Qualitative Evidence for Expression of *Klebsiella pneumoniae* *nif* in *Pseudomonas putida*

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Pseudomonas putida MT20-3 carrying the *Klebsiella pneumoniae* *nif* plasmids pRD1 or pMF250 showed highly O₂-sensitive aerobic acetylene reduction on low-N pyruvate or glucose agar. This finding confirms unequivocally that *K. pneumoniae* *nif* can be expressed in an obligate aerobe.

INTRODUCTION

The ability of members of the genus *Pseudomonas* to fix nitrogen, long subject to uncertainty (see discussion by Postgate *et al.*, 1987), was finally established by the report (Barraquio *et al.*, 1986) of O₂-sensitive diazotrophy in *P. saccharophila*. The ability of genetic constructs of *Pseudomonas* to express the *nif* gene cluster of *Klebsiella pneumoniae* has also been doubted. Mergeay & Gerits (1978) obtained transconjugants of a putative *P. fluorescens* carrying IncP or IncF plasmids bearing the *K. pneumoniae* *nif* cluster which reduced acetylene aerobically in stagnant culture. Their report had disquieting features because (a) the *Pseudomonas* had originally been isolated as a *Rhizobium* and (b) the IncF plasmid FN68, which is usually unstable, was accepted and expressed in this organism. Lehtinen & Mäntsälä (1981) found that the more stable IncP *K. pneumoniae* *nif* plasmid 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. They did not state the precise nature of their 'aerobic' conditions but they confirmed the presence of pRD1 in their derivatives by mating *P. putida*(pRD1) to *Escherichia coli* and demonstrating nitrogenase synthesis in the recipients. The question is important because, with the withdrawal of a claim that these genes are expressed in *nif* mutants of *Azotobacter vinelandii* (Cannon & Postgate, 1983), there is no clear evidence for their expression in an obligately aerobic host.

We now report unequivocal acetylene reduction by *P. putida* carrying plasmid-borne *K. pneumoniae* *nif*. The process is extremely O₂-sensitive in *P. putida* and satisfactory quantitative data are not available. This finding was briefly mentioned by Postgate *et al.* (1987).

METHODS

Organisms and culture. Organisms and plasmids are listed in Table 1. *P. putida* MT20-3 (Williams & Worsey, 1976) is a *his* derivative of *P. putida* WW20 which was cured of its Tol plasmid pWW20. The *nif* plasmid pMF250 is a descendant of RP4 via pRD1 (= RP41; Dixon *et al.*, 1976) and pMF100 (Merrick *et al.*, 1980) with the original drug resistance genes deleted and Km^r restored with Tn5. Plasmids were normally resident in *E. coli* JC5466 except for pWW20 which is naturally resident in *P. putida* MT20 and its derivatives. Bacteria were grown aerobically at 29 °C on minimal glucose agar (Davis & Mingioli, 1950) with 30 µg tryptophan ml⁻¹ for *E. coli* JC5466 (pMF250) or 30 µg histidine ml⁻¹ for *P. putida* MT20-3. *E. coli* was sometimes grown at 37 °C; *P. putida* MT20-3 did not grow at 37 °C. For mating, loops of donor *E. coli* and recipient *P. putida* were spread with 1 or 2 drops of saline phosphate buffer (SP; Krishnapillai & Postgate, 1980) on Luria's agar (LA; Kennedy, 1977) and incubated for 18 to 24 h at 29 °C. Cells resuspended in SP were spread on glucose minimal agar with 15 µg kanamycin ml⁻¹. To enable transfer of IncQ plasmids to *P. putida*, donor *E. coli* JC5466 derivatives were

Abbreviations: SB, saline phosphate buffer; LA, Luria's agar; NB, nutrient broth.

Table 1. *Bacteria and plasmids*

Bacterial strains	Genotype	Origin or reference
<i>Pseudomonas putida</i> MT20-3	<i>his</i>	P. A. Williams, University of Bangor, UK
<i>Escherichia coli</i> JC5466	<i>his trp recA rplE</i>	N. Willets
Plasmids		
pWW20	Tol	P. A. Williams
pRD1	Cb Km Tc Gnd Nif His ShiA Tra IncP	Dixon <i>et al.</i> (1976)
pMF250	Km Gnd His ShiA Tra IncP	M. Filser, see Postgate & Kent (1985)
pCK1	Sm Mob Km : <i>nifA</i> :Km IncQ	Kennedy & Robson (1983)
pRK2013	Sm Tra	Figurski & Helinski (1979)
pMD45	Sm Mob Km : <i>ntrC</i> :Km IncQ	Kennedy & Drummond (1985)
pMM22	Sm Mob Km : <i>ntrA</i> :Km IncQ	M. Merrick, University of Sussex, UK

constructed carrying both the IncQ plasmid and the Tra⁺ mobilizing plasmid pRK2013 (Figurski & Helinski, 1979). Presumptive transconjugant *Pseudomonas* isolates, selected with 100 µg streptomycin ml⁻¹, were checked for absence of donor by their failure to grow on LA at 37 °C. Plasmids in transconjugants were demonstrated by electrophoresis of alkaline lysates or boiled SDS extracts in agarose gels (Cannon, 1980) as appropriate; plasmid pWW20 was detected by Wheatcroft & Williams's (1981) method. Genetic evidence that the IncP plasmids retained relevant *Klebsiella* DNA in the new background was obtained by transconjugation from the *P. putida* derivatives to *E. coli* JC5466. Donor and recipient strains were mated in liquid NFDM (Cannon *et al.*, 1974) supplemented with aspartate (100 µg ml⁻¹) and Oxoid nutrient broth (NB; 2.5%, w/v) (Postgate & Kent, 1985) for 24 h at 30 °C, the culture selected for Km^rSpc^r *E. coli* transconjugants and their Nif⁺ phenotype checked by acetylene reduction in similar NFDM + 30 µg tryptophan ml⁻¹.

RESULTS

Plasmids pMF250 or pRD1 transferred from *E. coli* JC5466 to *P. putida* MT20-3 at about 3×10^{-4} transconjugants per total cell population mated with 100% co-transfer of Km^r and His⁺.

Slopes of *P. putida*(pRD1) or (pMF250) on glucose- or sodium pyruvate-based NFDM containing aspartate and NB, set with agar, were incubated in air until grown (18 to 24 h at 29 °C) and then sealed under N₂ with low O₂ (below). The cells grew as a thin film; they were clearly O₂-limited because growth was more abundant in air. Such cultures gave clear and qualitatively reproducible acetylene reduction after 2 to 5 d. All positive tubes were checked for absence of donor (see Methods); controls with wild-type *P. putida* were uniformly negative. A variety of formulations of the agar media was tested and, though the majority supported acetylene reduction, scatter between replicate tubes rendered the results quantitatively unsatisfactory. We concluded that glucose, pyruvate, succinate, citrate, acetate and mixtures thereof as carbon sources showed no consistent differences (except that acetate was not utilized by this strain at all) but that pyruvate was marginally the most consistently effective substrate. Aspartate and NB were desirable for derepression but increased concentrations were of no advantage, nor were addition of 10% (v/v) nutrient agar (Oxoid) or 10% LA. Activity was obtained at pO₂ values of 0.001 to 0.004 atm but no higher. This is a high level of O₂ sensitivity; for example, the obligately anaerobic diazotroph *K. pneumoniae* M5al, tested on the agar medium in Fig. 1, showed only 60% inhibition of its anaerobic acetylene reduction rate under 0.2 atm O₂.

In an effort to obtain standardized quantitative replication, the procedure and conditions given in Fig. 1 were adopted for routine use but an unacceptable scatter of reduction rates and lags occurred among triplicate tubes. We attribute this scatter partly to inhomogeneity of bacterial growth on the agar, partly to imperfect replication of oxygen tensions in the system used. However, in no test did *P. putida*(pMF250) or (pRD1) reduce acetylene if sealed under pure O₂-free N₂, nor if NH₄Cl (0.2%, w/v) were present in the agar. No anaerobic growth of this strain occurred with nitrate or fumarate as electron acceptors for glucose, pyruvate or fumarate.

Fig. 1. Acetylene reduction on low-N agar slopes at low pO_2 by *P. putida* MT20-3(pMF250) and derivatives. Organisms from 24 h plates of glucose minimal medium were suspended to similar densities (OD_{540} about 0.5) and inoculated in triplicate with a loop on 10 ml pyruvate-NFDM agar slopes in 30 ml tubes with kanamycin or kanamycin and streptomycin, as appropriate. After 7 h at 29 °C in air, tubes were tilted carefully to wet the whole agar surface. After 24 h in air, all tubes were gassed out aseptically with N_2 , sealed with a Suba-seal closure and air injected to 0.004 atm O_2 . After a further 21 h (time zero in the Figure), 1 ml C_2H_2 was injected. Selected tubes are illustrated of constructs carrying the *nif* plasmid pMF250 alone (○) or with pCK1 (■), pMD45 (□) or pMM22 (●) (see text and Table 1). Lags, turnover points and reduction rates were not consistent among triplicate tubes of the strains tested but all were qualitatively positive.

Constructs of *P. putida*(pMF250) were made carrying the plasmids pCK1 (*nifA*^c: Kennedy & Robson, 1983), pMD45 (*ntrC*^c: Kennedy & Drummond, 1985) and pMM22 (*ntrA*^c: M. Merrick, personal communication). None appeared better or worse than the parent (Fig. 1). The bacterial growth washed off six slopes, which had given clear, continuous acetylene reduction, into SP was assayed for protein by the Lowry method and specific activities were calculated. They were similar at about 0.3 nmol C_2H_4 formed (mg protein)⁻¹ min⁻¹ (cf. 70 to 180 for *K. pneumoniae*) but for one capricious tube of substantially higher activity which gave ordinary activity on subculture.

The Tol plasmid pWW20 carries the *xyIR* gene which regulates the *xyLABC* cluster. It does so via a promoter (OP1) which shows considerable homology to Ntr-activatable promoters of *nif* and other operons (see Dixon, 1986). However, slopes of *P. putida* MT20-3(pWW20, pMF250) showed no significant differences in acetylene-reducing activity from slopes of *P. putida* (pMF250). Induction of *xyIR* by including methylbenzyl alcohol in the medium decreased rather than augmented activity.

O_2 -sensitive aerobic acetylene reduction by *P. putida*(pMF250) growing dispersed in sloppy NFDM agar was mentioned by Postgate *et al.* (1987). That system had no advantage over agar for quantitative study. Considerable effort was made to obtain a homogeneous experimental system but no acetylene reduction by *P. putida*(pMF250) or (pRD1) was detected in numerous assays in liquid glucose or pyruvate-NFDM shaken gently in air, stagnant in air or stagnant in sealed vessels at initial pO_2 values ranging from 0.05 to 0.002 atm.

DISCUSSION

This work provides unequivocal evidence of aerobic expression of the *K. pneumoniae nif* cluster in a taxonomically defined aerobic *Pseudomonas* background. It confirms the claims of Mergeay & Gerits (1978) that *nif* from *K. pneumoniae* is expressed in the genus *Pseudomonas* and it indicates that there is no obstacle in principle to expression of this cluster in other aerobic physiological backgrounds. The extreme sensitivity of expression to O_2 -repression or inhibition suggests that ideal conditions for expression of *nif* from *K. pneumoniae* were not obtained in this work and implies that respiratory protection in these constructs is minimal. The apparent failure

of constitutive *ntrC*, *ntrA* or *nifA* plasmids to influence activity probably reflects this fact. The results also show that *P. putida* possesses a chromosomal *ntr*-like system sufficiently analogous to the *ntrA ntrBC* system of *K. pneumoniae* to activate *K. pneumoniae nif* and, further, that *xylR* is not involved.

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