Genetic Mapping of the *Rhodopseudomonas capsulata* Chromosome Shows Non-clustering of Genes Involved in Nitrogen Fixation

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(Received 19 March 1985; revised 7 July 1985)

The mutant R plasmid pTH10 was used to construct a circular linkage map of the *Rhodopseudomonas capsulata* B10 chromosome. Mutations affecting nitrogen fixation (*nif* mutations) were dispersed in several groups on the chromosome. Biochemical analysis of *nif* mutants allowed identification of the structural gene for the nitrogenase component II or Fe protein (*nifH*) and a putative regulatory gene, possibly *nifA*. These two genes appeared closely linked in conjugation experiments, but represented two distinct linkage groups in crosses mediated by gene transfer agent. Other mutants were affected in the synthesis and/or stability of the nitrogenase component I or MoFe protein; synthesis of component II was also affected, but to a lesser extent. In two of these mutants, nitrogenase activity and the content of component I was increased five- to sixfold by the addition of 1 mM-molybdate to the growth medium.

INTRODUCTION

The photosynthetic, purple, non-sulphur bacteria, in common with many other bacterial species, are able to fix N_2 . When derepressed for nitrogenase synthesis in the absence of N_2 , cultures of purple, non-sulphur bacteria show a light-dependent evolution of H_2 , a reaction that occurs at particularly high rates in *Rhodopseudomonas capsulata* (Hillmer & Gest, 1977; Jouanneau *et al.*, 1982). Nitrogenase synthesis in *R. capsulata* is repressed by NH_4^+ and by O_2 ; nitrogenase activity is also inhibited by NH_4^+ , as in other purple, non-sulphur bacteria, by a mechanism involving reversible covalent modification of the Fe protein [see Vignais *et al.* (1985) for a review].

As part of the study of the regulation of N_2 fixation and of H_2 production in *R. capsulata*, we wished to determine the organization of the genes involved in N_2 fixation and to identify regulatory genes. The organization and regulation of N_2 fixation (*nif*) genes has been studied in most detail in *Klebsiella pneumoniae*. Between 12 and 14 *nif* genes have been identified in addition to the structural genes for nitrogenase, *nifH*, *D* and *K*, which code respectively for component II, also known as the Fe protein, and for the α and β subunits of component I, the MoFe protein (Roberts & Brill, 1981; Dixon, 1984). These genes are clustered in a 24 kb region of the chromosome, and are organized in seven or eight transcriptional units or operons. The regulatory genes *nifL* and *nifA* form one of these operons: the *nifL* gene product has a repressor function and may be involved in O_2 regulation, while the *nifA* gene product is required for the expression of all other *nif* operons. Regulation of *nifLA* gene expression by fixed N is mediated by the unlinked N_2 regulation (*ntr*) genes which also regulate other genes involved in N metabolism (Magasanik, 1982; Ausubel, 1984; Dixon, 1984).

Abbreviations: Cma, chromosome-mobilizing ability; GTA, gene transfer agent.

Although a similar number of genes is probably involved in N_2 fixation in other organisms, relatively little is known about their organization. The *nifH*, *D* and *K* genes, and in some cases *nifV* or *nifS*, have been cloned from a number of bacteria by exploiting their homology with the corresponding genes from *K. pneumoniae*. These and neighbouring genes have been physically mapped in *Anabaena* sp. 7120 (Rice *et al.*, 1982), in several *Rhizobium* species (Ruvkun & Ausubel, 1981; Elmerich *et al.*, 1982; Corbin *et al.*, 1983; Downie *et al.*, 1983; Scott *et al.*, 1984; Fischer & Hennecke, 1984; Weinman *et al.*, 1984) and in *Azotobacter chroococcum* (Jones *et al.*, 1984). These studies have revealed differences in the organization and transcription of *nif* genes from that observed in *K. pneumoniae*. However, the presence of *nif* coding regions in the vicinity of the nitrogenase structural genes is frequently observed.

Among the purple, non-sulphur bacteria, genetic studies of N_2 fixation have been restricted to *R. capsulata*, although *Nif*⁻ mutants of some other species have been isolated (see Vignais *et al.*, 1985). The cloned *nifHDK* genes of *R. capsulata* are contiguous and transcribed in a single operon, as in *K. pneumoniae* (Avtges *et al.*, 1983). A 20 kb fragment of *R. capsulata* DNA has been isolated which contains several *nif* genes, although their function has not been identified (Pühler *et al.*, 1984). Finally, by analysing *Nif*⁻ mutants with gene transfer agent (GTA), a transduction-like system specific to *R. capsulata*, it has been possible to identify six linkage groups of *nif* mutations; however, the distance between these linkage groups could not be established (Wall & Braddock, 1984; Wall *et al.*, 1984).

In the present study, we have used R plasmid-mediated conjugation to construct a genetic map of the *R. capsulata* chromosome and to determine the approximate location of the *nif* genes. In parallel, we have biochemically analysed *Nif*⁻ mutants in order to obtain information on the function of the different *nif* genes. The results indicate that the *nif* genes are dispersed in at least four or five groups on the *R. capsulata* chromosome.

METHODS

Bacterial strains and plasmids. All the *R. capsulata* mutants were derived from the wild-type strain B10. However, the laboratory strain of B10 differed from the originally isolated strain (Marrs, 1974) in being a poor producer of GTA. Only strains derived from the His⁻ mutant J50 (obtained from Dr J. D. Wall, Department of Biochemistry, University of Missouri, USA) were good producers of GTA. The *Nif*⁻ mutants RC1 to RC35 have been described previously (Willison & Vignais, 1982). The *nif* or *ntr* allele numbers assigned to these mutants are the same as the strain number. Other strains and the plasmids used in this study are listed in Table 1.

Media and growth conditions. *R. capsulata* strains were grown and maintained as described previously (Willison, & Vignais, 1982). The minimal medium was RCV medium (Weaver *et al.*, 1975; Hillmer & Gest, 1977) containing either 30 mM-DL-malate or 30 mM-DL-lactate as carbon source and 7.5 mM-(NH₄)₂SO₄ as N source. N-free medium was prepared by omitting the (NH₄)₂SO₄. Growth factors for auxotrophs were added at the following concentrations: amino acids and nucleotide bases, 20 µg ml⁻¹; biotin, 50 ng ml⁻¹. The complex medium used was YPS medium (Weaver *et al.*, 1975) supplemented with 20 µg tryptophan ml⁻¹. *Escherichia coli* strains were grown in L-broth (Lennox, 1955), or in L-medium containing VB salts medium (Vogel & Bonner, 1956) instead of NaCl, and solidified with 1.5% (w/v) agar. Liquid cultures were grown aerobically at 30 °C, in 10 ml capacity stoppered tubes containing 3 to 5 ml medium agitated in a rotary shaker. Growth was followed by measuring the OD₆₆₀. The number of viable cells (c.f.u.) was determined by serially diluting the culture in dilution buffer (RCV medium without C or N source) and spreading 0.1 ml samples on agar plates. In exponential phase cultures, an OD₆₆₀ of 1.0 corresponded to approx. 3×10^8 c.f.u. ml⁻¹.

Isolation of auxotrophs. Wild-type or auxotrophic strains were mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) (Willison & Vignais, 1982) or ethyl methanesulphonate (Meynell & Meynell, 1970) and grown photosynthetically to early stationary phase in YPS medium. The cultures were diluted 10-fold in RCV medium, supplemented with the growth requirements of the parental strain, and incubated in the light until the OD₆₆₀ had doubled (2 to 3 h). The cells were then washed three times by centrifugation and resuspension in the same volume of RCV medium, diluted 10-fold in appropriately supplemented RCV medium, and incubated in the light until the culture became slightly turbid (4 to 6 h). Penicillin G was then added to a final concentration of 100 U ml⁻¹ and incubation continued for 24 h. The penicillin-treated cells were washed three times in RCV medium, diluted 10- to 100-fold and spread on YPS agar plates. Auxotrophs (0.1 to 1% of survivors) were identified by replica-plating of colonies after 2 to 3 d aerobic growth in the dark. The nutritional requirements of auxotrophic strains were subsequently identified by streaking onto media containing different combinations of growth factors, as described by Holliday (1956).

Isolation of carotenoidless mutants. Mutants affected in carotenoid biosynthesis (Crt^-) were obtained after NTG mutagenesis by plating appropriate dilutions of an early stationary phase culture onto YPS agar medium, and inspecting plates after 3 to 4 d aerobic incubation in the dark for colonies with altered pigmentation. Such colonies were observed at a frequency of 0.05 to 0.1%.

Isolation of Nif^- mutants. AP1, like strains AP29 and AP31, is a spontaneous mutant isolated from an N-limited continuous culture (P. Allibert, personal communication). Nif^- derivatives of strain J50 were isolated as described previously (Willison & Vignais, 1982) by spreading cultures onto RCV agar medium containing 7 mM-L-glutamate as major N source and supplemented with 20 μg histidine ml^{-1} and 200 μM -metronidazole. Between 200 and 500 metronidazole-resistant colonies appeared per 10^8 c.f.u. plated, of which 50 to 100% were Nif^- , irrespective of whether the culture had been treated with mutagen or not (cf. Wall *et al.*, 1984). This contrasted with the behaviour of strain B10, which yielded <2% Nif^- colonies in the absence of treatment with mutagen.

Isolation of spontaneous antibiotic-resistant mutants. Cultures grown to early stationary phase were plated on RCV or YPS agar medium containing streptomycin (100 μg ml^{-1}), rifampicin (40 μg ml^{-1}), erythromycin (10 μg ml^{-1}) or novobiocin (5 μg ml^{-1}). Only RCV medium was used for the isolation of erythromycin-resistant (Ery^r) mutants, due to the slow growth of Ery^r strains on YPS medium. Antibiotic-resistant colonies appeared after 3 to 4 d aerobic incubation in the dark, at the following frequencies (per c.f.u. plated): Str^r , 10^{-7} to 10^{-9} ; Rif^r , 10^{-6} ; Ery^r , 10^{-7} ; Nov^r , 10^{-6} to 10^{-7} .

Construction of plasmid-bearing strains. Plasmids were transferred between strains of *E. coli* and *R. capsulata*, and between different *R. capsulata* strains, by conjugation on YPS agar medium. For interspecific crosses, donor and recipient cultures were grown aerobically to exponential phase (2 to 3×10^8 c.f.u. ml^{-1}) in complex medium at 30°C and small samples (approx. 25 μl) mixed on the surface of a YPS agar plate. The drop was spread to cover an area of approx. 2 cm^2 , allowed to dry, and the plate incubated at 30°C in the dark for 16 to 24 h. The resultant growth was scraped off the plate, resuspended in 1.5 ml YPS medium, and 0.1 ml samples of a 10^{-4} dilution were plated on either RCV agar medium containing 10 μg kanamycin ml^{-1} (for transfer to *R. capsulata*) or L-agar medium containing 20 μg kanamycin ml^{-1} and 200 μg streptomycin ml^{-1} (for transfer to *E. coli* HB101). Different *R. capsulata* strains were crossed as above, except that cultures were generally grown overnight for 16 to 18 h. Km^r colonies of *R. capsulata* were grown aerobically in the dark for 4 to 6 d, and then purified on YPS agar medium containing 25 μg kanamycin ml^{-1} . The presence of other plasmid-encoded markers was tested on medium containing antibiotics at the following concentrations: for *E. coli*, 10 μg tetracycline ml^{-1} and 25 μg ampicillin ml^{-1} ; for *R. capsulata*, 1 μg tetracycline ml^{-1} (the R plasmids used in this study did not confer ampicillin-resistance to *R. capsulata* B10, for which the MIC was <0.1 μg ml^{-1}).

Transfer of chromosomal markers by R plasmids. The chromosome donor strains used in mapping experiments were generally auxotrophic and were counterselected on appropriately supplemented minimal medium. Donor and recipient strains were grown aerobically in YPS medium, except for strain G29, which was grown photosynthetically in RCV medium containing 12 mM-glutamine as major N source. A 1:1 mixture of YPS medium and dilution buffer, containing 10^6 to 10^8 donor c.f.u. ml^{-1} and 10^7 to 10^8 recipient c.f.u. ml^{-1} , was plated in 0.1 ml samples on RCV agar medium for the selection of prototrophic recombinants and on N-free RCV medium for the selection of Nif^+ recombinants. When the Crt^+ phenotype was selected, plates were incubated in anaerobic jars illuminated from one side only, at a distance of 60 cm, to minimize background growth of the recipient Crt^- strain. Mixtures containing recipient or donor cells alone were plated out as controls. Recombinant colonies were grown for 3 to 4 d in the light, or for 4 to 6 d in the dark, and were tested for cotransfer of unselected markers. Donor cell markers reverted at rates between 10^{-8} and 10^{-9} per c.f.u.; recipient cell markers, unless otherwise stated, reverted at rates between 5×10^{-7} and 10^{-8} per c.f.u. When antibiotic counterselection was used, e.g. for strain construction (see Table 1), crosses were done on YPS agar medium, and the suspension of the mating mixture plated, without dilution, on agar medium containing the appropriate antibiotic.

Complementation of Nif^- mutants with pRPA8. Triparental matings between HB101(pRPA8), HB101(pRK213) and *R. capsulata* Nif^- strains were done as described above for interspecific mating, except that 10 μl of each *E. coli* strain culture was mixed with 20 μl of *R. capsulata* culture. Mating mixtures were diluted 10^4 -fold and plated either on N-free RCV agar medium or on RCV medium containing 1 μg tetracycline ml^{-1} .

Mapping by GTA. GTA-producing strains containing *nif* markers were constructed either by isolating Nif^- mutants of strain J50 (see above) or by transferring *nif* markers into strain JP22 by conjugation, as indicated in Table 1. In the latter case, mating was done on YPS agar medium and the His^+ Str^r recombinants were selected and screened for Nif^- phenotype; *nif* markers were countertransferred with *his-1* $^+$ at a frequency of 0.3 to 1%. GTA-mediated crosses were done as described by Yen & Marrs (1976). Streptomycin resistance (Str^r) was used as the unlinked control marker in mapping experiments. Str^r recombinants were selected on RCV agar medium overlaid, after 4 h preincubation in the dark, with 5 ml RCV soft agar (0.6%, w/v) containing sufficient streptomycin to give a final concentration on the plates of about 100 μg ml^{-1} .

Agarose gel electrophoresis. Plasmids in *E. coli* and *R. capsulata* were visualized by a modification of the Eckardt procedure, as described by Rosenberg *et al.* (1982).

Table 1. *Bacterial strains and plasmids*

Strain	Genotype or phenotype*	Comments and source/reference†
<i>E. coli</i> strains		
TH471	F ⁻ , Thr ⁻ Leu ⁻ Thi ⁻ <i>tonA</i> <i>lacY supE</i> pTH10	Harayama <i>et al.</i> (1980)
HB101	F ⁻ , <i>recA</i> Pro ⁻ Leu ⁻ Thi ⁻ <i>lacZ</i> <i>rpsL supE</i>	Boyer & Roulland-Dussoix (1969)
<i>R. capsulata</i> strains		
B10	Wild-type	Marrs (1974); Weaver <i>et al.</i> (1975)
J50	<i>his-1</i>	Genthner & Wall (1984)
G29	<i>glnA1</i>	Wall & Gest (1979); Scolnik <i>et al.</i> (1983)
BY16510	<i>bchA165 crtB4 hsd-1 str-2</i>	Taylor <i>et al.</i> (1983)
AP1	<i>nif-200</i>	P. Allibert, Centre d'Etudes Nucléaires, Grenoble
AP29	<i>nif-201</i>	Allibert <i>et al.</i> (1984)
AP31	<i>nif-202</i>	Allibert <i>et al.</i> (1984)
RC1	<i>nif-1 (nifH1)</i>	Willison & Vignais (1982)
RC3	<i>nif-3 (nifH3)</i>	Willison & Vignais (1982)
RC4	<i>nif-4</i>	Willison & Vignais (1982); 'leaky'
RC7	<i>nif-7</i>	Willison & Vignais (1982)
RC8	<i>nif-8 (nifH8)</i>	Willison & Vignais (1982)
RC9	<i>nif-9 (nifH9)</i>	Willison & Vignais (1982)
RC10	<i>nif-10</i>	Willison & Vignais (1982); 'leaky'
RC11	Nif ⁻	Willison & Vignais (1982); 'leaky'
RC12	Nif ⁻	Willison & Vignais (1982); 'leaky'
RC15	<i>nif-15</i>	Willison & Vignais (1982)
RC16	<i>nif-16</i>	Willison & Vignais (1982)
RC17	<i>nif-17</i>	Willison & Vignais (1982)
RC18	<i>nif-18</i>	Willison & Vignais (1982)
RC19	<i>nif-19</i>	Willison & Vignais (1982)
RC20	<i>nif-20</i>	Willison & Vignais (1982)
RC21	<i>nif-21</i>	Willison & Vignais (1982)
RC22	<i>nif-22</i>	Willison & Vignais (1982)
RC23	<i>nif-23</i>	Willison & Vignais (1982)
RC24	<i>nif-24</i>	Willison & Vignais (1982)
RC26	<i>nif-26</i>	Willison & Vignais (1982)
RC27	<i>ntr-27</i>	Willison & Vignais (1982)
RC28	<i>ntr-28</i>	Willison & Vignais (1982)
RC29	<i>ntr-29</i>	Willison & Vignais (1982)
RC30	Nif ⁻	Willison & Vignais (1982); 'leaky'
RC31	Nif ⁻	Willison & Vignais (1982); 'leaky'
RC32	<i>nif-32</i>	Willison & Vignais (1982)
RC33	<i>nif-33 (nifH33)</i>	Willison & Vignais (1982)
RC34	<i>ntr-34</i>	Willison & Vignais (1982)
RC35	<i>nif-35</i>	Willison & Vignais (1982)
RC37	Wild-type, pTH10	Derived from B10 by conjugation with <i>E. coli</i> TH471
RC38	Wild-type, RP1	Derived from B10 by conjugation with <i>E. coli</i> HB101(RP1)
RC39	<i>ilv-10</i>	Derived from B10 by NTG
RC42	<i>ade-1</i>	Derived from B10 by NTG
RC58	<i>crt-4</i>	Blue-green mutant, derived from B10 by NTG
RC92	<i>ura-2</i>	Derived from B10 by NTG
RC94	<i>bio-2</i>	Derived from B10 by NTG
RC98	<i>ilv-10 str-6</i>	Spontaneous derivative of RC39
RC99	<i>ilv-10 nov-1</i>	Spontaneous derivative of RC39
RC100	<i>ilv-10 rif-1</i>	Spontaneous derivative of RC39
RC101	<i>ilv-10 rif-1</i> , pTH10	Derived from RC100 by conjugation with RC37
RC102	<i>ilv-10 rif-1</i> , RP1	Derived from RC100 by conjugation with RC38
RC103	<i>ilv-10 rif-1 str-1</i> , pTH10	Spontaneous derivative of RC101
RC126	<i>ilv-10 rif-1 str-1</i> , pTH10	Spontaneous derivative of RC103 with increased Cma
RC134	<i>tyr-1</i>	Derived from LS10 by GTA
RC135	<i>tyr-1 ery-1</i>	Spontaneous derivative of RC134
RC138	<i>tyr-1 ery-1 nov-9</i> , pTH10	Derived from RC135 by conjugation with GA100
RC149	<i>tyr-1 rif-1 str-1</i>	Derived from RC134 by conjugation with RC126
RC160	<i>ade-1 tyr-1</i> , pTH10	Derived from LS10 by conjugation with RC126
RC168	<i>ade-1 ura-1 rif-1 str-1</i> , pTH10	Derived from LS8 by conjugation with RC103

Table 1 (continued)

Strain	Genotype or phenotype*	Comments and source/reference†
RC183	<i>his-1, nif-85</i> , pTH10	Derived from GA108 by conjugation with GA100
RC188	<i>his-1 nif-85 str-14</i>	Derived from GA108 by GTA
RC189	<i>his-1 nif-88 str-14</i>	Derived from GA111 by GTA
RC192	<i>his-1 trp-1</i> , pTH10	Derived from LS2 by conjugation with GA100
GA100	<i>ilv-10 nov-1</i> , pTH10	Derived from RC99 by conjugation with RC37
GA108	<i>his-1 nif-85</i>	Derived from J50 by EMS
GA111	<i>his-1 nif-88</i>	Spontaneous derivative of J50
GA120	<i>ilv-10 rif-1 crt-10</i> , pTH10	Blue-green mutant, derived from RC392 by NTG
GA143	<i>his-1 nif-88 crt-13</i>	Orange mutant, derived from GA111 by NTG
JP1	<i>nif-1</i> , pTH10	Derived from RC1 by conjugation with RC101
JP13	<i>nif-3</i> , pTH10	Derived from RC3 by conjugation with RC101
JP16	<i>nif-16</i> , pTH10	Derived from RC16 by conjugation with RC101
JP22	<i>his-1 str-14</i>	Spontaneous derivative of J50
JP26	<i>nif-1 str-14</i>	Derived from JP22 by conjugation with JP1
JP27	<i>nif-3 str-14</i>	Derived from JP22 by conjugation with JP13
JP28	<i>nif-16 str-14</i>	Derived from JP22 by conjugation with JP16
LS2	<i>his-1 trp-1</i>	Derived from J50 by NTG
LS6	<i>his-1 ade-2</i>	Derived from J50 by NTG
LS8	<i>ade-1 ura-1</i>	Derived from RC42 by NTG
LS10	<i>ade-1 tyr-1</i>	Derived from RC42 by NTG
LS16	<i>ser-1</i>	Derived from B10 by NTG
LS27	<i>leu-3</i>	Derived from B10 by NTG
LS29	<i>leu-4</i>	Derived from B10 by NTG
<i>Plasmids</i>		
pTH10	Km ^r Tc ^r (Ap ^r)	Mutant of RP4 temperature-sensitive for maintenance in <i>E. coli</i> ; Harayama <i>et al.</i> (1980)
RP1	Km ^r Tc ^r (Ap ^r)	Wild-type plasmid, probably identical to RP4; Burkardt <i>et al.</i> (1979)
pRK292	Tc ^r	Plasmid vector; Avtges <i>et al.</i> (1983)
pRK2013	Km ^r	Mobilizing plasmid; Ditta <i>et al.</i> (1980)
pRPA8	Tc ^r	<i>R. capsulata nifHDK</i> genes clone in <i>Hind</i> III site of pRK292; Avtges <i>et al.</i> (1983)

* *E. coli* gene symbols are as defined by Bachmann & Low (1980). *R. capsulata* markers are abbreviated as follows. Biosynthetic markers: *ade*, adenine; *bch*, bacteriochlorophyll; *bio*, biotin; *crt*, carotenoids; *his*, histidine; *ilv*, isoleucine and valine; *leu*, leucine; *ser*, serine; *trp*, tryptophan; *tyr*, tyrosine; *ura*, uracil. Chromosomal antibiotic-resistance markers: *ery*, erythromycin; *nov*, novobiocin; *rif*, rifampicin; *str*, streptomycin. Others: *hsd*, host-specific defence; *nif*, N₂ fixation; *ntr*, N regulation. Plasmid markers: Ap^r, ampicillin resistance; Km^r, kanamycin resistance; Tc^r, tetracycline resistance.

† 'By NTG' and 'by EMS' refer to mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine or with ethyl methanesulphonate, respectively. Cma, chromosome-mobilizing ability (Holloway, 1979).

Biochemical techniques. Nitrogenase activity *in vivo* was assayed in resting cell suspensions as described previously (Willison & Vignais, 1982). Cell-free extracts for the determination of nitrogenase activity were prepared from 1-l cultures and assayed for activity and protein content as described previously (Hallenbeck *et al.*, 1982). Immunological assay of nitrogenase polypeptides was done on crude cell-free extracts which were prepared either as above, or by a small-scale method from 10 ml cultures (Jouanneau *et al.*, 1985), and centrifuged for 1 min at 15 lbf in⁻² (103.5 kPa) in a Beckman air-driven centrifuge to remove membrane fragments. Immunodiffusion was done on microscope slides coated with 3 ml 1% agarose dissolved in Tris/barbiturate buffer, pH 8.6 (LKB). Wells of 2 mm diameter were punched in a rosette configuration in the gel, 10 µl antiserum against either component I (RcI) or component II (RcII) of *R. capsulata* nitrogenase was placed in the central well and 10 µl of crude extract (5 to 10 µg protein) in each of the six peripheral wells. Immunodiffusion was done overnight at ambient temperature, and the precipitin arcs stained with Coomassie Blue R250. Rocket immunoelectrophoresis (Laurell, 1965) was done on 10 × 10 cm plastic sheets (LKB) coated with 10 ml 1% agarose containing 80 mM-Tris, 40 mM-sodium acetate, 1 mM-EDTA, 0.5 mM-calcium lactate, pH 8.6, and either 80 µl anti-RcI serum or 160 µl anti-RcII serum. Eight wells were punched in the gel and 10 µl cell-free extract, diluted to contain 0.05 to 0.5 mg protein ml⁻¹, was placed in each well. After electrophoresis for 4 h at 2.5 V cm⁻¹, gels were washed overnight in 0.1 M-NaCl, dried, and stained with Coomassie Blue R250. Rocket area was determined by photographing the gel and cutting out and weighing the rockets.

RESULTS AND DISCUSSION

Behaviour of plasmid pTH10 in R. capsulata

pTH10 and the wild-type plasmid RP1 could be transferred from *E. coli* to *R. capsulata* B10 at frequencies of up to 2×10^{-2} per recipient c.f.u. plated. Both plasmids conferred resistance in *R. capsulata* to kanamycin (Km^r) and tetracycline (Tc^r) but not to ampicillin (Ap^r). In *E. coli* K12, the plasmid pTH10 is temperature-sensitive for maintenance, and is lost from cells grown at 42 °C (Harayama *et al.*, 1980). Temperature-resistant derivatives can arise by transposition of the Ap^r gene (*TnI*) into the chromosome, followed by integration of the plasmid at the site of insertion. Such derivatives function as Hfr strains, transferring the *E. coli* chromosome from an origin at the site of insertion (Harayama *et al.*, 1980). In *R. capsulata*, however, pTH10 showed no evidence of temperature sensitivity or of integration into the chromosome, since the transfer frequency of pTH10 to other strains of *R. capsulata* was similar at 37 °C and at 30 °C, no detectable loss of Km^r ($<0.1\%$) occurred after aerobic growth in YPS medium at 37 °C, and the intensity of the plasmid band corresponding to pTH10 was similar in agarose gels of cell lysates from cultures grown at either 30 °C or 37 °C. (In *E. coli* TH471, this band was weak or undetectable when cultures were grown at 37 °C, and completely absent at 42 °C.) Transfer of pTH10 from *R. capsulata* RC37 to *E. coli* HB101 showed that the plasmid had retained its temperature sensitivity in *E. coli*. A similar lack of phenotypic expression of temperature-sensitive plasmids, including pTH10, has been reported for other Gram-negative bacteria (Hooykaas *et al.*, 1982).

Although the lack of temperature sensitivity of pTH10 in *R. capsulata* precluded the direct selection of Hfr strains, pTH10 was able to transfer chromosomal markers at frequencies as high as 5×10^{-3} per donor cell (see below), whereas RP1 transferred the same markers at frequencies of 10^{-8} to 10^{-9} . The mechanism of chromosome mobilization in *R. capsulata* by pTH10 is unknown. It has been suggested that the low frequency of chromosome mobilization by RP1 in *E. coli*, like the integration of pTH10 into the chromosome, is due to transposition of *TnI* (Harayama *et al.*, 1980, 1981). It is possible, therefore, that the frequency of transposition of *TnI* into the *R. capsulata* chromosome is greater in strains carrying pTH10 than in strains carrying RP1. Surprisingly, not all Km^r exconjugants containing pTH10 showed chromosome-mobilizing ability (Cma), as many as five in six colonies tested in each cross being Cma^- . However, after subsequent isolation and purification, Cma was maintained stably in positive strains (see below).

Characteristics of chromosome mobilization by pTH10

All genetic markers tested were transferred, at frequencies ranging from 10^{-6} to 2×10^{-4} per donor c.f.u. plated (transfer frequencies for a given marker varied up to fivefold between experiments and were roughly proportional to donor cell concentration in the range 10^6 to 10^7 donor c.f.u. per 0.1 ml plated). In crosses between the strains RC101 and RC1, the transfer frequency of *nif-1*⁺ (4×10^{-5} to 1×10^{-6} per donor c.f.u. plated) and the coinheritance frequency of *rif-1* ($5.5 \pm 2.8\%$ in 12 determinations), appeared to be independent of the growth phase of the donor and recipient strains, the growth medium (RCV or YPS) and growth conditions (aerobic or photosynthetic) of the recipient strain. Transfer and coinheritance frequencies were decreased twofold by washing the cells before mating, and up to 10-fold when mating was done on YPS agar medium or on freshly poured plates. The mating procedure described for *Rhodopseudomonas sphaeroides* (Sistrom, 1977) gave no improvement in conjugation efficiency over the method described here.

Donor strains of *R. capsulata* showed no detectable loss of Cma after being subcultured several times for 2 to 3 d on YPS agar medium containing $25 \mu\text{g}$ kanamycin ml^{-1} . In one such experiment, a derivative of RC103, strain RC126, was isolated which showed greatly increased transfer frequency of markers in region I of the chromosome map (see Fig. 1 and below), and increased coinheritance frequencies of these markers. For example, the transfer frequency of *nif-1*⁺ was proportional to the donor cell concentration in the range 10^4 to 10^5 donor c.f.u. per 0.1 ml plated, reaching a maximum value of 5×10^{-3} per donor c.f.u., and the *rif-1* allele was

Fig. 1. Circular linkage map of the *R. capsulata* chromosome. The map was constructed from data obtained in pTH10-mediated crosses (see Table 2, Fig. 2 and text), but excluding data obtained in crosses with RC126 as donor strain. The distance between genetic markers was calculated by assuming that $d = 1 - c^{1/3}$ where c is the coinheritance ratio and d is the map distance in arbitrary units (Kondorosi *et al.*, 1977). The scale of distance is marked inside the circle, with the position of the *tyr-1* locus being arbitrarily chosen as zero. The markers used to establish circularity of the chromosome (Table 2) are positioned on the circle itself, while the *ilv-10* locus is shown joined by a radial line to the circle, since the distance between the *ser-1* and *ilv-10* loci has not been established directly. The approximate position of other markers is shown outside the circle. Roman numerals indicate the four regions of the chromosome defined by their linkage to antibiotic-resistance determinants (see text).

contransferred at a frequency of 20 to 35%. Agarose gel electrophoresis showed that pTH10 was still present in the autonomous state in strain RC126, although this does not preclude the presence of a copy of the plasmid integrated in the chromosome. The enhanced Cma of strain RC126 appeared to be due to a host mutation rather than a plasmid mutation, since strains that had received pTH10 by conjugation from RC126 did not show enhanced Cma.

Mapping of the *R. capsulata* chromosome

A circular linkage of the *R. capsulata* chromosome (Fig. 1) was established by pTH10-mediated conjugation, using the data given in Table 2 and below. Auxotrophic donor strains containing pTH10 were counterselected on minimal medium, and selection was made for transfer of *nif*⁺ or biosynthetic markers. The apparent cotransfer frequency of unselected donor markers, e.g. antibiotic-resistance markers was then determined by replica plating. Attempts to select directly for transfer of antibiotic-resistance markers (either chromosomal or plasmid coded), using the method for transfer of Str^r by GTA, were unsuccessful, even when up to 8 h was allowed for phenotypic expression before addition of antibiotic to the selective medium. It is noteworthy that the chromosomal genes conferring resistance to rifampicin, streptomycin and erythromycin were closely linked, and two genes conferring resistance to novobiocin were identified (Fig. 1).

When each of a given pair of markers could be selected for, the two values for the cotransfer frequency of the unselected marker, expressed as a percentage, generally differed by less than 5%. An exception was the marker pair *his-1*⁺/*ade-2*⁺, since when selection was made for His⁺, the mean cotransfer frequency of Ade⁺ was 61.4%, whereas when selection was made for Ade⁺, the mean cotransfer frequency of His⁺ was only 14.1% (see Fig. 1). This difference was not due to a difference in plating efficiency of Ade⁺ and His⁺ recombinants, which was >90% in both cases. A possible explanation for the difference in cotransfer frequency is that *ade-2*⁺ could be transferred in a polar fashion from an origin located between the two markers. The overall pattern of transfer frequencies and linkage relationships suggests that transfer of the *R. capsulata* chromosome occurs from several origins.

Table 2. Summary of linkage data from genetic crosses between donor strains bearing the plasmid *pTH10* and different recipient strains

The values shown are the mean coinherence ratios (*c*) obtained in at least three independent crosses between each pair of strains. Either 100 (*c* > 0.10) or 200 (*c* < 0.10) recombinants from each cross were tested for the different unselected markers indicated. A dash indicates that no coinherence ratio could be determined either because the unselected allele (or a similar allele) was already present in the recombinants, or because the unselected marker was incompatible with the selected marker (e.g. *nif*⁺ and *crt*).

Recipient strain	Selected marker	Donor strain . . .					GA120 (<i>ilv-10 rif-1 crt-10</i>)	RC149 (<i>tyr-1 rif-1 str-1</i>)	RC160 (<i>ade-1 tyr-1</i>)	RC192 (<i>his-1 trp-1</i>)		
		RC103 (<i>ilv-10 rif-1 str-1</i>)			Other*							
		<i>rif-1/str-1</i>	<i>his</i> ⁺	<i>nif</i> ⁺		<i>rif-1</i>	<i>crt-10</i>	<i>rif-1/str-1</i>	<i>ade-1</i>	<i>tyr-1</i>	<i>his-1</i>	<i>trp-1</i>
GA111	<i>his-1</i> ⁺	0.02	—	0.10	—	0.03	0.58	0.02	0.02	<0.01	—	<0.01
	<i>nif-88</i> ⁺	0.03	0.17	—	—	0.04	—	0.09	<0.01	<0.01	—	<0.01
	<i>his-1</i> ⁺	0.03	—	0.29	0.48 ^a	0.01	—	0.03	<0.01	<0.01	—	<0.01
GA143	<i>nif-88</i> ⁺ <i>crt-13</i> ⁺	0.05	0.52	—	—	—	—	0.06	<0.01	<0.01	—	<0.01
	<i>crt-13</i> ⁺	0.04	0.50	0.59	—	—	—	0.05	<0.01	<0.01	—	<0.01
	<i>his-1</i> ⁺	0.05	—	—	<0.01 ^b	0.03	0.57	0.02	0.01	<0.01	—	—
LS2	<i>trp-1</i> ⁺	<0.01	<0.01	—	—	<0.01	<0.01	<0.01	0.03	0.04	—	—
	<i>ade-1</i> ⁺	<0.01	—	—	<0.01 ^c	<0.01	<0.01	<0.01	—	—	0.01	0.03
	<i>tyr-1</i> ⁺	<0.01	—	—	<0.01 ^d	<0.01	<0.01	<0.01	<0.01	—	<0.01	0.05
LS16	<i>ser-1</i> ⁺	<0.01	—	—	—	<0.01	<0.01	0.13	<0.01	0.04	<0.01	<0.01
LS27	<i>leu-3</i> ⁺	<0.01	—	—	—	<0.01	<0.01	<0.01	0.18	<0.01	0.09	0.02
RC1	<i>nif-1</i> ⁺ (<i>nifH</i> ⁺)	0.05	—	—	—	0.04	—	0.09	<0.01	<0.01	0.01	<0.01

* *a*, *crt-13*⁺; *b*, *trp-I*⁺; *c*, *tyr-I*⁺; *d*, *ade-I*⁺.

Fig. 2. Gene order and additive map distances in region I of the *R. capsulata* chromosome. (a) Summary of data from crosses between the donor strains RC103 and GA120, and the recipient strains RC1, RC58, GA111 and GA143 with selection being made for His⁺, Crt⁺ or Nif⁺ (NB in crosses with GA143, the Nif⁺ phenotype could not be selected independently of Crt⁺). The values shown are the map distances in arbitrary units, calculated from the mean coinheritance ratio $\pm$ SE. (b) As (a), but with RC126 as the donor strain. (c) Results of a single cross between strains RC138 and RC98, with selection being made for Ilv⁺. The percentage coinheritance of unselected markers is shown, with the calculated map distance in parentheses; 47% of Ilv⁺ Ery^r recombinants were Str^s, whereas 90% of Ilv⁺ Str^s were Ery^r. (d) As (c), but for a cross between strains RC168 and RC39. All Ilv⁺ Str^r recombinants were Rif^r; 62.5% of Ilv⁺ Str^r Rif^r were Ura⁻, whereas 100% of Ilv⁺ Ura⁻ were Rif^r and Str^r.

The map distances used in Fig. 1 were calculated by using a relationship derived for generalized transduction which is also applicable to plasmid R68.45-mediated conjugation in *Rhizobium meliloti* (Kondorosi *et al.*, 1977). In the latter case, large fragments of the chromosome are transferred from several points of origin. The data in Fig. 2 show that map distances calculated this way were approximately additive, at least for cotransfer frequencies greater than 10%. Analysis of mapping data was complicated by apparent variations in recombination frequency between certain markers. Thus, strain RC126 gave greatly increased cotransfer

frequencies compared to other donor strains (compare Fig. 2*a* with Fig. 2*b*), and with some pairs of markers (e.g. *ade-2*⁺/*his-1*⁺ and *his-1*⁺/*crt-13*⁺) cotransfer frequencies of 100% were sometimes observed. This could be due either to variations in the absolute frequency of recombination between cotransferred markers, or to variations in cotransfer frequency, with little or no recombination occurring between closely linked markers. The following observations support the latter explanation: (a) the increased cotransfer frequencies obtained with strain RC126 were correlated with an increase in the absolute transfer frequency of the markers concerned; (b) markers in region II of the chromosome (Fig. 1) appeared to be unlinked to the *rif-1* and *str-1* markers when the donor strain contained the *ilv-10* allele, but showed coinherance of these markers when the donor strain was *ilv-10*⁺, suggesting that little or no recombination occurs between the region II markers and *ilv-10*. Nevertheless, in crosses between RC126 and GA111, up to 50% of His⁺ Str^r recombinants were Nif⁻, suggesting either that crossing over does occur (between *his-1* and *nif-88* and between *nif-88* and *str-1*) or that *his-1*⁺ and *rif-1* can be transferred independently to the same recipient cell.

Organization and function of *N*₂ fixation genes

Groups I-A and I-B. A large number of *nif* mutations mapped in region I of the chromosome. These mutations were characterized by a high degree of linkage (>20% cotransfer) to *rif-1* and *str-1* in crosses with RC126, and were closely linked to the photosynthetic gene cluster, which contains the genes for carotenoid, bacteriochlorophyll and reaction centre biosynthesis (Fig. 1; cf. Yen & Marrs, 1976; Youvan *et al.*, 1982), and were also linked to a gene coding for histidine biosynthesis. The linkage between the *his-1* and *nif-88* markers in strain GA111 was variable, being as high as 30% when RC126 was the donor strain (see Fig. 2) and as low as 1% when either RC138 or RC192 was the donor. The proximity of the *nif-1*, *nif-3* and *nif-8* alleles to the photosynthesis genes was confirmed by crossing strains JP1, JP13 and JP16 with strain BY16510, and selecting Crt⁺ Bch⁺ Str^r recombinants. In each case, between 30% and 50% of such recombinants were Nif⁻ and had therefore received the donor *nif* allele. Finally, the wild-type alleles of all the mutations assigned to group I showed 28 to 37% coinheritance of *nov-9* and 6 to 16% coinheritance of *ery-1* in crosses with RC138 as donor strain.

The group I mutations were analysed in more detail with GTA (Fig. 3). They fell into two linkage groups (I-A and I-B) that are separated by at least 2700 bp (cf. Wall & Braddock, 1984). Group I-A mutants RC1, RC3, RC8 and RC33 were devoid of component II protein and contained variable amounts of active component I; in strain RC9, component II protein was present, but inactive (Table 3). We were unable to reproduce previous experiments that showed RC9 to have a low, but detectable nitrogenase activity (Willison & Vignais, 1982). The Nif⁻ phenotype of these mutants (but not that of group I-B mutants) was complemented by pRPA8, which contains the *R. capsulata nifHDK* genes. Group I-A mutations are therefore located in the *nifH* gene.

The group I-B mutants comprised 7 of 10 mutants isolated previously by direct plating onto RCV agar medium containing glutamate and metronidazole (Willison & Vignais, 1982), 23 of 24 mutants, including some spontaneous mutants, isolated in the present study by the same method, and 2 of 3 spontaneous mutants isolated from N-limited continuous culture. (Strains RC23 and AP31, which mapped in group I by conjugation, showed a similar phenotype to the group I-B mutants, but gave very low numbers of Nif⁺ recombinants in GTA-mediated crosses, and also failed to revert; they may therefore contain more than one *nif* mutation, or a deletion.) These mutants lacked detectable nitrogenase activity, as assayed by acetylene reduction, and were devoid of both nitrogenase components (data not shown). They appeared to be specifically affected in *N*₂ fixation, since growth on other N sources and on low concentrations of NH₄⁺ (<2 mM) was unaffected. Moreover, strains AP29 and AP31 contain normal levels of the NH₄⁺-assimilating enzymes glutamine synthetase and glutamine:oxoglutarate aminotransferase (Allibert *et al.*, 1984). Wall *et al.* (1984) have described a similar group of mutations that appear spontaneously in malate/glutamate medium and can be selected with metronidazole. Most of these mutations mapped in a single linkage group, which was different from the five *nif* linkage

Fig. 3. Fine-structure mapping of *nif* mutations in region I of the *R. capsulata* chromosome. (a) Group I-A (*nifH*); (b) group I-B (*nifA*-like gene). GTA-mediated crosses were done as described in Methods, using the donor strains JP26 (*nif-1*), JP27 (*nif-3*), JP28 (*nif-16*) and RC189 (*nif-88*), all of which contained the *str-14* allele. The values shown are map distances, calculated as described by Yen & Marrs (1976), with the 95% confidence intervals in parentheses (Kimball, 1961). A map distance of 1.0 unit is equivalent to 4600 bp (Taylor *et al.*, 1983). The two groups of mutations showed no significant linkage to each other, and are therefore separated by at least 2700 bp (Wall & Braddock, 1984).

groups described previously (Wall & Braddock, 1984). These mutations appear to be located in a regulatory gene (or genes) that affects the expression of the *nifHDK* operon, and therefore resembles the *nifA* gene of *K. pneumoniae*. However, it remains to be determined whether the mutations in this gene affect the expression of *nif* genes other than the structural genes for nitrogenase.

Groups II-A and II-B. The wild-type alleles of the biosynthetic markers in region II of the chromosome showed 5 to 57% coinheritance of *nov-1* in crosses with GA100 as donor strain, and were linked to *rif-1* and *str-1* (5 to 22% coinheritance) only in crosses with strains that did not contain the *ilv-10* allele, i.e. RC149 and RC168. Four *nif* mutations mapped in this region, and could be divided into two groups (II-A and II-B) on the basis of their linkage to *nov-1*. Group II-A was represented by a single mutation, *nif-85*, the wild-type allele of which was linked 50% to *nov-1*. Since the apparent transfer frequency of *nif-85*⁺ (7.6×10^{-5} per recipient c.f.u.) was only twice the spontaneous reversion rate (3.9×10^{-5}), the true coinheritance frequency was probably closer to 100%. In crosses with strain RC183 as donor, the *leu-4*⁺, *ser-1*⁺, *ura-2*⁺ and *bio-2a*⁺ alleles showed 20 to 73% coinheritance of *nif-85*, and the coinheritance of *ilv-10*⁺ and *nif-85* was 93%.

The wild-type group II-B alleles, *nif-7*⁺, *nif-15*⁺ and *nif-32*⁺, showed 1 to 8% coinheritance of *nov-1*, a lower frequency than that observed for other markers in region II of the chromosome. However, the predicted linkage of these markers to *tyr-1* could not be demonstrated, since no Nif⁺ recombinants could be obtained in crosses between RC160 (*tyr-1*) and the group II-B Nif⁻ mutants. Finally, GTA-mediated crosses between strain RC188 (*nif-85*) and strains RC7, RC15 and RC32 provided no evidence for linkage between the two groups of mutations.

Table 3. *Nitrogenase activity and nitrogenase component concentrations in crude extracts of wild-type and Nif⁻ strains*

Crude extracts containing 10–1000 µg protein were assayed for nitrogenase activity as described in Methods, and titrated with purified RcI or RcII until no further increase in activity was observed. The concentration of nitrogenase components was determined by rocket immunoelectrophoresis, with samples containing 1–10 µg protein, and using purified RcI and RcII as standards. The molecular weight of the nitrogenase component was taken to be 230000 for RcI and 63000 for RcII (Hallenbeck *et al.*, 1982). Nitrogenase activities and concentrations varied by less than twofold between different cultures of the same strain (cf. Willison & Vignais, 1982); the values shown are the mean determination on extracts from two or three separate cultures. ND, Not determined.

Mutant group	Strain	Nitrogenase activity [µmol C ₂ H ₄ h ⁻¹ (mg protein) ⁻¹]			Concentration of nitrogenase components [nmol (mg protein) ⁻¹]	
		No addition	+ RcI	+ RcII	RcI	RcII
Wild-type	B10	3.96	5.59	20.0	1.18	2.13
I-A	RC1	<0.01	<0.01	5.02	0.39	<0.08
	RC3	<0.01	<0.01	0.89	0.10	<0.08
	RC8	<0.01	<0.01	4.14	0.23	<0.08
	RC9	<0.01	<0.01	6.16	0.32	0.57
	RC33	<0.01	ND	ND	<0.04	<0.08
II-B	RC7	0.06	0.29	0.89	0.05	0.43
	RC15	0.05	0.37	1.00	0.07	0.41
	RC32	0.14	0.90	1.20	0.07	0.48
III	RC4	0.69	1.07	3.69	0.63	1.83
	RC10	0.07	0.35	0.64	0.27	1.97

Biochemical analysis showed that strain GA108 lacked detectable nitrogenase activity and was devoid of both nitrogenase components (data not shown), whereas the group II-B mutants, although deficient in both components, were more deficient in component I than in component II (Table 3). Titration of crude extracts with purified nitrogenase components (Table 3) showed that both components in group II-B mutants were catalytically active. It is probable that the group II-B mutations are located in a gene (or genes) affecting the synthesis and/or stability of component I, and that synthesis and/or stability of component II is affected indirectly. Like group I and group III mutants, these mutants appeared to be unaffected in the utilization of N sources other than N₂.

Group III. Strains RC4, RC10 and RC26 were slightly 'leaky' for growth on N-free RCV agar medium, but background growth was sufficiently low to allow the visualization of Nif⁺ recombinants. The *nif-4*, *nif-10* and *nif-26* mutations were tentatively localized in region III of the chromosome, since like *tyr-1* and *trp-1* they showed no linkage either to *nov-1* in crosses with GA100, or to *rif-1* and *str-1* in crosses with RC126. However, we were unable to detect linkage between these markers and the *tyr-1* and *trp-1* alleles, possibly because of competition for the tyrosine or tryptophan added to the medium between any auxotrophic Nif⁺ transconjugants and the background growth of the Nif⁻ recipient strain. An extrachromosomal location for these mutations therefore cannot be excluded.

In crude extracts of strain RC4, the specific nitrogenase activity was about 20% of that found in the wild-type strain, but the level of component I was about 50%, and the level of component II between 65 and 85% of that in the wild-type (Tables 3 and 4). However, although titrations with purified component II indicated that component I is less active in the mutant than in the wild-type, titration with purified component I failed to stimulate nitrogenase activity to a level consistent with the immunologically determined content of component II (Table 3).

Strains RC10 and RC26 differed from other strains tested in that nitrogenase activity in derepressed cultures was stimulated five- to sevenfold by the addition of 1 mM-molybdate to the growth medium; addition of molybdate resulted in an approx. sixfold increase in the level of nitrogenase component I, but had little or no effect on the content of component II (Table 4). These mutants are clearly affected in the utilization of molybdate for incorporation into the

Table 4. *Effect of molybdate on in vivo nitrogenase activity and nitrogenase component concentration in wild-type and Nif⁻ strains*

Strains were grown photosynthetically for 40 h in 10 ml volumes of RCV medium containing 2.25 mM-NH₄⁺ as N source, with or without addition of 1 mM-sodium molybdate. Resting cells and crude extracts were prepared from these cultures and assayed for nitrogenase activity and nitrogenase components, respectively, as described in Methods and the legend to Table 3. The values shown are the mean determination on two or three separate cultures; nitrogenase activities and concentrations for a given strain varied up to twofold between different cultures (cf. Willison & Vignais, 1982). ND, Not determined.

Mutant group	Strain	Addition of molybdate	Nitrogenase activity [$\mu\text{mol C}_2\text{H}_4 \text{ h}^{-1}$ (mg cell dry wt) ⁻¹]	Concentration of nitrogenase components [nmol (mg protein) ⁻¹]	
				RcI	RcII
Wild-type	B10	—	1.98	0.87	1.97
		+	1.66	0.66	2.35
II-B	RC32	—	<0.01	0.07	0.57
		+	<0.01	ND	ND
III	RC4	—	0.17	0.48	1.29
		+	0.14	0.40	1.08
	RC10	—	0.11	0.07	1.05
		+	0.77	0.47	1.38
	RC26	—	0.16	0.10	1.02
		+	0.87	0.61	1.22
IV	RC17	—	<0.01	0.19	0.57
		+	<0.01	0.22	0.51
	RC34	—	<0.01	0.21	0.40
		+	<0.01	0.28	0.63

MoFe protein and phenotypically resemble the *nifQ* mutants of *K. pneumoniae* (Imperial *et al.*, 1984). The loss of function in these mutants results in a decrease in synthesis and/or stability of the MoFe protein.

Group IV. The markers in region IV of the chromosome were linked to *ade-1* (3 to 27.5% coinheritance) and to *trp-1* (2 to 19.5% coinheritance) but not to *tyr-1*, and showed linkage of 1 to 4% to *rif-1* and *str-1* in crosses with RC126. The Nif⁻ mutants RC26, RC27, RC28 and RC34 were unable to utilize glutamate, proline, arginine or alanine as sole N source, but grew on glutamine with a long lag period and a doubling time of approx. 8 h, compared to 2.2 h for the wild-type. The growth rate of these strains on NH₄⁺ was similar to that of the wild-type, even at growth-rate-limiting concentrations (<2 mM). The inability of these strains to utilize poor N sources other than N₂ for growth, and their inability to derepress nitrogenase synthesis when grown with growth-limiting concentrations of NH₄⁺ (Table 3; Willison & Vignais, 1982) suggest that their mutations may be located in a gene (or genes) analogous to the nitrogen regulation (*ntr*) genes of *K. pneumoniae* (cf. Magasanik, 1982; Dixon, 1984).

In *K. pneumoniae*, the *ntrB* and *ntrC* genes form a complex operon with the structural gene for glutamine synthetase, *glnA*. In *R. capsulata*, the *glnA* gene mapped in the same chromosome region as the *ntr*-type mutations. However, on the basis of the observed cotransfer frequencies, it appeared to be closer to *trp-1* (15.5% coinheritance vs 3%) and further from *ade-1* (3% coinheritance vs 7 to 13.5%) than the *ntr*-type mutations.

Two other mutations affecting N₂ fixation, *nif-35* and *nif-17*, mapped in this region. The *nif-35* mutation may not be a true *nif* mutation, since the derepressed level of nitrogenase activity in RC35 was similar to that of the wild-type, and in some experiments this strain was unable to form colonies on RCV agar plates containing NH₄⁺ as N source. Strains RC11, RC12, RC30 and RC31 were similar to RC35 but presented a leaky Nif⁻ phenotype and could not be used as recipients in genetic crosses. Strain RC17 resembled RC34 in containing equally decreased levels of both nitrogenase components (Table 4) but grew normally on all N sources tested, except N₂. In RC17, as in the Ntr⁻ and group II-B mutants, the nitrogenase activity relative to

that of the wild-type was higher *in vitro* than *in vivo* (Willison & Vignais, 1982; cf. Tables 2 and 3). The reason for this behaviour is not yet clear, although possible explanations include a deficiency in electron transport to nitrogenase, *in vivo* inhibition of nitrogenase activity, and modification of the kinetics of nitrogenase activity due to an alteration in the ratio of the nitrogenase components.

Mutations such as *nif-17* and the group I-B and II-A mutations, which results in a co-ordinate decrease in synthesis of the two nitrogenase components, may be located in *nif*-specific regulatory genes, but might also occur in genes encoding cryptic functions that indirectly affect expression of the nitrogenase structural genes. Further studies are needed to distinguish between these two possibilities, e.g. by cloning the relevant genes and determining whether their expression is *nif*-specific, or subject to repression by NH_4^+ and/or by O_2 .

We thank P. Allibert and Drs B. A. Haddock, S. Harayama, R. Haselkorn, D. Helinski and J. D. Wall for generously providing strains, Laurence Sueur for isolating mutants and Dr Y. Jouanneau for providing antiserum and purified nitrogenase components. We are grateful to Jacqueline Roux for typing the manuscript. This work was supported by research grants from the Centre National de la Recherche Scientifique (CNRS, APP PIRSEM).

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