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nif genes in alien backgrounds

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Since the original construction of diazotrophic *Escherichia coli* by conjugal transfer of *nif* genes from *Klebsiella pneumoniae* in 1972, the manipulation of *nif* genes into alien prokaryotic backgrounds has become routine: much of the basic genetics of the *K. pneumoniae nif* cluster was elucidated in an *E. coli* background. Gene transfers to new species and genera can give new information regarding the stability of *nif* genes and, particularly, conditions for their expression; recipients in which *nif* is partly expressed, or not expressed at all, are especially useful. Appropriate examples are discussed. New diazotrophic prokaryotes show little promise for practical exploitation but their construction should give forewarning of problems to be expected in the construction of diazotrophic eukaryotes, as well as hints concerning the ecology and evolution of diazotrophy.

1. Introduction

The first intentional transfer of functional nitrogen fixation (*nif*) genes to an alien genetic background was the generation, by plasmid-mediated conjugation, of nitrogen-fixing (diazotrophic) derivatives of *Escherichia coli* C-603 able to utilize the *nif* genes of *Klebsiella pneumoniae* M5a1 (Dixon & Postgate 1972). This advance was followed rapidly by the apparent mobilization of *nif* from *Rhizobium trifolii* to *Klebsiella aerogenes* (Dunican & Tierney 1974). The gene transfers to *E. coli* exploited the linkage of the histidine operon (*his*) with *nif* genes on the *K. pneumoniae* chromosome: in intraspecific conjugational and transductional crosses, *nif* was already known to co-transfer with *his* readily. The *his* and *nif* genes are now known to be contiguous, so recipients carrying mutations in *his* are particularly suitable for intergeneric transfer of *K. pneumoniae* DNA carrying *nif* because direct selection for Nif^+ is difficult.

The diazotrophic *E. coli* hybrids were of two types: one in which the *nif* genes of *K. pneumoniae* became integrated into the *E. coli* chromosome (Cannon *et al.* 1974*a*), and others in which genetic evidence, and the limited physical techniques then available, indicated that the *K. pneumoniae* DNA had formed plasmids in *E. coli* carrying at least some *nif* genes (Cannon *et al.* 1974*b*). Indirect evidence suggested that the *nif* genes from *R. trifolii* were on a plasmid, which had become transferred to *K. aerogenes*, a view which was supported in principle a decade later with the discovery of natural *nif* plasmids in rhizobia (Nuti *et al.* 1979). These experiments clearly adumbrated the possibility of transferring diazotrophy to other unusual hosts. Eukaryotic plants were a particularly popular choice as host (see, for example, Streicher *et al.* 1972; Postgate 1974, 1977*a*, *b*, 1980, 1987; Hardy 1976; Discussion 1976; Gibson *et al.* 1977; Dixon 1978; Postgate & Cannon 1981; Shanmugam 1982; Dixon *et al.* 1983; Merrick & Dixon 1984), although animals were not wholly neglected (see, for example, Postgate 1974, 1977*b*). Genetic studies on *nif*, however, concentrated on analysis of gene structure and regulation, first in *K. pneumoniae* and later in other prokaryotes such as cyanobacteria, azotobacters and rhizobia (see, for example, Evans *et al.* 1985*b*). As the structural complexity of the *nif* regulon became

 20-2

Table 1. Transconjugation frequencies of His$^+$ and Km^r phenotype in matings with the *nif* plasmids pRD1 and pMF250

(The plasmids are described in the text. Donor strains used *E. coli* JC5466 *his trp spc* carrying either pRD1 (carries *his nif* Km Tc Cb etc.) or pMF250 (carries *his nif* Km), recipient strains as below, over night mating on 'Luria' agar at 30 °C with subsequent selection for Km^r or Km^r + His$^+$; Sm^r + trp$^-$ counterselection of donor. Loss of His$^+$ is taken as implying loss of *Klebsiella* DNA carrying *nif*.

recipient	genotype	plasmid	counts per millilitre: His$^+$ Km^r	counts per millilitre: Km^r	counts per millilitre: unselected	approximate plasmid transfer[a] (% donor)	cotransfer of His$^+$ with Km^r (%)
Proteus mirabilis WR20	*his nic str*	pRD1	7×10^2	1.2×10^6	1.4×10^9	0.17	0.058
		pMF250	6×10^7	1.7×10^8	2.7×10^9	12	35
Erwinia herbicola Y741	*his*	pRD1	4	3	5.5×10^8	0.000001	75
		pMF250	3.2×10^1	< 1	7.6×10^8	0.000005	< 3
Pseudomonas putida MT20-3	*his* (Tol$^-$)	pRD1	2.5×10^5	2.4×10^5	3×10^8	0.8	96
		pMF250	1.9×10^5	1.6×10^5	4.5×10^8	0.4	84
E. coli 1533	*arg leu met his str*	pRD1	7×10^7	1.4×10^7	1.4×10^8	100	50
		pMF250	1×10^8	1.3×10^8	4.4×10^8	50	100

[a] Frequencies calculated assuming 50 % of mated population was donor.

revealed (see Postgate (1982) for a survey), together with its elaborate regulatory system (see Dixon 1984), the naivety of some of the early excitement over diazotrophic plants became obvious. Nevertheless, experiments on the expression of *nif* in alien genetic backgrounds continued, because it is a strategy which would give information on the prerequisites for stability, control and efficiency of *nif* in new conditions.

A valuable tool in such studies was the availability of self-transmissible genetic elements carrying *nif* genes. The first of these was the F-prime FN68 (Dixon 1974) which was a self-transmissible plasmid of the *incF* incompatibility group constructed in *E. coli* but carrying *K. pneumoniae* DNA including *his nif*; it was rather unstable in certain hosts (Cannon *et al.* 1976). It was superseded by the relatively stable plasmid pRD1, which carries the same segment of *K. pneumoniae* DNA and which has played a fundamental part in the genetic analysis of *nif* because it can carry stable mutations, deletions and fusions; it was also the parent of all recombinant clones of *K. pneumoniae nif* (*Kp nif*). (We shall use the abbreviation *Kp nif* for *nif* DNA originating in *Klebsiella pneumoniae*.) Plasmid pRD1 was derived from the *incP* plasmid RP4 (Dixon *et al.* 1976) (it was originally called RP41) and, in addition to its quota of *K. pneumoniae* DNA plus (probably) some *E. coli* DNA acquired during its construction (see Puhler *et al.* 1979), it carries the drug-resistance determinants of RP4: kanamycin (Km), tetracycline (Tc) and ampicillin/carbenicillin (Cb) resistance. A derivative, pMF100, prepared by Dr Mechthild Filser (Filser 1979) with these drug-resistance genes removed, was valuable in mapping *nif* (see, for example, Merrick *et al.* 1980). A derivative of pMF100, pMF250, in which kanamycin resistance has been restored by insertion of Tn5, has proved to be markedly more stable than pRD1 in intergeneric transfers to *Proteus mirabilis* (Postgate & Kent 1985) but less stable in transfers to *Erwinia herbicola* (table 1). Two multicopy recombinant plasmids carrying the whole *Kp nif* have been constructed: pWK120 (Puhler *et al.* 1979) and pEFC6 (mentioned by Cannon *et al.* 1985); the former conferred Nif^+ on *E. coli* C603.

2. *Kp nif* IN OTHER BACTERIA

In coliform hosts

The initial transfer of *Kp nif* to *E. coli* yielded diazotrophic hybrids which were both NH_4^+-repressed and O_2-sensitive (Dixon & Postgate 1972), thus implying the presence of appropriate regulatory apparatus in the new hosts. This was the earliest evidence for an operon-like structure for *nif*; in due course *Kp nif* became revealed as a contiguous cluster of operons which has been called a 'regulon' (see Postgate 1982), itself regulated by the three nitrogen regulation genes: *ntrA* and the *ntrBC* operon, the latter contiguous with the structural gene for glutamine synthetase *glnA* (see figure 1 and Dixon, this symposium). Interspecific *nif* transfer was used to show that *Kp nif*, transferred to non-diazotrophic *Klebsiella aerogenes* which had a mutation leading to constitutive (i.e. non-NH_4^+-regulated) expression of *glnA* (the '$GlnC^-$' phenotype), also escaped NH_4^+-repression of *nif* (Tubb 1974). Physiological studies with an anti-metabolite (Gordon & Brill 1974) and interspecific transduction of the '$GlnC^-$' phenotype into diazotrophic *K. pneumoniae* (Streicher *et al.* 1974) led to the same conclusion: that *nif* was regulated by a system common to the utilization of other N-sources such as histidine, proline and arginine as well as regulating the synthesis of glutamine synthetase. That system is now widely known as the *ntr* system (see Kustu *et al.* 1986).

Transfer of *Kp nif* on pRD1 (Kennedy & Postgate 1977) or FN68 (Skotnicki & Rolfe 1976)

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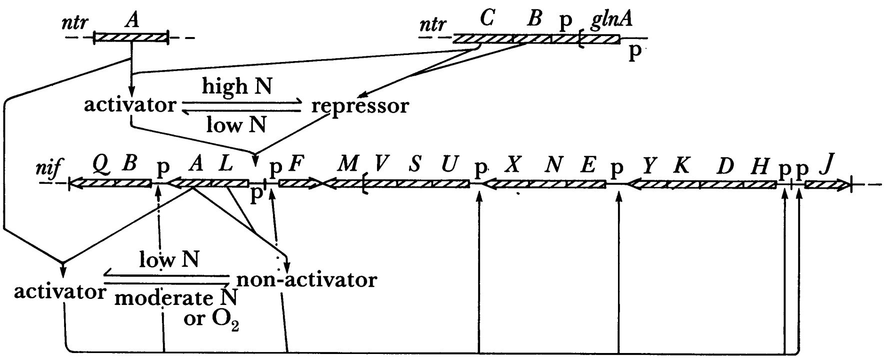

FIGURE 1. Model for *ntr* regulation of *nif* in *K. pneumoniae*. The diagram shows the products of *ntrC* and *ntrA* forming, in low-N conditions, an activator which acts at the promoter of the *nifLA* operon (the fact that it also activates the *glnA* promoter is omitted). In high-N conditions a repressor is formed by interaction of *ntrB*. Activation of *nifLAp* at low N in the presence of the *ntrA* product leads to an activator for all other promoters within the *nif* regulon. At intermediate levels of N, or as a result of sensing dissolved O_2, the *nifL* product intervenes, suppressing the activator function of the *nifA* product. (For details, see Dixon (1984).)

to *E. coli narD* (= *chlD*) mutants, which are defective in an early stage of processing Mo for nitrate reductase synthesis, showed that the *narD* gene product is also necessary for processing Mo for nitrogenase synthesis. The nitrogenase peptides were detected in the *narD*$^-$ background (see, for example, Kennedy & Postgate 1977) although they are unstable in Mo-starved *K. pneumoniae* (Brill *et al.* 1974). The phenotype of *narD*$^-$ *nif* hybrids resembles that of the *nifQ* mutation in that *nif* expression occurs at relatively high molybdate concentration (Imperial *et al.* 1984). Skotnicki & Rolfe (1979) reported that *fnr* mutants of *E. coli* did not express *Kp nif* on FN68 and proposed that *nif* regulation in response to O_2 might be linked to general control of the synthesis of redox enzymes. However, Hill (1985) reinvestigated the matter with the more stable pRD1 and obtained normal *nif* expression in five *fnr* mutants of *E. coli*.

Experiments on the expression of cloned fragments of *Kp nif* in *E. coli* have been informative in the context of gene function: by such experiments, Howard *et al.* (1986) and Berman *et al.* (1985 *a*, *b*) showed that the *nifM* and *nifH* products are necessary and sufficient for the synthesis of the Fe protein (Kp2) of *K. pneumoniae* and that the product is enzymically and immunologically active. Therefore no other *nif*-specific genes are involved in Kp2 synthesis, although the *nifA* product is necessary as an activator of *nifH* and *nifM* expression. Immunologically active Kp2 peptide is formed in the absence of *nifM*, so the processing role of the latter is unlikely to involve peptide modification. Sunderasan *et al.* (1983) used *E. coli* as the genetic background in which to show that *Kp nifA* activates the promoter of *Rhizobium meliloti nifH*; activation of *Azotobacter chroococcum nifH* by *Kp nifA* has likewise been shown in *E. coli* by Kennedy *et al.* (1985 *b*).

The readiness with which *E. coli* expresses *Kp nif* prompted at least one search for diazotrophic *E. coli* from the natural environment but, despite a false report, none has so far been reported (see Neilson 1979).

Transfer of *Kp nif* to *Salmonella typhimurium* revealed the problem of plasmid stability: plasmid FN68 lost *nif* (and the adjacent *his*) in this background and low nitrogenase (C_2H_2-reducing) activities (Cannon *et al.* 1976) were attributed to such segregation by Postgate & Krishnapillai (1977): even the relatively stable pRD1 required constant selection for His^+ to conserve *nif*.

Transfer of *Kp nif* on pRD1 to an *Erwinia herbicola his* mutant led to diazotrophy in the non-diazotrophic recipient chosen (Krishnapillai & Postgate 1980) but the novelty of this finding was vitiated somewhat by the nearly simultaneous report that some 22% of *E. herbicola* from the natural environment are diazotrophic (Papen & Werner 1979). Nevertheless, it is possible that acquirement of diazotrophy might confer pathogenicity on this usually harmless epiphyte. In unpublished tests, Dr Eve Billing of East Malling Research Station was unable to detect significant pathogenicity to pear fruits, apple shoots or tobacco leaves with *E. herbicola* Y74 alone or carrying pRD1, RP4 or pRD7 (a *nifA*$^-$ and therefore Nif$^-$ control for pRD1). Kozyrovskaya *et al.* (1984) studied the true pathogen *E. carotovora* and reported changes in its pathogenicity after introduction of pRD1. However, in most instances pathogenicity was apparently diminished. Controls with a *nif*$^-$ derivative of pRD1 were not included.

Kozyrovskaya *et al.* (1984) also obtained diazotrophic derivatives of the plant pathogen *Xanthomonas betica* by introducing pRD1, confirming presence of the plasmid by gel electrophoresis. They reported increased virulence in some transconjugants but provided no clear evidence that *nif* expression was responsible.

Kleeberger & Klingmuller (1980) transferred pRD1 to a strain of *Enterobacter cloacae* isolated from grass roots, with the possibility of generating a diazotrophic root association. They obtained Nif$^+$ transconjugants despite the fact that some transconjugants lost *nif* and, as in the earliest transfers to *E. coli*, some derivatives integrated *nif* into the *E. cloacae* chromosome and showed promising stability as regards Nif$^+$ even in association with grass roots. Klingmuller *et al.* (1983) reported that the chromosomally *nif*-integrated derivatives were nitrate-reduction negative (Nar$^-$) and hence chlorate-resistant (Chl^r), a pleiotropy which could be exploited to select for chromosomal *nif* derivatives. Using a derivative of pRD1 carrying temperature-sensitive (t/s) phage Mu, (pCE1), Nguyen *et al.* (1983) were able to induce chromosomal integration of *nif* by temperature shift. This research was reviewed by Klingmuller (1984). Gottfert & Klingmuller (1985) showed that t/s Mu phage in pCE1 could be exploited to obtain chromosomally integrated derivatives of *E. coli*.

Transfer of pRD1 to a *his* auxotroph of *Serratia marcescens* gave diazotrophic derivatives (Krishnapillai & Postgate 1980); a problem of sustaining their growth in chemostat culture is mentioned later. Coliform bacteria in which *Kp nif* is expressed presumably already have other determinants necessary for expression of *nif* in *K. pneumoniae*, such as *ntrA*, *ntrBC* and *narD*. Therefore the evidence that *Kp nif* was not expressed in *Proteus mirabilis* (Krishnapillai & Postgate 1980) might be due to lack of some such gene. Postgate & Kent (1985) successfully obtained expression of *Kp nif* from pMF250 in *P. mirabilis* but only if a plasmid were present which expressed *nifA*, the positive activator of the *nif* regulon, from a constitutive promoter (plasmid pCK1). Inadequacy of the *ntrBC* component of the *ntr* apparatus of *P. mirabilis* was thus implied, but a simple explanation in terms of an ineffective *ntrC* product was not established because a constitutive *Kp ntrC* plasmid (pMD45), which caused constitutive *nif* expression in *E. coli* or *K. pneumoniae*, did not substitute for pCK1 in *P. mirabilis*. Further complications were: (i) a need to precondition *P. mirabilis* (pMF250, pCK1) populations by aerobic growth on glucose before anaerobic expression of *Kp nif* was obtained; and (ii) the observation that pCK1 did not in fact confer NH_4^+-constitutive *nif* expression on *P. mirabilis* (pMF250, pCK1). The second of these problems is discussed further in the context of *Azotobacter* (below).

The need for extra *nifA* product for expression of *Kp nif* in *P. mirabilis* has not been

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satisfactorily explained. An interpretation which was not considered by Postgate & Kent (1985), is that the wild-type *nifL* product may normally be formed in a state which inactivates *nifA* product and that a so far unrecognized gene product may be needed to convert *nifL* product to an innocuous form. In that case a mutation which inactivates *nifL* but is not polar on *nifA* ought to give pCK1-independent *nif* expression in *P. mirabilis*. This proposition has been tested by one of us (S.H.), using the plasmid pMF337, a derivative of pMF100 carrying such a deletion in *nifL* which leaves *nifA* functional; it leads to partial oxygen constitutivity (Filser *et al.* 1983). This and the parent plasmid pMF100 were transferred into *P. mirabilis* WR19, the strain used by Postgate & Kent (1985), by using selection for His^+, and trans-conjugants were tested both in the conditions used by Postgate & Kent and with a modified procedure in which the assay vessels were set up under N_2. Neither *P. mirabilis* (pMF337) nor *P. mirabilis* (pMF100) gave activity, although *P. mirabilis* (pMF250, pCK1) showed its usual acetylene-reducing activity in similar test media and conditions.

A positive test for acetylene reduction is circumstantial evidence for derepression of *nif* and for nitrogenase activity, but its quantitative significance may be much influenced by manipulation of the samples. In some instances (*E. coli*, *S. typhimurium*, *E. herbicola*, *S. marcescens*) diazotrophy has been confirmed by ^{15}N incorporation from $^{15}N_2$. The physiological efficiency of the process in the new background has, however, rarely been evaluated. Table 2 records our

TABLE 2. YIELD COEFFICIENTS AND DIAZOTROPHIC EFFICIENCIES OF GENETICALLY CONSTRUCTED NITROGEN-FIXING BACTERIA IN GLUCOSE-LIMITED CHEMOSTAT CULTURE

organism	Y glucose (g mol^{-1})	efficiency μg N fixed mg^{-1} glucose	dilution rate h^{-1} (±0.002)
Klebsiella pneumoniae M5a1[a]	9.7	6.5	0.077
Escherichia coli CM74	7.5	5.0	0.04
E. coli JC5466 (pRD1)	7.1	5.3	0.06
Salmonella typhimurium LT7 1201 (FN68)	11.1	7.9	0.05

[a] Wild-type diazotroph from which the *nif* gene in *E. coli* CM74 and plasmids pRD1 and FN68 originated.

chemostat experiments in which diazotrophic constructs carrying *Kp nif* were compared with the parent *K. pneumoniae* in similar conditions of growth rate and nutritional status. All of the constructs were similar to *K. pneumoniae* as far as efficiency, steady-state population and nitrogen content were concerned; *S. typhimurium* carrying FN68 was the most efficient at the growth rates tested. The two *E. coli* strains differed in that strain CM74 had chromosomal *nif* (Cannon *et al.* 1974*a*) and *E. coli* JC5466 carried *nif* on pRD1. Judging from the nitrogen content (as a percentage of dry mass), all three constructs diverted carbon or energy sources into non-nitrogenous cell components, such as polysaccharide: analyses of cells for such macromolecular components are not available. It is important to record that, in this series of experiments, several careful attempts were made to set up diazotrophic chemostat populations of *E. herbicola* (pRD1) or *S. marcescens* (pRD1) without success: for reasons that are still not clear, steady states were not obtained in a variety of conditions, including those given in table 2.

Kp nif in pseudomonads

The question whether diazotrophy occurs within the genus *Pseudomonas* was a vexed one for many years. One reason was that Hill & Postgate (1969) showed that, of the two type strains then available, *P. azotocolligens* was not diazotrophic in their hands and *P. azotogensis* was not a pseudomonad (it was ultimately identified by Mr L. B. Perry (*personal communication*), of the National Collection of Industrial and Marine Bacteria, as a strain of the diazotrophic species *Bacillus polymyxa*). Another reason was the demonstration by Line & Loutit (1973) that presumptive diazotrophic 'pseudomonas' colonies from New Zealand soil were syntrophic mixtures of pseudomonads with anaerobic diazotrophs resembling *Clostridium butyricum*. Despite occasional reports of the isolation of taxonomically unassigned diazotrophic pseudomonads, a 'dogma' arose that the genus *Pseudomonas* did not include diazotrophs (see, for example, Meganathan 1979). However, an authenticated *P. saccharophila* exhibits very O_2-sensitive diazotrophy (Barraquio *et al.* 1986).

Mergeay & Gerits (1978) had earlier shown that, after conjugal transfer of pRD1 or FN68 to a *his* mutant of a putative *P. fluorescens*, O_2-dependent aerobic acetylene reduction could be observed, albeit at a remarkably slow rate. Their experimental conditions to obtain *nif* expression suggested that the process might be very O_2-sensitive, but their report was disquieting because (i) the unstable FN68 was expressed in this recipient, and (ii) the strain was originally isolated as a rhizobium. Lehtinen & Mäntsälä (1981) found that pRD1 was unstable in *P. fluorescens* but obtained three strains of *P. putida* (pRD1) which, however, did not reduce acetylene in anaerobic or aerobic conditions. The precise nature of their 'aerobic' conditions was not stated. Presence of pRD1 in the derivatives was confirmed by onward transfer from *P. putida* (pRD1) to *E. coli* and demonstration of nitrogenase protein synthesis in the latter recipient. In a reinvestigation of their question, using a *his* mutant of *P. putida* MT20, we have demonstrated unequivocal acetylene reduction by *P. putida* MT20-3 (pRD1) on the surface of low-N agar or as suspensions in sloppy agar under not more than 0.002–0.004 atm† O_2 in N_2 (see, for example, figure 2). For comparison, the 'anaerobic' diazotroph *K. pneumoniae* shows only 60 % inhibition of acetylene reduction under 0.2 atm O_2 on comparable pyruvate agar slopes. We have qualitative evidence that the process in *P. putida* is very O_2-sensitive, is repressed by NH_4 and is probably O_2-dependent, but satisfactory quantitative data are not yet available because of the leakiness of the experimental system used and the inhomogeneity of the bacterial growth. At the time of writing, replicable acetylene reduction by the constructs has not been obtained at any p_{O_2} in homogenous suspension, either in fluid or 'sloppy' media. However, these findings confirm expression of *Kp nif* in *P. putida* and establish its considerable O_2 sensitivity. Thus *Kp nif* can be expressed in the genetic background of an obligate aerobe.

A point of interest is that *nif* expression in *P. putida* is unaffected by whether or not the Tol plasmid pWW20 is present. This plasmid carried the *xylR* gene, which regulates the *xylABC* cluster, concerned in hydrocarbon catabolism *via* a promoter (OP1) showing considerable homology to *ntr*-activatable promoters of *nif* and other operons (see Dixon 1986). It follows that *P. putida* possesses a chromosomal *ntr* system sufficiently analogous to the *ntrA*, *ntrBC* system of *K. pneumoniae* to activate pRD1.

Kp nif in Azotobacteraceae

Earlier evidence for expression of pRD1 on an aerobe came from Cannon & Postgate's (1976) report of correction of the Nif$^-$ phenotype of non-regulatory *nif* mutants of *A. vinelandii*, but they were unable to repeat these observations some years later and duly withdrew their earlier

† 1 atm = 101 325 Pa.

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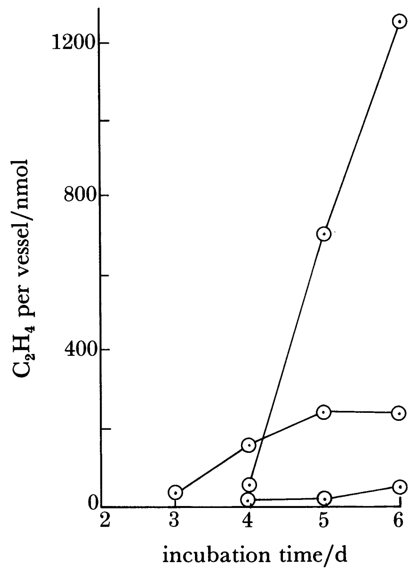

FIGURE 2. Replicate suspensions of *Pseudomonas putida* (pMF250) derepress *nif* in sloppy low-N agar under 0.5% O_2. Washed bacteria from aerobic glucose–nutrient broth culture were resuspended to about 0.2 mg (dry mass equivalent) ml^{-1} in 10 ml lots of N-free pyruvate salts medium with aspartic acid (200 μg ml^{-1}), nutrient broth (Oxoid, 2.5% by volume) and kanamycin (15 μg ml^{-1}), rendered 'sloppy' with 0.2% (by mass) agar in 27 ml Universal bottles. Cells and agar were mixed at 35 °C and left stagnant at 30 °C, sealed with a Suba-seal closure under N_2 + 0.4% O_2. C_2H_2 (1 ml) was injected after 2 d incubation and 0.2 ml samples were then analysed for C_2H_2 and C_2H_4 by gas chromatography. All cultures show positive acetylene reduction but the scatter among replicates is too great for useful quantitative conclusions to be drawn.

report (Cannon & Postgate 1983). They were unable to explain the discrepancy. The possibility that they fortuitously observed the now established alternative nitrogenase of *A. vinelandii* (see Bishop 1986) in their presumptively complemented mutants is not compatible with the data in their paper.

Although expression of the intact *Kp nif* regulon in *A. vinelandii* has not been demonstrated satisfactorily, evidence is available that several promoters within the *Kp nif* regulon are recognized by *A. vinelandii* and the cloned genes expressed. Thus *Kp nifL*:*lacZ* and *Kp nifF*:*lacZ* fusions were expressed in *A. vinelandii* but *Kp nifH*:*lacZ* was not (Kennedy & Drummond 1985). Toukdarian & Kennedy (1986) showed that expression from the *Kp nifL* promoter was activated by the *ntrC* gene of *A. vinelandii*, because the fusion was not expressed in *ntrC*: *Tn5* insertion mutants of *A. vinelandii*. The same authors showed that the *ntrC* product was not required for regular *nif* expression in *A. vinelandii*. Cloned *Kp nifA* expressed from a constitutive kanamycin resistance promoter on the plasmid pCK1 activated expression of *nif* in a regulatory mutant of *A. vinelandii* (Kennedy & Robson 1983) and there is now evidence for a *nifA*-like gene in *A. vinelandii* whose product activates *Kp nifU* and *nifE* (see Kennedy, this symposium).

The failure of the *nifH* promoter to be recognized probably underlies the non-expression of the intact regulon in *A. vinelandii* but the basic reason is still obscure: the *nifH* promoter regions of both *K. pneumoniae* and *A. vinelandii* are very similar in both the main and the upstream consensus sequences. However, the fact that promoters other than that of the *Kp nifHDKY* operon are recognized in *A. vinelandii* enabled Kennedy *et al.* (1985*a*, 1986) to use derivatives of pRD1 with a variety of *nif* mutations to identify *nifN* and presumptive *nifM* mutations of *A. vinelandii*.

The use of a constitutive *Kp nifA* clone has been valuable in the study of *nif* activation in *A. vinelandii*, but warning must be sounded because the behaviour of such plasmids is not wholly straightforward. For example, as mentioned earlier, pCKl gave NH_4^+-independent *nif* expression in a regulatory mutant in *A. vinelandii* as well as in a *nifA* mutant of *K. pneumoniae* (Kennedy & Robson 1983) but it did not do so in wild-type *K. pneumoniae* (Postgate & Kent 1985). Moreover, although pCK1 was necessary to obtain expression of wild-type *nif* on pRD1 in *Proteus mirabilis* (above), Postgate & Kent (1985) observed that such expression was not NH_4^+-constitutive.

Azospirilla are phylogenetically close to azotobacters. Plasmid pRD1 was transferred to *Azospirillum brasilense* to give strains presumably diploid for *nif* (Polsinelli *et al.* 1980) but its effect on *nif* expression was not tested. The integrity of pRD1 was checked by onward transfer of His^+ from *A. brasilense* (pRD1) to *E. coli*. Funayama *et al.* (1985) briefly reported correction of presumptive *nifA* mutants of *A. brasilense* by cloned *Kp nifA* on the *incP* plasmid pCK3.

Kp nif in Alcaligenes

Chen & Ye (1983) isolated diazotrophic *Alcaligenes faecalis* from the roots of rice plants and used pRD1 to complement a *nif* mutant. A curiosity was an apparent gene-dosage effect: pRD1 introduced into the wild-type diazotroph doubled its acetylene-reducing activity from *ca.* 34 to *ca.* 61 nmol C_2H_4 min^{-1} mg^{-1} bacterial protein. However, specific activities of populations of wild-type *K. pneumoniae* range from *ca.* 30 to *ca.* 200 according to cultural conditions, so a doubling of activity may arise for reasons only remotely related to *nif* expression.

Kp nif in Rhizobium and Agrobacterium

The original description of the construction of pRD1 (Dixon *et al.* 1976) reported that no diazotrophic growth or acetylene reduction took place after transfer to *Rhizobium meliloti* or *Agrobacterium tumefaciens* but that serologically detectable Kp1 peptide appeared in presumptively *nif*-derepressing conditions. Kozyrovskaya *et al.* (1984) confirmed non-expression of *nif* from pRD1 in *A. tumefaciens*. What genetic deficiency would lead to futile synthesis of a nitrogenase peptide is an interesting question which has not been resolved; in a preliminary report, Sastry *et al.* (1983) mentioned expression of *nifH–lac* and *nifL–lac* fusions in *A. tumefaciens*, implying that the promoters of both the regulatory and structural *nif* operons are recognized in this genetic background.

Kp nif in other prokaryotes

Plasmid pRD1 was transferred to *Zymomonas mobilis* by Skotnicki *et al.* (1980) without, apparently, testing for *nif* expression or for evidence that pRD1 had not reverted to RP4. Some strain-dependent instability of its drug-resistance markers was recorded. Similarly, Kuykendahl (1979) transferred pRD1 to *Bradyrhizobium japonicum* but presented no evidence that the plasmid had retained its *Klebsiella* DNA component. In view of its instability in *Salmonella* or *Proteus*, claims for stable conservation in alien backgrounds should be supported by onward transfer of pRD1 to *E. coli his* auxotrophs and demonstration of Nif^+.

Kp nif in eukaryotes

The possibility of constructing diazotrophic eukaryotes, notably crop plants of agricultural value, has interested scientists and excited discussion since the earliest *nif* gene transfers (see

Introduction above). The improbability of obtaining expression of a multi-operon cluster of at least 17 prokaryotic genes in a eukaryotic background raises daunting problems at the genetical level and, given success, the physiological problems then to be overcome are not inconsiderable. These matters have been well aired in the publications mentioned in the introduction and will not be discussed again here. However, two groups of workers have established the important point that the *Kp nif* cluster can be conserved intact in a eukaryotic background. Gerbaud *et al.* (1981) constructed a cosmid shuttle vector carrying *nif* and capable of transforming yeast (*Saccharomyces cerevisiae*). The cosmid was stably conserved for over 50 doublings of the yeast, shown by recovery of cosmid DNA and transformation of *E. coli* to Nif^+, but was 'silent' in yeast according to the acetylene test. Zamir *et al.* (1981) used a different approach: they co-mobilized the prokaryotic *nif* plasmid pWK220 into yeast with a yeast–*E. coli* shuttle vector and demonstrated integration of *nif* into the yeast chromosome by Southern hybridization with the *nif* plasmid. Two copies of *nif* were integrated; one was sometimes lost during multiplication but at least one was conserved for 40 doublings and showed Mendelian inheritance through meiosis and mitosis. The *nif* genes were 'silent' according to growth tests, acetylene reduction tests and, according to Maina *et al.* (1984), analysis for 'transcriptional products'.

The expression of single *nif* genes in a eukaryote presents less difficult problems in principle and can be achieved by placing such genes under the control of known yeast promoters. Maina *et al.* (1984) placed *Kp nifH* under the control of p-yeast-*adh*-1 and obtained *nifH*-specific mRNA in response to ethanol induction as well as observing material cross-reacting with antiserum to the purified *nifH* product, Kp2; Zilberstein *et al.* (1984) and Berman *et al.* (1985*a*, *b*) obtained similar cross-reacting material from a p-yeast-*ura*-3:*nifH* construct in yeast; Zilberstein *et al.* (1984) briefly reported material cross-reacting with anti-Kpl serum from a *padh*-1:*nifD* construct in yeast. Synthesis of enzymically active material was not obtained in any of these experiments but Berman *et al.* (1985*a*) reported that, as one might expect, expression of β-galactosidase from a *nifH*:*lacZ* fusion in yeast was independent of O_2 régime.

3. *Nif* genes from rhizobia in other bacteria

Nif genes were apparently mobilized from *R. trifolii* to *K. aerogenes* in a convoluted sequence of crosses in which the R factor Rldrd19 was transferred from *E. coli* to *Pseudomonas aeruginosa*, then into *R. trifolii* and finally to *K. aerogenes*. The Nif^+ phenotype was unstable in the recipient klebsiellae but was associated with a plasmid with an apparent molecular mass of 11 MDa (Dunican *et al.* 1976); this plasmid is too small to harbour a complete cluster of *Kp nif* genes. Stanley & Dunican (1979) obtained Nif^+ transconjugants in crosses between *R. trifolii* carrying plasmid RP1 and a deletion strain of *K. pneumoniae* lacking *nifQ*, *nifB* (genes involved in molybdenum uptake and FeMoco biosynthesis respectively), *nifA*, *nifL* (regulatory genes) and *nifF* (encoding a flavodoxin required for electron transport to nitrogenase). The *R. trifolii* (RP1) donor strain was also crossed with an avirulent strain of *Agrobacterium tumefaciens* and apparently gave rise to Nif^+ *A. tumefaciens* transconjugants. These early experiments with *R. trifolii* donors should be reconsidered in the light of more recent information concerning the organization of *nif* DNA in fast-growing rhizobia, which in many strains has been shown to be located with nodulation genes on a megaplasmid, designated pSym (Banfalvi *et al.* 1981, 1983; Hombrecher *et al.* 1981; Schofield *et al.* 1983). Transfer of the Sym plasmid from either *R. trifolii* (Hooykaas *et al.* 1984) or *R. meliloti* (Kondorosi *et al.* 1982) does not confer a Nif^+

phenotype on *A. tumefaciens*, even though the presence of *nif* genes in the recipient has been demonstrated. It is also important to note that, unlike *K. pneumoniae*, *nif* and *fix* genes are distributed into several non-linked clusters on the megaplasmids of fast-growing Rhizobiaceae (see Evans *et al.* 1985 *b*). Although it is possible that plasmid co-integrates were formed in the crosses between *R. trifolii* and Klebsiella strains, leading to the mobilization of large regions of the pSym plasmid, genes responsible for the Nif^+ phenotype of the recipients were not identified. It would certainly be useful to re-examine the original conclusions experimentally, now that defined *nif* and *fix* hybridization probes from rhizobia are available.

Page (1978) corrected structural *nif* mutants of *Azotobacter vinelandii* by transformation with genomic DNA from eight *Rhizobium* and *Bradyrhizobium* species; apparent correction of a non-structural *nif* mutant with DNA from two *Bradyrhizobium* species only was ambiguous for reasons which Page discussed. Page & von Tigerstrom (1978, 1979) and Page & Collinson (1982) described optimal conditions for such transformations.

Reports have been published of the transfer of *nod* and/or *sym* genes between species of *Rhizobium* (see, for example, Johnston *et al.* 1978) and also into *Agrobacterium*, *Azotobacter*, *Lignobacter*, *Pseudomonas* and *E. coli*, all with some degree of phenotypic expression (pseudo-nodulation or root curling) but no evidence of diazotrophy (Maier *et al.* 1978; Scott & Ronson 1982; Wong *et al.* 1983; Hirsch *et al.* 1984; Schofield *et al.* 1984; Plazinski & Rolfe 1985). Although rhizobial *nif* genes were probably co-transferred in these experiments, they provide few data pertaining to *nif* expression in the new backgrounds and they will not be reviewed further here. Cloning of rhizobial *nif* and *fix* genes in *E. coli* amounts, of course, to transfer of *nif* to an alien genetic background but for obvious reasons is not relevant to this particular contribution (see instead Johnston, this symposium).

4. Other instances of *nif* in alien backgrounds

Page (1985) reported transformational correction of nif^- mutants of *A. vinelandii* with genomic DNA from *Beijerinckia indica*. Derylo *et al.* (1981, 1982) studied the ligninolytic diazotroph *Lignobacter*, originally isolated as an agrobacterium, and confirmed that *nif* was located on one of its three natural plasmids, pUCS100, which could be transformed into *E. coli* and which rendered the recipient Nif^+ (growth and acetylene reduction). They quoted 17.5 or 19.8 MDa for the molecular mass of pUCS100, barely sufficient to carry a gene complement corresponding to the *nif* cluster of *K. pneumoniae* (23 ± 10 % kbp, equivalent to 15.5 MDa). A derivative with a Tn9 insertion expressed *nif* (growth on N-free medium) in *Salmonella typhimurium* but was unstable in this background.

Despite considerable research on the genetics of *nif* in *Azotobacter chroococcum* and *A. vinelandii* (see Kennedy *et al.* 1985 *b*) expression of a Nif^+ phenotype from azotobacter *nif* genes in an alien genetic background has not been reported. This is consistent with the fact that the *nif* genes corresponding to those identified in *K. pneumoniae* are not all clustered in a transmissible linkage group (see Kennedy, this symposium). However, correction of *Kp nif* mutants by cloned *A. chroococcum nif* DNA occurs and played an important part in the identification of *nifHD* and *K* (Jones *et al.* 1984) and *nifUSVM* (Evans *et al.* 1985 *a*) in *A. chroococcum*.

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5. Conclusions

Ecological

The transfer of *nif* genes to alien prokaryotic, or even eukaryotic, backgrounds is a reasonably facile laboratory operation, but obtaining nitrogen fixation by the recipients can be far from simple. It seems that the Princess Serendip looked favourably upon the earliest of these studies: in the *Klebsiella* → *E. coli* case an organism with a contiguous *nif* cluster was used as donor and the recipient possessed the *ntr* system essential for its expression. However, it now appears that such clustering of the *nif* genes is the exception rather than the rule: in most other diazotrophs that have been studied in sufficient detail, the *nif* genes are to some extent scattered. Several instances are now known in which the *nif* structural genes, and sometimes other genes, are plasmid-borne and therefore in principle transferable. So it is likely that conjugal or transformational transfer of some and sometimes all *nif* genes into new hosts, especially prokaryotes, is a fairly frequent event in the natural environment, particularly during the seasonal decay of diazotrophic plant associations or during microbial turnover in low-N environments such as decaying vegetation or compost heaps. However, expression of the transferred *nif* genes requires at least three important conditions to be satisfied:

(i) That the essential genes be present as a functional cluster in the donor, as in *K. pneumoniae*. Thus there is still no conclusive evidence that a rhizobial *sym* plasmid carries sufficient information to allow expression in a wholly new prokaryotic background, such as *E. coli*, nor that any reasonably sized contiguous fragment of the azotobacter chromosome could transform a heterogeneric recipient.

(ii) That a number of special genes be present in the recipient (the *narD* gene and its product, the *ntr* system and doubtless a variety of other determinants).

(iii) An O_2-excluding, micro-aerobic or anaerobic physiology.

Such genetic and physiological backgrounds are present among prokaryotes but not necessarily abundant. It is therefore reasonable to expect new types of prokaryotic diazotroph to appear where appropriate selection pressure exists but among a restricted range of donors and recipients: only organisms with a single linked *nif* cluster are likely to be effective donors but the range of potential recipients is clearly wider.

Evolutionary

Consideration of the position in *K. pneumoniae* led one of us (J. R. P.) to propose that the evolution of diazotrophy within the prokaryotes has taken place by lateral gene transfer among existing species and genera (see Postgate (1982) for a résumé). This view enables one to imagine that, in terms of biochemical evolution, diazotrophy is of relatively recent origin, thereby disposing of certain paradoxes concerning selection pressure in favour of the emergence of diazotrophy. In principle the view remains valid, with the modification that a complete *nif* cluster as in *K. pneumoniae* be regarded as more primitive than the dispersed arrangement of azotobacters or rhizobia. This proposal is consistent with more highly evolved state of the latter groups with regard to oxygen tolerance (Postgate 1982). The hypothesis that diazotrophy is a young property in terms of biochemical evolution has been challenged by Hennecke *et al.* (1985). They showed that the amino acid sequences of *nifH* products from six different diazotrophs (two others fell outside the comparison) could be arrayed in three pairs of similarity groups which gave a dendrogram approximately congruent to an array of the ‘fingerprints’ of the 16S

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rRNA of those diazotrophs or putative relatives. They argued that the evolutionary divergence of the *nifH* product in these organisms has followed that of the 16*S* rRNA and, because the latter is an accepted guide to prokaryotic evolutionary relationships, *nif* must have been part of their genomes since those genomes came into existence. This proposition is inconsistent with lateral diffusion of *nif* among prokaryotic genomes. However, congruence of dendritic trees of greater complexity is needed, as well as more knowledge of the factors influencing the evolution of acquired genetic characters within a given genetic background, before lateral diffusion can be discarded as a component of the evolution of diazotrophy.

Exploitation

With regard to the deliberate construction of diazotrophic organisms, the main prerequisites are now obvious: a genome capable of handling prokaryotic-type genetic information, means of assimilating and processing the component metals of nitrogenase and of regulating *nif*, means of generating adequate ATP and of excluding O_2. On top of these, the *nif* and any ancillary genes must be stabilized in the new background, perhaps by integration into the chromosome, as in *Enterobacter cloacae* and *E. coli* CM7, or perhaps by setting up internal selection, as in *Salmonella typhimurium his* deletions. Even so, the efficiency of this process may be influenced by unsuspected physiological factors, as was illustrated in the context of table 2: we were unable to establish *E. herbicola* (pRD1) or *S. marcescens* (pRD1) in continuous diazotrophic cultures at all, despite the fact that their diazotrophic activity, according to the acetylene test and $^{15}N_2$ incorporation, was not excessively low (Krishnapillai & Postgate 1980). We do not know why. However, such difficulties must not discourage attempts to transfer expressible *nif* to new organisms, but the scientist must be alert for problems which are likely to be recipient-specific, as with *Proteus mirabilis* and *Pseudomonas putida*.

The creation of new diazotrophs by manipulation of *nif* can yield valuable information of a fundamental character but it is not very likely to provide diazotrophs of practical value for the simple reason that diazotrophs already populate most ecological niches in which diazotrophy is advantageous. However, the transfer of expressible *nif* to eukaryotes, particularly crop cereals, presents a challenge which has fascinating fundamental and practical implications; the message of the limited research on intergeneric transfer conducted so far is that the genetic engineer should transfer as much of the prokaryotic genetic apparatus as possible, as an interlinked package (e.g. *ntr*+*nif* (Merrick & Dixon 1984)) and locate it in as prokaryote-like a part of the plant genome as can be found, the chloroplast being widely favoured. Essentially, the objective would be to convert the photosynthetic organelle into a diazotrophic organelle by manipulation of its genome; it is complementary to the less widely discussed strategy of taking an existing diazotroph and manipulating its genome such that it becomes a plant-dependent diazotrophic pseudo-organelle, a modification of the endocellular L-forms of Aloysius & Paton (1984).

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