes from *K. pneumoniae*. The findings with this system were then used as a model to investigate whether or not similar pathways of energy metabolism might be used in the symbiotic bacterium *Rhizobium trifolii*, both in free-living culture and in clover nodules.

CHAPTER 2

MATERIALS AND METHODS

Chapter 2 - Materials and Methods

2.1 Materials

Bacterial strains: The *E. coli* K12 strains used are listed in Table 2.1. Genetic symbols are those of Bachmann *et al.* [8]. *Klebsiella pneumoniae* strain M5a1 is a standard nif^+ strain [144]. *Rhizobium trifolii* strain T1, and a spectinomycin-resistant derivative, T1Sp, are prototrophs obtained from Schwinghamer [187] which effectively nodulate white, red and subterranean clovers. The genotypes of all strains were regularly checked before use.

Plasmids: The plasmids used are described in Table 2.2. Their genotypes were also checked before use.

Bacteriophages: ØT10, a phage specific for *R. trifolii* strain T1, and Tr8, a general *R. trifolii* phage, were obtained from Schwinghamer [187], as was bacteriocin T24 which is specific for *R. trifolii* [189].

Plants: The plants used to test for nodulation were *Trifolium repens* cv. New Zealand (white clover), *T. pratense* cv. Montgomery (red clover) and *T. subterraneum* cv. Mt. Barker (subterranean clover).

Gases: High purity grade gases from CIG (Australia) were always used to ensure that levels of contaminating O_2, CO and CO_2 were kept to a minimum. The contaminating concentrations of CO were listed by CIG as less than 1 vpm for H_2, N_2 and Ar, and less than 10 vpm for CO_2.

TABLE 2.1 *E. coli* K12 strains used

Strain	Description	Source and Reference
AN98	*men*$^+$ *met*$^-$ *arg*$^-$	Cox [49]
AN99	*menA*$^-$ *met*$^-$	Cox [49]
AN249	*uncA*$^-$ *arg*$^-$ *entA*$^-$	Cox [49]
AN259	*unc*$^+$ *arg*$^-$ *entA*$^-$	Cox [49]
AN283	*uncB*$^-$ *arg*$^-$ *entA*$^-$	Cox [49]
AN285	*uncD*$^-$ *arg*$^-$ *entA*$^-$	Cox [50]
AN771	*uncC*$^-$ *arg*$^-$ *entA*$^-$	Cox [50]
AN384	*ubiA*$^-$ *menA*$^-$ *thi*$^-$	Young [235]
AN385	*ubiA*$^-$ *men*$^+$ *thi*$^-$	Young [235]
AN386	*ubi*$^+$ *menA*$^-$ *thi*$^-$	Young [235]
AN387	*ubi*$^+$ *men*$^+$ *thi*$^-$	Young [235]
AN472	*frd*$^-$ *ilv*$^-$ *entA*$^-$	Rosenberg [84,181]
AN480	*frd*$^-$ *uncB*$^-$ *entA*$^-$	Rosenberg [84,181]
C111	*chlD*$^-$ mutant of W602	Venables [223]
C123	*chlD*$^-$ mutant of W602	Venables [223]
C181	*chlA*$^-$ mutant of R594	Venables [224]
C183	*chlB*$^-$ mutant of R594	Venables [224]
C197	*chlE*$^-$ mutant of R594	Venables [224]
C202	*chlE*$^-$ mutant of R594	Venables [224]
DD38	*chlG*$^-$ *bio*$^-$	Dykhuizen [72]
DD115	*chlB*$^-$ *gal*$^-$	Dykhuizen

TABLE 2.1 (cont'd) *E. coli* K12 strains used

Strain	Description	Source and Reference
KB70	*(bio-aroA)*$^{\Delta}$ *chlA*$^{-}$	Rolfe [178]
Puig 426	*chlC*$^{-}$ *thi*$^{-}$ *leu*$^{-}$ *suc*$^{-}$ *bio*$^{-}$ *gal*$^{-}$ *str*^{R}	Puig [169]
R594	*sup*$^{-}$ *str*^{R}	Venables [224]
SA291	*(gal-chlA)*$^{\Delta}$ *his*$^{-}$ *str*^{R}	Dykhuizen [72]
SB1801	Δ*(his gnd* RHA-2A*)* *str*^{R} λ^{-} λ^{R} *ara*$^{-}$ *gal*$^{-}$ *malA*$^{-}$ *xyl*$^{-}$ *mtl*$^{-}$	Dixon [42]
W602	*thi*$^{-}$ *gal*$^{-}$ *bio*$^{-}$ *leu*$^{-}$	Venables [223]

TABLE 2.2 Plasmids used

Plasmid	Description	Source and Reference
FN68	F' nif^{+}_{Kp} Cb^R. Carries *metG* to *shiA* region of *E. coli* K12 chromosome	Dixon [43]
R1-19 *drd*	Cm^R Sm^R Ap^R Km^R	Datta [60]
R68.45	Km^R Cb^R Tc^R Nm^R	Holloway [102]
RP4	Km^R Cb^R Tc^R Nm^R	Holloway [102]

Media: For all solid media, 1.5% Difco Bacto agar was used.

Luria broth with glucose (LBG) [147]

Yeast extract	5	g
Bacto tryptone	10	g
NaCl	5	g
Water	1	litre

(Final pH 7.0)

Glucose was added after autoclaving to a final concentration of 0.5%.

Nutrient agar with glucose and chlorate ($NAGClO_3$) [1]

Bacto nutrient broth	8	g
Glucose	2	g
$KClO_3$	2	g
Difco Bacto agar	15	g
Water	1	litre

Glucose and $KClO_3$ were autoclaved separately.

Nutrient agar with glucose and nitrate ($NAGNO_3$)

As for $NAGClO_3$, substituting 0.2% KNO_3 for $KClO_3$.

Eosin-methylene blue-sugar agar [147]

Tryptone	10	g
Yeast extract	1	g
KH_2PO_4	2	g

Eosin-methylene blue-sugar agar (cont'd)

NaCl	5	g
Eosin Y	400	mg
Methylene blue	65	mg
Difco Bacto agar	15	g
Water	1	litre

(Final pH 7.0)

100 ml 10% sugar solution was added to eosin-methylene blue agar after autoclaving.

EMBgal; EMB + galactose

EMBmal; EMB + maltose

Glucose peptone water [52]

Peptone	10	g
NaCl	5	g
Bromocresol purple	50	mg
Water	1	litre

(Final pH 7.2)

After autoclaving, glucose was added to give a final concentration of 0.5%.

Davis and Mingioli minimal medium (MM) [61]

K_2HPO_4	7	g
KH_2PO_4	3	g
Tri-sodium citrate $2H_2O$	500	mg
$MgSO_4.7H_2O$	100	mg
$(NH_4)_2SO_4$	1	g

Davis and Mingioli minimal medium (cont'd)

Water	1 litre

(Final pH 7.0)

After autoclaving, glucose was added to give a final concentration of 0.5%.

M9 minimal medium [147]

Na_2HPO_4	6 g
KH_2PO_4	3 g
NaCl	0.5 g
NH_4Cl	1 g
Water	1 litre

After autoclaving, $MgSO_4.7H_2O$ and $CaCl_2$ were each added to 1.0 mM final concentration.

Trifolii medium (TM)

KH_2PO_4	150 mg
$CaCl_2.2H_2O$	250 mg
$MgSO_4.7H_2O$	200 mg
NaCl	100 mg
$(NH_4)_2SO_4$	250 mg
Water	1 litre

The pH was adjusted to 6.8, and after autoclaving the following compounds were added (as filter-sterilized solutions).

Glucose	3.6 g
$FeSO_4.7H_2O$	2.5 mg

Trifolii medium (cont'd)

$Na_2MoO_4.2H_2O$	25	mg
Thiamine-HCl	1	mg
Biotin	0.5	mg
Nicotinic acid	1	mg
Pyridoxine-HCl	1	mg
Gamborg's trace elements	1	ml

TM succinate medium

As for TM, but with 20 mM Na succinate as sole carbon source.

TMY medium

1 g/l Difco yeast extract was added to TM medium.

Nitrogen-free medium (NFM) [41]

Glucose	20	g
$MgSO_4.7H_2O$	100	mg
K_2HPO_4	12.06	g
KH_2PO_4	3.4	g
$FeSO_4.7H_2O$	25	mg
$Na_2MoO_4.2H_2O$	25	mg
Water	1	litre

(Final pH 7.0)

Phosphate solution was separately autoclaved and added just before use.

Jensen's seedling medium [225]

$CaHPO_4$	1	g
K_2HPO_4	200	mg
$MgSO_4.7H_2O$	200	mg
NaCl	200	mg
$FeCl_3$	100	mg
Gibson's trace elements	1	ml
Difco Bacto agar	15	g
Water	1	litre

(Final pH 6.5)

After autoclaving, the medium was dispensed into plugged test tubes in 10 ml amounts, and allowed to set in long slopes.

Gamborg's trace element solution [76]

$MnSo_4.4H_2O$	10	g
H_3BO_3	3	g
$ZnSO_4.7H_2O$	3	g
$Na_2MoO_4.2H_2O$	250	mg
$CuSO_4.5H_2O$	250	mg
$CoCl_2.6H_2O$	250	mg
Water	1	litre

The solution was sterilized by filtration.

Gibson's trace element solution [78]

H_3BO_3	2.86	g
$MnSo_4.4H_2O$	2.03	g
$ZnSO_4.7H_2O$	220	mg
$CuSO_4.5H_2O$	80	mg
$H_2MoO_4.1H_2O$	90	mg
Water	1	litre

The solution was sterilized by filtration.

Buffers: All buffers were sterilized by autoclaving at 15 lb/in^2 for 20 min.

Saline phosphate buffer [41]

NaCl	8.5	g
K_2HPO_4	7	g
KH_2PO_4	3	g
Water	1	litre

(Final pH 7.2)

Protoplast dilution buffer (PDB) [83]

Sorbitol	0.25	M
Mannitol	0.25	M
K_2HPO_4	10	mM
$CaCl_2$	2	mM

in H_2O. pH 5.8

Antibiotics: Antibiotics were added to media as freshly-prepared filter-sterilized solutions. Solutions were stored for short periods at 4°C.

Antibiotics used were obtained from the following companies:

Ampicillin (Ap) — Beecham Research Laboratories

Carbenicillin (Cb) — Beecham Research Laboratories.

Neomycin (Nm) — Sigma Chemical Company

Kanamycin Sulphate (Km) — Sigma Chemical Company

Spectinomycin (Sp) — Upjohn Pty. Ltd.

Amino acids and Vitamins: Amino acid and vitamin solutions were sterilized by filtration, and were stored at 4°C. When required, amino acids were added to media at final concentrations of 25 μg/ml; vitamins other than biotin were added to 1 μg/ml; biotin was added at a final concentration of 1 μg/l.

Sugars: Sugar solutions were sterilized by filtration and were stored at 4°C.

2.2 Methods

Storage of bacterial cultures

Stock cultures were prepared in several different ways to ensure preservation [147]:

(a) Glycerol storage: An equal volume of sterile 80% glycerol was added to freshly grown overnight cultures in rich medium. The mixtures were then stored

at -20°C. To grow a fresh culture, a few drops of the glycerol suspension was added to fresh medium and incubated overnight.

(b) Storage of agar stabs: Freshly grown overnight cultures were stabbed into soft LBG or TMY agar in small bottles. After incubating for 24 h the bottles were sealed with Parafilm and stored either at room temperature or at 4°C.

(c) Storage on plates: For storage of less than one month, cultures were streaked on plates of rich or selective medium, sealed with Parafilm after incubation, and stored at 4°C. Single colonies were picked for regrowth.

(d) Storage of bacteriophages: Bacteriophage suspensions in liquid LBG or buffer were stored at 4°C.

Phage sensitivity tests

Rhizobia were tested for sensitivity to phages by adding 0.2 ml overnight culture to 3 ml soft agar at 45° and pouring on a TMY plate. When the top agar had set, various dilutions of the phages to be tested were spotted on the surface and allowed to dry into the agar. Plates were regularly examined during 24 hr incubation for zones of clearing and single plaques, indicating sensitivity of the bacterium to the phage.

Sensitivity to bacteriocins was tested similarly.

Conjugation experiments

Liquid matings were normally used for transfer of F' plasmids between *E. coli* strains. Filter matings were normally used for transfer of plasmids within *Rhizobium*.

Liquid matings: Overnight cultures of donor and recipient bacteria were subcultured 1:10 in fresh medium and grown to about 2 x 10^8 cells/ml. Cultures were then mixed, normally in the ratio 1 donor:1 recipient cell, and incubated without shaking for 2 hr. The mixture was then washed and diluted in saline phosphate buffer, and plated on appropriate selective media. Exconjugants were purified on similar media.

Filter matings: Overnight cultures of donor and recipient bacteria were centrifuged and the cells resuspended in saline phosphate buffer. The cells were mixed in the ratio of 10 donors:1 recipient cell, and were collected on a 0.22 μm membrane filter, which was incubated for up to 7 days on a plate of rich medium. The bacteria were then washed off the filter into buffer, and were plated on appropriate selective media. Exconjugants were purified on similar media.

Tests for nitrogen fixation

Growth on solid nitrogen-free medium: Cultures to be tested were grown to mid-log phase in rich medium,

and the cells resuspended in saline phosphate buffer, and then streaked on NFM plates. Plates were incubated for 5 days at 30°C under a continuously flowing atmosphere of 99% N_2/1% CO_2 in nylon bags [95]. The gas mixture was first sparged through 1M H_2SO_4 and then water to remove any traces of ammonia.

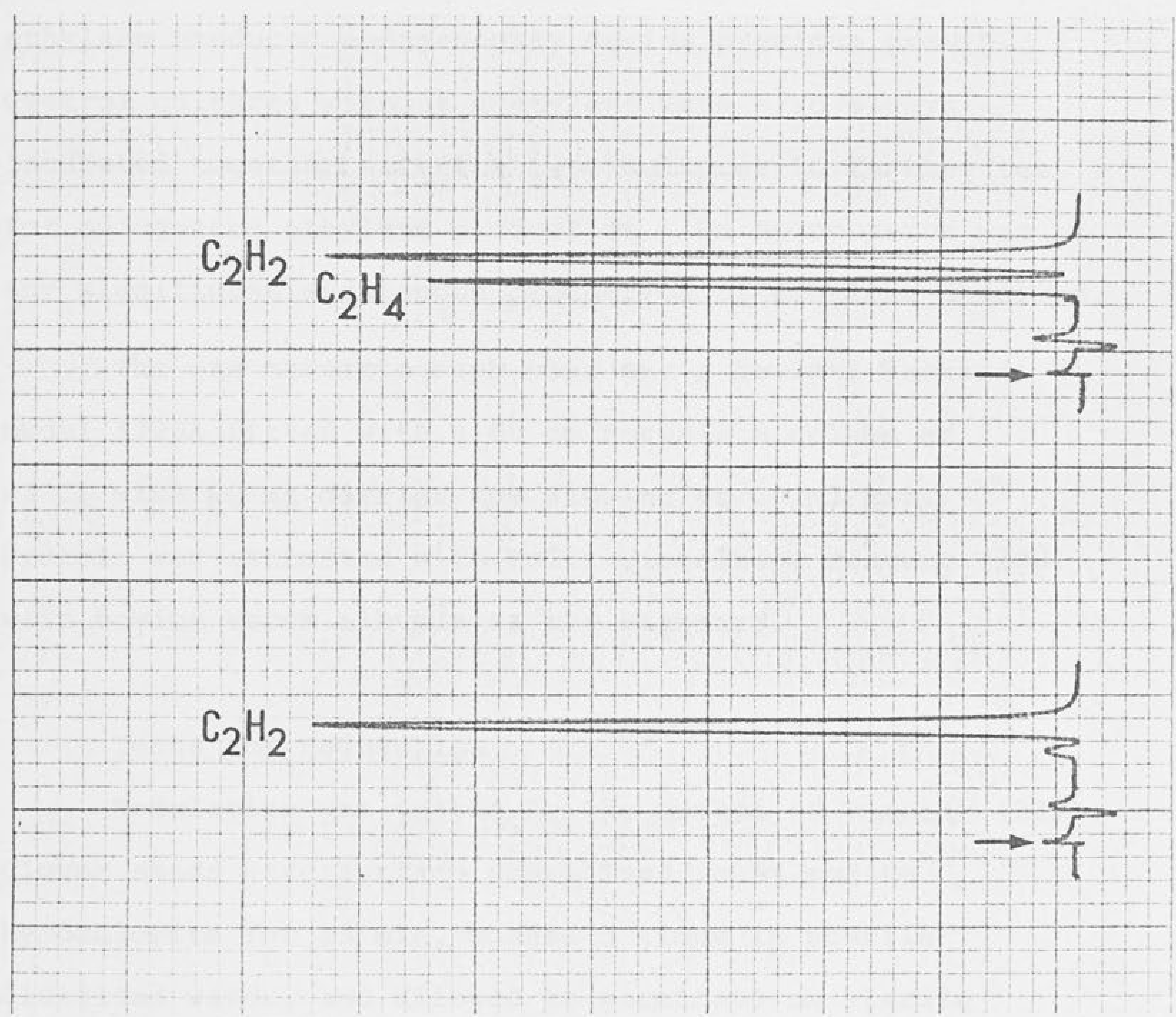
Growth in liquid nitrogen-free medium (Pankhurst tubes): Cultures were tested for growth in liquid NFM by the Pankhurst tube technique [40, 162]. A washed log-phase culture was inoculated (0.25 ml) into 10 ml NFM in a Pankhurst tube. Alkaline pyrogallol (1 ml saturated aqueous pyrogallol and 1 ml 10% NaOH + 15% K_2CO_3) was added to the sidearm tube, the tube was sealed with Suba seals, and after several hours 10 ml N_2 was injected to replace absorbed O_2. Growth was assessed by measuring the turbidity of the culture in a nephelometer.

Acetylene reduction tests

For qualitative tests, cultures were grown in NFM by the Pankhurst tube technique, with or without addition of 100 μg/ml vitamin-free Casamino acids to promote growth without repression of nitrogenase [220]. After 18 - 20 hr incubation, 10% acetylene was injected, and the gas phase was tested for the presence of ethylene at 2, 7 and 24 hr by gas chromatography (Figure 2.1). Presence of ethylene indicated reduction of acetylene by nitrogenase [64, 184].

FIGURE 2.1

Traces obtained from the Hewlett-Packard 5720A gas chromatograph injected with (a) C_2H_2, and (b) C_2H_2 + C_2H_4 in N_2. Other details were as described in the text.



C_2H_2
C_2H_4
C_2H_2

For quantitative tests, cultures were grown as above in Pankhurst tubes. 4 ml of an 18 hr culture was injected into a 50 ml flask flushed with argon and 10% acetylene; the flask was shaken gently and the rate of ethylene production was measured over 30 min [220].

Just prior to testing cultures for acetylene reduction, each was always tested for the presence of ethylene produced endogenously during previous growth. Control cultures without acetylene were also always incubated together with the test cultures to further test for endogenous ethylene production. On no occasion was any significant endogenous production of ethylene observed.

The gas chromatograph used was a Hewlett-Packard model 5720A fitted with a 60 cm Poropak R column at 42°C, with N_2 as carrier gas flowing at 20 ml/min. Protein was estimated with Folin-Ciocalteau reagent [138] with bovine serum albumin as the standard.

Tests for nodulation

Nodulation was tested by the method of Vincent [225]. Clover seeds were surface sterilized in 5% sodium hypochlorite for 15 min, washed 3 times in sterile distilled water, and allowed to germinate on sterile damp filter paper. Single seedlings were placed on slopes of Jensen's medium in cotton wool-plugged test tubes. After incubating overnight, in the dark, with tubes slanted to make the roots grow along the slope, each tube was inoculated with about 10^7 washed bacterial cells. The

tubes were incubated with roots in the dark with an 18 hr, 22° day, 6 hr, 19° night. Roots were regularly examined over 6 weeks for presence of nodules, which were either large, red and effective (nitrogen-fixing) or small, white and ineffective (non-nitrogen-fixing). Nodules were generally visible after about 2 weeks.

Alternatively, the seedlings were placed on Jensen's medium in Petri dishes, which were incubated vertically under the same conditions as used for tubes. This method allowed a similar extent of growth, with the advantages of being simpler to set up and allowing up to 6 plants per plate (depending on the species of clover used) (Figure 2.2).

Isolation of bacteria from nodules

Nodules were cut from the plant, surface sterilized for 15 min in 5% sodium hypochlorite, and thoroughly washed in sterile distilled water before being crushed in a small amount of sterile distilled water. The suspension obtained was plated on various rich and selective media and incubated at 30°C to enable bacteria to grow.

Nitrosoguanidine mutagensis. Mid-log phase cultures were washed twice in citrate buffer pH 5.5, and nitrosoguanidine (NTG) was added to give a concentration of 200 µg/ml. Mixtures were incubated for 60 min at 30° and the cells were centrifuged and washed once in saline phosphate buffer. Cells were then plated on appropriate

FIGURE 2.2

Appearance of white clover plants grown on Jensen's medium in upright Petri dishes, 3 weeks after inoculation. Left, uninoculated plants; right, plants inoculated and effectively nodulated with *R. trifolii* strain TlSp. Normally 3 plants were grown on each plate, but up to 6 plants could be accommodated with very little retardation of growth provided sufficient light was provided (2 plants were grown per plate for red and subterranean clovers, as these plants were larger than white clover).

selective media. This treatment caused a hundred-fold decrease in the number of viable cells.

Anaerobic jar technique

Inoculated plates were put in an anaerobic jar, the jar was evacuated and filled with H_2 twice, re-evacuated and filled with the test gas. A catalyst was present to remove any remaining traces of O_2. The jar was incubated for 2 - 5 days at the required temperature before opening. A strict anaerobe, *Bacteroides melaninogenicus* ss. *intermedius* was used as a control to check that jars were completely anaerobic, and grew under these conditions.

Hydrogenlyase assay

Tubes containing glucose peptone water and inverted Durham tubes were inoculated with the test bacteria, sealed with Suba seals and flushed with the required gas (e.g. H_2 or N_2).

After incubation, acid production was indicated by a change in colour of the medium from purple to yellow, or to very pale yellow if the dye had been reduced. Gas production was visible as small bubbles in the Durham tubes. Gases produced this way were analysed by gas chromatography.

Tests for nitrate reductase [1, 106]

(a) Cells were grown for 24 hr at 30° or 37° on NAG NO_3 plates, and then 0.5 ml each of solutions A, B, and C were pipetted into the medium. Solution

A contained KNO_3, 202 mg; $NaH_2PO_4.H_2O$, 1.38 g; propanol, 5 ml; chloramphenicol, 500 g; water, 95 ml. Solution B contained sulfanilamide, 1.0 g; 3 M HCl, 100 ml. Solution C contained N-napthyl-ethylenediamine-hydrochloride, 20 mg; water, 100 ml. Presence of nitrite, formed by reduction of nitrate by the enzyme nitrate reductase, was indicated by a red colouration of the medium, and was measured 5 min after addition of Solution C.

(b) Cells were streaked on NAG ClO_3 plates and incubated under H_2 at 30° for 1 - 3 days. Plates were further incubated aerobically at 30°. Growth indicated absence of a functional nitrate reductase system, since this enzyme system reduces chlorate to the toxic product chlorite.

Measurement of growth yield

Growth yields were measured by following the turbidity of cultures growing in a minimal medium (MM for *E. coli*, TM for *R. trifolii*) until constant turbidity was obtained. Cultures were grown with limiting amounts (1mM to 8 mM) of glucose, and the turbidity was measured in a nephelometer.

Measurement of growth rate

Growth rates were measured by following the increase in turbidity with time of cultures growing in media with

20 mM glucose as carbon source. MM was used for *E. coli* and TM for *R. trifolii*.

Determination of fermentation end-products

The end-products formed after fermentation of glucose by *E. coli* were measured in NFM and MM. The cells were removed by centrifugation after growth had ceased, and the spent medium was analysed by gas chromatography. A 6 ft x 1/8 in stainless steel column packed with Chromosorb W 60/80 mesh, loaded with 20% diethylene glycol adipate, was fitted to a Hewlett-Packard 5720A flame ionization gas chromatograph. Helium flowing at 100 ml/min was used as the carrier gas, with an oven temperature of 200°. 5 μl samples were injected and compared with peaks obtained with standard solutions [68].

ATPase assay (230)

Lysozyme solution

Lysozyme	20	mg
Sucrose	205.2	g
EDTA	372	mg
Tris-HCl buffer, 0.15 M, pH 6.5	1	litre

Mg^{2+}-Tris solution

$MgCl_2$	203	mg
Tris	2.42	g
H_2O	1	litre
pH 7.5		

ATP solution

Equal volumes of (a), (b) and (c) were mixed.

(a) 254 mg ATP in 10 ml total volume of water, adjusted to pH 7.0 with dilute NaOH (made up fresh each time).

(b) 636 mg Tris in 10 ml total volume of water, adjusted to pH 9.5.

(c) 31.8 mg $MgCl_2$ in 10 ml water.

TCA solution

Trichloroacetic acid	24	ml
H_2O	76	ml

$FeSO_4$ solution

$FeSO_4.7H_2O$	800	mg
H_2O	100	ml

Ammonium molybdate solution

$(NH_4)_6Mo_7O_{24}.4H_2O$	6.6	g
3.75M H_2SO_4	100	ml

Log phase cultures of *E. coli* strains grown in LBG and *R. trifolii* strains grown in TMY medium were spun down and resuspended in saline phosphate buffer. Cells were then placed on Millipore membrane filters (0.45 μ pore size), with about 2 x 10^8 cells per filter. The cells

were treated with 10 ml lysozyme solution for 15 min, and were then washed with 10 ml Mg^{2+}-Tris solution. The filters were removed to vials containing 0.3 ml ATP solution. The vials were incubated, with slow shaking, at 30° for 30 min. To complete the reaction, 0.05 ml TCA solution, 1.75 ml $FeSO_4$ solution and 0.15 ml ammonium molybdate solution was added to each vial. After vigorous mixing, and allowing the blue colour to develop for 5 min, the optical density was measured at 660 mμ in a spectrophotometer.

The amount of inorganic phosphate released was determined by comparison with a standard curve relating OD_{660} with known concentrations of inorganic phosphate.

Controls of filter without cells or ATP, filter without cells but with ATP, filter with cells but without ATP, and ATP alone were always included, to check the background levels of inorganic phosphate (always very low).

The protein content of cells used in ATPase assays was estimated by the Lowry method [138], and ATPase activity was expressed as μmol P_i released/min/mg protein.

Isolation of bacteroids for ATPase assays

White clover plants were grown under sterile conditions in pots containing 1 part loam and 3 parts sand in the glasshouse. Ten weeks after inoculation with *R. trifolii* strains, the plants were harvested. The roots were surface-sterilized in 5% sodium hypochlorite

for 10 min, and then thoroughly washed in sterile distilled water. Nodules, which were always plump and pink, were cut from the roots and digested overnight in an enzyme mixture containing 4% Cellulysin, 2% Macerase and 1% Driselase in PDB [83]. The nodule contents were then gently squeezed out and further digested for 1.5 hr, followed by a two-fold dilution of the enzyme mixture with PDB and further incubation for 1.5 hr to complete protoplasting. The nodule mantles were removed by filtering through a 100 μ sieve, and then the protoplasts were separated from free bacteria and cell debris by gently centrifuging through a 30% sucrose cushion. The purified bacteroid-containing protoplasts were then burst by forcing them through a 10 μ sieve. Free bacteroids were collected by centrifugation, and cell debris was removed by washing twice in PDB. Bacteroids were then resuspended in PDB, and their ATPase activity was assayed by the same method as used for free-living bacteria.

CHAPTER 3

THE PATHWAYS OF ENERGY METABOLISM REQUIRED FOR PHENOTYPIC EXPRESSION OF *KLEBSIELLA PNEUMONIAE* NITROGEN FIXATION GENES

CHAPTER 3 - THE PATHWAYS OF ENERGY METABOLISM REQUIRED FOR PHENOTYPIC EXPRESSION OF KLEBSIELLA PNEUMONIAE NITROGEN FIXATION GENES.

3.1 Introduction

Nitrogen fixation depends on appropriate electron transport reactions, adequate energy supplies, and probably on the incorporation of the nitrogenase enzyme into an organisational unit associated with membranes [166].

Klebsiella pneumoniae, which is closely related to *Escherichia coli*, has been shown to fix nitrogen under anaerobic and microaerophilic conditions [98, 125]. However, the precise mechanism of anaerobic electron transport and provision of energy for the nitrogenase complex in *Enterobacteriaceae* is not known, although a similarity has been suggested between *Klebsiella* and *E. coli* in the generation and transport of low potential reducing power from pyruvate [232].

Various hybrids between *K. pneumoniae* and *E. coli* C and K12 strains can fix nitrogen [43, 67]. Since *E. coli* strains are generally regarded as being genetically devoid of *nif* functions [194], nitrogen fixation by such hybrids has been taken as evidence for the transfer and expression of nif^{+}_{Kp} genes (*nif* genes from *K. pneumoniae*).

Because of the availability of genetically and biochemically well-characterized mutants in *E. coli* K12, various hybrids carrying an F' nif^{+}_{Kp} plasmid were constructed to study which energy pathways might be coupled to nitrogenase activity in *E. coli* K12.

For this study, groups of isogenic strains were used to avoid the possibility of other, unknown mutations affecting nif^{+}_{Kp} gene expression. All the oxidative phosphorylation (unc^{-}) and fumarate reductase (frd^{-}) mutants are in the isogenic background of AN259; menaquinone ($menA^{-}$) and ubiquinone ($ubiA^{-}$) mutants are in the isogenic background of AN387, except for AN99 $menA^{-}$ which is in another background of AN98 men^{+}. Thus, the need for functional oxidative phosphorylation, fumarate reduction, and the involvement of menaquinone and ubiquinone in expression of nitrogen fixation could be investigated.

E. coli K12 strain SB1801 $his^{-}mal^{-}$ carrying the FN68 plasmid (F' nif^{+}_{Kp} his^{+}_{Kp} Cb^{R}) which also has about 3 minutes of the *E. coli* K12 genome from *shiA* to *metG* [43] was used as the donor in conjugation experiments because the nif^{+}_{Kp} genes are stably linked to the carbenicillin resistance marker (Cb^{R}). All recipient strains were mal^{+}, and mal^{+} Cb^{R} hybrids were selected on EMB-maltose-carbenicillin (300 μg/ml) plates at 30°. Hybrid isolates were purified by restreaking three times on the selection medium, and were then tested for nitrogen fixation by growth on NFM and by acetylene reduction.

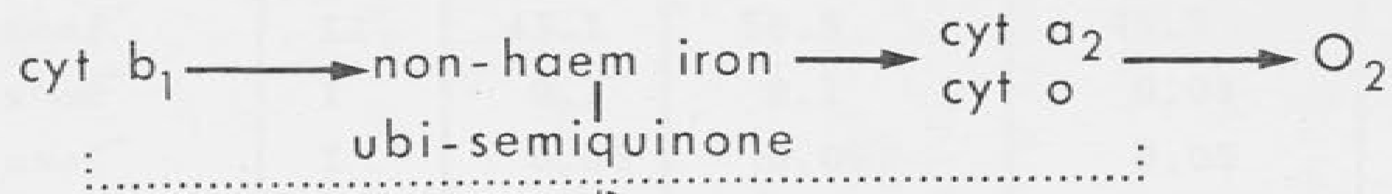
3.2 Phenotypic expression of nif^{+}_{Kp} genes in *E. coli* K12 unc^{-} mutants

The defects in the oxidative phosphorylation (unc^{-}) mutants are illustrated in Figure 3.1. The ATPase complex consists of the F_0 portion, which is located within the cell membrane, and the F_1 portion which is made up of five different subunits in a "knob" attached to the F_0 portion [88]. The *uncA401* mutant (AN249) has a defective α subunit in the F_1 extrinsic portion ("knob") of the ATPase complex, and the *uncD405* mutant (AN285) has a defective β subunit in the F_1 portion which prevents the rest of the "knob" from attaching to the membrane (Cox and Gibson, personal communication). The $uncC424^{-}$ mutant (AN771) and the $uncB402^{-}$ mutant (AN283) are both defective in proteins of the F_0 (intrinsic) portion of the ATPase complex, and thus are defective in coupling between the ATPase complex and the electron transport chain, but are of different complementation groups [81]. All four unc^{-} mutations are located in one operon between *pyrE* and *ilv* on the *E. coli* K12 chromosome, and map in the order *uncB uncA uncD uncC* (Gibson, personal communication).

When the FN68 plasmid (F' nif^{+}_{Kp} Cb^{R}) was transferred into the well-defined unc^{-} mutants of *E. coli* K12, two classes of hybrids were found (Table 3.1). Class I hybrids had a defective Nif phenotype; they grew slowly on nitrogen-free medium and reduced acetylene at a very slow rate of about 0.1% to 0.2% of that found with the isogenic unc^{+} hybrid. Strains in this class were the

OUTSIDE

MEMBRANE RESIDUE



uncB
uncC protein

transhydrogenase

INSIDE

uncD (knobless)

Mg-ATPase
(uncA protein)
and other
polypeptides

Mg-ATPase aggregate

ADP

ATP

TABLE 3.1 Phenotypic expression of *nif*$^{+}_{Kp}$ genes carried on plasmid FN68 in *unc*$^{-}$ mutants of *E. coli* K12

Hybrid strain	*unc* gene	Nif class	Acetylene Reduction†			Growth*	
			NFM	NFM + 20 mM fumarate	NFM + 20 mM succinate	NFM	NFM + 20 mM fumarate
AN259 (FN68)	*unc*$^{+}$	II	42.2	39.6	36.5	117	120
AN283 (FN68)	*uncB*$^{-}$	II	45.1	58.5	45.7	109	120
AN249 (FN68)	*uncA*$^{-}$	I	0.1	0.1	0.08	42	33
AN771 (FN68)	*uncC*$^{-}$	I	0.05	0.05	0.05	37	30
AN285 (FN68)	*uncD*$^{-}$	I	0.1	10.4	0.1	49	96

†Acetylene reduction in nmol C_2H_4/min/mg protein of cultures grown for 18 hr in nitrogen-free media supplemented with Casamino acids (100 μg/ml) and 20 mM fumarate or 20 mM succinate where indicated. All cultures were tested in duplicate.

*Growth in nitrogen-free medium supplemented with Casamino acids (100 μg/ml) was assessed after 20 hr incubation in Pankhurst tubes by measuring the increase in turbidity in a nephelometer. Turbidity is expressed in nephelometer units (initial nephelometer readings were between 8 and 10 units). No stimulation of growth by 20 mM succinate was observed for any hybrid. In the same experiment, all the parental strains (AN259, AN249, etc.) were included as controls, but none reduced acetylene under any condition.

FIGURE 3.1

Scheme summarizing mutants of *E. coli* uncoupled in oxidative phosphorylation, modified after Cox and Gibson [49].

uncA⁻ hybrid AN249 (FN68), the *uncC*⁻ hybrid AN771 (FN68) and the *uncD*⁻ hybrid AN285 (FN68). Class II hybrids, however, were phenotypically Nif^+ and reduced acetylene at rates comparable with *K. pneumoniae* strain M5a1, the bacterium from which the nif^+_{Kp} genes were derived. In this class, besides the isogenic unc^+ hybrid, was the *uncB*⁻ hybrid AN283 (FN68), which showed a high rate of acetylene reduction (Table 3.1).

The difference between the Class I and Class II responses was not solely due to growth differences. Although the Class I hybrids grew more slowly than the Class II hybrids, these cultures did increase considerably in turbidity with time (Table 3.1).

The Class I hybrids were routinely tested for acetylene reduction throughout the course of their growth, but at no time was any culture found to reduce acetylene at rates higher than those listed in Table 3.1.

All the unc^- mutants show an additional defect of a diminished formation of fumarate from glucose under anaerobic conditions, the effect being most marked in those strains having a defective Mg^{2+}-ATPase aggregate [181]. This explains why mutants AN249 ($uncA^-$) and AN285 ($uncD^-$), which are of this latter type, have been shown to require added fumarate for growth under H_2 in minimal media with glucose as sole carbon source, as well as for the anaerobic uptake of inorganic phosphate and serine [181].

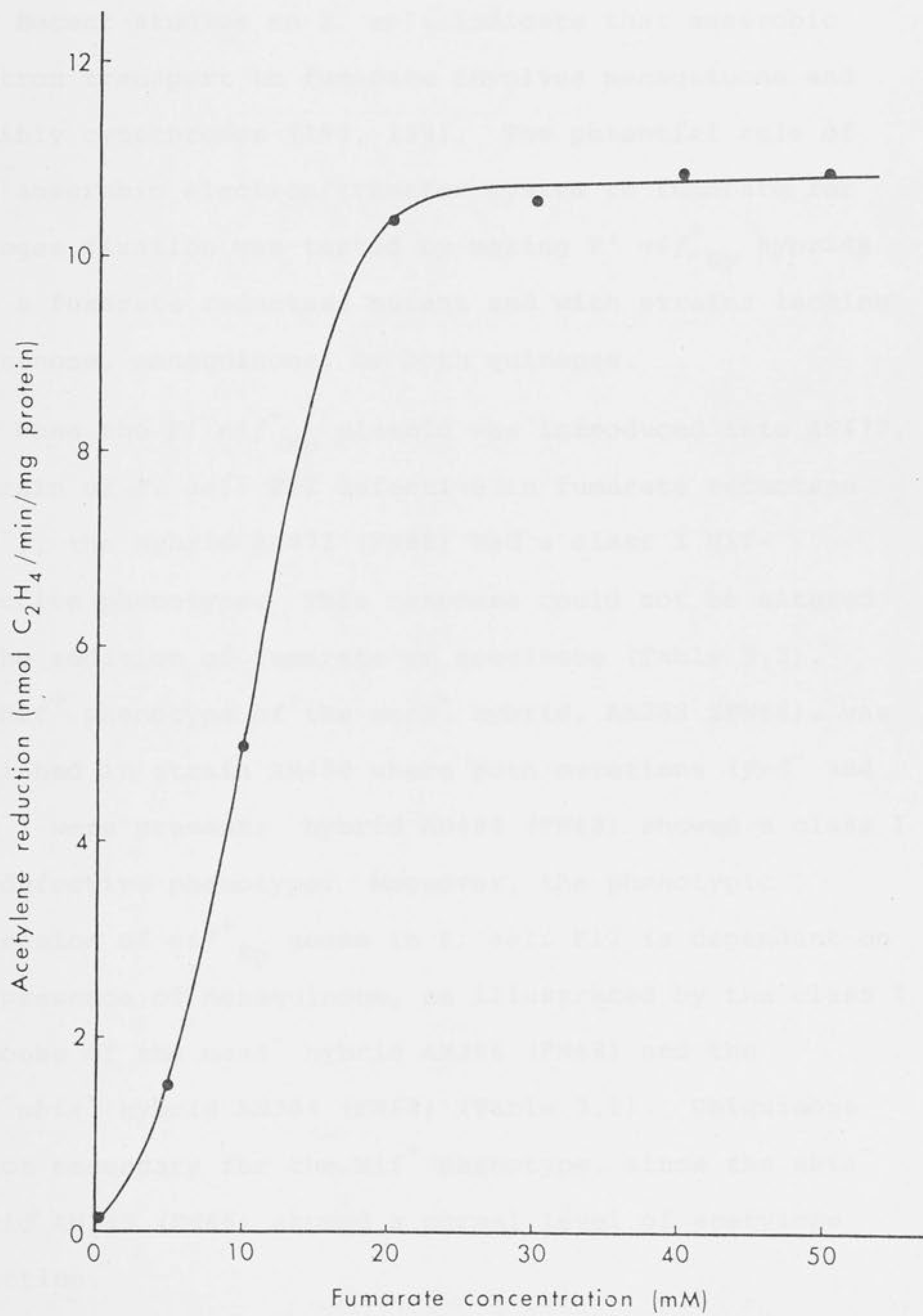
When fumarate was added to the *uncD*$^{-}$ hybrid AN285 (FN68), a stimulation of up to one hundred-fold in its level of nitrogen fixation (acetylene reduction) was observed, depending on the amount of fumarate added (Figure 3.2 and Table 3.1). In contrast, the *uncA*$^{-}$ and *uncC*$^{-}$ hybrids, AN249 (FN68) and AN771 (FN68), showed no response to added fumarate (Table 3.1).

Although strain AN283 is defective in coupling between the Mg^{2+}-ATPase aggregate and the electron transport chain [49, 181] its hybrid, AN283 (FN68) showed a high rate of nitrogen fixation. AN283 (*uncB*$^{-}$) does not require fumarate for anaerobic growth, nor for phenotypic expression of the *nif*$^{+}_{Kp}$ genes carried on the FN68 plasmid, but does need addition of fumarate for anaerobic uptake of inorganic phosphate and serine [181].

Fumarate did, however, slightly stimulate acetylene reduction by AN283 (FN68) when added at 20 mM, but not when added at 5 or 10 mM [201]. Succinate addition was also tested in these experiments to check whether the important role played by fumarate in nitrogen fixation was to supply succinate. The results show that stimulation of acetylene reduction is specific for fumarate and not succinate (Table 3.1), and indicate a requirement for an anaerobic electron transport system involving fumarate for *nif*$^{+}_{Kp}$ phenotypic expression in *E. coli* K12.

FIGURE 3.2

Acetylene reduction by the *uncD*$^-$ hybrid AN285 (FN68) growing in NFM with various levels of added fumarate.



Acetylene reduction (nmol C_2H_4/min/mg protein)
0
2
4
6
8
10
12
0
10
20
30
40
50
Fumarate concentration (mM)

3.3 Phenotypic expression of *nif*$^{+}_{Kp}$ genes in other *E. coli* K12 mutants

Recent studies on *E. coli* indicate that anaerobic electron transport to fumarate involves menaquinone and possibly cytochromes [198, 199]. The potential role of this anaerobic electron transfer system to fumarate for nitrogen fixation was tested by making F' *nif*$^{+}_{Kp}$ hybrids with a fumarate reductase mutant and with strains lacking ubiquinone, menaquinone, or both quinones.

When the F' *nif*$^{+}_{Kp}$ plasmid was introduced into AN472, a strain of *E. coli* K12 defective in fumarate reductase (*frd*$^{-}$), the hybrid AN472 (FN68) had a class I Nif-defective phenotype. This response could not be altered by the addition of fumarate or succinate (Table 3.2). The Nif^{+} phenotype of the *uncB*$^{-}$ hybrid, AN283 (FN68), was abolished in strain AN480 where both mutations (*frd*$^{-}$ and *uncB*$^{-}$) were present; hybrid AN480 (FN68) showed a class I Nif-defective phenotype. Moreover, the phenotypic expression of *nif*$^{+}_{Kp}$ genes in *E. coli* K12 is dependent on the presence of menaquinone, as illustrated by the class I response of the *menA*$^{-}$ hybrid AN386 (FN68) and the *menA*$^{-}$*ubiA*$^{-}$ hybrid AN384 (FN68) (Table 3.2). Ubiquinone is not necessary for the Nif^{+} phenotype, since the *ubiA*$^{-}$ hybrid AN385 (FN68) showed a normal level of acetylene reduction.

When the slightly leaky *menA*$^{-}$ hybrid AN99 (FN68) was tested, a low level of acetylene reduction was detected. This nitrogenase activity was stimulated

TABLE 3.2 Phenotypic expression of nif^{+}_{Kp} genes carried on plasmid FN68 in ubi^{-}, men^{-} and frd^{-} mutants of *E. coli* K12.

Hybrids	Relevant genotype		Acetylene reduction[†]			Growth*	
		Nif class	NFM	NFM + 20 mM fumarate	NFM + 20 mM succinate	NFM	NFM + 20 mM fumarate
AN259 (FN68)	frd^{+}	II	42.2	39.6	36.5	117	120
AN472 (FN68)	frd^{-}	I	<0.01	<0.01	<0.01	24	32
AN480 (FN68)	frd^{-} $uncB^{-}$	I	<0.01	<0.01	<0.01	4	5
AN387 (FN68)	ubi^{+} men^{+}	II	39.9	41.2	40.3	95	100
AN385 (FN68)	$ubiA^{-}$ men^{+}	II	43.8	45.0	43.2	87	91
AN386 (FN68)	ubi^{+} $menA^{-}$	I	0.06	0.08	0.06	46	47
AN384 (FN68)	$ubiA^{-}$ $menA^{-}$	I	<0.01	0.02	<0.01	40	39
AN98 (FN68)	men^{+}	II	29.7	25.2	28.6	120	135
AN99 (FN68)	$menA^{-}$	I	1.04	5.4	1.05	46	75

[†]Acetylene reduction in nmol/C_2H_4/min/mg protein; cultures were grown as in Table 3.1

*Growth is in nephelometer units, measured as in Table 3.1. No stimulation of growth by 20 mM succinate was observed for any strain. In the same experiment, all the parental strains (AN472, AN480, etc.) were included as controls, but none reduced acetylene under any condition.

five-fold by the addition of 20 mM fumarate (Table 3.2), suggesting that menaquinone may play a role in fumarate biosynthesis as well as in its reduction.

3.4 Retransfer of the FN68 plasmid from class I hybrids

The Nif-defective phenotype of class I hybrids could have resulted by segregation of the nif^{+}_{Kp} genes from the FN68 plasmid. To check this possibility, the Nif-defective hybrids were used as donor strains in conjugation experiments with recipient strain SB1801 (Table 3.3). When the plasmid was transferred back into SB1801 from any of the Nif-defective hybrids it was possible to recover Nif^{+}, His^{+} and Cb^{R} colonies which showed that nif^{+}_{Kp} genes had not been lost by segregation of the plasmid in the class I hybrids.

3.5 Complementation by the FN68 plasmid

The presence of the FN68 plasmid made no difference to the Suc^{-} phenotype exhibited by various *unc*$^{-}$ mutants, both aerobically and anaerobically [49], so that no function is present on the FN68 plasmid to complement this defect (Table 3.4).

The ability of *uncA*$^{-}$ and *uncD*$^{-}$ hybrids to grow anaerobically contrasts with previously published results, where it was shown that AN249 (*uncA*$^{-}$) and AN285 (*uncD*$^{-}$) were unable to grow anaerobically under H_2 with glucose as sole carbon source [49, 50]. To investigate whether this difference in anaerobic growth was due to the

TABLE 3.3 Retransfer of plasmid FN68 from Nif-defective hybrids back into recipient strain SB1801

Strains	Phenotype		
	Cb^R	His^+	Nif^+ (C_2H_2 reduction†)
SB1801	-	-	<0.01
SB1801 (FN68)	+	+	50.0
SB1801-249 (FN68)††	+	+	45.0
SB1801-285 (FN68)	+	+	43.0
SB1801-771 (FN68)	+	+	34.1
SB1801-472 (FN68)	+	+	42.0
SB1801-480 (FN68)	+	+	43.6

†Ethylene production in nmol C_2H_4/min/mg protein.

††Plasmid FN68 transferred from class I hybrid, AN249 (FN68) back to strain SB1801.

TABLE 3.4 Utilization of succinate as sole carbon source under aerobic and anaerobic conditions.

Strains	Growth on minimal succinate medium	
	Aerobically	Anaerobically[†]
AN259	+++	+
AN259 (FN68)	+++	+
AN249	-	-
AN249 (FN68)	-	-
AN283	-	-
AN283 (FN68)	-	-
AN285	-	-
AN285 (FN68)	-	-
AN771	-	-
AN771 (FN68)	-	-

-, no growth; +, poor growth; +++, good growth.

[†]Incubation was under H_2 and N_2 atmospheres.
All tests were done on solid MM, with 20 mM succinate as sole carbon source.

presence of the FN68 plasmid or to the different atmospheres above the cultures (N_2 as opposed to H_2), growth of the *unc*$^-$ strains was tested under various anaerobic atmospheres (Table 3.5). On no occasion did the hybrids containing the FN68 plasmid give results different from their parental *unc*$^-$ strains. Thus the growth differences were not due to the presence of the FN68 plasmid, but rather to the anaerobic atmosphere above the cultures (Table 3.5). Under H_2, the inability of the *uncA*$^-$ and *uncD*$^-$ mutants to grow on glucose without fumarate [49, 50] was confirmed, although addition of 1% CO_2 partially alleviated this inability (Table 3.5). However, all *unc*$^-$ strains grew as well as the parental *unc*$^+$ strain under N_2 and Ar (Table 3.5), which demonstrates that H_2 has a positive effect on preventing growth in the absence of added fumarate.

Both AN283 (*uncB*$^-$) and its hybrid AN283 (FN68) produced acid in glucose peptone water under H_2,N_2 or Ar as indicated by a change in the colour of the dye bromocresol purple to yellow. In addition, AN283, but not its hybrid, bleached the dye under these anaerobic conditions. Since this dye could be also bleached by sodium dithionite or by a culture supernatant containing ferredoxin from *E. coli* but not by ascorbic acid, AN283 must overproduce a redox system which is poised at a level between ascorbic acid and dithionite. Thus the presence of the F' *nif*$^+_{Kp}$ plasmid AN283 (*uncB*$^-$) appears to cause a loss of overproduction of the redox system, presumably by

TABLE 3.5 Anaerobic growth of *E. coli* K12 *unc*$^-$ mutants on minimal medium

	Growth on: Minimal medium +							
	Glucose (0.2% or 0.4%)				Glucose (0.2%) + fumarate (0.2%)			
	H_2	H_2/CO_2	N_2	Ar	H_2	H_2/CO_2	N_2	Ar
AN259 *unc*$^+$	++++	++++	++++	++++	++++	++++	++++	++++
AN249 *uncA*$^-$	-	++	++++	++++	+	+++	++++	++++
AN283 *uncB*$^-$	++	+++	++++	++++	++	+++	++++	++++
AN285 *uncD*$^-$	-	++	++++	++++	+	+++	++++	++++

-, no growth; +, ++, +++, ++++, increasing degrees of growth from poor to good.

Both MM and M9 solid minimal media were used. Both media gave the same results. The presence of the FN68 plasmid in any of the four strains listed made no difference to the results. H_2/CO_2 = 99% H_2/1% CO_2.

complementation between the mutant and some function(s) on the plasmid. The presence of the plasmid did not effect the response of the other *unc*$^-$ strains in these hydrogenlyase assays; all produced acid and gas and did not bleach the dye.

3.6 Hydrogenlyase activity of *E. coli* K12 *unc*$^-$ mutants

The *unc*$^-$ mutants were further characterized in glucose peptone water to see (a) whether they produced H_2 and CO_2 like the parental *unc*$^+$ strain, and (b) whether the anaerobic atmosphere above the cultures affected the pathways used for energy metabolism for growth in this rich medium as well as in minimal media (Table 3.5), by measuring the amounts of H_2 and CO_2 produced. It was found that all the *unc*$^-$ mutants produced H_2 and CO_2, but that they differed from the *unc*$^+$ strain and from each other in the amounts of these gases formed (Tables 3.6 and 3.7), although all cultures grew to the same extent.

Under both atmospheric conditions, the *unc*$^-$ mutants produced more H_2 than their *unc*$^+$ parent, the effect being most marked under N_2 (Table 3.6). Also, under both conditions the *unc*$^-$ mutants produced less CO_2 than the *unc*$^+$ strain, with the effect again being most marked under N_2 (Table 3.7). However, the one exception was AN283 (*uncB*$^-$) which produced more CO_2 than AN259 (*unc*$^+$) and the other *unc*$^-$ mutants under H_2, and less CO_2 than any other strain under N_2. Thus the various *unc*$^-$ mutations have an additional pleiotropic effect on regulation of hydrogenlyase activity under anaerobic conditions, with

TABLE 3.6 H_2 production by *unc*$^-$ strains grown anaerobically in glucose peptone water

Atmo-sphere	AN259*unc*$^+$	AN249*uncA*$^-$	AN283*uncB*$^-$	AN771*uncC*$^-$	AN285*uncD*$^-$
H_2	1.87	2.72	2.31	2.36	2.34
N_2	1.12	2.03	2.26	1.57	2.30

H_2 production is expressed as μmol H_2 collected in an inverted Durham tube in a 10 ml glucose peptone water culture incubated for 24 hr at 30° under various anaerobic atmospheres. H_2 was measured by gas chromatography. All cultures grew to the same extent, as assessed by measuring the turbidity.

TABLE 3.7 CO_2 production by *unc*⁻ strains grown anaerobically in glucose peptone water

Atmo-sphere	AN259*unc*$^+$	AN249*uncA*$^-$	AN283*uncB*$^-$	AN771*uncC*$^-$	AN285*uncD*$^-$
H_2	44.04	41.67	50.60	42.86	35.42
N_2	85.71	48.81	44.04	51.19	57.14

CO_2 production was measured by gas chromatography, using the same cultures described in Table 3.6. CO_2 production is expressed in nmol/Durham tube.

the *uncB*$^-$ mutation having a different effect from the other *unc*$^-$ mutations. Since this was also the only *unc*$^-$ mutation to cause bleaching of the dye, it may be that this result can also be explained by the altered hydrogenlyase regulation of this strain.

3.7 The relationship between nitrogenase and nitrate reductase

In *Rhizobium*, it has been suggested [44] that nitrate reductase and nitrogenase may share a common molybdoprotein subunit because the molybdenum-containing subunit of the nitrate reductase enzyme of *Neurospora crassa* could be replaced by the molybdenum-containing subunit of nitrogenase enzyme from soybean nodule bacteroids [153]. In addition, some nitrate reductaseless mutants of *Rhizobium meliloti* have been found to be ineffective and lacked nitrogenase activity [128, 200]. Pagan *et al.* [159], after examining 48 nitrate reductase mutants of the cowpea strain 32H1 of *Rhizobium*, concluded that there was little evidence to support commonality between nitrate reductase and nitrogenase in cowpea rhizobia. This has also recently been confirmed by Kiss *et al.* [123], after testing 25 nitrate reductase mutants of *R. meliloti* which were mapped at 4 different chromosomal sites, but all of which had nitrogenase activity. However, genetic studies of *nif*$^-$ *chl*$^-$ mutants of strain M5al of *Klebsiella pneumoniae* indicate that there may be a region within the *nif*$^+$ cluster of genes where particular pleiotropic mutants affecting both systems occur [210, 213].

The nitrate respiratory complex has been well-studied in *Escherichia coli* K12 [129, 139, 140, 141, 197], and mutants defective in nitrate reductase have been isolated on the basis of resistance to chlorate, as nitrate reductase can also reduce chlorate to toxic chlorite [1, 223, 224]. Mutations which lead to defects in the nitrate reductase system (*chl*) map in 7 genetic loci around the *E. coli* K12 chromosome (*chlA-G*) [8, Figure 3.3]. Most chlorate-resistant mutants show pleiotropic effects and have reduced levels of the components of both the formate-nitrate reductase and formate hydrogenlyase systems [129].

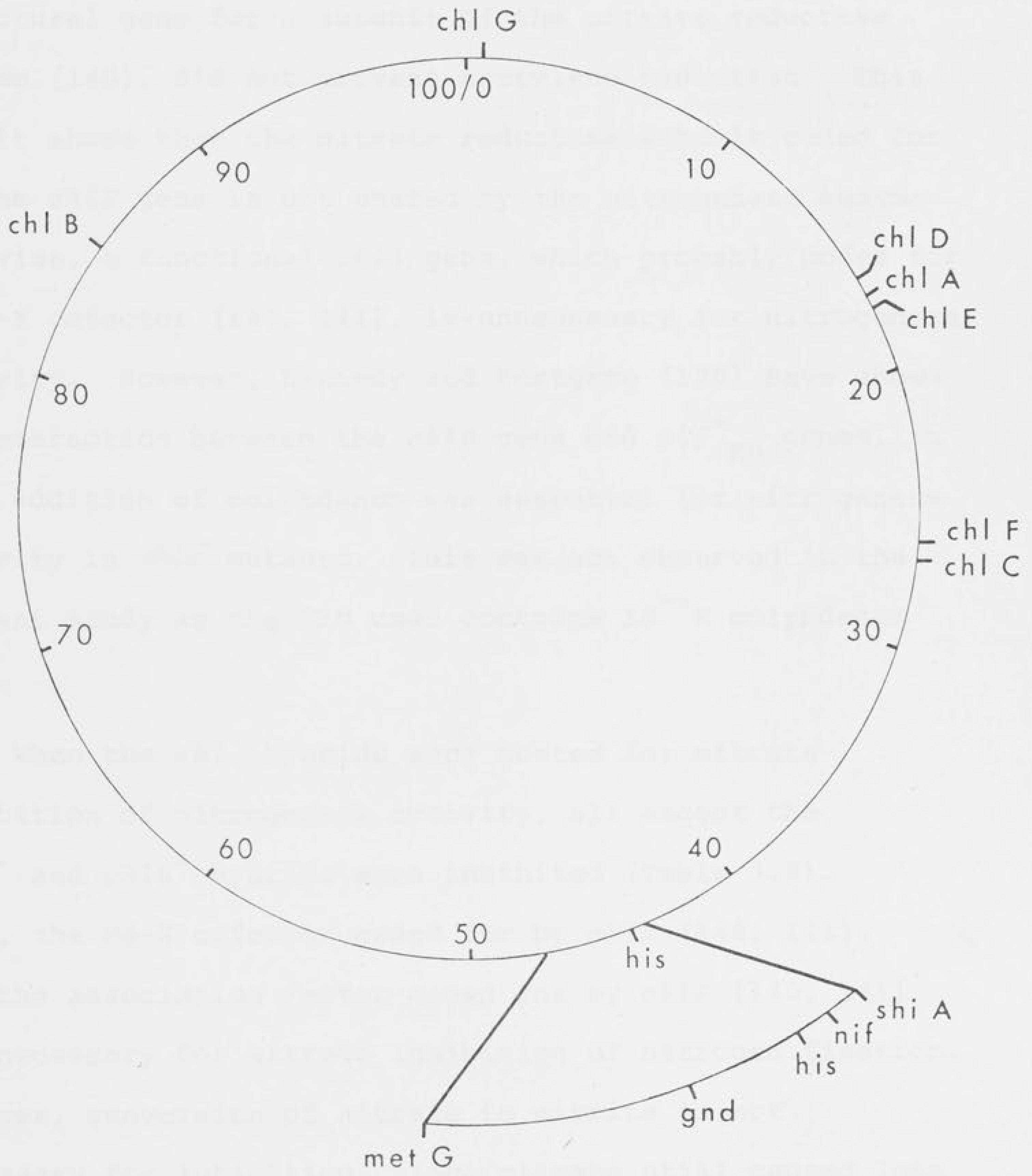
3.8 Phenotypic expression of nif^{+}_{Kp} genes in *E. coli* K12 chl^{-} mutants

Because the nitrate reductase system can act to energize the membrane in a manner similar to the fumarate reductase system, the possible relationship between the nitrate respiratory system and the nitrogen-fixing complex of *Enterobacteriaceae* was investigated by using hybrid strains constructed between various *E. coli* K12 mutants defective in nitrate respiration and the FN68 plasmid containing the nif^{+} cluster of genes from *K. pneumoniae*. As in the previous section, isogenic strains were used as far as possible.

Deletion and point mutants of *E. coli* K12 defective in six of the seven nitrate reductase genes were used to make hybrids with the F' nif^{+}_{Kp} plasmid. It was

FIGURE 3.3

E. coli K12 chromosome map showing the location of the various nitrate reductase (*chl*) mutations [8]. The region of the *K. pneumoniae* chromosome incorporated into plasmid FN68 is also shown.


chl G
100/0
90
10
chl B
chl D
chl A
chl E
80
20
chl F
chl C
70
30
60
40
50
his
shi A
nif
his
gnd
met G

not possible to test the effect of a mutation in *chlF* because such mutants were not longer available (DeMoss, personal communication). However, none of the mutations tested prevented or reduced expression of nitrogen fixation in these hybrids (Table 3.8). Even loss of *chlC*, the structural gene for a subunit of the nitrate reductase enzyme [140], did not prevent acetylene reduction. This result shows that the nitrate reductase subunit coded for by the *chlC* gene is not shared by the nitrogenase enzyme. Likewise, a functional *chlA* gene, which probably codes for a Mo-X cofactor [140, 141], is unnecessary for nitrogenase activity. However, Kennedy and Postgate [120] have shown an interaction between the *chlD* gene and nif^{+}_{Kp} genes, in that addition of molybdenum was essential for nitrogenase activity in *chlD*$^{-}$ mutants. This was not observed in the present study as the NFM used contains 10^{-4}M molybdenum ions.

When the *chl*$^{-}$ hybrids were tested for nitrate inhibition of nitrogenase activity, all except the *chlA*$^{-}$ and *chlB*$^{-}$ hybrids were inhibited (Table 3.8). Thus, the Mo-X cofactor coded for by *chlA* [140, 141], and the association factor coded for by *chlB* [140, 141] are necessary for nitrate inhibition of nitrogen fixation. However, conversion of nitrate to nitrite is not necessary for inhibition, since nitrate still caused loss of acetylene reduction in the *chlC*$^{-}$ hybrid. Similarly in the *chlD*$^{-}$, *chlE*$^{-}$, and *chlG*$^{-}$ hybrids, nitrate still inhibited nitrogen fixation (Table 3.8).

The necessity for functional *chlA* and *chlB* genes

TABLE 3.8 Phenotypic expression of *nif*$^{+}_{Kp}$ genes on plasmid FN68 in nitrate reductase mutants of *E. coli* K12.

E. coli K12 hybrid	*chl* mutation	Acetylene reduction		
		NFM	NFM + KNO_3	NFM + KNO_2
C181 (FN68)	*chlA*$^{-}$	39.0	38.0	0.9
KB70 (FN68)	*chlA*$^{\Delta}$	54.4	58.6	0.6
C183 (FN68)	*chlB*$^{-}$	43.0	40.8	0.08
DD115 (FN68)	*chlB*$^{-}$	31.2	32.2	0.08
Puig 426 (FN68)	*chlC*$^{-}$	35.4	0.08	<0.01
C111 (FN68)	*chlD*$^{\Delta}$	46.0	<0.01	<0.01
C123 (FN68)	*chlD*$^{-}$	39.1	<0.01	<0.01
C197 (FN68)	*chlE*$^{-}$	49.0	<0.01	<0.01
C202 (FN68)	*chlE*$^{-}$	45.0	<0.01	<0.01
DD38 (FN68)	*chlG*$^{-}$	56.8	0.3	0.1
SA291 (FN68)	*chlA*$^{\Delta}$*chlD*$^{\Delta}$	40.2	40.2	0.2

Acetylene reduction was measured in nmol C_2H_4/min/mg protein.
KNO_2 and KNO_3 were added with the inoculum at final concentrations of 50 mM.

-, point mutation; $^{\Delta}$, deletion

In the same experiment, none of the parental strains (C181, KB70, etc.) reduced acetylene under any condition.

for nitrate inhibition indicates that nitrogenase and nitrate reductase do share some molybdenum-processing functions, a conclusion also reached by Kennedy and Postgate [120].

The presence of the FN68 plasmid in *chl*$^-$ mutants of *E. coli* K12 did not restore nitrate reductase activity, as indicated by production of nitrite and sensitivity to chlorate, to any of the hybrids. Nitrate was not reduced to nitrite (as measured by pink colouration on addition of nitrite reagents) by any *chl*$^-$ hybrid (except those defective in *chlD*), after growth in either NFM with 50 mM nitrate or in nutrient agar with nitrate. Nitrite was only detected for *chlD*$^-$ hybrids when the medium contained MoO_4^{2-} ions, which overcomes the *chlD*$^-$ defect. Thus, no genes carried on the plasmid (including *nif*$^+_{Kp}$ genes) can complement defects in the nitrate reductase system.

3.9 The source of ATP for phenotypic expression of *nif*$^+_{Kp}$ genes

Nitrogenase is known to require large amounts of ATP, but can still function in the absence of a coupled oxidative phosphorylation system. This suggests that substrate level phosphorylation can provide sufficient ATP from glycolysis for both cellular growth and nitrogen fixation. Therefore this possibility was investigated in some detail using F' *nif*$^+_{Kp}$ hybrids of the *uncB*$^-$ mutant, AN283, and its *unc*$^+$ parent strain AN259.

The growth yield of a culture is proportional to the amount of ATP which can be obtained from the supplied energy source, provided that this is supplied in limiting amounts [209]. Therefore, measurement of the final growth yields of different strains can determine whether they can derive the same amount of ATP from the carbon source.

When the *uncB*$^-$ hybrid AN283 (FN68) and the *unc*$^+$ hybrid AN259 (FN68) were grown in minimal medium with glucose as sole carbon source present at the limiting level of 1 to 8 mM, it was found that the final growth yields differed aerobically, but were the same anaerobically [49] (Figure 3.4). Clearly, under aerobic conditions the *unc*$^+$ hybrid uses a coupled oxidative phosphorylation system to produce more ATP than the *uncB*$^-$ hybrid, leading to a greater growth yield of the *unc*$^+$ hybrid [49]. This result again demonstrates that there are no genes on the FN68 plasmid capable of complementing the *uncB*$^-$ defect.

Under anaerobic conditions, the two hybrids grew to the same extent (Figure 3.4), which suggests that either coupling through the Mg^{2+}-ATPase complex is not used anaerobically, or that some gene on the FN68 plasmid allows the *uncB*$^-$ hybrid to grow as well as the *unc*$^+$ hybrid. The latter hypothesis was eliminated by comparing the *unc*$^+$ and *uncB*$^-$ strains AN259 and AN283, without the FN68 plasmid. These two strains grew to the same extent as each other (Figure 3.5) under anaerobic conditions.

The end-products of glucose fermentation by the two hybrids AN259 (FN68) and AN283 (FN68) were tested after

FIGURE 3.4

Final growth yields of *unc*$^+$ hybrid AN259 (FN68) and *uncB*$^-$ hybrid AN283 (FN68) in minimal medium (MM) with glucose as sole carbon source. All cultures were grown in Pankhurst tubes, and growth was measured by following the turbidity in a nephelometer until it became constant. The constant turbidity reading was taken as the final growth yield, and plotted against the initial glucose concentration of the medium.

—○— AN259 (FN68) aerobic; —□— AN259 (FN68) anaerobic; —●— AN283 (FN68) aerobic; —■— AN283 (FN68) anaerobic.

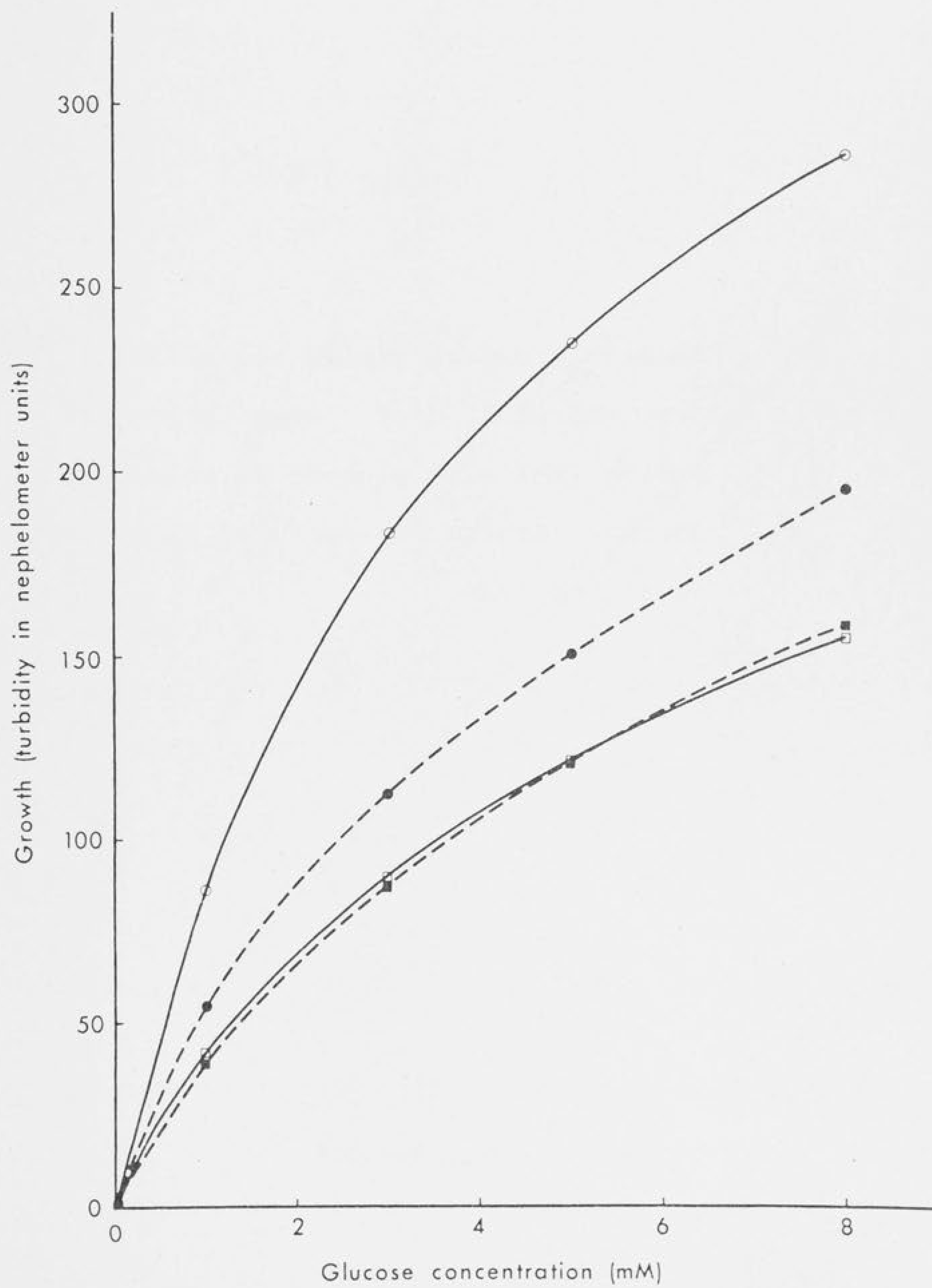
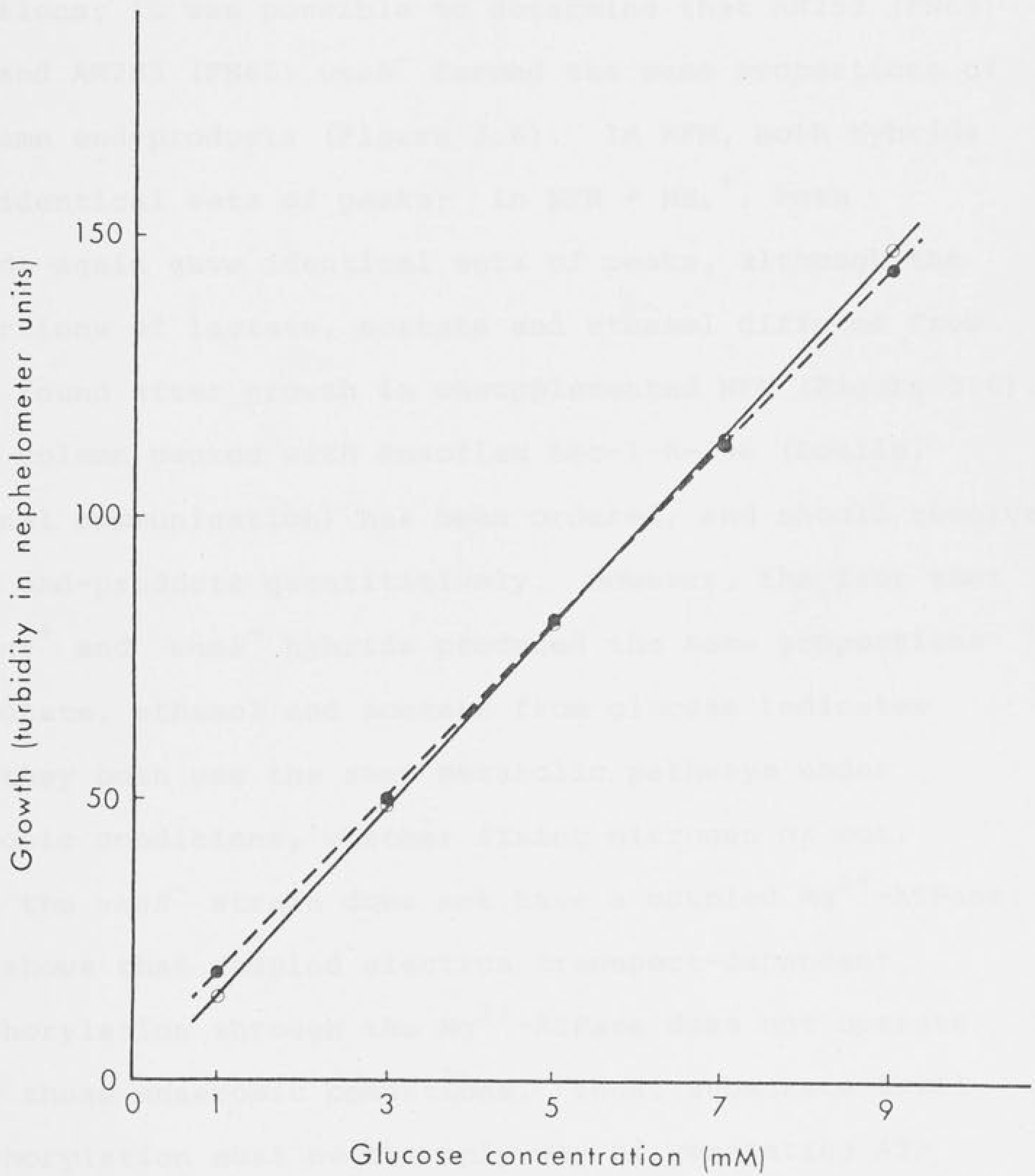

300
250
200
150
100
50
0
Growth (turbidity in nephelometer units)
0
2
4
6
8
Glucose concentration (mM)

FIGURE 3.5

Anaerobic growth yields of AN259 *unc*$^+$ (-○-) and AN283 *uncB*$^-$ (-●-) in minimal medium (MM) with glucose as sole carbon source. Growth was measured as described in Figure 3.4.



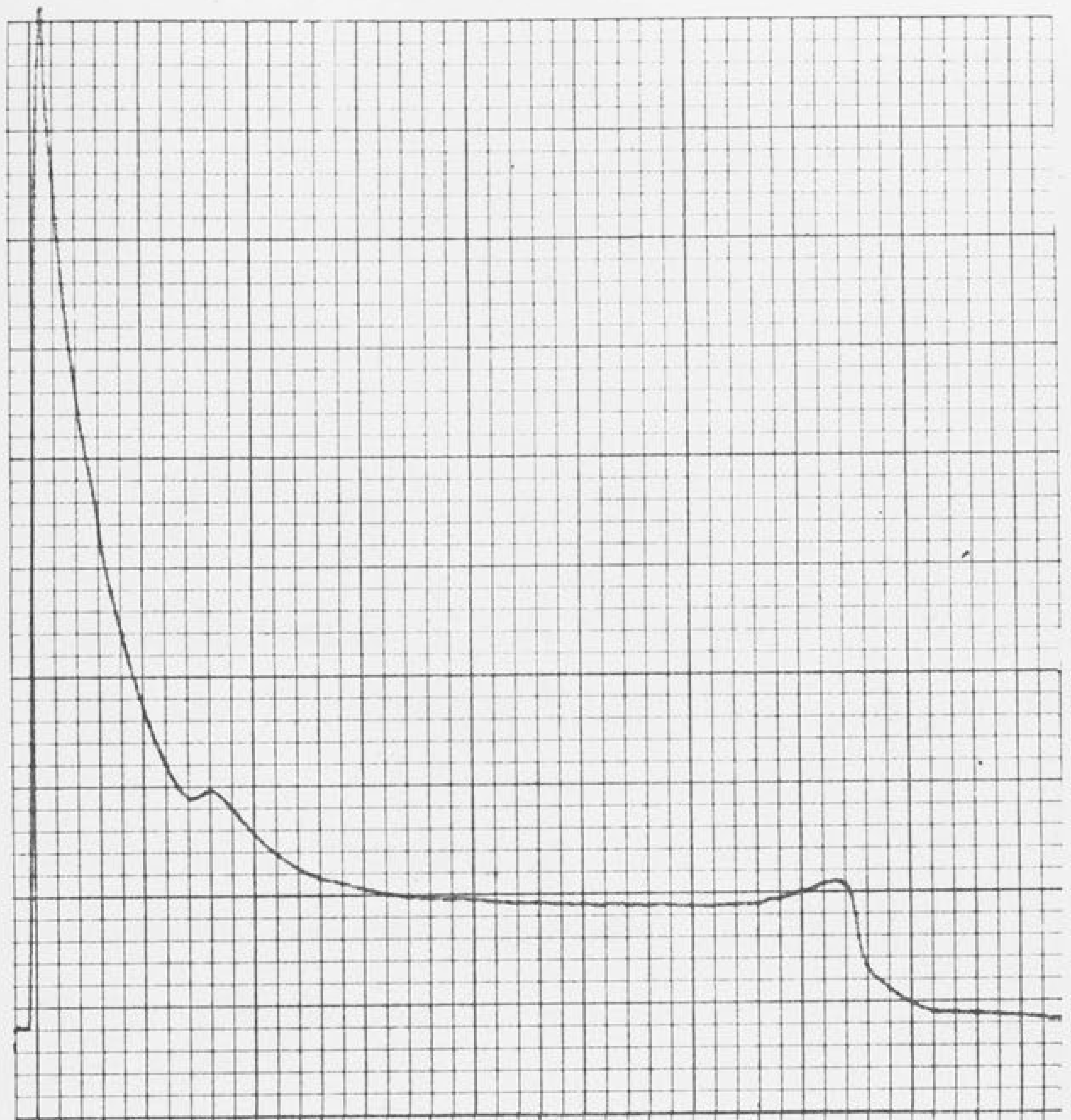
Growth (turbidity in nephelometer units)
150
100
50
0
0
1
3
5
7
9
Glucose concentration (mM)

anaerobic growth in Pankhurst tubes containing NFM with and without NH_4^+. End-products were analysed by gas chromatography [68]. Although the peaks obtained could not be separated well enough to estimate the amounts of lactate, acetate and ethanol formed under the two conditions, it was possible to determine that AN259 (FN68) *unc*$^+$ and AN283 (FN68) *uncB*$^-$ formed the same proportions of the same end-products (Figure 3.6). In NFM, both hybrids gave identical sets of peaks; in NFM + NH_4^+, both hybrids again gave identical sets of peaks, although the proportions of lactate, acetate and ethanol differed from those found after growth in unsupplemented NFM (Figure 3.6). A new column packed with Resoflex Lac-1-R-296 (Doelle, personal communication) has been ordered, and should resolve these end-products quantitatively. However, the fact that the *unc*$^+$ and *uncB*$^-$ hybrids produced the same proportions of lactate, ethanol and acetate from glucose indicates that they both use the same metabolic pathways under anaerobic conditions, whether fixing nitrogen or not. Since the *uncB*$^-$ strain does not have a coupled Mg^{2+}-ATPase, this shows that coupled electron transport-dependent phosphorylation through the Mg^{2+}-ATPase does not operate under these anaerobic conditions. Thus, substrate level phosphorylation must be the only way of generating ATP for growth under these conditions. It was also found that the *unc*$^+$ and *uncB*$^-$ hybrids grew to the same extent as each other, anaerobically in NFM, although the growth yield was about 55% less than in NFM with excess ammonium (Figure 3.7) [98]. When log-phase cultures of AN283

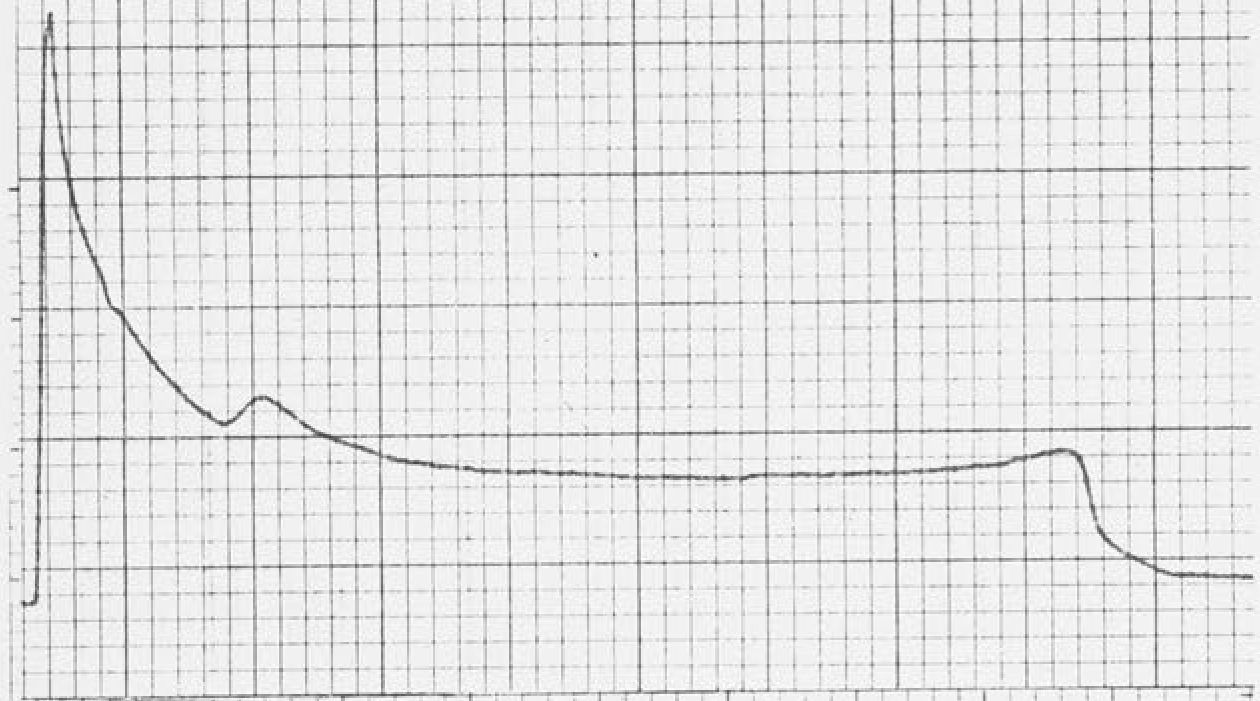
Figure 3.6

Traces obtained by gas chromatographic analysis of end-products of glucose fermentation. (a) AN259 (FN68) *unc*$^+$, grown anaerobically in NFM (10 µl sample). (b) AN283 (FN68) *uncB*$^-$, grown anaerobically in NFM (5 µl sample). (c) AN259 (FN68), grown anaerobically in NFM + 5 mM NH_4^+ (5 µl). (d) AN283 (FN68), grown anaerobically in NFM + 5 mM NH_4^+ (5 µl). All cultures were grown in Pankhurst tubes until the turbidity became constant. The cells were then removed by centrifugation, and the supernatant medium was analysed. The first peak (left) is due to ethanol, the second is lactate and the third is acetate, but the separation is not sufficient for quantification.

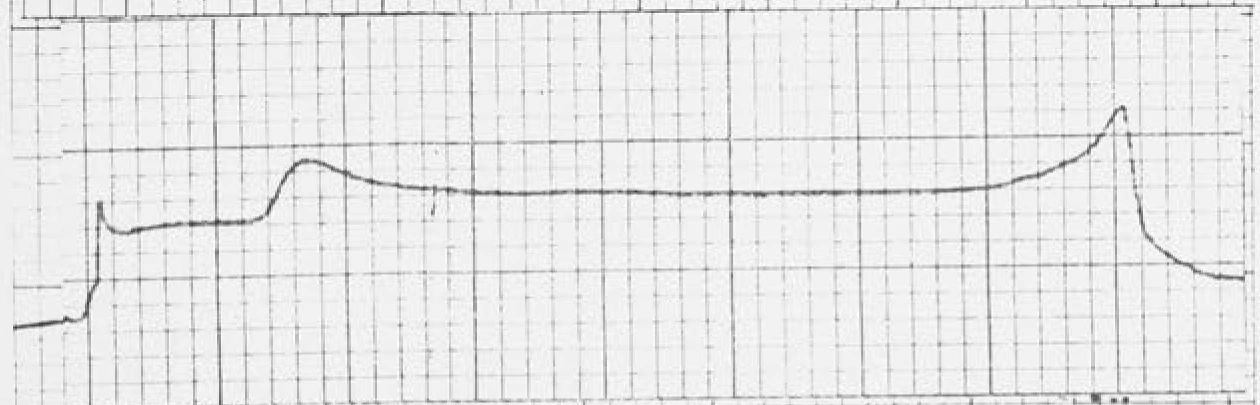
a



b



c



d

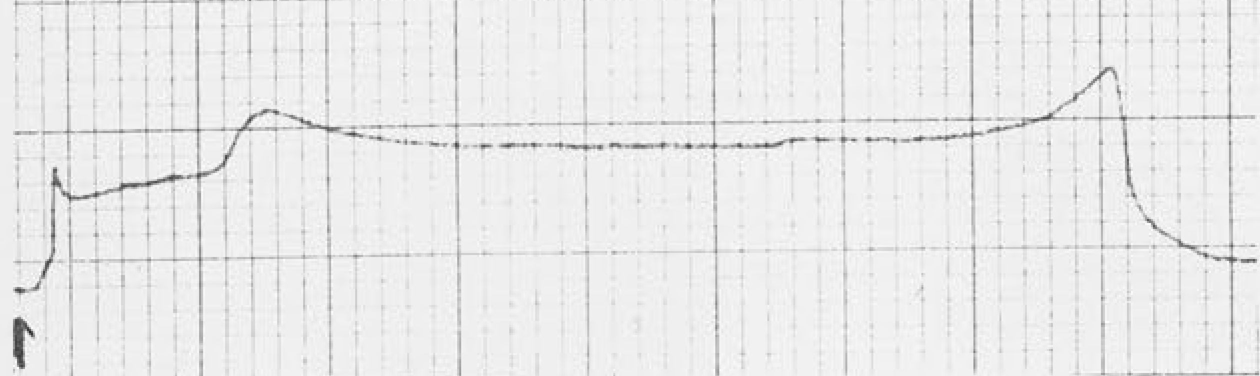
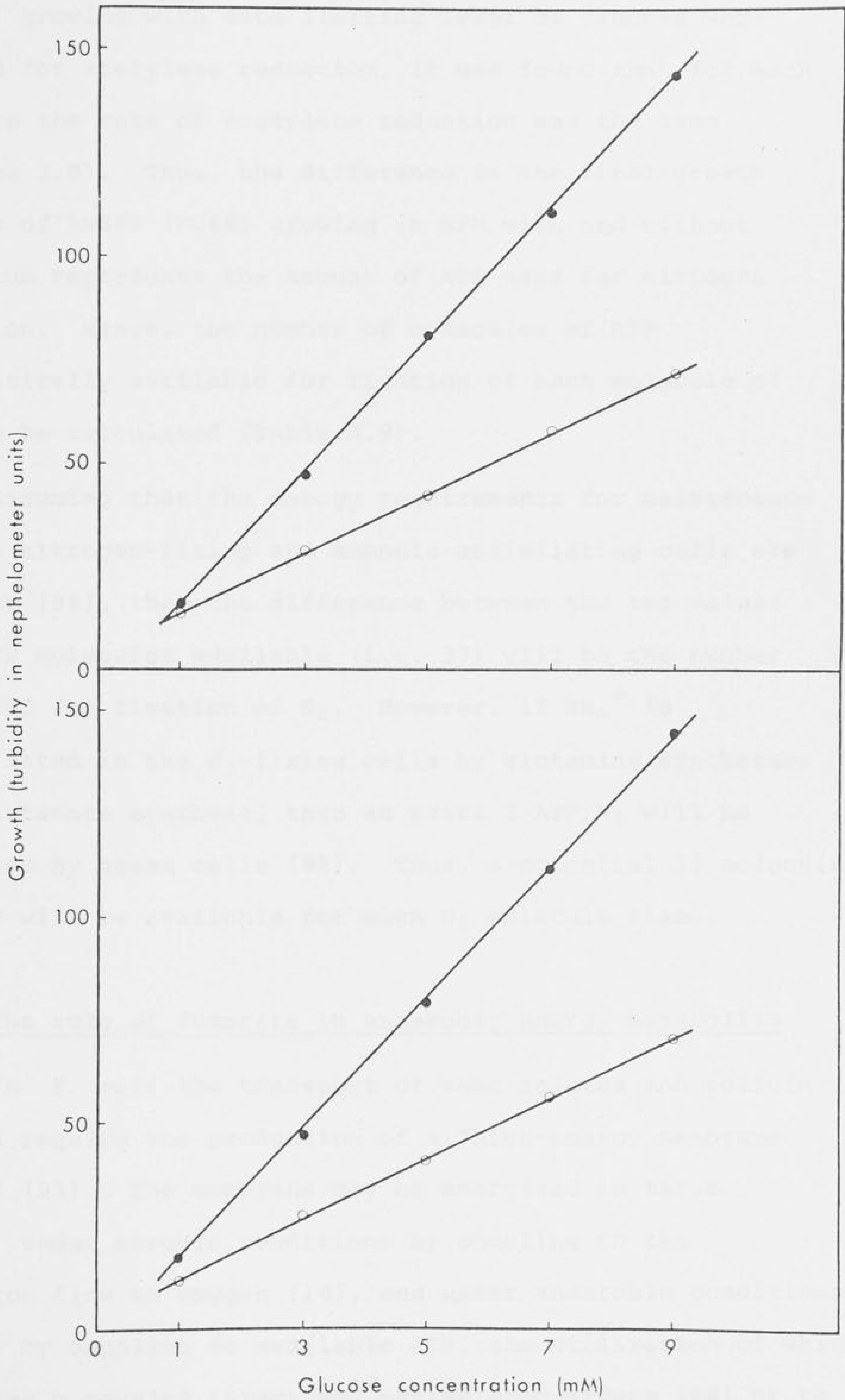


FIGURE 3.7

Anaerobic growth yields of AN259 (FN68) *unc*$^{+}$ (upper graph) and AN283 (FN68) *uncB*$^{-}$ (lower graph) in Pankhurst tubes containing NFM (-○-) or NFM with 20 mM ammonium (-●-), with limiting levels of glucose. Growth was measured as described in Figure 3.4.



Growth (turbidity in nephelometer units)
150
100
50
0
150
100
50
0
0
1
3
5
7
9
Glucose concentration (mM)

(FN68) growing with each limiting level of glucose were tested for acetylene reduction, it was found that for each culture the rate of acetylene reduction was the same (Figure 3.8). Thus, the difference in the final growth yields of AN283 (FN68) growing in NFM with and without ammonium represents the amount of ATP used for nitrogen fixation. Hence, the number of molecules of ATP theoretically available for fixation of each molecule of N_2 can be calculated (Table 3.9).

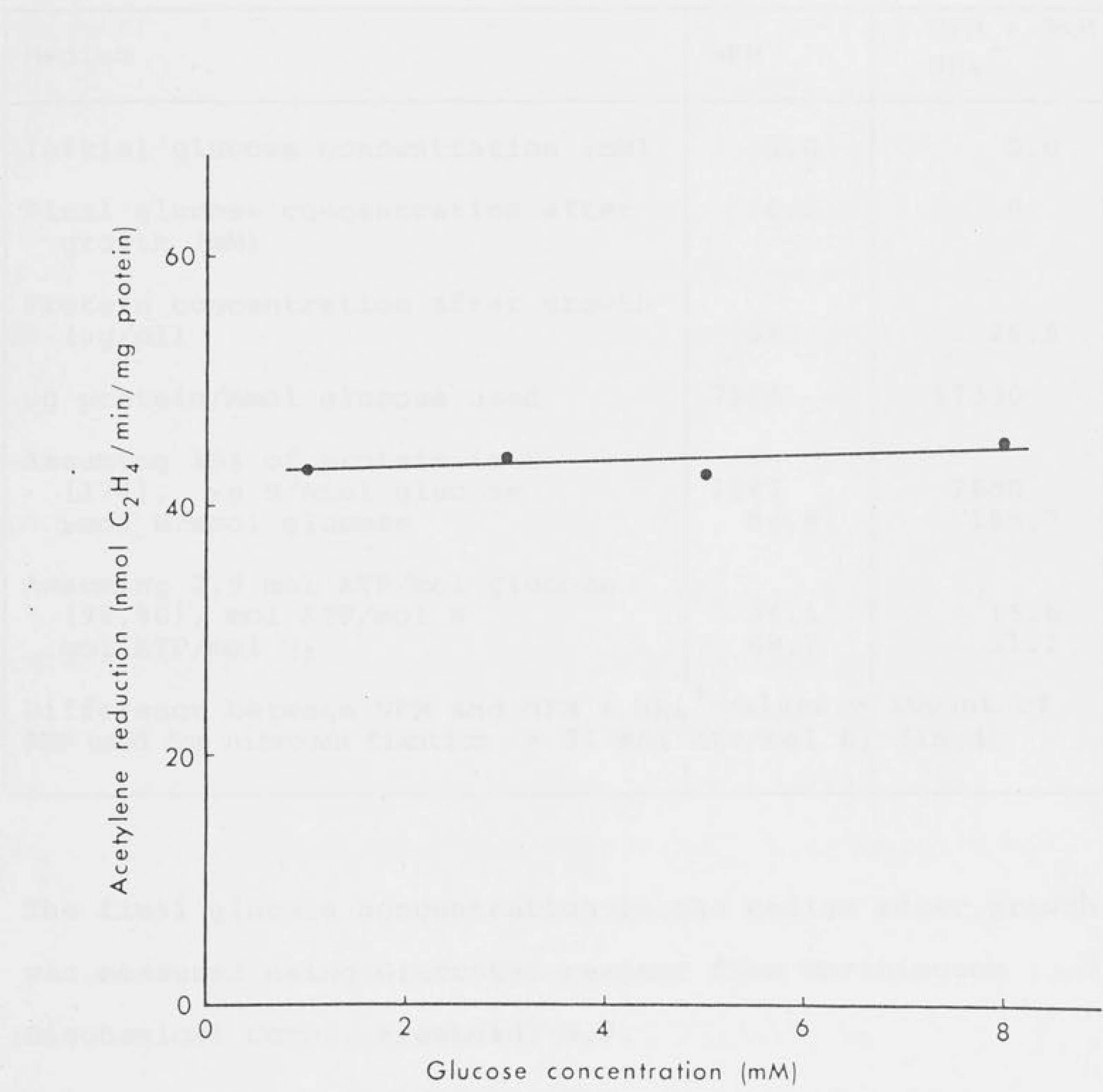
Assuming that the energy requirements for maintenance in the nitrogen-fixing and ammonia-assimilating cells are similar [98], then the difference between the two values for ATP molecules available (i.e. 37) will be the number required for fixation of N_2. However, if NH_4^+ is assimilated in the N_2-fixing cells by glutamine synthetase and glutamate synthase, then an extra 2 ATP/N_2 will be consumed by these cells [98]. Thus, a potential 35 molecules of ATP will be available for each N_2 molecule fixed.

3.10 The role of fumarate in anaerobic energy metabolism

In *E. coli* the transport of some solutes and colicin action require the production of a "high-energy membrane state" [93]. The membrane may be energized in three ways; under aerobic conditions by coupling to the electron flow to oxygen [10], and under anaerobic conditions either by coupling to available ATP, the utilization of which requires a coupled intact Mg^{2+}-stimulated ATPase [84] or to the electron flow to the acceptors nitrate or fumarate [130]. When the Mg^{2+}-stimulated ATPase activity was

FIGURE 3.8

Acetylene reduction by the *uncB*$^-$ hybrid AN283 (FN68). Log phase cultures growing in NFM with limiting glucose were tested quantitatively for acetylene reduction, which is expressed as nmol C_2H_4/min/mg protein.



Acetylene reduction (nmol C2H4/min/mg protein)
60
40
20
0
0
2
4
6
8
Glucose concentration (mM)

TABLE 3.9 Calculation of ATP used for nitrogen fixation for AN283 (FN68) growing under N_2 in Pankhurst tubes

Medium	NFM	NFM + 5mM NH_4^+
Initial glucose concentration (mM)	5.0	5.0
Final glucose concentration after growth (mM)	0.2	0
Protein concentration after growth (µg/ml)	38	86.5
µg protein/mmol glucose used	7916	17300
Assuming 15% of protein is N [174], µg N/mmol glucose	1187	2600
µmol N/mmol glucose	84.8	185.7
Assuming 2.9 mol ATP/mol glucose [98,90], mol ATP/mol N	34.1	15.6
mol ATP/mol N_2	68.2	31.2
Difference between NFM and NFM + NH_4^+ values = amount of ATP used for nitrogen fixation = 37 mol ATP/mol N_2 fixed		

The final glucose concentration in the medium after growth was measured using Glucostat reagent from Worthington Biochemical Corp., Freehold, N.J.

abolished by mutation, the Nif$^+$ phenotype still occurred. Since the addition of nitrate abolishes the Nif$^+$ phenotype, this indicates that nitrogen fixation in *E. coli* K12 hybrids depends on the electron transport system to the acceptor fumarate being functional. This conclusion was confirmed by the construction of hybrids with particular *E. coli* mutations: (a) AN472 (FN68), defective in fumarate reductase; (b) AN386 (FN68), which does not produce menaquinone; (c) AN285 (FN68), an *unc*$^-$ mutant also defective in the synthesis of fumarate from glucose. All these hybrids are altered in either the synthesis or the reduction of fumarate, and gave a Nif-defective phenotype.

To test whether the fumarate reduction system is necessary for the presence of a "high-energy membrane state" [93], which in turn is necessary for the phenotypic expression of nif^+_{Kp} genes in *E. coli* K12, the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was used. CCCP acts by uncoupling electron transport in the membrane, thereby dissipating the "high-energy membrane state". Thus, CCCP inhibits the uptake systems of various compounds such as proline, serine and inorganic phosphate which require the membrane to be energized [84, 181].

Therefore, CCCP was added to various nitrogen-fixing hybrids to test whether the membrane must be energized for nitrogen fixation (Table 3.10). It was observed that addition of the uncoupler inhibited expression of

TABLE 3.10 Effect of addition of uncoupler CCCP on the Nif^+ phenotype of hybrid strains

Strain and relevant genotype	Addition	Acetylene reduction (Percent of control without CCCP)
AN259 (FN68) *(unc$^+$)*	O CCCP fumarate fumarate + CCCP	100 0 100 2
AN283 (FN68) *(uncB$^-$)*	O CCCP fumarate fumarate + CCCP	100 12 100 20
AN285 (FN68) *(uncD$^-$)*	fumarate fumarate + CCCP	100 5

20 mM fumarate was added to NFM in Pankhurst tubes at the start of the experiment; after 18 hr incubation, rates of acetylene reduction were determined by injecting samples of cultures into flasks with 90% argon/10% acetylene, with or without CCCP at a final concentration of 40 μM, and ethylene production was then measured.

the Nif^+ phenotype by 80 to 100% (depending on the particular hybrid) in the presence or absence of fumarate (Table 3.10). Since CCCP does affect the Nif^+ phenotype, it can be concluded that the "high-energy membrane state" is necessary for nitrogenase activity. Also, for nitrogen fixation to occur in the background of *E. coli*, the fumarate reductase system must be functional so that the membrane may be energized.

3.11 Summary

1. Phenotypic expression of nif^+_{Kp} genes in *E. coli* K12 does not require coupling of the electron transport-dependent phosphorylation system.

2. For phenotypic expression of the nif^+_{Kp} genes in *E. coli* K12, the anaerobic electron transport system to fumarate must be functional, i.e. a functional fumarate reductase and menaquinone (but not ubiquinone) are obligatory.

3. The role of the fumarate reduction system is to energize the membrane for nitrogen fixation.

4. Substrate level phosphorylation can provide sufficient ATP for anaerobic growth and nitrogen fixation, provided the fumarate reduction system is functional.

5. The nif^+_{Kp} gene cluster does not contain functions able to complement a defective Mg^{2+}-ATPase aggregate, but does contain a function which appears to interact with the *uncB*⁻ mutant to correct

overproduction of a redox system.

6. The nitrate respiration pathway of *E. coli* K12 is not necessary for phenotypic expression of the nif^{+}_{Kp} genes.

7. The nitrate reductase genes *chlA* and *chlB* but not *chlC*, *chlD*, *chlE* or *chlG*, are necessary for inhibition by nitrate.

8. Addition of nitrate to $ubiA^{-}$, $menA^{-}$ or unc^{-} *E. coli* K12 (FN68) hybrids inhibited nitrogen fixation. Thus, nitrate inhibition does not appear to act through these quinones or the coupled electron transport-dependent phosphorylation system.

CHAPTER 4

ISOLATION AND CHARACTERIZATION OF MUTANTS UNCOUPLED IN OXIDATIVE PHOSPHORYLATION OF *RHIZOBIUM TRIFOLII* STRAIN T1

CHAPTER 4 - ISOLATION AND CHARACTERIZATION OF MUTANTS UNCOUPLED IN OXIDATIVE PHOSPHORYLATION OF *RHIZOBIUM TRIFOLII* STRAIN T1

4.1 Introduction

The establishment of a nitrogen-fixing symbiosis between *Rhizobium* and its legume host involves complex physiological and biochemical interactions which are still not well understood. It is only recently that nitrogen fixation has been demonstrated in certain strains of cultured rhizobia outside the nodule [116, 132, 146, 158, 216] and also that plant cells can produce diffusable factors which induce nitrogenase activity in free-living rhizobia [11, 170]. However, these results have allowed the initiation of many genetic and physiological studies of rhizobial nitrogen fixation, and permit comparisons between the free-living rhizobia and their bacteroid forms. Although physiological studies of the mechanism by which rhizobia derive energy for nitrogen fixation have advanced rapidly over the last few years, the lack of well-defined mutants in pathways of energy metabolism has limited these investigations.

For nitrogen fixation, legume root nodules require oxygen [13, 24]. However, oxygen can also inactivate bacteroid nitrogenase and act as an inhibitor of this system [13, 229]. Leghaemoglobin, which is a characteristic haemoprotein found only in nitrogen-fixing legume root nodules [5, 19], plays an important role in maintaining a high flux of O_2 to the bacteroids at very low free O_2

concentrations [6, 22, 228], so that nitrogen fixation can occur. It has also been suggested that bacteroids generate steep gradients of O_2 concentration close to their membranes, which is the most probable location of bacteroid oxidases, leading to more efficient oxidase function [22, 228].

There is now considerable support for the concept of two different terminal oxidases in bacteroids from soybean nodules [4, 7, 23, 228]. One of these oxidase systems has a high affinity for O_2, operating with maximum efficiency near 0.1 μm free O_2, with little activity above 1 to 3 μM O_2 [23]. It is sensitive to inhibition by N-phenylimidazole and CO, and is probably related to cytochrome P-450 [7]. Bacteroid ATP concentrations are increased when levels of free O_2 are such that this high affinity oxidase is functional [23]. The low affinity oxidase system has little activity below 1 μM free O_2, is insensitive to N-phenylimidazole and CO, and bacteroid ATP concentrations are much lower when this oxidase is functional [23].

For nitrogenase activity in suspensions of bacteroids from soybean root nodules, the optimum O_2 concentration appears to be about 0.1 μM free O_2 [22, 23]. This concentration is also optimum for production of ATP [23]; the decline in nitrogenase activity at higher concentrations of free O_2 may be due to decreased ATP supply rather than inhibition by excess O_2 [23].

Using soybean bacteroids suspended in a solution

of leghaemoglobin, it has been shown that a linear relationship exists between the total bacteroid ATP/ADP ratio and bacteroid nitrogenase activity [6, 7]. This result has led to the proposal [6, 7, 228] that leghaemoglobin-facilitated O_2 diffusion causes a general increase in the bacteroid ATP/ADP ratio, by increasing the efficiency of bacteroid oxidative phosphorylation, with a consequent stimulation of nitrogenase activity. Thus, at least in *Rhizobium japonicum*, oxidative phosphorylation would seem to be the most obvious method for production of the large amounts of ATP needed for nitrogen fixation.

In bacteroids, therefore, oxidation of substrates appears not only to produce ATP by oxidative phosphorylation in a coupled respiratory pathway with O_2 as terminal electron acceptor, but can also provide reducing power for the reduction of N_2 to NH_3 [16]. In intact nodules, reducing power and energy are derived from photosynthetic products supplied by the host plant, and several studies have been aimed at elucidating the metabolic pathways used by rhizobia to produce energy. It has been demonstrated that there is little or no change in pathways of substrate oxidation between the bacterium and bacteroid forms [12, 226]. However, it was also found [38, 221] that hexoses such as glucose and sucrose were scarcely oxidized by bacteroids, which led to the conclusion that bacteroids receive organic acids rather than sugars from the host plant [221]. In addition, recent evidence has shown that intact bacteroids of *R. leguminosarum* take up glucose very

poorly, whereas cultured bacteria transport it rapidly [63].

Although initially one study suggested that the tricarboxylic acid (TCA) cycle may not operate in *Rhizobium* due to the failure of *R. japonicum* to completely oxidize intermediates of this cycle [12], another study indicated the existence of the TCA cycle in *R. japonicum* since this bacterium readily oxidized TCA cycle intermediates [221].

Subsequent studies of *R. japonicum* have provided evidence for the operation of a TCA cycle [111, 115, 149]. Evidence has also been obtained, in various species of both fast- and slow-growing rhizobia, for the function of the Embden-Meyerhof glycolytic pathway [111, 114, 149, 221], the Entner-Douderoff pathway [113, 115, 149], the pentose-phosphate pathway [111, 114], and the potential for, but probable inactivity of, the glyoxylate cycle [110].

Thus it seems very likely that *Rhizobium* possesses all the necessary enzymes for aerobic catabolism of the products of photosynthesis supplied by the host, for production of reducing power and ATP by a coupled oxidative phosphorylation system. However, it is not known whether oxidative phosphorylation is essential for provision of sufficient ATP for nitrogen fixation by *Rhizobium*, or whether substrate level phosphorylation could provide all the necessary ATP (as found with the *K. pneumoniae/E. coli* hybrids. The use of mutants uncoupled in oxidative phosphorylation is the simplest way of analysing these possibilities, and of resolving whether changes to energy

conservation mechanisms take place during the differentiation of bacteria to bacteroids.

Rhizobium trifolii strain T1 was chosen for this study because it is a fast-grower which effectively nodulates a relatively broad range of clovers (red, white and subterranean) [187], and because nodulation tests on clover are simple to set up and give results very quickly compared with other *Rhizobium*-legume associations. Strain T1 is also sensitive to several bacteriophages and bacteriocins [189], and there is a phage which is apparently specific for this strain of *R. trifolii* [187]; these properties are useful for confirming the identification of derivatives of strain T1. Strain T1 had also been well-studied with respect to drug sensitivity and the effect of drug resistance on nodulation, as well as being used for isolation of metabolic mutants [161, 185, 186, 190].

4.2 Attempts to transfer nif^{+}_{Rt} genes to *E. coli* K12

In Chapter 3, well-defined mutants of *E. coli* K12 had enabled an analysis of the pathways used to provide energy for the phenotypic expression of nif^{+} genes from *K. pneumoniae*. Initially, it was hoped to use these observations as a model for investigating the pathways of energy metabolism used for nitrogen fixation in *R. trifolii*. Dunican and Tierney [69, 71] had reported the transfer of nif^{+} genes from strain T1K, a derivative of *R. trifolii* strain T1, by means of a transferable drug-resistance plasmid. The nitrogen-fixing phenotype was transferred, with expression, to *Klebsiella aerogenes* [69,71].

Since *K. aerogenes* is closely related to *E. coli*, it seemed probable that a similar transfer of nif^+ genes from strain TlK to *E. coli* could be obtained. In this background the control of nif^+_{Rt} gene expression could then be analysed by methods similar to those used for nif^+_{Kp} genes (Chapter 3).

Although it was possible to transfer nif^+ genes from strain TlK to *E. coli* K12 with expression of a Nif^+ phenotype, it was also found that strain TlK could not have arisen as a derivative of *R. trifolii* strain Tl (see Appendix, sections 2 and 3). Instead, TlK is a very interesting bacterium with characteristics of both *Rhizobium* and *Agrobacterium*. However, because its origin was not known, it could not be used for analysis of the control of nif^+_{Rt} genes.

Attempts were then made to transfer nif^+ genes directly from *R. trifolii* strain Tl to *E. coli* K12, by means of a transferable drug-resistance plasmid. Three different drug-resistance plasmids were used: Rl-19*drd*, and F-like R plasmid reported by Dunican [71] to transfer nif^+ genes from strain TlK; RP4, a P-type R plasmid with a wide host range [102]; and R68.45, another P-type R plasmid derived from R68 which transfers chromosomal regions at random [86]. The donor strains used were *E. coli* K12 J53(Rl-19*drd*); *A. tumefaciens* LBA57(RP4); *Ps. aeruginosa* PA025(R68.45). All were auxotrophs, whereas Tl is a prototroph, so selection was based on use of TM minimal medium containing kanamycin (20 μg/ml). Resistance to this antibiotic is conferred

by all three plasmids. Each plasmid was transferred to T1 from its original host by filter matings; the frequency of plasmid transfer was 10^{-6} per donor cell above a very low background of spontaneous mutation to Km^R in T1 (Table 4.1).

The presence of any of these three plasmids in strain T1 did not appear to affect the physiology in any way, other than making it resistant to the drugs specified by the plasmids (Table 4.1). All hybrids nodulated normally on white, red and subterranean clovers (Table 4.1).

Using a mutant of T1 resistant to spectinomycin and streptomycin as the recipient strain, it was possible to transfer RP4 and R68.45 from T1 (RP4) and T1 (R68.45) by conjugation, at a frequency of 10^{-1} to 10^{-2} per donor cell (selecting for transfer of *Km*^{R}).

Strains of T1 containing each of the drug-resistance plasmids were mated with *E. coli* K12 strain SB1801(*his*$^{\Delta}$) in liquid or on filters. Selection was for transfer of *Km*^{R}, *nif*$^+$ and *his*$^+$ genes. In liquid, *Km*^{R} was transferred at a low frequency of about 10^{-8}, but no transfer of *his*$^+$ or *nif*$^+$was observed, even when mating mixtures were incubated for up to 36 hr. In filter matings incubated for between 16 and 72 hr, *Km*^{R} was always transferred at a frequency of 10^{-2} - 10^{-3}, but no transfer of *his*$^+$ or *nif*$^+$ markers were observed, even if the donor was irradiated with UV light for 90s to promote DNA recombination prior to conjugation (as done by Dunican for strain T1K [71]).

TABLE 4.1 Properties of *R. trifolii* strain T1 carrying various R plasmids

Property	Strain			
	T1	T1(RP4)	T1(R68.45)	T1(R1-19*drd*)
Frequency/donor cell	-	10^{-6}	10^{-6}	10^{-6}
Resistance to drugs:				
Kanamycin (20 μg/ml)	-	+	+	+
Carbenicillin (50 μg/ml)	-	+	+	-
Chloramphenicol (12.5 μg/ml)	-	-	-	+
Sensitivity to:				
phage ØT10	+	+	+	+
phage Tr8	+	+	+	+
bacteriocin T24	+	+	+	+
Growth on:				
TMY	+	+	+	+
LBG	-	-	-	-
MM	-	-	-	-
Effective nodulation of clovers:				
white	+	+	+	+
red	+	+	+	+
subterranean	+	+	+	+

The failure to transfer a Nif^+ phenotype from *R. trifolii* strain T1 to *E. coli* K12 may be due to the nif^+_{Rt} genes not being clustered together on the chromosome, or to nif^+_{Rt} genes not being expressed in *E. coli* K12. However, since no transfer of his^+ genes was detected, another possibility is that no chromosome mobilization by these plasmids occurred.

4.3 Isolation of *R. trifolii* mutants defective in energy conservation

Since it was not possible to transfer nif^+ genes from *R. trifolii* strain T1 to *E. coli* K12, attempts were made instead to directly isolate mutants of strain T1 defective in energy conservation.

Uncoupled (unc^-) mutants of *E. coli* have been isolated by their resistance to the antibiotic neomycin, which allows direct selection [112, 183], together with their inability to grow on succinate as sole carbon source. Sufficient energy for cell growth can only be derived from succinate by oxidative phosphorylation. Hence unc^- mutants uncoupled in oxidative phosphorylation cannot grow on this carbon source. Since this method had worked well in *E. coli*, the same method was applied to *R. trifolii*.

For isolation of neomycin-resistant mutants of *R. trifolii*, two strains were used: T1 and T1Sp, an effectively nodulating derivative of strain T1 resistant to 100 µg/ml spectinomycin. The spectinomycin-resistant strain was used because it had this additional marker for later identification of bacteria isolated from nodules.

For isolation and characterization of large numbers of neomycin-resistant colonies, it was necessary to devise a medium which would reduce the amount of extracellular polysaccharide produced by strain T1 on many *Rhizobium* media.

TM is a defined minimal medium specifically designed to allow good growth of *R. trifolii* strain T1 with ammonium as sole nitrogen source and glucose as sole carbon source (see Appendix, Section 4). Ammonium was used as nitrogen source rather than compounds which have previously been used in *Rhizobium* media, such as glutamate, to avoid possible complications of the nitrogen source also being used as the carbon source. In TM medium, strain T1 uses glucose as carbon source very well, and TCA cycle intermediates such as succinate and fumarate can also be used, when present as sole carbon source.

TM medium also reduces the amount of polysaccharide produced by strain T1. This is useful when using replica plating techniques or picking of colonies on to TM media containing various carbon sources, since more colonies can be tested per plate and the extent of growth of colonies is not obscured by large amounts of polysaccharide. TMY is the same as TM medium, except for the addition of 1 g/l yeast extract, which allows faster growth of strain T1.

A freshly-grown culture of strain T1Sp was mutagenized with 200 μg/ml nitrosoguanidine for one hour, and after washing in buffer the cell suspension was divided into two, and either plated directly on TMYNm (TMY

containing 20 μg/ml neomycin) or grown overnight in liquid TMY (to allow phenotypic expression) before being plated on TMYNm.

Colonies resistant to 20 μg/ml neomycin appeared at a frequency of 10^{-6} per viable cell after 2-3 days' incubation. 1000 Nm^R colonies from each half of the experiment (i.e. 2000 colonies) were picked on to fresh TMYNm plates and also on to TM plates with 20 mM succinate as sole carbon source. Fourteen colonies (seven from each half of the experiment) were found to be Nm^RSuc^-. Thus, Nm^RSuc^- colonies arose at the low frequency of 7×10^{-9}. These colonies were purified by restreaking twice on TMYNm plates before being rechecked for inability to use succinate as sole carbon source.

Strain RT2 (Nm^RSuc^-) was isolated by the same procedure, but in the parental background of strain T1.

The mutants were next tested for sensitivity to bacteriocin T24, for sensitivity to the general *R. trifolii* phage, Tr8, and to the phage specific for strain T1, ØT10. All of the mutants, like the parent strain T1Sp, were still sensitive to T24, Tr8 and ØT10, and were also still resistant to 100 μg/ml spectinomycin (Table 4.2).

In test tube culture, it was found that Suc^+ revertants of the 5 Nm^RSuc^- mutants only appeared at a very low frequency of about 10^{-8}. These revertants were also Nm^S, had normal levels of ATPase activity, and effectively nodulated white clover. Growth of cultures in

TABLE 4.2 Properties of Nm^R Suc^- mutants of *R. trifolii*

Property	Strain			
	T1	RT2	T1Sp	T1Sp Nm^R Suc^- mutants
Resistance to drugs:				
spectinomycin (100 μg/ml)	-	-	+	+
neomycin (20 μg/ml)	-	+	-	+
Growth on glucose	+	+	+	+
Growth on succinate	+	-	+	-
Sensitivity to:				
phage Tr8	+	+	+	+
phage Ø10	+	+	+	+
bacteriocin T24	+	+	+	+

the presence of neomycin was sufficient to ensure that revertants did not arise.

Mutants of *E. coli* K12 which are uncoupled in oxidative phosphorylation (*unc*$^-$) are unable to derive sufficient energy for growth by other means on TCA cycle intermediates or on D-lactate [49, 58]. However, they can grow on glucose or on glycerol, since energy can be derived from these compounds by substrate level phosphorylation [58]. Since all 15 Nm^{R}Suc$^-$ strains had proved to be completely unable to use succinate, they were tested for growth in TM media with other TCA cycle intermediates and related compounds as sole carbon source.

The 15 Suc$^-$ mutants of strains T1 and T1Sp were streaked on plates of TM medium with various 2 mM or 20 mM carbon sources, and growth was assessed after 5 days' incubation at 30°. The results shown in Table 4.3 indicate that 5 of the mutants, RT2, RT18, RT20, RT21 and RT49 can grow slowly on glucose and glycerol, but not on TCA cycle intermediates or on D-lactate, and may therefore be uncoupled in oxidative phosphorylation.

Another characteristic of the well-defined *E. coli* K12 *unc*$^-$ mutants is their reduced growth rate on glucose when compared with the *unc*$^+$ parent strain [181]. The growth rates of strains T1, T1Sp, and the 5 possible *unc*$^-$ mutants were therefore measured in tubes of liquid TM medium with 20 mM glucose. All 5 mutants do share the reduced growth rate in common with *E. coli* K12 *unc*$^-$ mutants, as illustrated by the doubling times shown in

TABLE 4.3 Growth of Nm^R Suc^- mutants of *R. trifolii* on various carbon sources.

Mutant	Growth on:					
	Glucose	Glycerol	Succinate	Fumarate	Malate	D-lactate
T1Sp	++++	++++	++++	+++	++	++
RT2	++	++	-	-	-	-
RT6	+++	++	-	+	+	+
RT9	+++	+++	-	++	+	-
RT11	+++	++	-	+	+	+
RT16	++	++	-	+	+	+
RT17	++	++	-	+	+	-
RT18	++	++	-	-	-	-
RT20	++	+++	-	-	-	-
RT21	++	+++	-	-	-	-
RT26	+++	++	-	+	+	-
RT30	+++	+++	-	+	-	-
RT34	++	+++	-	+	+	-
RT44	+++	+++	-	+	-	-
RT48	+++	++	-	-	+	-
RT49	++	++	-	-	-	-

Growth was assessed on plates of TM medium with the various compounds as sole carbon source, present at 20 mM (except for malate and D-lactate which were present at 2 mM, since 20 mM inhibits the growth of strain T1 (see Appendix, Section 4)). Plates were incubated aerobically for 5 days at 30°.

-; no growth

+, ++, +++, ++++; increasing degrees of growth from poor to very good, assessed both in regions of confluent growth and on size of isolated colonies.

Table 4.4 and the growth curves of two mutants shown in Figure 4.1.

E. coli K12 *unc*$^-$ mutants also give a much-reduced growth yield on limiting glucose, due to the fact that they can only make ATP from glucose by substrate level phosphorylation. When the five Nm^RSuc^- mutants of strains T1 and T1Sp were grown to stationary phase in tubes containing TM medium with 1 mM to 5 mM glucose as sole carbon source, all 5 mutants had greatly reduced growth yields (Table 4.5, Figure 4.2).

After growth of the mutants had ceased in the TM medium, the cells were removed by centrifugation, and the end-products of glucose fermentation were measured by gas chromatography. All five mutants produced the same proportions of the same end-products as the parent strains T1 and T1Sp, although the precise amounts and compounds produced could not be determined with the column used [68]. However, it was clear that large amounts of lactate, as found in the end-products of ubiquinone mutants [49, 51], were not produced.

4.4 Respiration of *R. trifolii* Nm^RSuc^- mutants

It was possible that some of the pleiotropic Nm^RSuc^- phenotype observed in the ATPase mutants could have been due to a defect in the aerobic electron transport chain, for example in quinones or cytochromes. To test this possibility, the ability of these mutants to respire O_2 in the presence of succinate (i.e. to pass electrons from

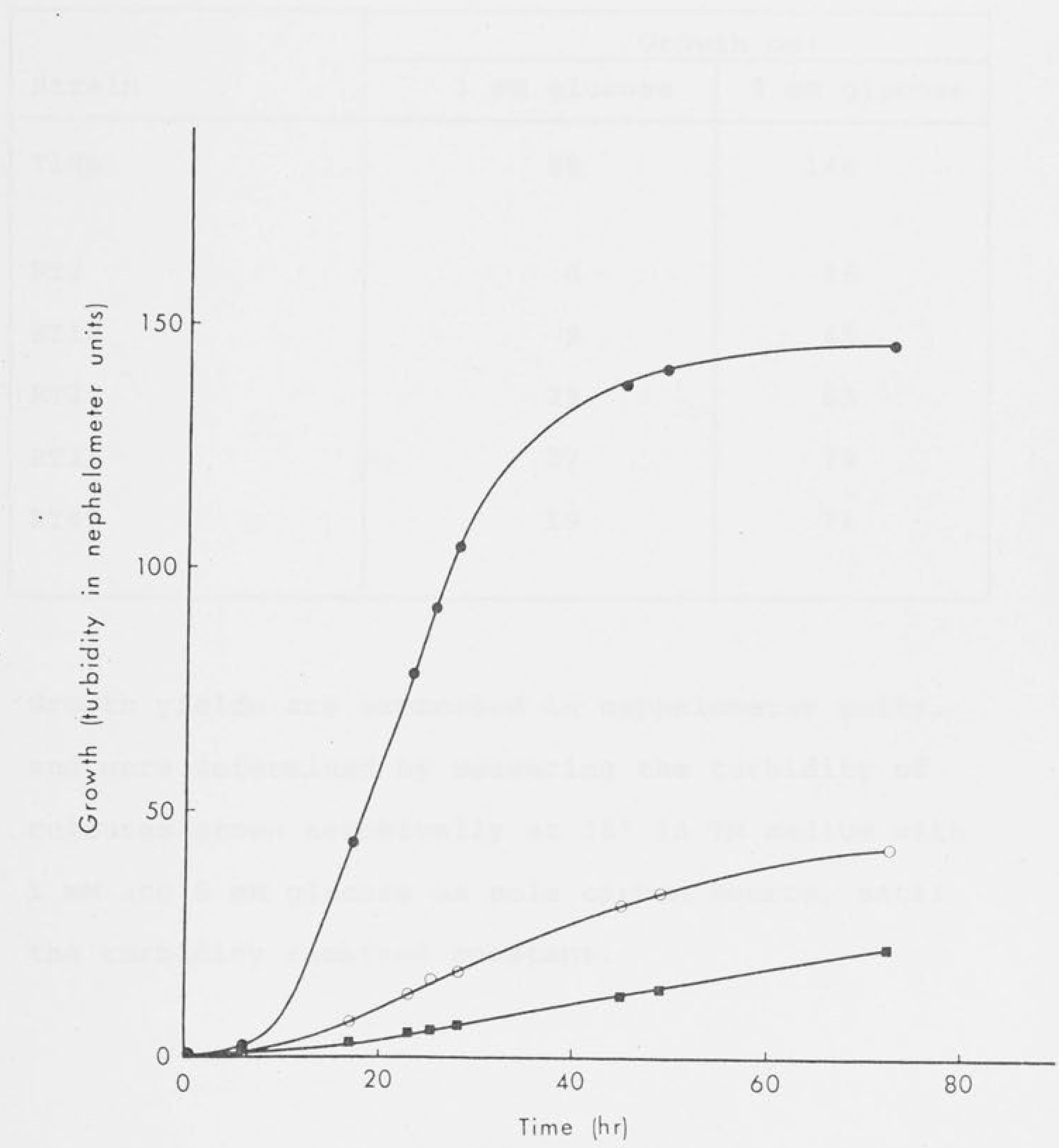
TABLE 4.4 Doubling times of *R. trifolii* T1, T1Sp and $Nm^R Suc^-$ mutants in TM medium with glucose as carbon source

Strain	Doubling time (hr)
T1	3.20
RT2	34.40
T1Sp	3.42
RT18	13.73
RT20	10.38
RT21	9.52
RT49	11.42

Doubling times were measured in TM liquid medium with 20 mM glucose as carbon source. The increase in turbidity in tubes shaken aerobically at 30° was measured in a nephelometer.

FIGURE 4.1

Growth rates of *R. trifolii* T1Sp (●) and 2 $Nm^R Suc^-$ mutants, RT2 (■) and RT18 (o). Cells were grown in liquid TM medium with 20 mM glucose as carbon source, aerobically at 30°, and the increase in turbidity of cultures was measured using a nephelometer.



Growth (turbidity in nephelometer units)
150
100
50
0
0
20
40
60
80
Time (hr)

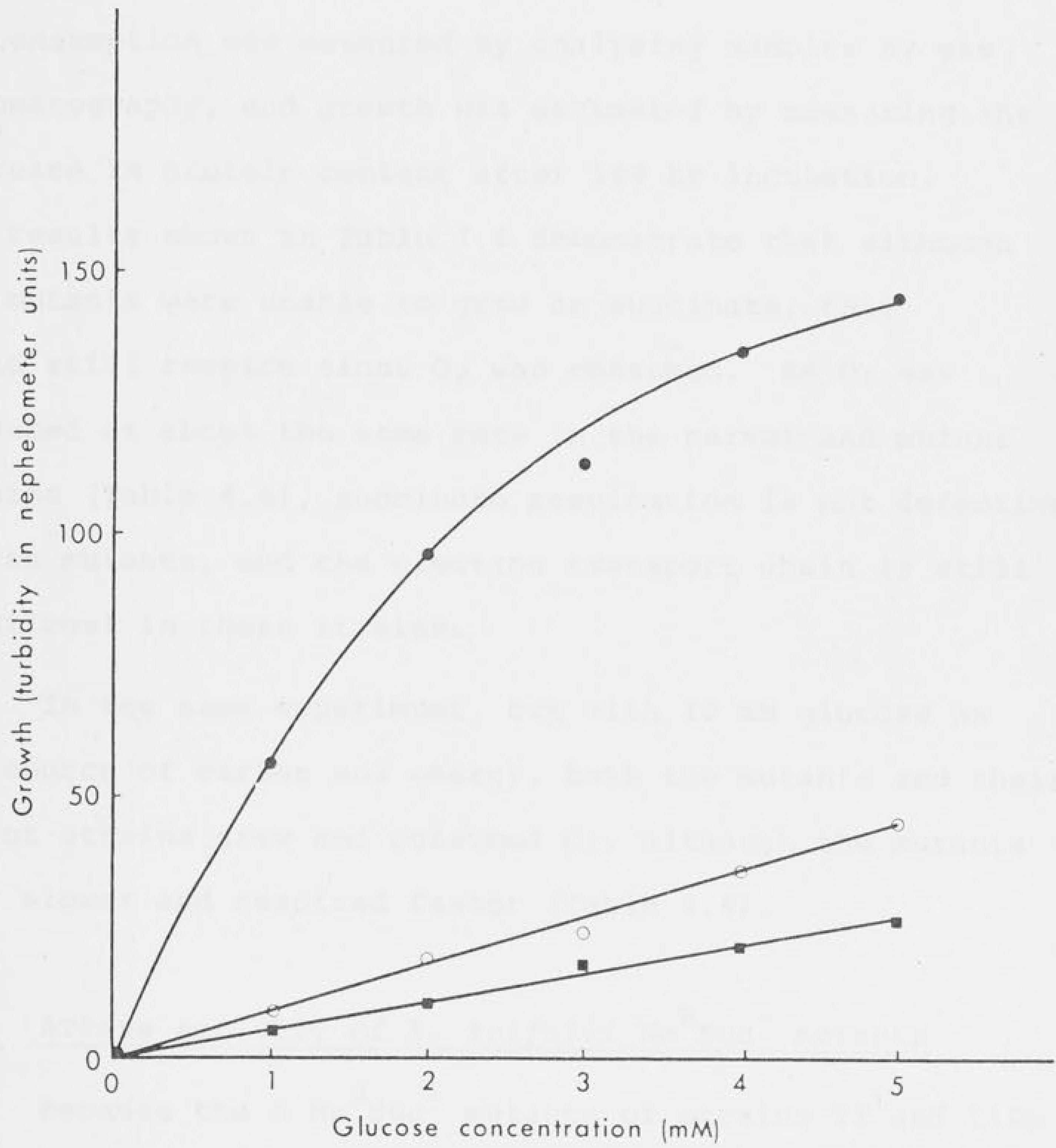
TABLE 4.5 Growth yields of *R. trifolii* T1Sp and Suc^- mutants on limiting glucose

Strain	Growth on:	
	1 mM glucose	5 mM glucose
T1Sp	58	146
RT2	6	26
RT18	9	45
RT20	29	83
RT21	27	79
RT49	19	71

Growth yields are expressed in nephelometer units, and were determined by measuring the turbidity of cultures grown aerobically at 30° in TM medium with 1 mM and 5 mM glucose as sole carbon source, until the turbidity remained constant.

FIGURE 4.2

Growth yields of *R. trifolii* strain T1Sp (●), $Nm^R Suc^-$ mutants, RT2 (■) and RT18 (○). Growth yields were determined aerobically at 30° in TM medium with 1 to 5 mM glucose as sole carbon source, by measuring the turbidity of cultures in a nephelometer until constant turbidity was attained.


Growth (turbidity in nephelometer units)
150
100
50
0
0
1
2
3
4
5
Glucose concentration (mM)

succinate down the electron transport chain to the terminal acceptor O_2) was tested.

Fresh TMY-grown cells were diluted and incubated in Valera and Alexander's medium [222] with 10 mM succinate as carbon source, under argon with 2% O_2. O_2 consumption was measured by analysing samples by gas chromatography, and growth was estimated by measuring the increase in protein content after 100 hr incubation. The results shown in Table 4.6 demonstrate that although the mutants were unable to grow on succinate, they could still respire since O_2 was consumed. As O_2 was consumed at about the same rate in the parent and mutant strains (Table 4.6), succinate respiration is not defective in the mutants, and the electron transport chain is still functional in these strains.

In the same experiment, but with 10 mM glucose as the source of carbon and energy, both the mutants and their parent strains grew and consumed O_2, although the mutants grew slower and respired faster (Table 4.6).

4.5 ATPase activity of *R. trifolii* Nm^RSuc^- mutants

Because the 5 Nm^RSuc^- mutants of strains T1 and T1Sp exhibited all the growth characteristics of *E. coli* K12 *unc*$^-$ mutants, they were all tested for the presence or absence of a functional ATPase. Assays for ATPase are based on the conversion of ATP to ADP and Pi by this enzyme; the inorganic phosphate can then be assayed colorimetrically.

TABLE 4.6 Respiration of *R. trifolii* ATPase mutants on glucose and succinate

Strain	Glucose		Succinate	
	O_2 consumption	Growth	O_2 consumption	Growth
T1	47	0.190	50	0.262
RT2	116	0.132	67	0.021
T1Sp	44	0.229	80	0.134
RT18	70	0.210	62	0.032
RT20	81	0.189	83	0.023

O_2 consumption was measured by gas chromatography 50 hr after inoculation of vials, and is expressed as μmol O_2 consumed/50 hr/mg protein.

Growth is expressed as mg protein/vial, measured after 100 hr incubation.

Initially, the 30 ml vials contained about 0.02 mg protein (bacteria), and 2% O_2 in the gas phase. In general, the amount of O_2 consumed did not increase after 50 hr incubation.

These results were obtained by Dr. M. Reporter and Dr. B. Rolfe.

The ATPase assay developed by Yamamoto *et al.* [230], which requires spheroplasts to be made from whole cells by treatment with lysozyme so that the ATPase on the inner cell membrane is more accessible to ATP, allows an accurate determination of ATPase activity while eliminating lengthy cell breakage procedures for collection of cell membranes. Since 5 mutants as well as the parent strains were to be tested for ATPase activity under various conditions the faster method of Yamamoto *et al.* [230] was employed.

As this ATPase assay had not previously been applied to *Rhizobium* (having been developed mainly for *E. coli* [215, 230]), it was first necessary to determine the conditions which would allow maximum ATPase activity in the parent strains of *R. trifolii*, strains T1 and T1Sp. Also, to check that this assay method worked accurately, the *E. coli* K12 *unc*$^-$ mutants were tested (Table 4.7), and it was found that this method did give accurate repeatable results for these mutants. Although the % ATPase activities of the various *E. coli* K12 *unc*$^-$ mutants closely corresponded with published values, the actual activities were considerably higher than published values. This could be due to the differences in the methods used, such as whole cells rather than cell membranes being assayed, different growth conditions (rich medium instead of minimal), and different incubation temperatures. It is known that adding only 5% LB to minimal medium can increase the ATPase activity of AN259*unc*$^+$ three-fold [48, 75]; the

TABLE 4.7 ATPase activity of *E. coli* K12 *unc*⁻ mutants and *R. trifolii* strain T1Sp

Culture	Mg^{2+}-ATPase activity (μmol Pi/min/mg protein)	% ATPase activity of AN259 *unc*$^{+}$	Published value for* % ATPase activity of AN259 (*unc*$^{+}$)
AN259 *unc*$^{+}$	3.110	100	100
AN249 *uncA*$^{-}$	0.471	15.1	16
AN283 *uncB*$^{-}$	3.140	101	104
AN285 *uncD*$^{-}$	0.986	31.7	32
T1Sp	2.546	80.8	-

*See ref. [80]

ATPase activities were measured as described in Chapter 2.

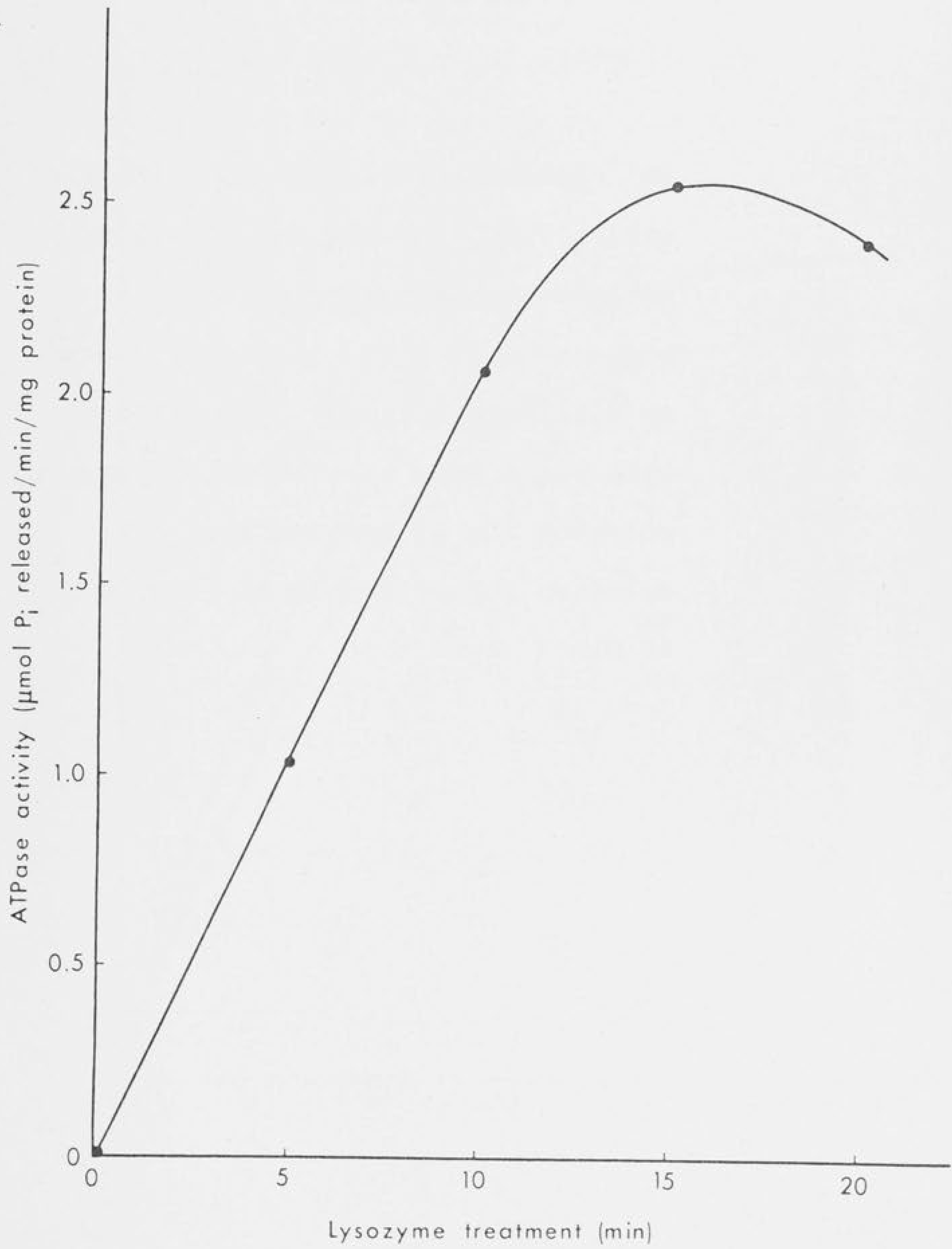
cultures described here were grown in 100% LBG, or in TMY which is also a rich medium. Also, the presence of an active membrane Mg^{++}-activated ADP hydrolyzing activity capable of releasing additional Pi from ADP, the product of ATPase action [75, 230], represents a source of potential additional Pi production when such preparations are assayed. Once it was established that this method of assaying ATPase activity worked for *E. coli* K12, *R. trifolii* strain T1Sp was tested. First, the optimum length of lysozyme treatment was determined for strain T1Sp. Using a constant volume (200 µl) of a suspension containing about 2 x 10^9 log phase, freshly-washed cells, strain T1Sp was treated with the lysozyme solution for 5 - 20 minutes before washing and incubating with ATP.

It was found that 15 minutes' exposure to lysozyme gave spheroplasts which subsequently showed the highest ATPase activity (Figure 4.3).

Next, the number of cells per Millipore filter, for maximum activity, was determined. Log-phase, freshly-washed cells were always used to ensure maximum activity. Between 25 and 300 µl of cells of strain T1Sp containing about 2 x 10^9 cells/ml were placed on Millipore filters, and treated with lysozyme for 15 minutes, ATP solution for 30 minutes and then assayed for release of inorganic phosphate. It was found that 200 µl of cells was optimum (Figure 4.4); above this, very little increase in ATPase activity was obtained.

FIGURE 4.3

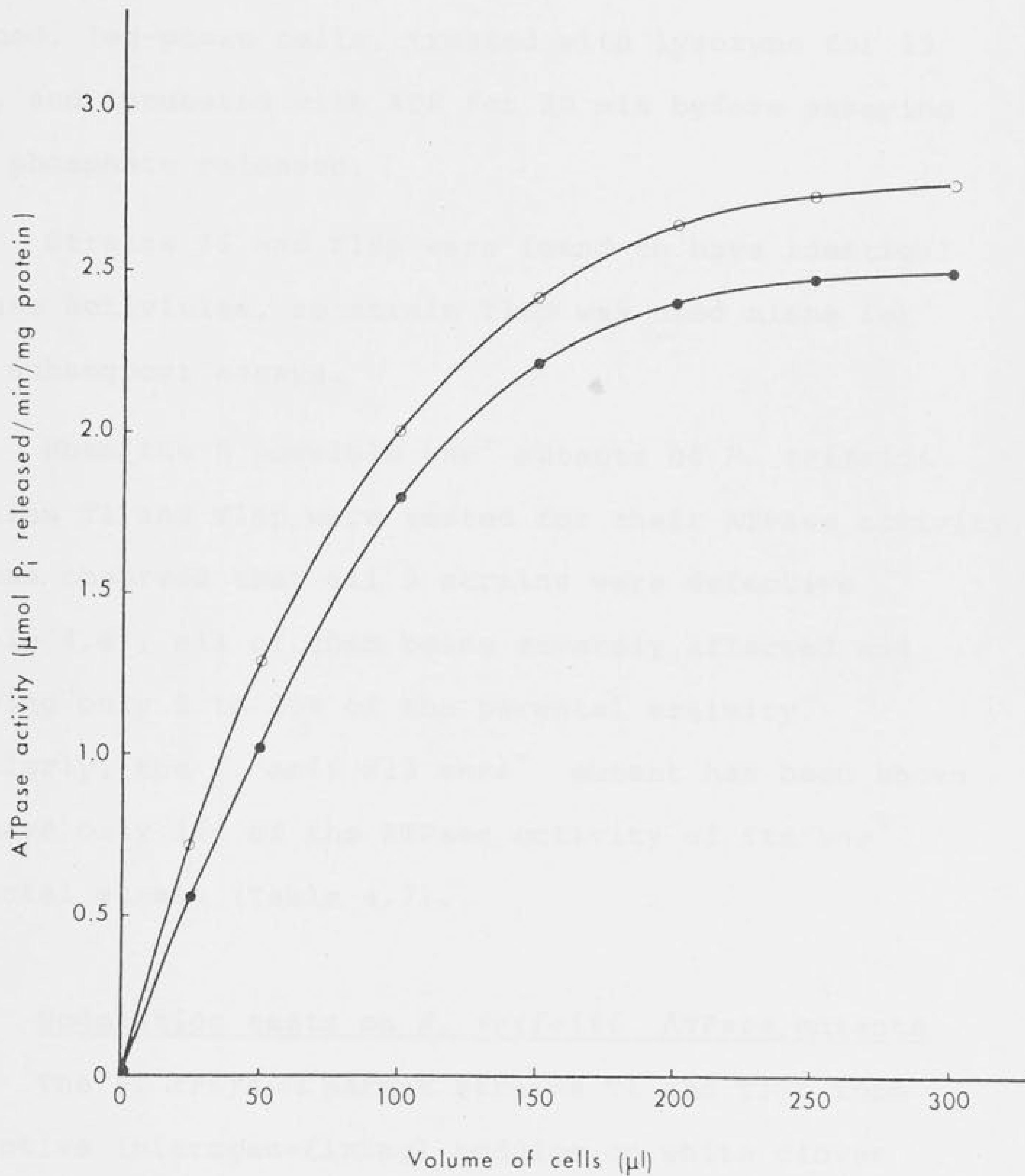
Mg^{2+}-ATPase activity of *R. trifolii* strain T1Sp, after exposure of the log-phase, freshly-washed cells on Millipore filters to lysozyme solution for increasing lengths of time.



2.5
2.0
1.5
1.0
0.5
0
ATPase activity (μmol P$_i$ released/min/mg protein)
0
5
10
15
20
Lysozyme treatment (min)

FIGURE 4.4

Mg^{2+}-ATPase activity of *R. trifolii* strain T1Sp, showing the effect of increasing the volume of the cell suspension (2 x 10^9 cells/ml) placed on Millipore filters. All filters with cells were treated with lysozyme solution for 15 min and then ATP solution for either 30 min (●) or 60 min (○).


ATPase activity (μmol P_i released/min/mg protein)
3.0
2.5
2.0
1.5
1.0
0.5
0
0
50
100
150
200
250
300
Volume of cells (μl)

In the same experiment, it was found that incubation with ATP for 60 min rather than 30 min produced little extra ATPase activity (Figure 4.4). Therefore, in all subsequent experiments the following conditions were used: 200 μl of a suspension containing about 2×10^9 freshly-washed, log-phase cells, treated with lysozyme for 15 min, and incubated with ATP for 30 min before assaying the phosphate released.

Strains T1 and T1Sp were found to have identical ATPase activities, so strain T1Sp was used alone for all subsequent assays.

When the 5 possible *unc*$^-$ mutants of *R. trifolii* strains T1 and T1Sp were tested for their ATPase activity, it was observed that all 5 strains were defective (Table 4.8), all of them being severely affected and showing only 8 to 20% of the parental activity. Similarly, the *E. coli* K12 *uncA*$^-$ mutant has been shown to have only 16% of the ATPase activity of its *unc*$^+$ parental strain (Table 4.7).

4.6 Nodulation tests on *R. trifolii* ATPase mutants

The *R. trifolii* parent strains T1 and T1Sp form effective (nitrogen-fixing) nodules on white clover (Figure 4.5), red clover and subterranean clover. Therefore these three species were used to test nodulation by the defective ATPase mutants. Tests were done both in plugged test tubes and in Petri dishes, using Jensen's medium. Initially, plants in upright Petri dishes grew

TABLE 4.8 ATPase activity of *R. trifolii* strain T1Sp and $Nm^R Suc^-$ mutants

Strain	Mg^{2+}-ATPase activity (µmol Pi/min/mg protein)	%	Ca^{2+}-ATPase activity (µmol Pi/min/mg protein)	%
T1Sp	2.546	100	3.13	100
RT2	0.280	11.0	0.291	9.3
RT18	0.255	10.0	0.257	8.2
RT20	0.224	8.8	0.391	12.5
RT21	0.514	20.2	0.319	10.2
RT49	0.323	12.7	0.235	7.5

ATPase activities were determined as described in Chapter 2, using 200 µl cells, treated with lysozyme solution for 15 min and exposed to ATP solution for 30 min. All cells on filters were washed with Mg^{2+}-Tris buffer after lysozyme treatment, and were then incubated in a solution containing 5 µmoles ATP, 20 µmoles Tris-HCl buffer and 1.5 µmoles $MgCl_2$ or $CaCl_2$.

FIGURE 4.5

Comparison of white clover plants uninoculated (left) and inoculated (right) with *R. trifolii* strain T1Sp one month after inoculation. Plants were grown on Jensen's medium in upright Petri dishes.

less well than those in tubes, but by increasing the intensity of light reaching the tops of plants in Petri dishes, similar growth (and rates of acetylene reduction) was obtained by the two methods. Subsequently, only the Petri dish method was used, as this is a much simpler and quicker way to set up nodulation tests.

All 5 ATPase mutants of strains T1 and T1Sp effectively nodulated white, red and subterranean clovers. No mutant was discernably different from its parent strain in nodulation tests on any of the 3 clover species with respect to such properties as when nodules were first visible, how many nodules were formed, size, shape and colour of nodules, and health and vigour of plants. Also, plants nodulated with each of the mutants reduced acetylene at approximately the same rate as the parent strain T1Sp (results with white clover are shown in Table 4.9 and Figure 4.6).

4.7 Reisolation of *R. trifolii* ATPase mutants from nodules

In order to ensure that the effective nitrogen-fixing nodules formed on clover plants after inoculation with the ATPase mutants of strain T1Sp were not simply due to reversion of some cells to wild type, the nodule contents were isolated and tested for their growth characteristics. For each mutant, and the parent strain T1Sp, every nodule was removed from 3 effectively-nodulated 5-week-old white clover plants. The nodules were surface sterilized in sodium hypochlorite, washed,

TABLE 4.9 Acetylene reduction by white clover plants inoculated with *R. trifolii* strain T1Sp and $Nm^R Suc^-$ mutants

Strain used to inoculate plants	Acetylene reduction (nmol C_2H_4/hr/ plant)
T1Sp	63
RT2	58
RT18	64
RT20	71
RT21	64
RT49	61

6 plants were tested for each strain, and the mean is shown.

Nodulated, one month-old white clover plants grown on Jensen's medium in Petri dishes were transferred with attached agar to 1 oz McCartney bottles, and the bottles were sealed with Suba seals. After evacuation, 70% Ar, 20% O_2 and 10% acetylene were injected, and ethylene production was measured by gas chromatography. No endogenous ethylene production was observed in controls without acetylene. Plants grown in tubes gave similar results to those grown in Petri dishes. Since these plants had an average of 7 nodules, the rates of acetylene reduction are comparable with those of Pankhurst *et al.* [160], who obtained a rate of about 300 nmol C_2H_4/hr/plant for plants with 30 to 40 nodules.

FIGURE 4.6

Comparison of white clover plants grown on Jensen's medium in upright Petri dishes, one month after inoculation. From left: uninoculated plant; typical plant inoculated with *R. trifolii* strain T1Sp; typical plant inoculated with *R. trifolii* mutant RT2; typical plant inoculated with *R. trifolii* mutant RT18.

and crushed in sterile buffer. The mixtures were then plated on TMY, TMYNm and TM succinate media at various dilutions. After several days' incubation at 30°, colonies were counted. For each mutant, large colonies appeared on TMY and TMYNm, and minute colonies on TM succinate plates at similar frequencies. Although approximately 10^7 colonies from the nodule preparations appeared on TM succinate plates for each of the mutants, not a single revertant able to grow well on succinate was observed. The appearance of minute colonies was most probably due to some growth on other carbon sources present in the nodule preparations. These colonies did not continue to grow, and did not grow at all when picked off and restreaked on TM succinate plates (Table 4.10). Strain T1Sp, similarly isolated from nodules, grew well on succinate, producing large colonies on TM succinate plates. T1Sp colonies also grew well on TMY, but not at all on TMYNm plates. Thus, none of the ATPase mutants had reverted in the effective white clover nodules.

To check that the bacteria in nodules formed by the ATPase mutants really were derivatives of strain T1Sp, several typical colonies from reisolated bacteria were tested for various characteristics (Table 4.10). All bacteria tested had the characteristics of the original inoculum.

4.8 ATPase activity in bacteroids of *R. trifolii* ATPase mutants

All five ATPase mutants of *R. trifolii* strains T1

TABLE 4.10 Properties of *R. trifolii* T1Sp and Nm^RSuc^- mutants reisolated from nodules on white clover plants

Property	Bacteria reisolated from nodules on white clover plants inoculated with:						
	T1	RT2	T1Sp	RT18	RT20	RT21	RT49
Resistance to drugs:							
Neomycin (20 μg/ml)	−	+	−	+	+	+	+
Spectinomycin (100 μg/ml)	−	−	+	+	+	+	+
Sensitivity to phages:							
ØT10	+	+	+	+	+	+	+
Tr8	+	+	+	+	+	+	+
Sensitivity to bacteriocin T24	+	+	+	+	+	+	+
Growth on sole carbon source:							
Succinate	+	−	+	−	−	−	−
Fumarate	+	−	+	−	−	−	−
D-lactate	+	−	+	−	−	−	−
Growth rate on 20 mM glucose (doubling time in hr)	3.2	35.1	3.4	14.0	10.5	9.4	11.7
Growth yield on 5 mM glucose (nephelometer units)	149	24	147	44	82	80	71

After isolation from nodules, 5 typical single colonies for each strain were picked from TM succinate plates (minute colonies for Nm^RSuc^- mutants). These colonies were grown overnight in liquid TMY medium, washed once in buffer, and then tested for their characteristics. All colonies tested retained the characteristics of the original inoculum. Growth rates and yields on glucose are the mean for the five colonies tested.

and T1Sp formed effective, nitrogen-fixing nodules on clover roots. Thus, the mutants could derive sufficient energy for nitrogen fixation within the nodule. There were several possibilities to explain how this energy might be derived, namely (a) substrate level phosphorylation within the bacteroid; (b) use of ATP equivalents rather than ATP itself; (c) provision of ATP by the plant; (d) provision of some ATPase component by the plant; (e) amplification of a partially defective bacterial ATPase, or (f) differentiation of a new bacteroid ATPase. To start to analyse these possibilities, ATPase activity was measured in bacteroids prepared from nodules containing *R. trifolii* strain T1Sp, and two ATPase mutants, RT2 and RT18.

White clover plants were grown under sterile conditions in pots to ensure that nodules were as large as possible. All three strains tested, T1Sp, RT2 and RT18, gave large, healthy green plants compared with uninoculated controls, although the plants inoculated with RT2 and RT18 had slightly smaller leaves than those inoculated with strain T1Sp. Ten weeks after inoculation, the nodules were large and pink, and were harvested for bacteroid isolation. Fifty plants for each strain were used, and the nodules were cut from the roots after surface sterilization. It was observed that plants inoculated with RT2 and RT18 produced more nodules than those inoculated with T1Sp (about 1500 nodules for RT18, 1300 for RT2 and 1200 for T1Sp were picked from the same number of plants). However, the overall increase in nodule

volume for the two mutants was only slight, since RT2 and RT18 produced slightly smaller nodules than T1Sp.

After overnight digestion in enzyme mixture, the bacteroid-containing protoplasts were gently squeezed from the nodules. Bacteroids were then carefully isolated from the protoplasts, washed to remove cell debris, and collected in PDB (see Chapter 2 for methods). All preparation of bacteroids was done aerobically, to ensure inactivation of nitrogenase, which is very sensitive to oxygen, since nitrogenase itself has ATPase activity [136]. This gentle method enabled isolation of bacteroids which partially retained their peribacteroid membranes (Figure 4.7).

After checking that nitrogenase was inactivated after exposure to O_2, by the inability of the bacteroids to reduce acetylene, these bacteroid preparations were used to assay ATPase activity, using the same method as for the free-living bacteria. A freshly-grown culture of T1Sp in TMY was included as a control in ATPase assays.

It was found that the T1Sp bacteroid suspension had ATPase activity, and this activity was about 10% greater than that of the free-living culture of strain T1Sp (Table 4.11). The RT2 and RT18 bacteroid preparations also had ATPase activity, of about 70% of T1Sp bacteroids (Table 4.11).

It was possible that the ATPase activity observed in bacteroid preparations of ATPase mutants was due to plant enzymes contaminating the preparation [177].

FIGURE 4.7

Electron micrograph of bacteroids of *R. trifolii* strain T1Sp from white clover nodules, showing retention of some of the peri-bacteroid membranes. Magnification: X44000. This micrograph was obtained by Ms. J. Hughes.

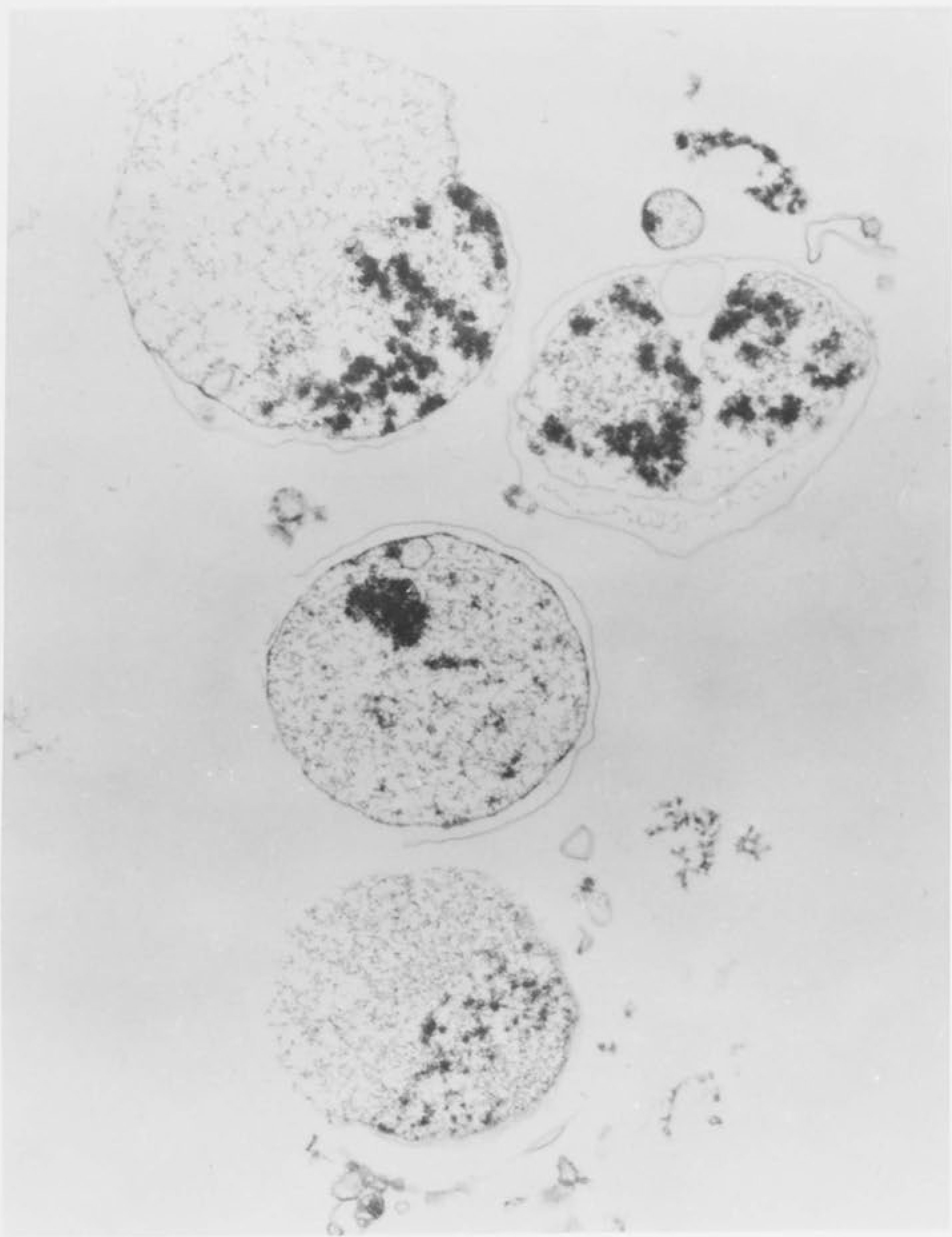


TABLE 4.11 ATPase activity of bacteroid suspensions prepared from white clover nodules

	Mg^{2+}-ATPase activity (µmol Pi/min/mg protein)	% ATPase activity of T1Sp bacteroids
T1Sp (cultured cells)	2.42	89
T1Sp (bacteroids)	2.71	100
RT2 (bacteroids)	1.91	70
RT18 (bacteroids)	1.89	70

These same four suspensions of cultured cells and bacteroids were used in all subsequent experiments described in the following tables. ATPase activity of all suspensions gradually declined with time, but suspensions still had active ATPase after two weeks' storage at room temperature.

However, it is known that ATPases associated with plant plasma membranes are stimulated by monovalent cations such as Na^{+} and K^{+}, and by TCA cycle intermediates such as succinate and malate [93, 101]. Stimulation also occurs with Mg^{++}, but this is partially due to Mg^{2+}-ATP being the substrate for the enzyme; Ca^{++} inhibits plasma membrane ATPase [101]. Bacterial ATPases, however, are stimulated by Ca^{++} and Mg^{++}, with monovalent cations such as Na^{+} and K^{+} having very little effect [75, 93]. Therefore, the effect of the cations Na^{+}, K^{+}, Mg^{++} and Ca^{++} on ATPase activity of the bacteroid preparations was tested.

The bacteroids of all three strains, TlSp, RT2 and RT18, behaved similarly in that they were stimulated most by Ca^{++} ions, but were also markedly stimulated by Mg^{++} ions (Table 4.12). The effect of Na^{+} and K^{+} ions, however, was only slight (Table 4.12).

The bacteroids of all 3 strains, as well as TlSp cultured cells, were all stimulated by the same proportion by each of the cations tested (Table 4.12). However, no matter which cation was used, TlSp bacteroids had about 10% more activity than TlSp cultured cells, and RT2 and RT18 bacteroids had about 30% less activity than TlSp bacteroids (Table 4.13).

Since these preparations were stimulated very much more by divalent than monovalent cations, like the TlSp cultured cells, there does not appear to be much contamination by plant ATPase enzymes. In this case, the plant

TABLE 4.12 Stimulation of ATPase activity in bacteroid suspensions by monovalent and divalent cations

	ATPase activity (μmol Pi/min/mg protein	Stimulation of ATPase activity with added cations (% of control without cations)			
	O	Na^+	K^+	Mg^{++}	Ca^{++}
TlSp (cultured cells)	1.21	101	106	187	246
TlSp (bacteroids)	1.33	103	100	184	253
RT2 (bacteroids)	0.97	100	106	182	248
RT18 (bacteroids)	0.97	101	106	177	242

ATPase activity in the control was measured on cells which were treated with lysozyme, washed with Mg^{2+}-Tris buffer, and then incubated for 60 min at 30° with 5 μmoles ATP and 20 μmoles Tris-HCl buffer. Stimulation by the various cations was measured by treating cells in the same way as the control, but incubating in a mixture of 5 μmoles ATP, 20 μmoles Tris-HCl buffer and 1.5 μmoles of the particular cation.

TABLE 4.13 % ATPase activities of bacteroids of *R. trifolii* strain T1Sp

	% ATPase activity				
	O	Na^{+}	K^{+}	Mg^{++}	Ca^{++}
T1Sp (cultured cells)	91	90	95	92	88
T1Sp (bacteroids)	100	100	100	100	100
RT2 (bacteroids)	73	71	77	72	71
RT18 (bacteroids)	73	76	77	70	70

ATPase activity with each cation is expressed as % activity of T1Sp bacteroids, using the same data as for Table 4.12.

enzymes should be present in the fraction not pelleted with the bacteroids. To check this, the supernatants from the first centrifugation to separate bacteroids from plant cell debris were also assayed for ATPase activity (Table 4.14). Although the supernatants did contain some bacteroids and free bacteria, which formed colonies on TMY plates, these preparations were very much less stimulated by Ca^{++} and Mg^{++} than the bacteroid suspensions, but were stimulated to about the same extent as bacteroids by Na^{+} and K^{+} ions (Tables 4.12 and 4.14).

Also, protoplasts made from white clover callus were treated in precisely the same way as nodule protoplasts. The pellet and supernatant so obtained were then tested for ATPase activity in the same way as bacteroid preparations (Table 4.15). However, lower levels of ATPase activity were detected in comparison with bacteroid preparations, and these activities were affected much more by K^{+} ions than the bacteroid activities had been (Table 4.15). Also, the activity of the supernatant was inhibited by Mg^{++} and Ca^{++}, and that of the pellet was stimulated less than in the bacteroids (Table 4.15). These data therefore indicate that the ATPase activities observed in bacteroid preparations is indeed due to bacteroid ATPase, rather than to contaminating plant enzymes.

It is also known (Robertson, personal communication) that ATPase activity of plant origin associated with the peribacteroid membrane [101, 177] is stimulated by TCA cycle intermediates such as succinate and fumarate. When these compounds were added to the bacteroid

TABLE 4.14 Stimulation of ATPase activity in supernatants containing plant cell debris and free rhizobia

Supernatant from nodules of:	ATPase activity (μmol Pi/min/mg protein)	Stimulation of ATPase activity with added cations (% of control without cations)			
	O	Na^+	K^+	Mg^{++}	Ca^{++}
TlSp	3.43	105	100	103	151
RT2	2.90	107	103	110	125
RT18	3.17	105	105	114	155

ATPase activity, and stimulation of activity by the various cations, was measured as for Table 4.12

TABLE 4.15 ATPase activities of white clover protoplasts after treating in the same way as nodule protoplasts

	ATPase activity (μmol Pi/min/mg protein)	Stimulation of ATPase activity with added cations (% of control without cations)			
	O	Na^+	K^+	Mg^{++}	Ca^{++}
Pellet	0.12	108	283	142	208
Supernatant	0.83	100	87	76	67

ATPase activity, and stimulation of activity by the various cations, was measured as for Table 4.12.

TABLE 4.16 Stimulation of ATPase activity in bacteroid suspensions by succinate, fumarate and glucose

	Mg^{2+}-ATPase activity (µmol Pi/min/mg protein) with added compounds (12.5 mM)			
	0	succinate	fumarate	glucose
T1Sp (cultured cells)	2.24	2.44	2.44	2.52
T1Sp (bacteroids)	2.45	2.06	2.05	2.63
RT2 (bacteroids)	1.76	1.26	1.16	1.94
RT18 (bacteroids)	1.94	1.63	1.81	2.32

suspensions, a reduction in ATPase activity was observed (Table 4.16), whereas the T1Sp cultured cells were stimulated (Table 4.16). However, when glucose was added, all suspensions were stimulated in their ATPase activity by 10 to 20% (Table 4.16). Although there is some evidence that bacteroids cannot take up and utilize glucose [38, 63, 221], presumably these bacteroids can do so because they have been made permeable by treatment with lysozyme, which was used in preparation of the cells for ATPase assays.

4.9 Summary

1. Mutants of *R. trifolii* strains T1 and T1Sp were isolated which were uncoupled in oxidative phosphorylation (*unc*$^-$).

2. An assay for ATPase activity in *R. trifolii* was developed.

3. *R. trifolii unc*$^-$ mutants respired O_2 in the presence of succinate even though they could not use it as sole carbon source.

4. *R. trifolii unc*$^-$ mutants effectively nodulated red, white and subterranean clovers, and reduced acetylene in the nodules.

5. Bacteroids of *unc*$^-$ mutants had 70% of the ATPase activity found in T1Sp bacteroids.

6. A functional oxidative phosphorylation system appears necessary to provide ATP for nitrogen fixation in *R. trifolii* strain T1.

CHAPTER 5

General Discussion

Chapter 5 - General discussion

5.1 The provision of energy in nitrogen-fixing bacteria

The central aim of this study was to determine which biochemical pathways provide the energy for bacterial nitrogen fixation. Although biological nitrogen fixation is an exergonic process, additional energy supplied by hydrolysis of ATP to ADP and Pi is required for nitrogenase activity. The provision of ATP for nitrogenase activity has been studied physiologically in four different nitrogen-fixing bacteria: *Rhizobium* (a symbiotic organism), *Azotobacter* (an aerobe), *Clostridium pasteurianum* (an anaerobe), and *Klebsiella pneumoniae* (a faculative anaerobe). For *Rhizobium*, it has been postulated [67, 16, 228] that bacteroids within the nodule make ATP by a coupled oxidative phosphorylation system, with leghaemoglobin of plant origin maintaining a high flux of O_2 to the bacteroids at very low O_2 tension. In *Azotobacter*, ATP is synthesized both by oxidative phosphorylation and by substrate level phosphorylation by glycolysis and the phosphoroclastic cleavage of pyruvate[241] .In *Clostridium*, ATP is also generated by glycolysis and the phosphoroclastic cleavage of pyruvate [136]. However, the manner in which ATP for nitrogen is synthesized in the facultative anaerobe *K. pneumoniae* has not been resolved.

Strains of *K. pneumoniae* are usually tested for nitrogen fixation under anaerobic conditions, although

some strains can produce active nitrogenase in the presence of O_2 [125, 96, 97]. In glucose-limited chemostat cultures of *K. pneumoniae* strain M5al, the introduction of low levels of O_2 causes an increase in the rate of nitrogen fixation and the growth yield [97]. Also, in samples not supplied with glucose, O_2 is required for nitrogenase activity [97]. Therefore, under low atmospheric O_2 concentrations, some of the ATP required for growth and nitrogen fixation is probably generated by oxidative phosphorylation [97].

In the study presented in this thesis, the question of how energy is provided for nitrogen fixation was approached by examining the phenotypic expression of nitrogen fixation genes from *K. pneumoniae* in various mutants of *E. coli* K12. *E. coli* K12 is the most suitable organism for combining genetic and biochemical approaches. The extensive background information on its genetic constitution and the ease of genetic manipulation have been widely used to genetically "tailor" this bacterium to answer specific questions. Moreover, a wide range of well-characterized mutants defective in pathways of energy metabolism is available.

The results obtained with *E. coli* K12 were then applied to *R. trifolii*, to investigate the biochemical pathways used to provide energy in this organism. *R. trifolii* strain T1 was chosen because it is a fast-grower which nodulates clovers, and because it is better characterized than many other strains of *R. trifolii*.

5.2 The necessity for electron transport-dependent phosphorylation in *E. coli* K12 (F'nif^{+}_{Kp}) hybrids

This study shows that in the *E. coli* K12 (F'nif^{+}_{Kp}) hybrids, under anaerobic conditions, ATP for nitrogen fixation is not generated by coupled electron transport-dependent phosphorylation. The *uncB*$^{-}$ hybrid, AN283 (FN68), reduces acetylene at the same rate and grows to the same extent as the *unc*$^{+}$ hybrid. This demonstrates that not only is the coupled electron transport-dependent phosphorylation system unnecessary, but also that its presence has no effect on nitrogen fixation under the anaerobic conditions used.

The different *unc*$^{-}$ mutations also affect, to varying degrees, other electron transport systems in *E. coli* K12 [181]. Because of this, some of the F'nif^{+}_{Kp} hybrids made with *E. coli* K12 *unc*$^{-}$ mutants do not express a Nif^{+} phenotype. If the phenotypic expression of nif^{+}_{Kp} genes in the *E. coli* K12 *unc*$^{-}$ mutants is considered together with their ATPase activity then these mutants can be classified into their various complementation groups (Table 5.1).

This is by far the most simple and convenient method available for phenotypically classifying these particular *unc*$^{-}$ mutants, and can be used to classify mutants defective in either the F_0 intrinsic portion or the F_1 extrinsic portion of the ATPase complex (Table 5.1).

TABLE 5.1 Phenotypes of *unc*⁻ *E. coli* K12 (F'nif^{+}_{Kp}) hybrids

Phenotype	*unc*⁻ mutation of hybrid				
	unc^{+}	*uncA*$^{-}$	*uncB*$^{-}$	*uncC*$^{-}$	*uncD*$^{-}$
ATPase activity	+	-	+	+	-
Acetylene reduction	+	-	+	-	-
Stimulation of acetylene reduction by fumarate	-	-	+	-	+
Defective in F_0 portion of ATPase complex	-	-	+	+	-
Defective in F_1 portion of ATPase complex	-	+	-	-	+

+ and - indicate presence and absence of gross phenotype in each hybrid

Several other *uncA*$^-$ and *uncD*$^-$ mutants are now available, and examination of phenotypic expression of *nif*$^+_{Kp}$ genes in these mutants will determine whether the phenotypic classification can be applied to all four groups of *unc*$^-$ mutants, or only to the four particular mutants so far tested.

Under the anaerobic growth conditions used, the *unc*$^+$ and *uncB*$^-$ hybrids grow and fix nitrogen to a similar extent and produce the same end-products. Thus, under these conditions the ATPase complex apparently does not function to make ATP. However, the complex must still be present, since the various *unc*$^-$ mutations have pleiotropic effects on the hydrogenlyase system. All of the *unc*$^-$ mutations affect gas production, which demonstrates that the ATPase complex is involved in regulating the activity of the membrane-bound hydrogenlyase system. In addition, the *uncB*$^-$ mutation has a further effect in that it causes overproduction of a redox system. This defect can be partially complemented by the F'*nif*$^+_{Kp}$ plasmid.

5.3 The necessity for fumarate reduction in *E. coli* K12 (F'*nif*$^+_{Kp}$) hybrids

The *E. coli* K12 *unc*$^-$ mutations also have a pleiotropic effect on the regulation of another membrane-bound electron transport system to the terminal acceptor fumarate [181]. All of the *unc*$^-$ mutations diminish the

formation of fumarate from glucose. Therefore, it is possible that this secondary effect on the fumarate reduction system in the *uncA*$^-$, *uncC*$^-$ and *uncD*$^-$ mutants is the reason why their F'*nif*$^+_{Kp}$ hybrids express a defective Nif phenotype.

This is indeed the most likely explanation, since no acetylene reduction occurs when an additional mutation in fumarate reductase (*frd*$^-$) is present in the *uncB*$^-$ hybrid (i.e. hybrid AN480 (FN68)). Also, addition of fumarate to the growth medium of the *uncD*$^-$ hybrid AN285 (FN68) partially overcomes the defect in this strain, and allows acetylene reduction to occur.

When the level of fumarate added to the growth medium of the *uncD*$^-$ hybrid AN285 (FN68) is increased from 5 mM to 20 mM, the rate of acetylene reduction is increased from 1.5 to 10.4 nmol C_2H_4/min/mg protein. Further increases to the rate of 42.2 nmol C_2N_4/min/mg protein found with the *unc*$^+$ hybrid are not obtained with higher levels of added fumarate. Thus, the additional defect in the *uncD*$^-$ strain is more complex than a simple inability to form fumarate.

The additional defect concerned with fumarate reduction in the *uncA*$^-$ strain AN249 is known to be more complex than that of the *uncD*$^-$ strain AN285 (Cox, personal communication). Addition of up to 100 mM fumarate to the growth medium of hybrid AN249 (FN68) does not allow any detectable acetylene reduction.

Presumably, there is a similar defect in the recently isolated *uncC*⁻ strain AN771 [81], since acetylene reduction is not observed with added fumarate in this hybrid either.

The requirement for added fumarate could be for induction of the fumarate reduction system, or for the metabolism of fumarate by a fumarate reduction system which is already present but non-functional, or for a combination of both these possibilities. The fumarate requirement is not simply for biosynthesis of succinate (the first product of oxidative metabolism of fumarate), since addition of succinate to cultures has no effect.

In the *E. coli* K12 (F'nif^{+}_{Kp}) hybrid made with a strain defective in fumarate reductase, AN472*frd*⁻, no acetylene reduction occurs. Also, in hybrids defective in production of menaquinone (which is used in the fumarate reduction pathway (Figure 1.5)), acetylene is only reduced very slowly. Thus it is a functional fumarate reduction system which is essential for nitrogen fixation in the *E. coli* K12 (F'nif^{+}_{Kp}) hybrids.

Addition of fumarate or succinate to the *frd*⁻ hybrid AN472(FN68) has no effect on its inability to reduce acetylene. This again demonstrates that it is the fumarate reduction system itself which must be functional for nitrogen fixation to occur in *E. coli*.

5.4 Role of the fumarate reduction system in *E. coli* K12 (F'nif^{+}_{Kp}) hybrids

There are several possible roles for the fumarate

reduction system in nitrogen fixation under anaerobic conditions, namely (a) in the provision of energy, in the form of ATP equivalents, to the nitrogenase complex; (b) in maintaining the energized state of the membrane; or (c) in the passaging of electrons from NAD(P)H to the nitrogenase complex.

It is unlikely that the fumarate reduction system provides energy for nitrogen fixation in the form of ATP equivalents, since nitrogenase is known to be very specific in its requirement for ATP, and in uncoupled mutants substrate level phosphorylation can provide sufficient ATP for nitrogen fixation.

The membrane may be energized, according to the chemiosmotic hypothesis [238, 239], by ordering of the electron carriers in the membrane in such a way as to separate protons and electrons across the membrane. This leads to the generation of a proton-motive force and an electrical potential difference across the membrane.

Aerobically, the membrane may be energized by coupling to the electron flow to oxygen [10, 128]. Under anaerobic conditions, it can be energized either by coupling to available ATP, the utilization of which requires a coupled intact ATPase [84], or by coupling to the electron flow to the acceptors nitrate or fumarate [130].

Carbonylcyanide *m*-chlorophenylhydrazone (CCCP) is an uncoupler which acts by enhancing the rate of

proton diffusion across the membrane by several orders of magnitude. This results in dissipation of the high-energy membrane state and uncoupling of oxidative phosphorylation [88, 93].

In *E. coli* K12 (F'nif^{+}_{Kp}) hybrids, CCCP inhibits nitrogenase activity by 80 - 100%. It is possible that CCCP may have some undefined inhibitory effect on nitrogenase itself, since it is known that CCCP can also inhibit glutamine uptake (which is independent of the high-energy membrane state [84]) in some undefined manner. However, this possibility is most unlikely as CCCP should then inhibit equally well in unc^{+} and unc^{-} cells. In fact, CCCP inhibits the $uncB^{-}$ hybrid less than the $uncD^{-}$ and unc^{+} hybrids. Therefore it appears that CCCP does affect nitrogen fixation by dissipating the energized state of the membrane.

In unc^{+} cells, this inhibition by CCCP could be caused by a diminished supply of ATP for nitrogen fixation, due to hydrolysis of ATP by the ATPase complex in attempts to re-energize the membrane. However, similar inhibition of nitrogenase activity by CCCP occurs in the unc^{+} hybrid AN259 (FN68) and the $uncD^{-}$ hybrid AN285 (FN68). Since the $uncD^{-}$ hybrid lacks normal ATPase activity, CCCP does not inhibit nitrogen fixation merely by draining the available ATP in the cell.

Nitrogenase activity is therefore dependent on the high-energy membrane state. Presumably this state is required either for the passage of electrons to

nitrogenase, or for the uptake of some component(s) required for nitrogen fixation, which is dependent on the membrane being energized (such as amino acids or inorganic phosphate).

Since the electron transport system to fumarate must be functional in nitrogen-fixing cells, this system will help to maintain the membrane in an energized state. However, this is unlikely to be the only role of the fumarate reduction system, as there is no apparent advantage gained in energizing the membrane by this system rather than by hydrolysis of ATP from substrate level phosphorylation.

The number of ATP molecules from each glucose molecule available for nitrogen fixation is probably similar, whether the membrane is energized by hydrolysis of ATP or by the fumarate reduction system. If glucose is fermented to pyruvate and then converted to acetate by the phosphoroclastic reaction, a maximum of 4 ATP/ glucose can be made by substrate level phosphorylation, although the actual number is closer to 3 [98, 90]. However, some of this ATP is then hydrolysed to energize the membrane.

If glucose is fermented to pyruvate (yield of 2 ATP/glucose) and is then converted to fumarate *via* oxaloacetate and asparate [47, 205], 1 ATP/pyruvate is used up. Thus, a yield of 0 ATP/glucose can result. However, the membrane can be energized by reduction of

fumarate, with the possibility of generating 1 ATP/ fumarate (or 2 ATP/glucose) by coupled electron-transport dependent phosphorylation [85, 88, 240].

Therefore it does not appear that a great advantage in the number of residual ATP molecules is gained by energizing the membrane by fumarate reduction rather than ATP hydrolysis.

It seems most likely, therefore, that the main role of the fumarate reduction system in nitrogen fixation is in passaging of electrons from NAD(P)H to the nitrogenase complex.

In nitrogen-fixing bacteria, electrons from NAD(P)H are donated directly to the nitrogenase complex by ferredoxin, which is an iron-sulphur protein [136, 166]. Under iron-limiting conditions, however, ferredoxin can be replaced by a flavoprotein, flavodoxin. Similarly, in the fumarate reduction system, an iron-sulphur protein or a flavoprotein can pass electrons from the donor NADH to menaquinone, and then on to fumarate reductase (see Figure 1.5). Thus it seems probable that the same electron carriers are shared by the nitrogen fixation and fumarate reduction systems, and that the fumarate reduction system must be induced so as to provide an appropriate electron carrier for nitrogenase.

5.5 The necessity for nitrate reduction in *E. coli* K12 (F'*nif*$^+_{Kp}$) hybrids

It has been proposed [88, 129] that the nitrate

reduction system also shares some electron carriers with the fumarate reduction system, including the iron-sulphur protein or flavoprotein. Nitrate reductase, like fumarate reductase, can energize the membrane by chemiosmotic coupling. Nitrate is also known to regulate anaerobic electron transport pathways, by inducing the nitrate reductase system [197], repressing the formate dehydrogenase and hydrogenlyase systems [182], and probably repressing the fumarate reductase system [129]. Nitrate also inhibits nitrogen fixation, although the precise mechanism is not clear. Therefore, the effect of mutations in the nitrate reduction system (*chl*$^-$ mutations) on nitrogenase activity was studied in *E. coli* K12 (F'*nif*$^+_{Kp}$) hybrids.

The nitrate reductase and formate hydrogenlyase systems are not essential for phenotypic expression of *nif*$^+_{Kp}$ genes in *E. coli* K12. Similarly ubiquinone, which is also involved in nitrate reduction, is unnecessary.

Nitrogenase and nitrate reductase do, however, share some molybdenum-processing functions. The *chlD*$^-$ hybrids, which are defective in transport of molybdenum (Table 5.2), only reduce acetylene in the presence of added molybdenum ions [120 and Appendix, Section 1]. It is known that *chlD*$^-$ hybrids make inactive nitrogenase which can be activated by addition of molybdenum ions [120].

The *chlA* and *chlB* genes, which are also involved in the processing of molybdenum (Table 5.2), also affect

TABLE 5.2 Effect of various *chl*$^-$ mutations on *E. coli* K12 mutants defective in nitrate reduction.

Mutation	Effect of mutation	Effect on nitrogen fixation
chlA$^-$	Lacks Mo-cofactor Defective in formate hydrogenlyase	No inhibition by nitrate
chlB$^-$	Lacks association factor for insertion of Mo-cofactor into nitrate reductase Defective in formate hydrogenlyase	No inhibition by nitrate
chlC$^-$	Lacks α subunit of nitrate reductase	None
chlD$^-$	Defective in transport of molybdenum Defective in formate hydrogenlyase	No nitrogen fixation unless molybdenum added
chlE$^-$	Lacks β subunit of nitrate reductase Defective in formate hydrogenlyase	None
chlF$^-$	Lacks formate dehydrogenase?	Not known
chlG$^-$	Defective in formate hydrogenlyase	None

Data presented is from references 88, 129, 139, 140, 141, 178.

the phenotypic expression of $nif^{+}{}_{Kp}$ genes in the presence of nitrate. Thus all three nitrate reductase genes necessary for molybdenum processing can affect nitrogenase activity.

5.6 Nitrate inhibition of nitrogenase activity

Nitrate, like ammonia, inhibits nitrogen fixation. From studies with *E. coli* K12 hybrids carrying the $nif^{+}{}_{Kp}$ genes, Kennedy and Postgate [120] conclude that nitrate must be metabolized to nitrite in order to repress the synthesis of nitrogenase. However, their conclusions are based only on the absence of nitrate inhibition in *chlA*$^{-}$ and *chlB*$^{-}$ hybrids (these genes being necessary for processing of molybdenum).

From the study presented in this thesis, it is apparent that it is nitrate itself, rather than the product nitrite or the process of passing electrons to nitrate, which causes inhibition of nitrogenase activity. Even in the *chlC*$^{-}$ and *chlE*$^{-}$ hybrids, which are defective in a subunit of the nitrate reductase enzyme (Table 5.2), acetylene reduction is still inhibited by nitrate. Similarly, nitrate still inhibits nitrogenase activity in *chlD*$^{-}$ and *chlG*$^{-}$ hybrids. Since no production of nitrite can be detected by the *chlC*$^{-}$, *chlE*$^{-}$ and *chlG*$^{-}$ hybrids in NFM even with 50 mM nitrate added, conversion of nitrate to nitrite is not necessary for inhibition of nitrogen fixation.

Nitrate does not directly repress the formation

or activity of nitrogenase, since acetylene reduction still occurred in several *chl*$^{-}$ *E. coli* K12 (F'*nif*$^{+}$$_{Kp}$) hybrids in the presence of nitrate.

As various *chl*$^{-}$ mutations also cause loss of the formate hydrogenlyase system (Table 5.2), inhibition of nitrogenase activity by nitrate does not act through this electron transporting system. Also, nitrate completely inhibits nitrogenase activity in *unc*$^{-}$ *E. coli* K12 (F'*nif*$^{+}$$_{Kp}$) hybrids. Thus nitrate inhibition does not occur by affecting the electron transport-dependent phosphorylation system.

It seems most likely, therefore, that nitrate acts indirectly, by affecting the fumarate reduction system which must be functional for nitrogen fixation, or by acting through a regulator protein.

In *E. coli* K12, it has been suggested that a functional or organisational relationship exists between the nitrate reduction and fumarate reduction systems [133]. This suggestion is based on the characterization of single mutations (*fnr*$^{-}$) which cause defects in nitrate reduction, fumarate reduction and formate hydrogenlyase [133]. It has also been proposed that competition between electron carriers involved in these electron transport pathways can prevent efficient electron transport to fumarate.

Therefore, one model for the mechanism of nitrate inhibition of nitrogen fixation is that the proposed competition between electron carriers can prevent sufficient electron transport to nitrogenase.

Alternatively, less efficient electron transport to fumarate may lead to expression of a Nif$^-$ phenotype.

Another model for the effect of nitrate is based on regulation of the various electron-transporting systems by regulator proteins. Further examination of the *fnr*$^-$ mutation has established that it maps in the *nirA* (nitrite reductase A) gene [242]. It has been proposed [242] that the *nirA* gene codes for a positive regulator protein which is essential for the synthesis of a variety of enzymes involved in anaerobic redox reactions. These include nitrate reductase, nitrite reductase, fumarate reductase, hydrogenase and formate hydrogenlyase (Figure 5.1) [133, 242].

It seems likely, therefore, that protein A may also regulate nitrogenase, since nitrogen fixation is also an anaerobic redox reaction.

It has also been proposed [242] that the *chlA* and *chlB* genes of *E. coli* K12 make another regulator, factor B, which prevents the activation of genes by protein A (Figure 5.1). Factor B is made under anaerobic conditions in the presence or absence of nitrate or nitrite, and is absent or defective in *chlA*$^-$ and *chlB*$^-$ strains [242].

Since *E. coli* K12 (F'nif^+_{Kp}) hybrids made with strains defective in *chlA* or *chlB* reduce acetylene at the same rate as *chl*$^+$ hybrids, factor B is not necessary for activity of the fumarate reduction or nitrogen fixation systems. This factor is, however,

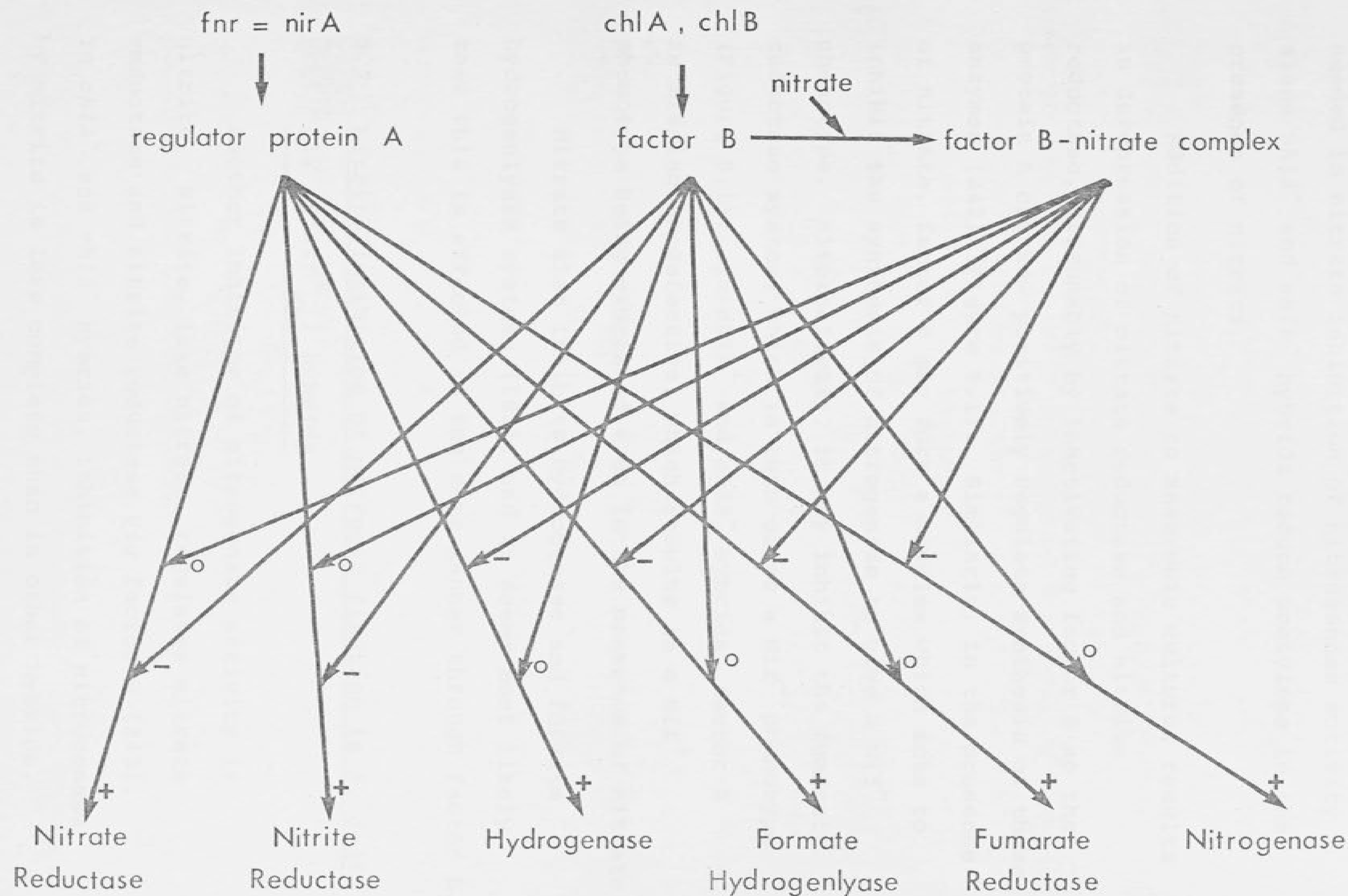
FIGURE 5.1

Scheme summarizing the possible role of *nirA*, *chlA* and *chlB* genes in the regulation of anaerobic redox reactions in *E. coli* K12 [242].

+; positive regulatory effect

-; negative regulatory effect

O; no regulatory effect observed


fnr = nirA
chlA , chlB
nitrate
regulator protein A
factor B
factor B-nitrate complex
Nitrate
Reductase
Nitrite
Reductase
Hydrogenase
Formate
Hydrogenlyase
Fumarate
Reductase
Nitrogenase

needed in nitrate inhibition of nitrogenase activity since *chlA*$^-$ and *chlB*$^-$ hybrids reduce acetylene in the presence of nitrate.

Addition of nitrate to anaerobic cultures results in derepression of nitrate reductase and nitrite reductase, presumably by inactivating factor B so that protein A can now positively regulate synthesis of these enzymes [242] (Figure 5.1). Similarly, in the presence of nitrate, factor B may form a complex which acts to inhibit the synthesis of nitrogenase to give a Nif$^-$ phenotype. Alternatively, it may inhibit the fumarate reductase system, which in turn gives a Nif$^-$ phenotype (Figure 5.1). In *chlA*$^-$ and *chlB*$^-$ hybrids, factor B is missing or defective, which results in a Nif$^+$ phenotype being expressed even in the presence of nitrate.

Nitrate also inhibits hydrogenase and formate hydrogenlyase systems [182], and it seems most likely that this is effected in the same manner through factor B.

5.7 Nitrite inhibition of nitrogen fixation in *E. coli* K12 (F'*nif*$^+_{Kp}$) hybrids

Another inhibitor of nitrogenase activity is nitrite. Nitrite, like nitrate, regulates nitrate reductase and nitrite reductase *via* factor B [242]. In *chlA*$^-$ and *chlB*$^-$ hybrids, inhibition of nitrogenase by nitrite is less complete than in other hybrids. Therefore, it is likely that nitrite, like nitrate,

inhibits nitrogenase *via* a factor B-nitrite complex. However, nitrogenase activity of *chlA*$^-$ and *chlB*$^-$ hybrids is inhibited much more by nitrite than nitrate. Also, in a kinetic study in the *E. coli* K12 hybrid AN259 (FN68), nitrite completely inhibits nitrogenase activity immediately, whereas with nitrate no effect on nitrogenase activity is observed for about 30 minutes, followed by a gradual decline in activity. Thus, nitrite has some additional inhibitory effect on nitrogenase activity, possibly by conversion to ammonia.

5.8 Derivation of ATP in *E. coli* K12 (F'*nif*$^+_{Kp}$) hybrids

This study shows that the coupled electron transport-dependent phosphorylation system is not necessary in *E. coli* K12 (F'*nif*$^+_{Kp}$) hybrids, and that substrate level phosphorylation must, therefore, be able to provide all the ATP needed for both nitrogen fixation and growth. For *K. pneumoniae* it has been estimated that 15 to 29 ATP molecules are required for each molecule of N_2 fixed [2, 98]. Moreover, if the additional energy used for ATP-dependent reduction of H^+ to H_2 by nitrogenase is taken into account, as well as the energy required for maintenance and synthesis of the cell, it has been estimated that an extra 20 to 30 ATP molecules are used up per molecule of N_2 fixed [2, 99, 166].

The calculation from results obtained in this study with an *E. coli* K12 (F'*nif*$^+_{Kp}$) hybrid shows that a possible 35 molecules of ATP are available for each molecule of N_2 fixed (excluding the "maintenance" energy, but including

the energy used for H_2 evolution by nitrogenase). In addition, about 31 molecules of ATP are available per N_2 fixed for maintenance and synthesis of the cell. Therefore, substrate level phosphorylation is indeed able to provide sufficient ATP for nitrogen fixation and growth in *E. coli* K12 (F'nif^{+}_{Kp}) hybrids.

5.9 Isolation of *R. trifolii* mutants defective in pathways of energy conservation

In the symbiotic bacterium *Rhizobium*, as in other free-living nitrogen fixers such as *K. pneumoniae*, large amounts of ATP are needed for nitrogenase activity. Studies of how this requirement for energy might be satisfied in *Rhizobium* bacteroids, and more recently in free-living nitrogen-fixing rhizobia, have so far been limited to physiological and biochemical assays. However, the possibility now exists of extending studies of the pathways of energy metabolism used in *Rhizobium*, by the use of specific mutants.

It is not yet possible to transfer nif^{+}_{Rt} genes from *R. trifolii* strain T1 to *E. coli* K12. Therefore, the pathways used to provide energy for phenotypic expression of nif^{+}_{Rt} genes cannot be studied in the same was as for nif^{+}_{Kp} genes.

Instead, mutants defective in particular pathways of energy conservation can be isolated in strain T1 using the neomycin selection technique [112, 183]. Because the phenotypic expression of nif^{+}_{Kp} genes in *E. coli* K12 does not depend on electron transport-dependent

phosphorylation, and because it has been proposed [6, 7, 228] that this coupled system is the main source of ATP for nitrogen fixation in *Rhizobium*, the neomycin selection technique was used to isolate mutants of *R. trifolii* defective in oxidative phosphorylation and other electron transport pathways.

Using the neomycin selection technique, 15 mutants of *R. trifolii* strains T1 and T1Sp have been isolated which are unable to grow on succinate. Ten of these mutants have various growth properties which, by analogy with well-defined *E. coli* K12 mutants, suggest they may be mutant in electron transport components such as quinones, fumarate reductase, D-lactate dehydrogenase or the energy coupling factor (Table 5.3). Such mutants can be isolated in *E. coli* K12 by the neomycin selection technique.

Several of these 10 mutants, such as RT6 and RT11, may be *sdh*$^-$ mutants defective in succinate dehydrogenase (the aerobic equivalent of fumarate reductase). These mutants, which grow on TCA cycle intermediates other than succinate and have normal ATPase activity, form ineffective nodules on red, white and subterranean clovers. This suggests that succinate dehydrogenase may be necessary for nitrogen fixation in *Rhizobium* bacteroids. Succinate dehydrogenase might be needed either for metabolism of succinate supplied by the plant, or for passaging electrons to nitrogenase as proposed for fumarate reductase under anaerobic conditions in *E. coli* K12 (F'nif^+_{Kp}) hybrids, or for both

TABLE 5.3 Properties of various *E. coli* K12 mutants defective in pathways of energy metabolism

Mutation	Property
uncA$^-$, *uncD*$^-$	Defective in F_1 extrinsic portion of ATPase complex. Lack ATPase activity.
uncB$^-$, *uncC*$^-$	Defective in F_0 intrinsic portion of ATPase complex. Retain normal ATPase activity.
	All *unc*$^-$ mutants have the following properties: Uncoupled in oxidative phosphorylation. Grow with reduced rate and yield on glucose. Cannot grow on TCA cycle intermediates or D-lactate. Respiration unimpaired.
etc$^-$	Defective in electron transfer coupling. Uncoupled in oxidative phosphorylation. Have normal ATPase activity. Grow with reduced rate and yield on glucose. Cannot grow on TCA cycle intermediates or D-lactate. Can respire with succinate, D-lactate and NADH.
ecf$^-$	Defective in energy coupling factor. Uncoupled in oxidative phosphorylation. Have normal ATPase activity. Grow with reduced rate and yield on glucose. Cannot grow on TCA cycle intermediates or D-lactate. Can respire with succinate and D-lactate. Defective in active transport of amino acids and sugars.
ubi$^-$	Defective in ubiquinone. Have normal ATPase activity. Grow with reduced rate and yield on glucose. Much lactate produced from glucose. No electron transport to O_2.

TABLE 5.3 (cont'd)

Mutation	Property
sdh$^-$	Defective in succinate dehydrogenase. Have normal ATPase activity. Grow with reduced rate and yield on glucose. Cannot grow on succinate, but can grow on D-lactate. Cannot respire on succinate.
frd$^-$	Defective in fumarate reductase. Have normal ATPase activity. Grow with reduced rate and yield anaerobically on glucose. Grow aerobically but not anaerobically on succinate. Cannot use fumarate as terminal electron acceptor.
dld$^-$	Defective in D-lactate dehydrogenase. Have normal ATPase activity. Grow with reduced rate and yield on glucose. Cannot grow on D-lactate, but can grow on succinate. Cannot respire on D-lactate.

Data presented is from references 37, 47, 48, 49, 50, 51, 58, 81, 85, 103, 134, 181, 203, 204.

these possibilities.

5.10 Characterization of *unc*$^-$ mutants of *R. trifolii* strain T1

Five of the 15 Nm^RSuc^- mutants of strains T1 and T1Sp were studied in detail. These five mutants show several pleiotropic changes, which are all due to a single mutation since reversion to Suc^+ also reverts all of the other alterations.

By comparing the properties of the five mutants with those of *E. coli* K12, it is possible to determine which pathway of energy metabolism is defective (Table 5.3).

The outstanding feature of the five *R. trifolii* mutants is their lack of normal ATPase activity. This feature, together with their growth properties and respiration demonstrates that they must be *unc*$^-$ mutants uncoupled in oxidative phosphorylation (Table 5.3).

By analogy with the *E. coli* K12 *unc*$^-$ mutants, the five *R. trifolii* mutants are unlikely to be defective in the F_0 portion of the ATPase complex, since such mutants (*uncB*$^-$ and *uncC*$^-$) retain normal ATPase activity. Instead, the five mutants are like *uncA*$^-$ or *uncD*$^-$ mutants of *E. coli* K12 (Table 5.3). Such mutants (which are defective in the F_1 portion of the ATPase complex) exhibit about 10 - 30% of normal ATPase activity.

Similarly, the *R. trifolii unc*$^-$ mutants retain 10 - 20% ATPase activity.

The situation is, however, more complex in the *R. trifolii unc*$^-$ mutants, since they acquire a high level of ATPase activity in the nitrogen-fixing bacteroid form.

5.11 ATPase activity in bacteroids of *R. trifolii unc*$^-$ mutants

In nodulation tests in tubes or Petri dishes, using 3 clover species (red, white and subterranean), the five *unc*$^-$ mutants cannot be distinguished from their parent strains. In pots, however, where plants grow much better than in tubes or Petri dishes, the mutants produce more, slightly smaller nodules than their parent strains.

Also, the leaves of plants inoculated with *unc*$^-$ mutants are slightly smaller than those of plants inoculated with the parent strain T1Sp, although all leaves are very much greener than those of uninoculated controls. This indicates that the *unc*$^-$ mutants do, in fact, produce slightly less effective nodules than their parent strains.

When gently isolated from white clover nodules, bacteroids of the *unc*$^-$ mutants RT2 and RT18 have about 70% of the ATPase activity of T1Sp bacteroids. The ATPase activity of both mutant and parental bacteroids, like that of cultured cells, is stimulated most by the divalent cations Mg^{2+} and Ca^{2+} and only slightly by the

monovalent cations Na^{+} and K^{+}. It is interesting to note that the ATPase activity of *E. coli* K12 is stimulated more by Mg^{2+} than Ca^{2+} [37, 215], whereas in *R. trifolii* strain T1 and mutants Ca^{2+} is more stimulatory.

When these bacteroids of *unc*$^{-}$ mutants are redifferentiated into the bacterial form by growth on TMY media, they reacquire the characteristics of the original inoculum. The redifferentiated bacteria are unable to grow on succinate, are resistant to neomycin, and have a very low level of ATPase activity. Thus, the presence of ATPase activity in bacteroids is not simply due to a reversion of the mutants to *unc*$^{+}$.

It is clear that bacteroids of *unc*$^{-}$ mutants do acquire a functional ATPase enzyme, which is not due to contaminating plant ATPases. Whether this ATPase activity is due to (a) the plant supplying some component(s) which enables the defective bacterial ATPase to function, or (b) some form of amplification of the defective bacterial ATPase in the bacteroids, or (c) differentiation of a new ATPase in the bacteroids, cannot be distinguished at this stage.

There is now some evidence that the ATPase complex can indeed have an altered composition after growth under different atmospheric conditions [180 and Cox, personal communication). In *E. coli* K12, anaerobic growth results in synthesis of an ATPase complex with

components different from those formed under anaerobic conditions. There may be synthesis of a new peptide in the intrinsic F_0 portion of the complex, or modification of an existing peptide under anaerobic conditions [180].

This production of a different ATPase complex in *E. coli* K12 under anaerobic conditions, together with the induction of different electron transport pathways such as the fumarate reduction system, can be thought of as a type of differentiation analogous to that which occurs when *Rhizobium* differentiates from the bacterial to bacteroid form.

5.12 The necessity for oxidative phosphorylation and differentiation of a new electron-transporting system in *Rhizobium* bacteroids

This study shows that the provision of ATP for nitrogen fixation probably occurs by different mechanisms in *K. pneumoniae* and *Rhizobium*. In the *E. coli* K12 (F'nif^{+}_{Kp}) hybrids, nitrogen fixation occurs under anaerobic conditions, and glucose can be fermented to provide fumarate as well as sufficient ATP by substrate level phosphorylation. In contrast, nitrogen-fixing *Rhizobium* bacteroids cannot utilize glucose [38, 221, 63], but instead use TCA cycle intermediates such as succinate and fumarate which are supplied by the plant. Sufficient ATP cannot be derived from such compounds by substrate level phosphorylation [88, 93, 58, 240]. In addition, the bacteroids are

constantly supplied with oxygen by leghaemoglobin [7, 22, 23]. Therefore, it is reasonable to assume [6, 7, 228] that in *Rhizobium* bacteroids oxidative phosphorylation is indeed the main pathway for provision of ATP for nitrogen fixation. Also, because the physiological studies have been done on bacteroids of the slow-growing *R. japonicum* [6, 7, 12, 22, 23, 228] whereas *unc*$^-$ mutants have been isolated in the fast-growing *R. trifolii* strain T1, it is very likely that similar pathways of energy metabolism for nitrogen fixation occur in both groups of *Rhizobium*.

When rhizobia differentiate into the nitrogen-fixing bacteroid form, the cytochrome pattern is altered, with the absence of cytochromes a, a_3 and o (which are present in the bacterial form), and the presence of cytochromes b, P-450 and P-420 (which are absent in bacteria) [18]. In bacteroids, the amounts of nitrate reductase and flavin co-enzymes are increased [18], and nitrogenase is present. The cell wall composition and morphology is also different in bacteroids of fast-growing rhizobia. Therefore, it appears that *Rhizobium* differentiates a new electron-transporting system, possibly including a new or altered ATPase complex, in the bacteroid form.

Rhizobium bacteroids within the nodule are in a very different environment from the free-living form, with different carbon sources and oxygen supply.

Therefore, a new system for transporting electrons is probably necessary to ensure that sufficient ATP is generated for nitrogen fixation, and to ensure maintenance of a chemiosmotic gradient across the membrane for passaging of electrons to nitrogenase, and for uptake of compounds such as succinate supplied by the plant and used for respiration by the bacteroids.

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Appendix

While the experiments described in this thesis were being done, several other aspects of the physiology of the nitrogen-fixing bacteria used were also investigated. Some of these studies are described in the publications included in this appendix.

SECTION 1

While the effect of nitrate on the phenotypic expression of nif^{+}_{Kp} genes was being investigated in *E. coli* K12 (FN68) hybrids, it was observed that nitrate and nitrite could also affect the growth of these hybrids, having either a stimulatory or inhibitory effect depending on the particular *chl*⁻ mutation.

When nitrate was added to chl^{-} *E. coli* K12 hybrids, all four possible combinations of inhibition of growth and the Nif^{+} phenotype were observed. Thus, nitrate inhibition of nitrogenase activity does not appear to act through the assimilatory nitrate reductase pathway, but is more likely to act through the nitrate respiratory pathway.

The results obtained showed that metabolism of nitrate to nitrite is not necessary for inhibition of nitrogenase activity. This contrasts with the findings of Kennedy and Postgate [120], who claimed that nitrate must be metabolized to nitrite in order to repress the synthesis of nitrogenase. Similarly, Pagan *et al.* [159] using nitrate reductase mutants of *Rhizobium* sp. 32H1, concluded that the major inhibitory effect of nitrate on nitrogenase activity was a consequence of its reduction to nitrite,

although they did not exclude the possibility that nitrate or nitrite was competing with nitrogenase for electrons, thereby lowering nitrogenase activity. These possibilities could not be distinguished in *Rhizobium* because of the lack of well-defined mutants available in *E. coli* K12, which have allowed a clearer analysis of this problem.

INTERACTION BETWEEN THE NITRATE RESPIRATORY SYSTEM OF ESCHERICHIA COLI K12 AND THE NITROGEN FIXATION GENES OF KLEBSIELLA PNEUMONIAE

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SUMMARY: Hybrids were constructed between *E. coli* K12 *chl*$^-$ mutants defective in nitrate respiration and an F' plasmid carrying nitrogen fixation genes from *K. pneumoniae*. Examination of these hybrids showed that expression of nif^+_{Kp} genes does not require a functional nitrate respiratory system, but that nitrate reductase and nitrogenase do share some Mo-processing functions. For nitrate repression of nitrogenase activity, reduction of nitrate to nitrite is not necessary, but the Mo-X cofactor encoded by *chl* genes is essential. Nitrate probably inhibits nitrogen fixation by affecting the membrane relationship of the nitrate and fumarate reduction systems such that the membrane cannot be energized for nitrogenase activity.

INTRODUCTION:

Evidence suggesting that the membrane-bound nitrate reductase and nitrogenase enzyme complexes may share a common molybo-protein subunit has been presented (1, 2, 3), although other studies have provided little support for this proposed commonality(4).

The nitrate respiratory complex has been well-studied in *E. coli* K12 (5, 6, 7). Mutants defective in their inducible formate-nitrate reductase complex have been isolated because of resistance to chlorate, which nitrate reductase can reduce to toxic chlorite (8, 9).

Therefore, the possible relationship between the nitrate respiratory system and the nitrogenase of *K. pneumoniae* was investigated by using hybrids constructed between various *E. coli* K12 mutants defective in nitrate respiration (*chl*$^-$) and the FN68 plasmid which carries the nitrogen fixation genes from *K. pneumoniae* (nif^+_{Kp}).

MATERIALS AND METHODS

Bacterial strains: *E. coli* K12 strain SB1801 *his*$^-$ *mal*$^-$ carrying the

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FN68 plasmid (F'nif^+_{Kp} his^+_{Kp} Cb^R) (10) was used as the donor in conjugation experiments with nitrate reductase (chl^-) mutants of E. coli K12, and also with pleiotropic nif^- chl^- mutants of K. pneumoniae strain M5al.

Media: Nitrogen-free medium (NFM) was that of Cannon et al. (11). Growth in liquid NFM was measured by following turbidity in a nephelometer. When necessary, NFM was supplemented with vitamin-free Casamino acids at 100 μg/ml, amino acids at 25 μg/ml and vitamins at 10^{-3} μM.

Conjugation: E. coli K12 strain SB1801 (FN68) was used as the donor in conjugation experiments with mal^+ recipient strains. Mal^+ Cb^R hybrids were selected and purified as described previously (12), and were then tested for nitrogen fixation by growth on NFM and by acetylene reduction.

Acetylene reduction assay: Rates of acetylene reduction to ethylene were measured by the method of Tubb & Postgate (13). In all cases, addition of 50 mM $(NH_4)_2SO_4$ to the medium repressed nitrogenase activity as measured by acetylene reduction.

RESULTS AND DISCUSSION

None of the nitrate reductase mutants prevented or reduced phenotypic expression of nif^+_{Kp} genes (Table 1). Even the loss of chlC, the structural gene for a subunit of the nitrate reductase enzyme (5), did not prevent nitrogen fixation. Likewise, a functional chlA gene, coding for a Mo-X cofactor (5) is unnecessary for nitrogenase activity. However addition of 10^{-4}M MoO_4^{2-} ions was essential for full nitrogenase activity in $chlD^-$ mutants indicating an interaction between chlD and nif^+ functions.

To examine the mechanism by which nitrate inhibits nitrogen fixation, hybrids carrying the FN68 plasmid were grown in NFM with KNO_3 or KNO_2, and were tested for acetylene reduction. Only the $chlA^-$ and $chlB^-$ hybrids showed no nitrate inhibition of nitrogenase activity; these hybrids gave similar levels of acetylene reduction in NFM with or without nitrate (Table 1).

Thus, the Mo-X cofactor coded for by chlA (5) and the association factor coded for by chlB (5) are necessary for nitrate inhibition of nitrogen fixation, indicating that molybdenum-processing functions are shared by nitrogenase and nitrate reductase. However, conversion of nitrate to nitrite is not necessary for inhibition, since nitrate still caused loss of acetylene reduction in the $chlC^-$ hybrid. Similarly in the $chlD^-$, $chlE^-$, and $chlC^-$ hybrids, nitrate still inhibited nitrogen fixation. Since the

TABLE 1. Phenotypic expression of nif^{+}_{Kp} genes on plasmid FN68 in nitrate reductase mutants of *E. coli* K12

E. coli K12 hybrid	chl mutation	Acetylene Reduction		
		NFM	NFM+ KNO_3	NFM+ KNO_2
C181(FN68)	$chlA^{-}$	39.0	38.0	0.9
KB70(FN68)	$chlA^{\Delta}$	54.4	58.6	0.6
C183(FN68)	$chlB^{-}$	43.0	40.8	0.08
DD115(FN68)	$chlB^{-}$	31.2	32.2	0.08
Puig 426(FN68)	$chlC^{-}$	35.4	0.08	<0.01
C111(FN68)	$chlD^{\Delta}$	46.0	<0.01	<0.01
C113(FN68)	$chlD^{-}$	46.3	<0.01	<0.01
C123(FN68)	$chlD^{-}$	39.1	<0.01	<0.01
C197(FN68)	$chlE^{-}$	49.0	<0.01	<0.01
C202(FN68)	$chlE^{-}$	45.0	<0.01	<0.01
DD38(FN68)	$chlG^{-}$	56.8	0.3	0.1
SA291(FN68)	$chlA^{\Delta}$ $chlD^{\Delta}$	40.2	40.2	0.2

Acetylene reduction was measured in nmol C_2H_4/min/mg protein. KNO_2 and KNO_3 were added at final concentrations of 50 mM.

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various chl^{R} mutations also cause loss of the formic hydrogenlyase system (6), inhibition of nitrogenase activity by nitrate is unlikely to be acting through this system.

Nitrogenase activity in all hybrids tested was inhibited by KNO_2. However, in the $chlA^{-}$, $chlB^{-}$ and $chlG^{-}$ hybrids, there was a reproducible low level of acetylene reduction which may indicate incomplete repression by nitrite (Table 1).

TABLE 2. Growth of *E. coli* K12 (F'nif^{+}_{Kp}) hybrids in NFM with KNO_3 or KNO_2

Hybrid	*chl* mutation	Growth		
		NFM	NFM+ KNO_3	NFM+ KNO_2
C181(FN68)	*chlA*$^{-}$	100	47	64
KB70(FN68)	*chlA*$^{\Delta}$	100	93	49
C183(FN168)	*chlB*$^{-}$	100	220	106
DD115(FN68)	*chlB*$^{-}$	100	110	75
Puig 426(FN68)	*chlC*$^{-}$	100	172	57
C111(FN68)	*chlD*$^{\Delta}$	100	155	45
C113(FN68)	*chlD*$^{-}$	100	192	75
C123(FN68)	*chlD*$^{-}$	100	148	46
C197(FN68)	*chlE*$^{-}$	100	44	61
C202(FN68)	*chlE*$^{-}$	100	46	73
DD38(FN68)	*chlG*$^{-}$	100	109	37

Growth in Pankhurst tubes was measured in a nephelometer after 24 hr incubation, and is expressed as a percentage of the NFM control. KNO_3 and KNO_2 were added to give final concentrations of 50 mM.

All four possible classes of growth response and acetylene reduction were observed when hybrids were grown in NFM with nitrate (Tables 1 and 2):

(a) inhibition of both growth and nif^{+}_{Kp} phenotypic expression (*chlE*);

(b) inhibition of growth but not of nif^{+}_{Kp} phenotypic expression (*chlA*);

(c) inhibition of nif^{+}_{Kp} phenotypic expression but not of growth (*chlC*, D, G);

(d) no inhibition of either growth or nif^{+}_{Kp} phenotypic expression (chlB).

Genetic and biochemical studies indicate that only one nitrate reductase exists, and that it is a complex of two distinct components: the assimilatory nitrate reductase pathway and the nitrate respiration system (14).

All the chlorate-resistant mutants are defective in their nitrate respiration system, but because of their good growth in NFM and nitrate appear to have retained a functional assimilatory nitrate reductase pathway. However, the pleiotropic mutations in the $chlA^{-}$ and $chlE^{-}$ mutants appear to have perturbated both complexes.

When the same hybrids were grown in NFM + nitrite, generally the Nif^{+} phenotype was completely inhibited, and except for the $chlB^{-}$ mutants, the extent of growth was also reduced (Table 2).

Nitrate inhibition of nitrogenase activity was examined in hybrids made with mutants of *E. coli* K12 defective in particular systems for anaerobic energization of the membrane, such as mutants defective in oxidative phosphorylation, mutants affecting the fumarate-nitrate reductase complex, and quinone mutants important in either fumarate or nitrate reduction, but in all cases nitrate completely inhibited nitrogenase activity. Thus these systems are not required for nitrate inhibition of nitrogenase activity.

The FN68 plasmid did not restore any nitrate reductase activity to chl^{-} *E. coli* K12 hybrids, and thus does not carry functions to complement defects in the nitrate respiratory system.

Two nif^{+} mutants of *K. pneumoniae* strain M5al, *nif*88 and *nif*105, which are probably defective in nitrogenase component II, and which are also defective in nitrate reduction (15), were used as recipients for the FN68 plasmid and were then tested for nitrogen fixation (Table 3). In the *nif*88(FN68) hybrid no acetylene reduction was detected, which indicates that phenotypic expression of the plasmid-borne nif^{+}_{Kp} genes is abolished by the chromosomal nif^{-} mutation. As expected, no acetylene reduction was obtained by this

TABLE 3. Phenotypic expression of nif^{+}_{Kp} genes on plasmid FN68 in mutants of *K. pneumoniae* defective in both nitrogen fixation and nitrate reduction

Hybrid	Medium	Acetylene reduction ($nmol\ C_2H_4/min/mg$ protein)	Growth
nif88(FN68)	NFM	<0.01	100
	NFM + NO_3^-	<0.01	77
	NFM + NH_4^+	<0.01	208
nif105(FN68)	NFM	8.0	100
	NFM + NO_3^-	30.0	98
	NFM + NH_4^+	<0.01	141
M5a1 nif^+	NFM	51.3	100
	NFM + NO_3^-	<0.01	84
	NFM + NH_4^+	<0.01	201

NO_3^- and NH_4^+ were added to NFM in Pankhurst tubes to give final concentrations of 50 mM. Growth was assessed after 24 hr incubation, by measuring turbidity in a nephelometer, and is expressed as a percentage of the NFM control.

hybrid in the presence of either nitrate or ammonia (Table 3). In marked contrast, hybrid nif105(FN68) reduced acetylene at a low level in NFM, but also showed a nitrate-dependent, four-fold stimulation of acetylene reduction (Table 3). This result could not be explained simply on the basis of better growth with nitrate, since growth was similar in NFM with or without nitrate (Table 3). A similar stimulation of nitrogenase activity by nitrate has been reported for soybean bacteroids (16) and also for *Spirillum lipoferum* (17). The F'nif^{+}_{Kp} plasmid was expressed normally in other nif^{-} chl^{S} mutants of *K. pneumoniae*.

These results, using nif^{+}_{Kp} hybrids defective in nitrate respiration,

show that regulation of growth and nitrogenase activity is complex. Nitrate inhibition of nitrogen fixation may be manifested by affecting the proposed functional or organizational relationship between the fumarate and nitrate reductase systems (18) such that competition by electron carriers in the membrane prevents efficient electron transport to fumarate. Without a functional fumarate reduction system, a Nif$^-$ phenotype occurs (19). This interpretation would also explain the results obtained with a chl^R mutant of A. vinelandii (20), where nitrate inhibited the activity but not the formation of nitrogenase. Since the chlA$^-$ and chlB$^-$ hybrids reduced acetylene even in the presence of nitrate, these functions must directly affect the proposed competition by electron carriers. However, the situation is more complicated, since nitrate actually stimulated nitrogenase activity in Klebsiella pneumoniae nif105(FN68) hybrid. The simplest explanation of this result is that the hybrid nitrogenase enzyme complex formed is less able to be activated by the normal electron transport system of Klebsiella, but addition of nitrate presumably affects the proposed competition by electron carriers such that the modified nitrogenase complex can now be fully activated.

The results obtained with chlA$^-$, chlB$^-$ and chlD$^-$ hybrids suggest that nitrate reductase and nitrogenase share Mo-processing functions. This commonality could explain the pleiotropy observed in Rhizobium meliloti (1), where some nitrate reductase mutants also lacked nitrogenase activity. Since these mutants were also altered in their nodulation capacity, it appears that defects in the nitrate respiration pathway may also affect the Rhizobium-plant interactions.

Pagan et al. (4), using nitrate reductase mutants of Rhizobium sp. 32H1, concluded that nitrate probably inhibited nitrogenase activity by its reduction to nitrite, although they did not exclude the possibility that nitrate or nitrite competed with nitrogenase for electrons, thereby lowering nitrogenase activity. These two possibilities could not be distinguished in Rhizobium due to the lack of well-defined mutants available in E. coli

K12, which have allowed a clearer analysis of the problem.

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SECTION 2

When *E. coli* K12 hybrids carrying the F'nif^{+}_{Kp} plasmid were stored in dimethyl sulphoxide (DMSO), a marked effect on expression of the Nif^{+} phenotype was observed. This can probably be explained on the basis of perturbations on the organization of electron carriers within the membrane, such that electron transport to fumarate no longer occurred normally. DMSO was found to cause differential killing of hybrids, and at least four regions on the *E. coli* chromosome was associated with resistance to DMSO. Because the mutants resistant to DMSO were altered in particular inner membrane proteins, the killing effect of DMSO is probably due to membrane damage.

An important group of membrane proteins associated with energy metabolism are the ferredoxins. It is known that DMSO can cause a shift in the absorption spectra of HIPIP, a ferredoxin-like molecule, which is probably due to a conformational change induced in the protein [39]. This is analagous to the change induced in ferredoxins by a variety of denaturing agents, and denaturation of ferredoxin from *Clostridium pasteurianum* by unfolding has also been observed in DMSO [39].

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

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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	$unc405^- \ arg^- \ entA^-$	Cox *et al.* (1974)
	C111	$chlD^{\Delta} \ thi^- \ bio^- \ gal^- \ leu^-$	Venables and Guest (1968)
	C113	$chlD^{\Delta} \ thi^- \ bio^- \ gal^- \ leu^-$	
	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	$(gal \ chlD \ bio)^{\Delta} \ pro^- \ his^-$	Dykhuizen collection
	DD38	$chlG^- \ bio^-$	Dykhuizen (1973)
	DD96	$(att\lambda \ chlA \ bis)^{\Delta} \ his^-$	
	DD182	$(gal \ chlD \ bio \ chlA)^{\Delta}$	Dykhuizen collection
	KS60	$(gal \ chlA)^{\Delta}$	Shimada *et al.* (1972)
	KS649	$(gal \ chlA)^{\Delta} \ his^- \ gnd^- \ som^-$	
	KS650	$(gal \ chlA)^{\Delta}$	
	SB1801	$(his \ gnd \ RHA\text{-}2A)^{\Delta} \ gal^- \ chl^-$	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 $(gnd \ his \ nif \ shu)^{\Delta}$	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, λcI, λcI857 (a heat sensitive *c*I repressor mutant of λ), λvir (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.

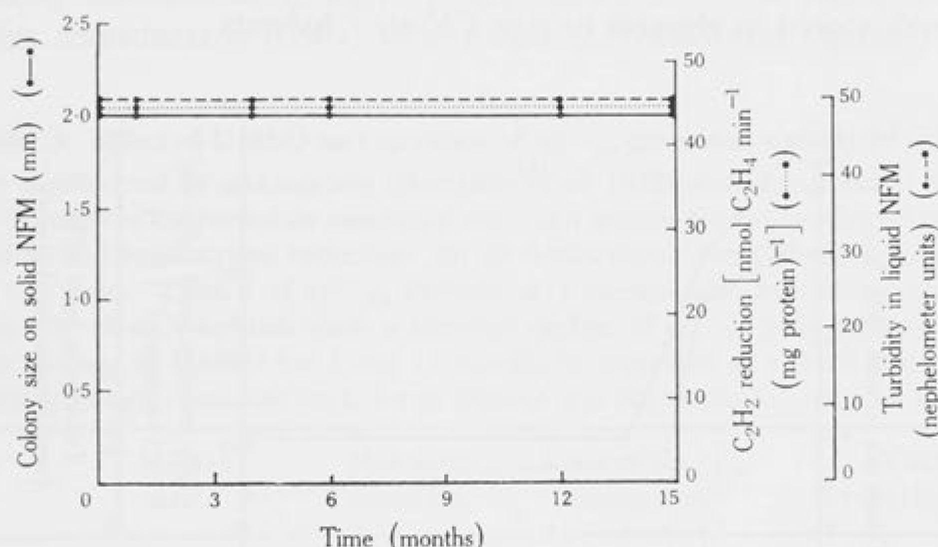


Fig. 1. Expression of *nif* $^{+}_{\mathrm{Kp}}$ genes in *E. coli* K12 hybrid strains over a period of 15 months storage in DMSO was tested by growth on solid NFM under 99% N_2–1% CO_2 (Hill 1973) after 7 days (●——●), by growth in liquid NFM in Pankhurst tubes (Campbell and Evans 1969; Postgate 1972)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

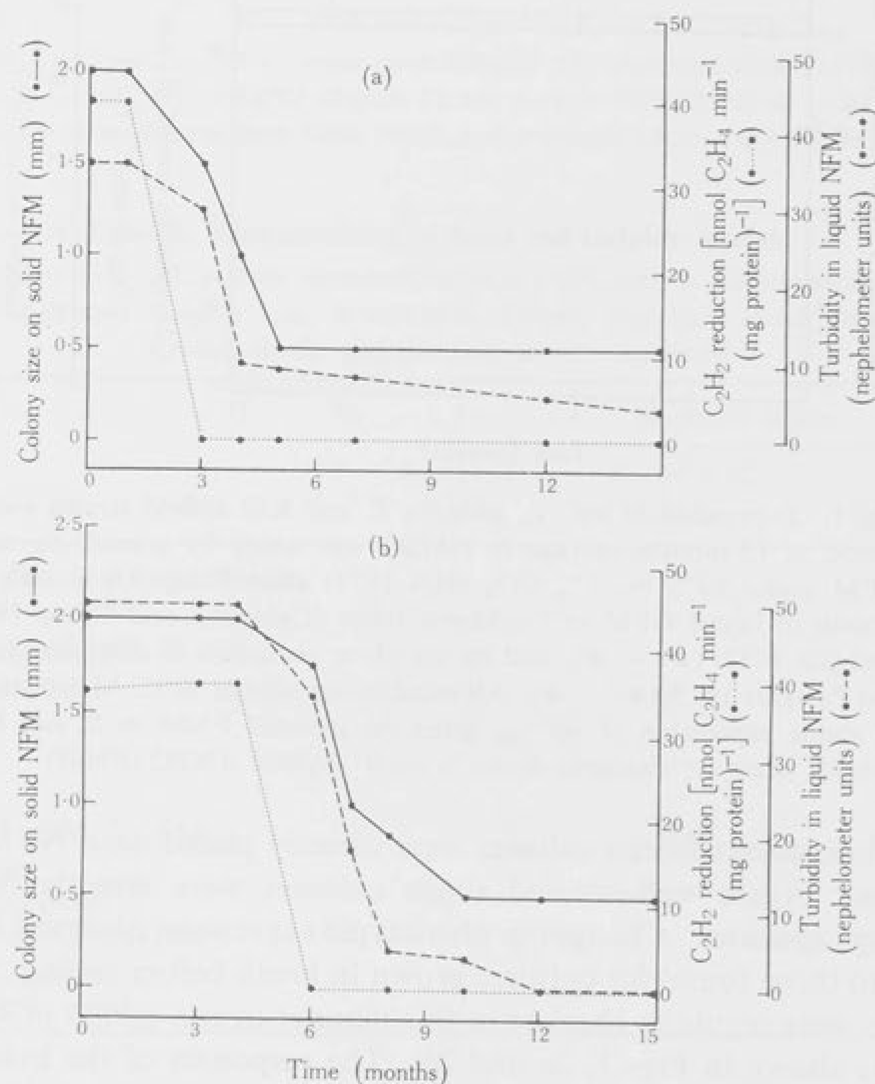


Fig. 2. Gradual decline of expression of *nif*$^+_{Kp}$ genes observed in class II hybrids. The decline is shown first by loss of acetylene reduction, then poor or no growth in liquid NFM, resulting finally in hybrids which can only form small colonies on solid NFM. See Fig. 1 legend for methods. (*a*) 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

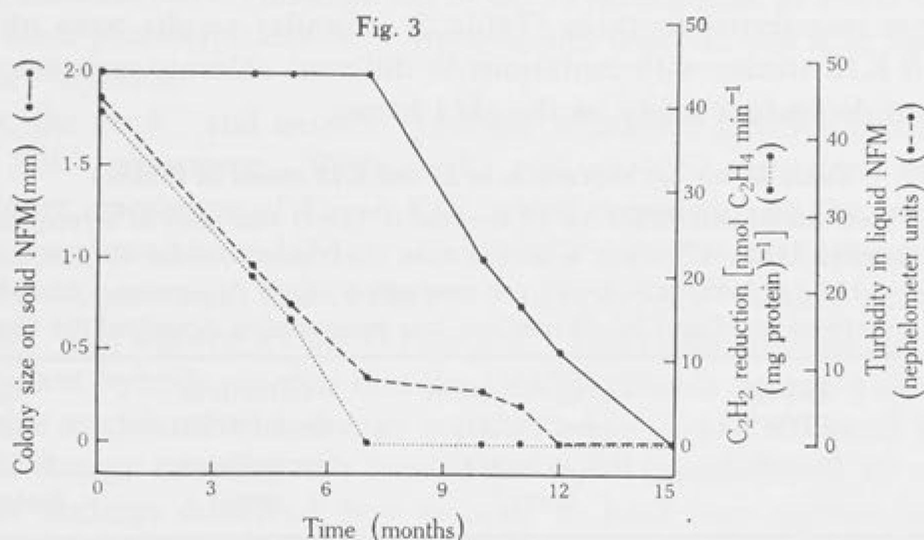
analysis were hybrids RB95 and RB96, which were largely *E. coli*-like in their properties. Hybrids RB95 and RB96 were stored in DMSO and their properties were checked regularly; the results are summarized in Fig. 4. The expression of the transferred nitrogen-fixing capacity from *R. trifolii* in this *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



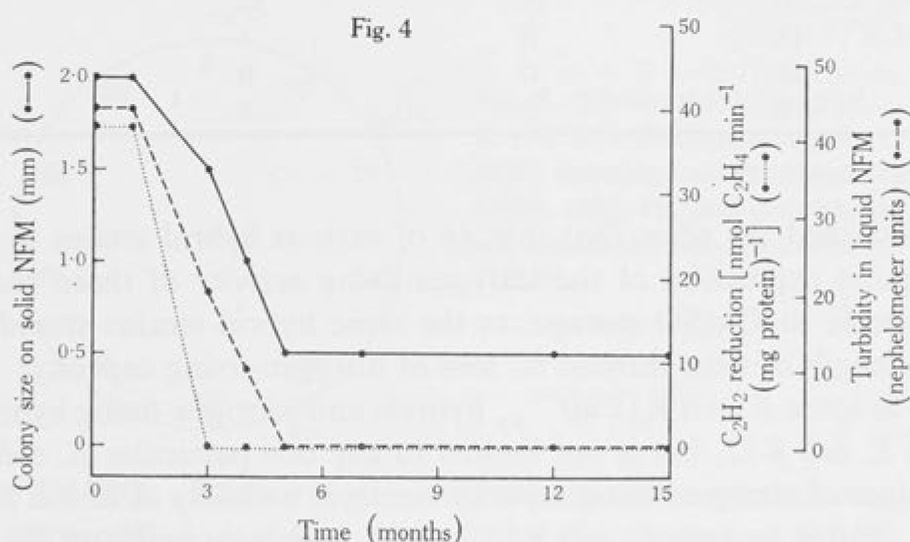


Fig. 3. Decline of expression of nif^{+}_{Kp} genes in transductant strain RB101—an *E. coli* K12 hybrid between C-M7 and *E. coli* K12 strain KS649 with the *his nif*$_{\text{Kp}}$ region stably integrated into the chromosome. This strain was constructed by P1 transduction from host C-M7. At 15 months, 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 $nif^{+}_{\text{Kp}}\,Cb^{\text{R}}$ 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 $unc405^-$ 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 $unc405^-$ 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

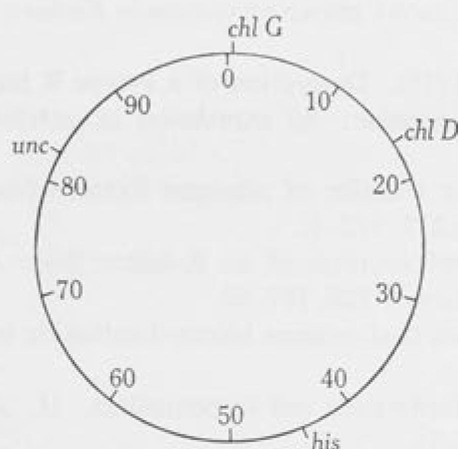


Fig. 5. Chromosomal map of *E. coli* K12 (Bachmann *et al.* 1976) showing four proposed regions (*chlD*, *chlG*, *his*, and *unc*) which are associated with resistance to DMSO.

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).

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Section 3

In attempts to transfer the nif^{+}_{Rt} genes from *R. trifolii* strain T1 to *E. coli* K12, so as to study which biochemical pathways provide energy for rhizobial nitrogen fixation, strain T1K was used. Strain T1K is a reputed derivative of strain T1, carrying a derepressed R plasmid which can mobilize the nif_{Rt} genes [69, 71]. However, although it was possible to transfer a Nif^{+} phenotype from strain T1K to *E. coli* K12, T1K could not have originated from strain T1 as its physiology differed markedly and was not simply due to introduction of the R plasmid. However, the nif^{+} genes transferred from strain T1K were able to replace the complete nif^{+} cluster of genes deleted in *K. pneumoniae* strain Δ17. Therefore, it is conceivable that strain T1K has somehow "cloned" a cluster of nif^{+} genes from another organism such as *Klebsiella*. This might also explain the presence of other genes corresponding to a region of the *E. coli/Klebsiella* chromosome in strain T1K.

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Transfer of Nitrogen Fixation Genes from a Bacterium with the Characteristics of Both *Rhizobium* and *Agrobacterium*

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Strain T1K, reported to be *Rhizobium trifolii* strain T1 carrying the drug resistance plasmid R1-1*drd*, was able to transfer a cluster of *nif*$^+$ genes to *Escherichia coli* K-12. Additional genetic material, resembling the *gal-chlA* region of *E. coli*, was also transferred from strain T1K. The segregation pattern of these transferred genes suggested that they were on a plasmid. Although strain TIK was able to nodulate red and white clover, it also formed very slow-growing galls on tomato stems and shared many physiological properties with *Agrobacterium tumefaciens*, to which it seemed more closely related than to *R. trifolii*. The *R. trifolii* hybrid T1(R1-19*drd*), constructed by conjugation, did not share any of these properties with *A. tumefaciens*. Thus, strain T1K appears to be a bacterium with properties of both *A. tumefaciens* and *R. trifolii* and with the capacity to transfer *nif*$^+$ genes and other functions which it may have "cloned" from another bacterium such as *Klebsiella*.

In 1973, Dunican and Tierney reported transformation of *Rhizobium trifolii* strain T1 with the drug resistance plasmid R1-19*drd* (9). Plasmid R1-19*drd* was first transferred by conjugation from its *Escherichia coli* host to a strain of *Pseudomonas aeruginosa*. The DNA was extracted from this hybrid and was then used to transform *R. trifolii* T1. After selection for kanamycin resistance (determined by the R1-19 plasmid), a hybrid, T1K, was obtained. This hybrid had also acquired the other drug resistances determined by the R1-19 plasmid, ampicillin and chloramphenicol (9).

After irradiation of T1K with ultraviolet light to promote recombination between R1-19*drd* and nif^+_{Rt} genes (nitrogen-fixing genes from *R. trifolii*), T1K was used as the donor in conjugation experiments with recipient *Klebsiella aerogenes* strain 418 *rif* (10). Dunican reported successful cotransfer of the R1-19 plasmid and nif^+_{Rt} genes, which were directly selected for in the *K. aerogenes* recipient. In two experiments, transfer frequencies of about 10^{-5} for Km^r and 10^{-6} to 10^{-7} for Km^r *nif*$^+$ were obtained (10).

Dunican et al. concluded from these results that *R. trifolii* does possess all the necessary genetic material for nitrogen fixation, and that such genes may be located on a resident plasmid in *R. trifolii* strain T1 (8).

Although *K. aerogenes* is closely related to *E. coli*, it has been much less widely studied and is therefore less well genetically characterized than *E. coli*. Since *nif*$^+$ genes could be transferred from T1K to *K. aerogenes*, it seemed probable that similar transfer could be obtained with *E. coli* recipients with the advantage that in this background the control of nif^+_{Rt} genes could be studied by methods similar to those used for nif^+_{Kp} genes (nitrogen-fixing genes from *K. pneumoniae*) (28).

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used are listed in Table 1, and bacteriophages and bacteriocins are listed in Table 2. One particular strain of *E. coli* K-12, strain KS650 (deleted in the *gal-chl* region), was chosen as a recipient to test whether *nif*$^+$ genes could be transferred from strain T1K and expressed in this background. Three separate isolates of strain T1K were obtained from Dunican, and all behaved similarly on all occasions.

Media and growth conditions. Luria broth with glucose (LBG) and eosin-methylene blue medium (EMB) have been described previously (20). Minimal medium (MM) was that of Davis and Mingioli (5). Nitrogen-free medium (NFM) was that of Cannon et al. (4). The *Rhizobium* medium used was Bergersen's modified medium (BMM) (2).

Antibiotics were added to media as freshly prepared, filter-sterilized solutions. Octopine was kindly provided by J. Schell since nopaline was unavailable.

All cultures were grown at 30°C.

Tests for nitrogen fixation. Cultures were grown to mid-log phase in rich media, washed in saline phosphate buffer (4), and either plated on solid NFM and incubated under continuously flowing 99% N_2-1% CO_2 (15), or inoculated (0.25 ml) into 10 ml of NFM in anaerobic Pankhurst tubes (3, 22) with or without 100 μg of Casamino Acids per ml.

TABLE 1. *Bacterial strains used*

Bacterium	Strain	Characteristics[a]	Source
R. trifolii	T1	Prototroph; nodulates white, red, and subterranean clovers effectively	E. A. Schwinghamer
	T1K	Nodulates white clover effectively and red clover ineffectively	L. K. Dunican
K. aerogenes	418 *rif*	*rif* recipient used by Dunican in matings with T1K	F. C. Cannon
K. pneumoniae	M5a1	nif^{+} prototroph	F. C. Cannon
K. pneumoniae	Δ17	Δ*nif* Δ*his* Δ*shu* mutant of M5a1	R. C. Valentine
E. coli K-12	KS650	Δ(*gal-chl*) *his*	M. Gottesman
	SA291	Δ(*gal-chlA*) *his str*	D. Dykhuizen
	J53(R1-19)	Carries R1-19 Km^r Ap^r Cm^r plasmid used by Dunican to transform T1	N. Datta
A. tumefaciens	B6S3	oct^{+} prototroph	J. Schell
	C58	nop^{+} prototroph	J. Schell

[a] Genetic symbols: *gal* (ability to ferment galactose), *chl* (defective in nitrate reductase and thus resistant to chlorate), *uvrB* (resistance to ultraviolet irradiation), *att*λ (attachment site for phage λ), *nif* (nitrogen fixation), *his* (histidine biosynthesis), *oct* (ability to use octopine), *nop* (ability to use nopoline), Δ (deletion). The deletion of strain KS650 was shown to extend into *chlA*.

TABLE 2. *Bacteriophages and bacteriocins used*

Phage or bacteriocin	Host bacterium	Source
Bacteriophage		
λ^{+}	*E. coli*	B. G. Rolfe
P1	*E. coli*	B. G. Rolfe
φ80	*E. coli*	B. G. Rolfe
T4	*E. coli*	B. G. Rolfe
φT10	*R. trifolii* strain T1	E. A. Schwinghamer
Tr8	*R. trifolii*	E. A. Schwinghamer
GS1	*A. tumefaciens*	J. Schell
GS2	*A. tumefaciens*	J. Schell
GS5	*A. tumefaciens*	J. Schell
GS6	*A. tumefaciens*	J. Schell
GS18	*A. tumefaciens*	J. Schell
ψ	*A. tumefaciens*	J. Schell
Bacteriocin		
S1005	*A. tumefaciens*	J. Schell
K84	*A. tumefaciens* nop^{+}	A. Kerr
T24	*R. trifolii*	E. A. Schwinghamer

Nitrogenase activity was measured as reduction of acetylene to ethylene by Pankhurst tube cultures, by the method of Tubb and Postgate (29).

Phage sensitivity and lysogeny experiments. Sensitivity to phages was tested by spotting the phage suspensions onto test bacteria poured in soft agar on LBG or BMM plates. Zones of clearing and single plaques for diluted phage suspensions indicated sensitivity of the bacterium to the phage.

Bacteriocin sensitivity was determined in a similar manner, with clear zones indicating sensitivity to the particular bacteriocin.

Lysogeny of *E. coli* K-12 strains and hybrids by phage λ was determined by the method of Miller (20).

Nodulation and gall formation tests. Nodulation of white, red, and subterranean clovers was tested by the method of Vincent (32).

Gall formation on *Datura*, tomato (*Lycopercison esculentum* cultivar Grosse Lisse), peas, French beans, wheat, maize, and sorghum was tested by dipping a sterile needle into a washed overnight culture of the test bacterium and then stabbing the needle through the stem of the test plant. Gall formation was first visible after about 2 weeks, and the galls continued to grow from then on.

Conjugation between T1K and *E. coli* K-12 strain KS650. T1K and *E. coli* K-12 strain KS650 were grown to mid-log phase in LBG at 30°C. The donor strain, T1K, was irradiated with ultraviolet light (80% kill) and mixed with recipient KS650 in the ratio 1 part T1K (3×10^8 cells/ml)-9 parts KS650 (3×10^8 cells/ml)-9 parts fresh broth. The mixture was incubated without shaking at 30°C for 4.5 h. The cells were washed twice in saline phosphate buffer, plated on NFM with biotin (2 μg/ml), and incubated under a nitrogen atmosphere for 5 days at 30°C. Presumptive nitrogen-fixing clones, which arose at a frequency of about 10^{-7}, were picked and purified by restreaking twice on similar medium.

RESULTS

Characteristics of hybrids. Conjugation experiments between strain T1K and *E. coli* K-12 strain KS650 yielded nitrogen-fixing hybrid clones at a low frequency (about 10^{-7}) on NFM under anaerobic conditions (27). Two examples of these hybrid clones which were picked for more detailed analysis were hybrids RB95 and RB96. The criteria for classifying these hybrids as *E. coli* rather than *R. trifolii* are listed in Table 3.

Like several other T1K × KS650 hybrids tested, both hybrids RB95 and RB96 grew well on solid and liquid NFM under N_2; both reduced acetylene at rates comparable to that of the standard strain *K. pneumoniae* M5a1 (Table 4). None of these properties was observed for recipient strain KS650, so it was concluded that *nif*$^+$ genes could be transferred from strain T1K to *E. coli* K-12 and give full expression, in the absence of any plant material.

On further examination of these hybrids, it was found that whereas KS650 had required biotin for growth, they no longer needed biotin. Since the *bio* gene is deleted in KS650, it could not have reverted in the hybrids and therefore could only have been acquired from strain T1K during conjugation. Other genes deleted by the same mutation that removed the *bio* gene in strain KS650 are *gal chlD pgl att*λ *bio uvrB chlA*. Thus, hybrids RB95 and RB96 were examined to see whether any of these other deleted genes had also been acquired by conjugation. Both hybrids were found to be *gal*$^+$ *chl*$^+$ *bio*$^+$ and *uvr*$^+$. For hybrids RB95 and RB96 to regain sensitivity to chlorate, they had to acquire functions equivalent to the *chlD*$^+$ and *chlA*$^+$ loci from strain T1K.

Since the genes acquired by hybrids RB95 and RB96 correspond so closely to the chromosomal region deleted in recipient KS650, tests were carried out for another locus in this region (the integration site for phage λ). If the λ integration site (*att*λ) is present, lysogens of *E. coli* K-12 strain HfrH can be made at a frequency of about 61%. However, in KS650, which is derived from HfrH but deleted for *att*λ, the frequency of lysogeny is only 0.05% (Table 5). When tested for ability to lysogenize phage λ, both hybrids RB95 and RB96 had greatly increased frequencies of lysogeny of 15 and 27%, respectively (Table 5), indicating that they had also acquired an *att*λ site from strain T1K. Thus a completely unrelated species (supposedly *R. trifolii*) possessed an attachment site for a bacteriophage specific for *E. coli*.

Hence, a region equivalent to the *gal-chlA* chromosomal region deleted in KS650 and amounting to about 15 genes in length, had been acquired in hybrids RB95 and RB96 from strain T1K by conjugation.

Other genes acquired from strain T1K were those determining resistance to the drugs ampicillin, streptomycin, chloramphenicol, and kanamycin. Resistance to a variety of other drugs, including tetracycline, erythromycin, and rifampin was not observed. These acquired resistances correspond to those of strain T1K and are presumably carried on the plasmid R1-19 (9).

Another characteristic of hybrids RB95 and RB96 was their inability to grow on rich media at 42°C. Whereas KS650 grows well at both 30 and 42°C, strain T1K can only grow at 30°C. Incubation at 42°C has a bacteriostatic effect on T1K: if a plate is incubated for several days

TABLE 3. *Characteristics of donor and recipient strains*

Characteristic	Bacterial strain			
	T1K	*E. coli* KS650	Hybrids	
			RB95	RB96
Excessive production of polysaccharides	+	−	−	−
Urease activity	+	−	−	−
Sensitivity to phages:				
λ, φ80, T4, P1	−	+	+	+
Indole formation	−	+	+	+
Fermentation of:				
Glucose	−	+	+	+
Melibiose	−	+	+	+
Growth on NFM under N_2	−	−	+	+
Acetylene reduction	−	−	+	+
Resistance to drugs:				
Kanamycin (20 μg/ml)	+	−	+	+
Chloramphenicol (12.5 μg/ml)	+	−	+	+
Ampicillin (50 μg/ml)	+	−	+	+
Streptomycin (250 μg/ml)	+	−	+	+

TABLE 4. *Expression of nitrogen fixation genes from T1K in E. coli K-12 hybrids RB95 and RB96*

Determination	T1K	*E. coli* K-12 KS560	Hybrids		*K. pneumoniae* M5a1[a]
			RB95	RB96	
Growth on solid NFM[b]	0	0	2	2	2
Growth in liquid NFM[c]	0	0	45	43	60
Acetylene reduction (nmol of C_2H_4/min per mg of protein)	<0.01	<0.01	38.0	40.1	51.0

[a] *K. pneumoniae* strain M5a1 is included as an *nif*$^+$ standard strain.
[b] Colony diameter (millimeters) after 5 days of incubation under N_2 at 30°C.
[c] Turbidity in Pankhurst tubes (nephelometer units) after 19 h of incubation.

at 42°C, there is no growth, but on further incubation at 30°C normal growth occurs. Both RB95 and RB96 behaved like T1K in this respect, although on each occasion tested a few colonies per plate grew at 42°C, as though segregation of the gene or genes responsible for 42°C inhibition was taking place.

Segregation of transferred genes in hybrids RB95 and RB96. Further examination of the ability to grow at 42°C showed that segregation of the "heat inhibition" marker was dependent on the previous growth condition and on the particular hybrid. For example, if RB96 was streaked on NFM and single colonies from this medium were then tested for their ability to grow at 42°C, all colonies were inhibited; i.e., they had the heat inhibition marker. If, however, this hybrid was grown first on EMBgal, then almost all colonies tested grew at 42°C, indicating loss or segregation of the heat inhibition marker.

Since segregation of the heat inhibition marker was occurring, hybrids RB95 and RB96 were examined for segregation of other genes acquired from strain T1K.

Hybrids were grown in liquid NFM in Pankhurst tubes to select for those cells still *nif*$^+$, and then these cells were plated onto different media under various growth conditions. Both hybrids RB95 and RB96 showed segregation of all known acquired genes from donor strain T1K. The frequency of loss of individual acquired markers depended on the previous growth medium on which the hybrid was plated (Fig. 1). This segregation was so frequent that the genes in question were most probably located on a plasmid rather than incorporated into the bacterial chromosome.

To test whether genes were permanently lost by segregation, *gal* colonies of RB96 that had arisen from a *gal*$^+$ culture were restreaked several times onto EMBgal media at 30°C. On no occasion did any *gal*$^+$ revertant colonies grow, indicating permanent loss of the *gal*$^+$ genes by segregation.

Characteristics of plasmid R1-19*drd*. It seemed strange that an organism, supposedly a strain of *R. trifolii*, should possess a piece of DNA with a possible gene order similar to that of the phylogenetically distant bacterium *E. coli*. One possibility was that the plasmid R1-19*drd* already carried the *gal-chlA* region from *E. coli* K-12 before it was transferred into strain T1 of *R. trifolii*. This possibility was eliminated by mating plasmid R1-19*drd* from the original *E. coli* K-12 strain J53 used by Datta (9) into strain SA291, another *E. coli* K-12 strain deleted between the *gal* and *chlA* loci. However, there was no evidence for cotransfer of any of the *gal-chlA* region by plasmid R1-19*drd*, although the plasmid was transferred to this recipient strain as detected by transfer of drug resistance markers (Table 6).

Retransfer of Nif$^+$ phenotype from hybrid RB96. A conjugation experiment was set

FIG. 1. *Segregation of genes in hybrid RB96. RB96 was first grown in liquid NFM and then streaked onto different media under various conditions as described in the figure. Single colonies from each medium were then tested for the presence of each of the genes listed in the figure. Between 40 and 100 single colonies were picked from each medium and resuspended in small amounts of saline phosphate buffer. Drops of each suspension were then spotted onto media to test for presence or absence of genes. All plates were incubated at 30°C except for those testing for the heat inhibition marker, which were incubated at 42°C. Symbols: solid line, gene present; dotted line, gene absent. amp, Ampicillin; str, streptomycin; kan, kanamycin; cml, chloramphenicol; HK, heat killing or inhibition.*

TABLE 5. *Frequency of lysogeny by phage λ in E. coli K-12 and hybrids*

E. coli K-12 host	No. of lysogens/100 infected cells
HfrH	61
KS650	0.05
RB95	15
RB96	27

TABLE 6. *Properties of a Δ(gal-chlA) strain of E. coli K-12, SA291, containing drug resistance plasmid R1-19drd*

Property	SA291	SA291 (R1-19*drd*)
Fermentation of galactose	–	–
Sensitivity to chlorate	–	–
Growth without biotin	–	–
Resistance to ultraviolet light	–	–
Resistance to drugs:		
Kanamycin (20 μg/ml)	–	+
Chloramphenicol (12.5 μg/ml)	–	+
Ampicillin (50 μg/ml)	–	+

up between hybrid RB96 and deletion strain Δ17 to check whether a cluster of *nif*$^+$ genes had been transferred to KS650 from strain T1K, or whether there was complementation between genetic material transferred from strain T1K and genes of the *E. coli* chromosome. Strain Δ17 is a derivative of *K. pneumoniae* M5a1, deleted for the *his-nif* gene cluster-*shu* region of the chromosome (26). Since this strain cannot revert to Nif$^+$, any Nif$^+$ exconjugants from the mating experiments with hybrid RB96 must be derived by transfer of *nif*$^+$ genes from the donor.

Strains RB96 and Δ17 were grown to mid-log phase in LBG at 37°C, mixed and incubated statically for 7 h at 37°C, washed in saline phosphate buffer, and plated on NFMhis with 0.5% citrate as sole carbon source (*K. pneumoniae* but not *E. coli* can use citrate as sole carbon source [17]). Nif$^+$ exconjugants were obtained at a frequency of about 10^{-5}, indicating a transfer of a *nif*$^+$ cluster of genes from hybrid RB96.

Nodulation of T1K × KS650 hybrids. Eleven hybrids from the T1K × KS650 cross, including RB95 and RB96, were tested for nodulation by the method of Vincent (32). Strain T1K nodulated white clover effectively and red clover ineffectively, did not nodulate subterranean clover, and could be reisolated from white clover nodules. Strain KS650 and all the hybrids tested did not nodulate any of the three species of clover, so that either the genes responsible for nodulation were not transferred from strain T1K or else they could not be expressed in the background of *E. coli* K-12 strain KS650.

Properties of strain T1K. Because strain T1K apparently possessed genes similar to those on the *E. coli* chromosome, in particular, a lambdoid phage attachment site, this strain was investigated in detail.

R. trifolii strain T1 requires biotin for growth and is unable to grow on a wide range of media used for enterobacteria, e.g., LBG, EMB, MM, and NFM. Strain T1K, however, no longer required biotin for growth and was able to grow on all media tested, including LBG, EMB, and MM (Table 7). It was unable to grow on NFM under N_2 (the growth conditions used for the conjugation experiment with KS650) but did grow on NFM plates under 99% N_2-1% CO_2 or under air. *Agrobacterium tumefaciens* strains B6S3 and C58 behaved like strain T1K on NFM under the various atmospheres, whereas *R. trifolii* strain T1 did not grow on NFM under any condition. However, since no acetylene reduction by strain T1K, or any *A. tumefaciens* strain, could be detected, the mucoid translucent growth under these conditions was presumably due to scavenging of trace amounts of nitrogen from the agar. Moreover, strain T1K did not grow in liquid NFM under air, N_2, or 99% N_2-1% CO_2 (neither did strain T1 nor any *A. tumefaciens* strain).

Thus strain T1K differs greatly from strain T1 in its ability to grow on various media, and it is difficult to see how a simple transformation of strain T1 with R1-19*drd* plasmid DNA could lead to such radical alterations.

To check whether strain T1K was really equivalent to strain T1 containing the plasmid R1-19*drd*, a conjugation experiment to transfer plasmid R1-19*drd* into strain T1 was attempted. *E. coli* K-12 strain J53 (R1-19*drd*) was grown in LBG at 37°C to mid-log phase and was mixed 1:1 with strain T1 grown in BMM at 30°C to mid-log phase. One part BMM and one part LBG was added to the mating mixture, which was then incubated without shaking at 30°C for 7 h. The cells were washed in saline phosphate buffer and plated on *Rhizobium* minimal medium (16) plus kanamycin (20 μg/ml) and incubated at 30°C for 3 days. Recombinants arose at a frequency of 2×10^{-6}, and several were

TABLE 7. *Comparison of R. trifolii strain T1 with strains T1K and T1 (R1-19drd)*

Property	T1	T1K	T1 (R1-19*drd*)
Growth on:			
BMM	+	+	+
LBG	–	+	–
EMBgal	–	+	–
MM	–	+	–
NFM	–	+	–
Requirement for biotin	+	–	+
Resistance to drugs:			
Kanamycin (20 μg/ml)	–	+	+
Chloramphenicol (12.5 μg/ml)	–	+	+
Ampicillin (50 μg/ml)	–	+	+

picked and purified on the same medium. Four potential strain T1(R1-19*drd*) colonies were further tested, and their properties are summarized in Table 7. Except for resistance to drugs determined by plasmid R1-19*drd*, strain T1(R1-19*drd*) colonies retained all the characteristics of strain T1 and did not acquire any properties characteristic of strain T1K.

Thus, unless the R1-19*drd* plasmid picked up some DNA from *E. coli* or *P. aeruginosa* before being used to transform *R. trifolii* strain T1, strain T1K could not have arisen as a T1(R1-19*drd*) derivative.

Strains T1, T1(R1-19*drd*), and T1K were tested for sensitivity to phages Tr8 and T10. Phage Tr8 is a general *Rhizobium* phage, whereas T10 is specific for strain T1 (25). Strains T1 and T1(R1-19*drd*) were sensitive to both of these phages, but strain T1K was not (Table 8). Because strain T1K appeared similar to *A. tumefaciens* on morphological and physiological criteria, it was tested for sensitivity to several *Agrobacterium* phages (31). Unlike strains T1 and T1(R1-19*drd*), which were resistant to all the *Agrobacterium* phages, strain T1K was sensitive to phage GS18, forming clear plaques (Table 8). Phage GS18 was thought to be specific for strain Kerr 14, a nopaline-utilizing strain of *A. tumefaciens* known to have at least one "silent" plasmid besides the tumor-inducing plasmid (31; Schell, personal communication).

Although strain T1K effectively nodulated white clover and ineffectively nodulated red clover, it appeared to be much more closely related to *A. tumefaciens* than to *R. trifolii.* For this reason, strain T1K was tested for its ability to form crown gall on a range of plants and for other *Agrobacterium*-like properties.

It was found that strain T1K induced very slow-growing galls when stabbed through stems of tomato plants, but did not induce galls on *Datura,* peas, French beans, or wheat, maize, and sorghum (Fig. 2). Strain T1K could be reisolated from galls on tomato by surface sterilizing a piece of gall tissue in 5% sodium hypochlorite, crushing the tissue in sterile water, and plating on LBG or BMM. The cells retained sensitivity to phage GS18 and could still effectively nodulate white clover.

Octopine-utilizing strains of *A. tumefaciens* grow well on octopine as sole carbon and nitrogen source. Nopaline-utilizing strains can give rise to mutants constitutive for nopaline utilization; such mutants can also use octopine as sole carbon and nitrogen source (19). Since strain T1K gave rise to octopine-utilizing mutants at a low frequency above a background of octopine nonutilizers, this is indicative of strain T1K being a nopaline-utilizing strain.

TABLE 8. *Sensitivity of T1, T1K, and T1 (R1-19drd) to phages*

Phage	Specific for bacteria:	T1	T1K	T1 (R1-19*drd*)
Tr8	*R. trifolii*	+	−	+
T10	*R. trifolii* strain T1	+	−	+
GS1	*A. tumefaciens*	−	−	−
GS2	*A. tumefaciens*	−	−	−
GS3	*A. tumefaciens*	−	−	−
GS5	*A. tumefaciens*	−	−	−
GS6	*A. tumefaciens*	−	−	−
GS18	*A. tumefaciens*	−	+	−
λvir, λ^+	*E. coli*	−	−	−
$\phi 80$	*E. coli*	−	−	−
P1	*Enterobacteriaceae*	−	−	−

Another test for nopaline-utilizing *A. tumefaciens* strains is their sensitivity to agrocin 84 (11, 18, 31). Strain T1K was found to be sensitive to agrocin 84 at a level similar to that for strain C58, a nopaline-utilizing strain of *A. tumefaciens.* Strain T1K was also sensitive to agrocin S1005, another bacteriocin specific for *A. tumefaciens* (11).

R. trifolii strain T1, unlike strain T1K, did not give rise to octopine-utilizing mutants, did not induce galls on any similar test plants, and was not sensitive to agrocin S1005, although it was very slightly sensitive to agrocin 84.

DISCUSSION

These findings show that genetic information coding for a Nif^+ phenotype (ability to grow on NFM under N_2 and a capacity to reduce acetylene) can be transferred at a low frequency from strain T1K to *E. coli* K-12. Since this Nif^+ phenotype can be retransferred to a *nif* deletion mutant of *K. pneumoniae,* a cluster of nif^+ genes was transferred from strain T1K to *E. coli* strain KS650 rather than there being a complementation between some *nif* genes from strain T1K and some genes from *E. coli.* The same situation could also apply to the hybrids which occurred in conjugation experiments between strain T1K and *K. aerogenes* strain 418 rif^r described by Dunican and Tierney (10).

It was also found that additional genetic material, resembling the *gal-chlA* region of *E. coli,* was cotransferred with the nif^+ genes from strain T1K to *E. coli* K-12. When examined, both hybrids RB95 and RB96 showed segregation of all their known acquired genes. However, which genes were lost depended on the previous growth medium on which the hybrids were grown. Drug resistance markers were most frequently lost, whereas the *bio* genes were usually retained. The segregation pattern indicates that these ac-

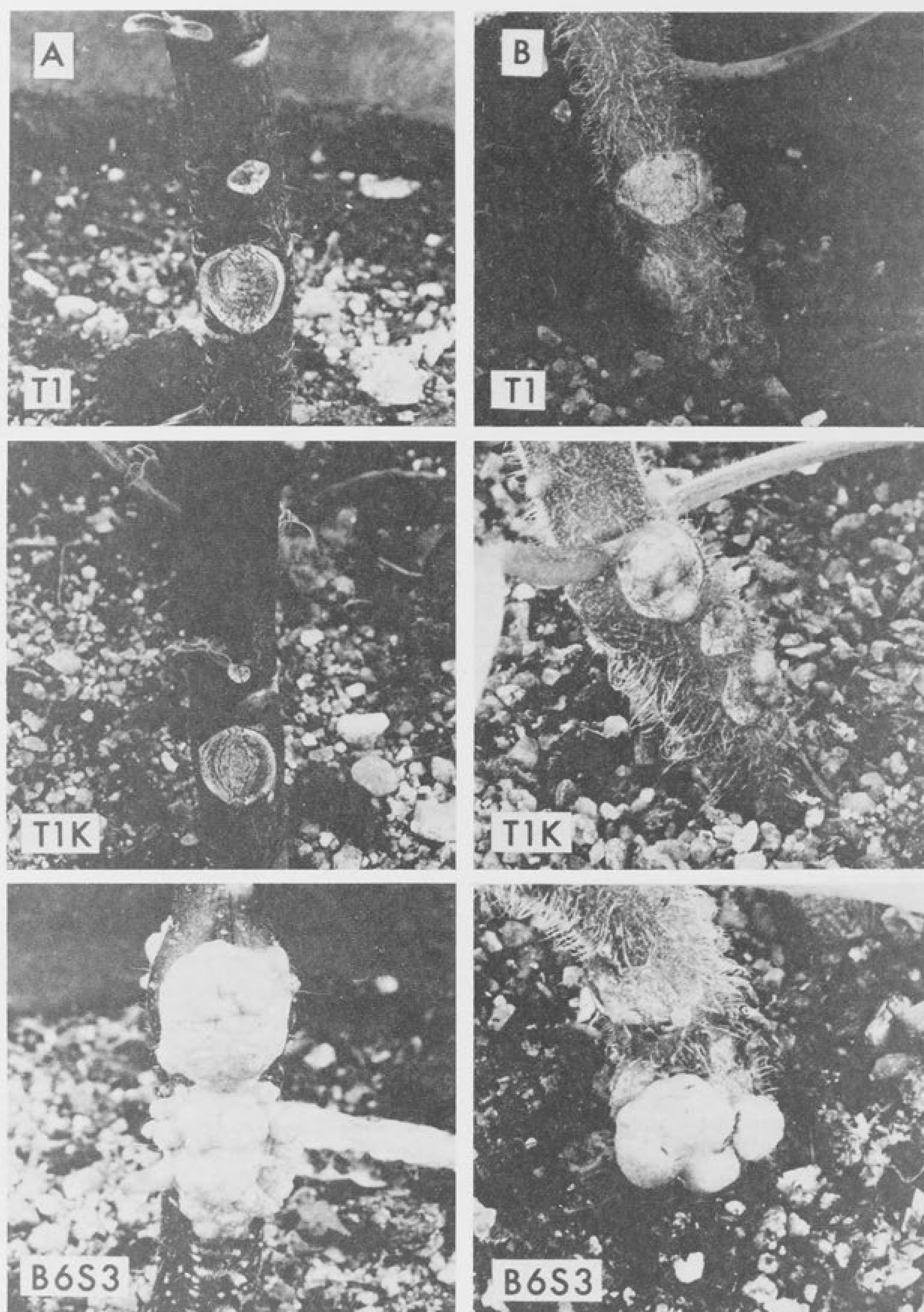

FIG. 2. *Induction of galls on (A) Datura and (B) tomato by R. trifolii strains T1 and T1K, and A. tumefaciens strain B6S3. T1 did not induce galls to form on Datura or tomato; T1K induced very slow-growing galls on tomato only. B6S3 induced galls on both Datura and tomato.*

quired genes were on a plasmid and suggests a gene order similar to that of the *gal-chl*A region of *E. coli*. It is conceivable that strain T1K has somehow "cloned" a cluster of *nif*$^+$ genes and other functions from another organism such as *Klebsiella*. Such an explanation would account for the other genes corresponding to the *gal-chl*A region of the *E. coli/Klebsiella* chromosome in strain T1K.

The findings described here show that strain

T1K is not a derivative of *R. trifolii* strain T1, but that it is a very interesting bacterium having characteristics of both *Agrobacterium* and *Rhizobium.* Many comments on the similarities of the genera *Rhizobium* and *Agrobacterium* have been made, and it has even been suggested that the fast-growing group of rhizobia should be amalgamated with *Agrobacterium* to form a single genus, *Rhizobium,* leaving the slow-growing rhizobia in a separate genus, the *Phytomyxa* (7, 13, 14, 21). *Agrobacterium* and the fast-growing rhizobia share many characteristics not found in the slow-growing rhizobia, such as similar guanine-cytosine ratios and utilization of the same carbohydrates and nitrogen sources (6, 13, 14, 34), as well as sharing a common phage (24). The main distinguishing characteristic is that whereas *Agrobacterium* forms galls and related diseases on stems of a wide range of dicotyledonous plants, *Rhizobium* forms nodules on a restricted range of legumes. The ability to form crown gall (and possibly also to nodulate) is believed to be coded for by genes carried on extrachromosomal plasmids in these bacteria (12, 19, 31), and loss of the plasmid leads to loss of ability to form crown gall (30, 33). Strain T1K, however, appears to be a bacterium bridging the gap between the fast-growing rhizobia and the agrobacteria. It is thought to have at least one plasmid (8) and may contain others. It is conceivable that the so-called silent plasmids found in many strains of *A. tumefaciens* at one stage in evolution allowed such strains to nodulate. Although plasmids of *Rhizobium* have not been examined in great detail, it is also possible that similar silent tumor-inducing plasmids could exist in some strains. A recent report has shown that it is possible to construct a gall-forming, nodulating hybrid bacterium of *R. trifolii* by transfer of the tumor-inducing plasmid from *A. tumefaciens* (16).

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SECTION 4

In order to develop a defined minimal medium which would allow good growth of *R. trifolii* strain T1 on glucose and TCA cycle intermediates without production of large amounts of extracellular polysaccharide, the physiology of this strain was investigated. This study allowed the development of such a suitable medium (TM), and also revealed that strain T1 may have a defective TCA cycle, possibly due to an altered NAD:NADH ratio. In many bacteria the enzymatic reactions leading to the synthesis of citrate, oxaloacetate, malate and pyruvate are inhibited by α-ketoglutarate [239]. Moreover, the enzyme citrate synthetase which makes citrate from acetyl CoA and oxaloacetate (first step of the TCA cycle) can also be inhibited by α-ketoglutarate [239]. It may be that to set up the nitrogen-fixing phenotype of the bacteroid within the nodule, strain T1 has to maintain a high reducing potential and elevated levels of NADH which could consequently maintain a control of the TCA cycle.

MICROBIOS (in press, 1977)

DIFFERENTIAL STIMULATION AND INHIBITION OF GROWTH OF *RHIZOBIUM TRIFOLII* STRAIN T1 AND OTHER *RHIZOBIUM* SPECIES BY VARIOUS CARBON SOURCES

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ABSTRACT

The physiological properties of *Rhizobium trifolii* strain T1 were studied in detail, since this strain has many useful characteristics and appears ideal for development as a reference strain for *R. trifolii*. Some tricarboxylic acid cycle intermediates and related compounds were found to stimulate growth in the presence of sucrose and arabinose, while others inhibited growth partially or completely. Other *R. trifolii* strains behaved likewise. Moreover, similar responses were also observed with other *Rhizobium* species, both fast-growing and slow-growing.

On the basis of these growth responses, the various species of fast-growing and slow-growing rhizobia could be differentiated. Of the fast-growers tested, *R. trifolii* and *R. leguminosarum* are much more closely related to each other than either is to *R. meliloti*. Similarly, the slow-growing cowpea rhizobia are more closely related to *R. japonicum* than either group is to *R. lupini*. It is proposed that strain T1 should be developed as the reference strain for *Rhizobium trifolii*.

INTRODUCTION

The rhizobia can be divided into two major groups, the 'fast-growers' and the 'slow-growers' (Bergey, 1974). Although the fast-growing and slow-growing rhizobia share the common characteristic of nodulation of legumes, in other properties, including DNA homology, physiology and morphology, the two groups are very different.

The fast-growing rhizobia include the species *Rhizobium trifolii*,

R. meliloti, *R. phaseoli* and *R. leguminosarum*, which in general produce acid and grow to a dense culture in 2-3 days. *R. trifolii* forms nodules on clover, *R. meliloti* on lucerne, *R. phaseoli* on beans and *R. leguminosarum* on peas (Bergey, 1974). Of all the fast-growers, only one strain of *R. leguminosarum* has so far been demonstrated to fix N_2 in the free-living state (Kurz and La Rue, 1975). Of the slow-growing, alkali-producing rhizobia, strains of both *R. japonicum* and the 'cowpea' group of *Rhizobium* have been shown to have the ability to fix N_2 in the free-living state (Keister, 1975; Kurz and La Rue, 1975; McComb *et al.*, 1975; Pagan *et al.*, 1975; Tjepkema and Evans, 1975; Gibson *et al.*, 1976).

Until now the lack of a good system for genetic mapping of defined mutants in *Rhizobium* has prevented a genetic analysis of the nitrogen-fixing symbiosis between *Rhizobium* and legumes. Recently, genetic linkage maps have been reported for the fast-growers *R. leguminosarum* and *R. meliloti* (Beringer and Hopwood, 1976; Meade and Signer, 1977; Kondorosi *et al.*, 1977). It has also been demonstrated by genetic mapping that *R. trifolii* and *R. leguminosarum* are more closely related to each other than either is to *R. meliloti*, and that *R. trifolii/R. leguminosarum* hybrids can be constructed (Johnston and Beringer, 1977; Meade, personal communication). However, because nodulation tests for *R. trifolii* on clover plants are simpler and more rapid than tests on lucerne and peas, *R. trifolii* appears to be more suitable than *R. leguminosarum* or *R. meliloti* for studying the mechanisms of host specificity and the genetics of symbiosis.

We chose *R. trifolii* strain T1 for development as a reference strain for this species because it grows very rapidly, nodulates a relatively braod range of clovers, is sensitive to specific and general bacteriocins and phages, and had been reported as able to transfer nitrogen fixation genes by means of an enterobacterial drug-resistance plasmid (Dunican and Tierney, 1974; Schwinghamer, 1970; Schwinghamer and Belkengren, 1968).

Because strain T1 had not been shown to fix nitrogen outside the nodules, attempts were made to devise a medium (based on those used successfully for slow-growing rhizobia) which would induce expression of nitrogen fixation genes in the free-living state.

Although no such induction was detected, it was observed that certain TCA cycle intermediates supplied in the media could completely inhibit growth of strain T1.

To check whether these inhibitions were peculiar to strain T1, or were a more general characteristic of *Rhizobium* possibly involved in regulation of the nitrogen-fixing bacteroid state, the growth responses of several strains of other species of fast and slow-growing rhizobia were also tested.

MATERIALS AND METHODS

Bacterial strains

Of the fast-growing rhizobia, the following strains were tested: *R. trifolii* strains T1, TA1, NA30, WA67, UNZ29 and CC2480a; *R. meliloti* strains SU47, SU431 and U45; and *R. leguminosarum* strains NA525, NA503-2, NA504-4 and L4. Of the slow-growing rhizobia, the following strains were tested: *R. japonicum* strains CB1809, CC714, CC720 and USDA129, *R. lupini* strains W72, A13 and 606A; and strains CB746, NRG231 and 177A6 of the 'cowpea' group of *Rhizobium*.

Media

A modified Bergersen's synthetic medium (BMM) (Bergersen, 1961) was used initially to grow all strains before testing. The *Rhizobium* basal medium (RBM) used for testing growth with various compounds contained: KH_2PO_4, 150 mg; $CaCl_2.2H_2O$, 150 mg; $MgSO_4.7H_2O$, 200 mg; NaCl, 100 mg; $FeSO_4.7H_2O$, 25 mg; $Na_2MoO_4.2H_2O$, 25 mg; thiamine-HCl, 1 mg; biotin, 0.5 mg; nicotinic acid, 1 mg; pyridoxine-HCl, 1 mg; Gamborg's trace elements (Gamborg and Eveleigh, 1968), 1 ml; Difco Bacto agar, 15 g; water, 1.1. The pH was adjusted to 5.5. All media contained 2 mM $(NH_4)_2SO_4$ as nitrogen source; sucrose (10 g/1) and arabinose (10 g/1) were added after autoclaving where indicated. All tricarboxylic acid (TCA) intermediates and related compounds (as sodium salts) were added to the medium after autoclaving to give final concentrations of 2 mM and 20 mM.

Growth conditions

All strains were grown up in liquid BMM at 30°C, washed once in saline phosphate buffer (Cannon *et al.*, 1974), and plated (0.1 ml/plate) on the various media. All plates were incubated at 30°C,

and growth was recorded after 3 and 7 days. As no differences except in extent of growth were observed between these two times, only the 7-day results are described in this communication.

To test for anaerobic growth, inoculated plates were put into an anaerobic jar which was evacuated and filled with H_2 twice, re-evacuated and filled with N_2, or in controls, air. A catalyst for anaerobic jars, (Made by Gallenkamp, (London)) was present to remove remaining traces of O_2. The jar was incubated for 2-5 days at $30^{o}C$ before opening. A strict anaerobe *Bacteroides melaninogenicus* ss *intermedius* was included as a control to check that jars were completely anaerobic, and grew on all occasions tested. The possibility of there being sufficient oxygen dissolved in the medium to support growth was also minimized by using freshly prepared medium.

RESULTS

Effect of TCA cycle intermediates on *R. trifolii* strain T1

During examination of growth of strain T1 on various carbon sources, it was observed that certain TCA cycle intermediates and related compounds could partially or completely inhibit growth, while others stimulated growth (Table 1). Of the TCA cycle intermediates, all but succinate and fumarate completely inhibited growth when supplied at 20 mM. It was observed that succinate stimulated aerobic growth slightly more than fumarate, but in anaerobic growth the opposite effect was produced.

Of the carbon compounds which feed into the TCA cycle, lactate completely inhibited and acetate partially inhibited growth. To investigate further the influence of lactate on the metabolism of strain T1, uptake of ^{3}H-glucose was studied, and it was found that both D- and L-lactate could stimulate uptake of ^{3}H-glucose (Figure 1).

Effect of TCA cycle and related compounds on fast-growing rhizobia

Since strain T1 showed some interesting growth responses with TCA cycle intermediates, several strains of *R. trifolii* and of other fast-growing rhizobia, were examined for the same phenomenon.

Although some variations between strains within a species were observed, generally each of the three species (*R. trifolii*, *R. leguminosarum* and *R. meliloti*) showed characteristic patterns of growth stimulation and inhibition by TCA cycle intermediates (Table 2) and related compounds (Table 3). In general, the effects of these

compounds on strain T1 were found also on other *R. trifolii* species, in that all compounds, except succinate, fumarate, pyruvate, and in some strains glyoxylate, inhibited growth.

In these experiments, the strains of *R. leguminosarum* behaved like *R. trifolii* except strain NA503-2 which was stimulated rather than inhibited by 20 mM oxaloacetate (Table 2). Since otherwise this strain behaved as expected, the reason for oxaloacetate stimulation is not known.

The three strains of *R. meliloti* tested behaved slightly differently from *R. trifolii* and *R. leguminosarum*. Although these strains shared the same pattern of growth stimulation and inhibition by most of the compounds tested, a marked stimulation rather than inhibition was observed with citrate, lactate and acetate.

When the compounds of the TCA cycle and related compounds were tested as sole carbon source, none was used well by any strain although growth of all these species on pyruvate was greater than on any other compound (Table 4). Generally, the compounds which completely inhibited growth in combination with sucrose and arabinose allowed poor growth when present as sole carbon source.

Effect of TCA cycle and related compounds on slow-growing rhizobia

Similar patterns of growth stimulation and inhibition by compounds of the TCA cycle and related compounds were also observed for strains of three species of slow-growing rhizobia, *R. japonicum*, *R. lupini* and the cowpea group of *Rhizobium*. All the slow-growers were completely inhibited by the same TCA cycle intermediates which inhibited *R. trifolii* and *R. leguminosarum*. However, the slow-growers were also partially inhibited by 20 mM succinate and fumarate (Table 5), in contrast to the stimulation of fast-growers by these compounds.

Of the compounds which feed into the TCA cycle, both lactate and glyoxylate (20 mM) completely inhibited all the slow-growers; acetate had little effect (Table 6).

R. japonicum seemed more closely related to the cowpea rhizobia than either group was to *R. lupini*, since strains of *R. japonicum* and the cowpea group were markedly stimulated by all TCA cycle intermediates, by lactate, and by pyruvate all at 2 mM (Tables 5 and 6). In contrast, the *R. lupini* strains tested were not stimulated by any of these compounds, and were actually inhibited by pyruvate (Table 6).

Very little growth, either of fast-growers or of slow-growers, occurred on TCA cycle intermediates and related compounds as sole carbon source (Table 7). Again, pyruvate gave slightly better growth than other compounds, at least for the cowpea group.

DISCUSSION

Utilization of various carbon sources has been used in the subdivision of *Rhizobium* into species (Fred *et al.*, 1932; Graham, 1964). Although no detailed studies have been made of the various metabolic pathways which operate in rhizobia, circumstantial evidence suggests the presence of the oxidative pentose phosphate pathway, the Embden-Meyerhof glycolytic pathway and the glyoxylate cycle in rhizobia (Johnson *et al.*, 1966; Jordan, 1962; Martinez-De Drets and Arias, 1972). A study of bacteroids of one strain of *R. japonicum* which failed to completely oxidize intermediates of the TCA cycle, led Bergersen (1958) to conclude that this cycle may not operate in *R. japonicum*. However, a subsequent study (Tuzimura and Meguro, 1960) demonstrated the probable existence of a TCA cycle in another strain of *R. japonicum*, since it was able to oxidize TCA cycle intermediates and related compounds. Similarly, Graham (1964) showed that many strains of seven species of *Rhizobium*, both fast- and slow-growing, could utilize pyruvate and the intermediates fumarate, malate, succinate and citrate. Because we had found that *R. trifolii* strain T1 was inhibited by at least some of these compounds, we have studied effects of TCA cycle intermediates on *Rhizobium* species to ascertain whether this cycle might operate and be involved in providing energy for nitrogen fixation in bacteroids.

None of the rhizobia tested were readily able to use TCA cycle intermediates or related compounds other than pyruvate as sole carbon source. However, marked stimulations and inhibitions of growth were observed when these compounds were used in combination with sucrose and arabinose. Some TCA cycle intermediates (succinate and fumarate) and the related compound pyruvate stimulated growth of both fast- and slow-growing *Rhizobium* species in the presence of sucrose and arabinose, while related compounds (oxaloacetate, citrate, α-keto glutarate, malate, lactate, acetate and glyoxylate) partially or completely inhibited growth. The amount of these compounds supplied is also important, since low concentrations of most compounds stimulated, while higher concentrations inhibited

growth.

Thus a general feature shared by both fast- and slow-growing rhizobia is an apparently defective TCA cycle; the simplest explanation would be a block at some step in the cycle. As pointed out by Bergersen (1958), a block in the TCA cycle may lead to an accumulation of one or more of the acids concerned in the preceding steps, or these acids may be diverted to other processes. Since maintenance of the TCA cycle requires adequate levels of oxidized NAD and NADP, it is also possible that the growth inhibition observed with the various TCA cycle intermediates is due to an imbalance of the oxidized to the reduced levels of NAD and NADP caused by addition of these TCA cycle intermediates (Sanwal, 1970).

Alternatively, it may be that the TCA cycle intermediates can act as chelating agents, particularly for such metals as calcium, iron or molybdenum in the medium. This could explain the growth stimulations observed when low levels of the TCA cycle intermediates were supplied, and when higher concentrations were supplied these might inhibit growth by strongly chelating the ions and thus preventing their use by the rhizobia, thereby inhibiting growth. This might also explain the response of *R. trifolii* strain T1 to high concentrations of lactate and acetate. Although the simplest explanation for stimulation of ^{3}H-glucose uptake by 1 mM lactate is that lactate is taken up and metabolized to provide energy for glucose uptake, it is also possible that lactate could have the same effect at the outer cell surface.

Whether or not the apparently defective use of TCA cycle intermediates and related compounds by *Rhizobium* is fortuitous or has a consequence for symbiosis is not known, but an inability to use these compounds might be important in the provision of energy for nitrogen fixation in the nodule.

It has recently been shown (Skotnicki and Rolfe, 1977) that the phenotypic expression of the nitrogen fixation genes from *Klebsiella pneumoniae* in *E. coli* K12 does not require coupling of oxidative phosphorylation, but does require a functional fumarate reduction system to energize the membrane and thus provide the energy necessary for nitrogen fixation. Aerobically, growth of strain T1 of *R. trifolii* was better on succinate than on fumarate, but under N_2 fumarate stimulated growth to the same extent as succinate did aerobically. As strain T1 also appears to be defective in its use of TCA cycle compounds, fumarate reductase could provide succinate, a very good source of carbon for growth of this strain. By analogy with *K. pneumoniae*,

fumarate reductase in strain T1 might also be involved in the provision of energy for nitrogen fixation in the nodule.

Because strain T1 has physiological properties in common with other strains of *R. trifolii* and with *Rhizobium* in general, and because this strain has several other useful characteristics, including a very fast growth rate, a broad host range and sensitivity to specific phages, it is proposed that this strain be further developed as a reference strain for the species *R. trifolii*.

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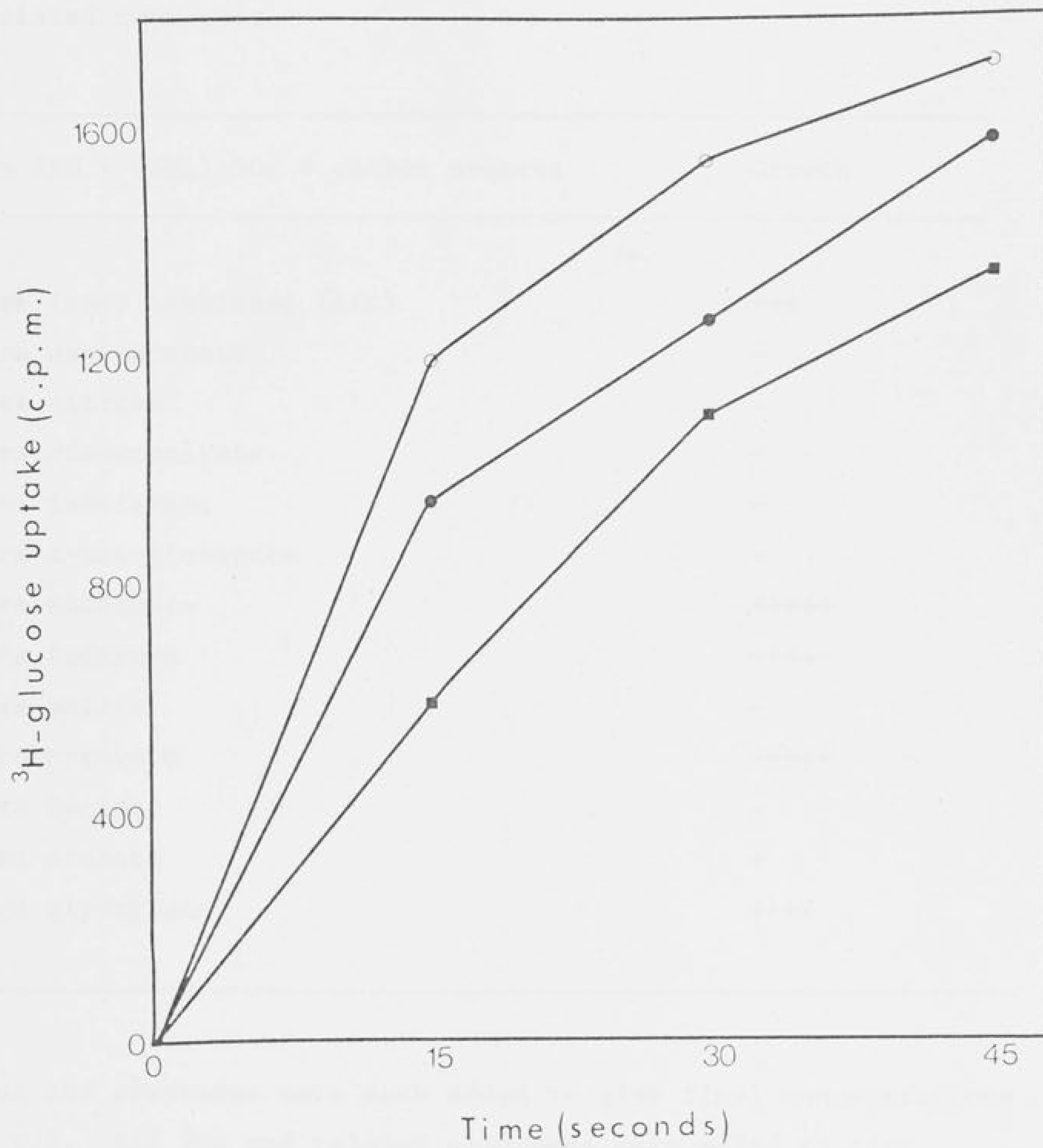
Effect of carbon sources on growth of *Rhizobium*

Microbios Mary L. Skotnicki and Barry G. Rolfe



1600
1200
800
400
0
0
15
30
45
³H-glucose uptake (c.p.m.)
Time (seconds)

Figure 1 Stimulation of ^{3}H-glucose uptake by D- and L-lactate. Equal molarities (10^{-3} M) of all compounds were used. ■ , ^{3}H-glucose alone; ● , ^{3}H-glucose and L-lactate; ○ , ^{3}H-glucose + D-lactate.

Table 1 Growth of *R. trifolii* strain T1 on TCA cycle intermediates and related compounds

Medium RBM + $(NH_4)_2SO_4$ + carbon source:	Growth
sucrose (suc) arabinose (ara)	+++
suc ara oxaloacetate	-
suc ara citrate	-
suc ara *cis*-aconitate	-
suc ara isocitrate	-
suc ara *α*-ketoglutarate	-
suc ara succinate	+++++
suc ara fumarate	++++
suc ara malate	-
suc ara pyruvate	+++++
suc ara lactate	-
suc ara acetate	+
suc ara glyoxylate	++++

Sucrose and arabinose were each added to give final concentrations of 10 g/l. All TCA and related compounds were added to give final concentrations of 20 mM. In this and all subsequent tables: - = no growth, + = trace to poor growth, ++ = fair growth, +++ = fairly good growth, ++++ = good growth, and +++++ = vigorous growth.

Table 2 Growth of fast-growing rhizobia with TCA cycle intermediates

Medium RBM + $(NH_4)_2SO_4$ + carbon source	Bacterium: *R. trifolii*						*R. leguminosarum:*				*R. meliloti:*		
	T1	TA1	NA30	WA67	UNZ29	CC2480a	NA525	NA503-2	NA504-4	L4	SU47	SU431	U45
sucrose (suc) arabinose (ara)	+++	++	++	+++	++	+++	+++	+++	+	+++	+++	+++	+++
suc ara oxaloacetate 2 mM	++++	+++	+++	++	++	+++	++	++	++	+++++	+++	+++	+++
suc ara oxaloacetate 20 mM	–	–	–	–	–	–	–	++++	–	–	–	–	–
suc ara citrate 2 mM	+++++	+++++	+++	++	++++	++++	+++	++++	+++	+++++	+++	+++	++++
suc ara citrate 20 mM	–	–	–	–	–	–	–	–	–	–	+++++	++++	+++
suc ara α-keto-glutarate 2 mM	+++	+++	+++	++	++	++	++	++	++	++++	+++	+++	++++
suc ara α-keto-glutarate 20 mM	–	–	–	–	–	–	–	–	–	–	–	–	–
suc ara succinate 2 mM	++++	+++	+++	+++	++++	++++	++	+++	+++	+++++	++	++	+++
suc ara succinate 20 mM	+++++	+++++	++++	+++	++++	++	+++++	+++++	+++	+++	+++++	+++++	+++++
suc ara fumarate 2 mM	+++++	++++	++	+++	++++	+++	++	+++	++	+++++	++	++	++++
suc ara fumarate 20 mM	++++	++++	++	+++	++++	++	++++	+++++	+	++	+++++	+++++	+++++
suc ara malate 2 mM	++++	+++	++	++	+++	++	++	+++	+++	++++	++	++	+++
suc ara malate 20 mM	–	–	–	–	–	–	–	–	–	–	–	–	–

Table 3 Growth of fast-growing rhizobia with TCA cycle-related compounds

Medium RBM + $(NH_4)_2SO_4$ + carbon source	Bacterium: *R. trifolii* T1	TA1	NA30	WA67	UNZ29	CC2480a	*R. leguminosarum:* NA525	NA503-2	NA504-4	L4	*R. meliloti:* SU47	SU431	U45
sucrose (suc) arabinose (ara)	+++	++	++	+++	++	+++	+++	+++	+	+++	+++	+++	+++
suc ara pyruvate 2 mM	+++++	++++	++	+++	++++	+++	+++	+++	++	++++	+++++	++++	++++
suc ara pyruvate 20 mM	+++++	+++++	++	+++	+++++	++++	+++++	++++	+++++	+++++	+++++	+++++	+++++
suc ara lactate 2 mM	++++	+++	+	+	++	+	++	++	+	+	++	+++	+++
suc ara lactate 20 mM	-	-	-	-	-	-	-	-	-	-	+++++	+++++	+++++
suc ara acetate 2 mM	+	+	+	+	+	+	-	-	-	-	++	++	++
suc ara acetate 20 mM	+	-	-	++	+	+	-	-	-	++	+++++	+	+++++
suc ara glyoxylate 2 mM	+++	+++	++	++	+++	+++	++	+++	++	+++++	+++++	+++++	+++
suc ara glyoxylate 20 mM	++++	++	+++	+	-	-	++++	++++	+++	-	+++	+++	+++

Table 4 Growth of fast-growing rhizobia on TCA cycle and related compounds as sole carbon source

Medium RBM + $(NH_4)_2SO_4$ + carbon source (2 mM):	Bacterium: *R. trifolii*						*R. leguminosarium:*				*R. meliloti:*		
	T1	TA1	NA30	WA67	UNZ29	CC2480a	NA525	NA503-2	NA504-4	L4	SU47	SU431	U45
oxaloacetate	+	+	+	+	+	+	+	+	+	+	+	−	+
citrate	+	+	+	+	+	+	+	+	+	+	+	+	+
α-ketoglutarate	−	−	−	−	+	+	+	+	+	+	+	−	+
succinate	+	+	+	+	+	+	+	+	+	+	+	+	+
fumarate	+	+	+	+	+	+	+	+	+	+	+	+	+
malate	+	+	+	+	+	+	+	+	+	+	+	+	+
pyruvate	++	++	+++	++	+++	++	++	+++	+	++	+++	+++	+
lactate	+	+	+	+	+	+	+	+	+	+	+	−	+
acetate	+	+	+	+	+	+	−	+	−	+	+	+	+
glyoxylate	−	−	−	−	+	+	+	+	−	+	+	+	+

Table 5 Growth of slow-growing rhizobia with TCA cycle intermediates

Medium RBM + $(NH_4)_2SO_4$ + carbon source:	Bacterium: *R. japonicum:* CB1809	CC714	CC720	USDA129	*'Cowpea' Rhizobium:* CB746	NGR231	177A6	*R. lupini:* W72	A13	606A
sucrose (suc) arabinose (ara)	+++	++	+	+	++	+++	++	+	+	+
suc ara oxaloacetate 2 mM	+++++	++++	+	++	++	+++	+++	+	+	+
suc ara oxaloacetate 20 mM	-	-	-	-	-	-	-	-	-	-
suc ara citrate 2 mM	+++++	++++	++	++	++++	++	+++	+	+	+
suc ara citrate 20 mM	-	-	-	-	-	-	-	-	-	-
suc ara α-ketoglutarate 2 mM	+++++	+++	++	+++	++++	+++++	+++++	+	+	+
suc ara α-ketoglutarate 20 mM	-	-	-	-	-	-	-	-	-	-
suc ara succinate 2 mM	+++++	+++	++	++	+++	++++	++++	++	++	++
suc ara succinate 20 mM	++	+++	+	+	+	+	++	+	+	-
suc ara fumarate 2 mM	+++++	++++	+	++	++	++	++	+	+	+
suc ara fumarate 20 mM	++	+++	+	+	+	+	++	+	+	+
suc ara malate 2 mM	+++++	++++	++	++	+++	++++	++++	+	+	+
suc ara malate 20 mM	-	-	-	-	-	-	-	-	-	-

Table 6 Growth of slow-growing rhizobia with TCA cycle-related compounds

Medium RBM + $(NH_4)_2SO_4$ + carbon source	Bacterium: *R. japonicum*:				*'Cowpea' Rhizobium*:			*R. lupini*:		
	CB1809	CC714	CC720	USDA129	CB746	NGR231	177A6	W72	A13	606A
sucrose (suc) arabinose (ara)	+++	++	+	+	++	+++	++	+	+	+
suc ara pyruvate 2 mM	+++	++	+	++	+	+++	++++	+	+	-
suc ara pyruvate 20 mM	+++++	++++	+	++	+	+++++	++	-	-	-
suc ara lactate 2 mM	++++	++++	+	++	++	+++	++++	+	+	+
suc ara lactate 20 mM	-	-	-	-	-	-	-	-	-	-
suc ara acetate 2 mM	++++	++	+	++	+++	+++	+++	+	+	-
suc ara acetate 20 mM	++++	++	+	+	+++	+++	+++	+	+	-
suc ara glyoxylate 2 mM	++++	+++	+	+	++	+++	+++	+	+	+
suc ara glyoxylate 20 mM	-	-	-	-	-	-	-	-	-	-

Table 7 Growth of slow-growing rhizobia on TCA cycle and related compounds as sole carbon source

Medium RBM + $(NH_4)_2SO_4$ + carbon source (2 mM)	Bacterium: *R. japonicum*: CB1809	CC714	CC720	USDA129	*'Cowpea' Rhizobium*: CB746	NGR231	177A6	*R. lupini*: W72	A13	606A
oxaloacetate	+	+	+	+	+	+	+	+	+	+
citrate	+	+	+	+	+	+	+	-	-	-
α-ketoglutarate	+	+	-	+	+	+	+	-	-	-
succinate	+	+	-	+	-	-	-	+	-	-
fumarate	+	+	-	+	+	+	+	+	+	+
malate	+	+	+	+	+	+	+	+	+	+
pyruvate	+	+	+	+	+	++	++	+	+	-
lactate	+	+	+	+	+	+	+	+	+	+
acetate	+	+	-	+	-	+	+	+	-	-
glyoxylate	-	-	-	-	-	-	-	-	-	-