Ammonia-dependent growth (Adg^-) mutants of *Rhodobacter capsulatus* and *Rhodobacter sphaeroides*: comparison of mutant phenotypes and cloning of the wild-type (*adgA*) genes

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Mutants of *Rhodobacter capsulatus* deficient in growth on nitrogen sources other than NH_4^+ were compared with mutants of a similar phenotype isolated from *Rhodobacter sphaeroides*. In addition to N_2 and some amino acids (glutamate, alanine, proline, arginine), mutants of *R. sphaeroides* and *R. capsulatus* strain AD2 were unable to utilize NO_3^- as sole nitrogen source for growth. Under conditions of nitrogen starvation, mutants of both species lacked the methylammonium (ammonium) uptake system, which was found in the wild-type strains under these conditions. The wild-type (*adgA*) genes complementing these mutants were isolated from gene banks of the respective species and localized to a 2.9 kb *Bam*HI–*Sal*I fragment in *R. sphaeroides* and to a 1.7 kb *Sma*I fragment in *R. capsulatus*. These two fragments hybridized strongly with each other, showing that they contain homologous sequences. Furthermore, the *adgA* gene from *R. capsulatus* fully restored the wild-type phenotype to Adg^- mutants of *R. sphaeroides* and vice versa. Inactivation of the chromosomal *adgA* gene by insertion of an antibiotic-resistance cassette resulted in a severe inhibition of growth in rich medium and in minimal medium containing NH_4^+ . This suggests that the *adgA* gene is required for normal growth under all growth conditions.

Introduction

The purple, non-sulphur photosynthetic bacteria are generally able to use a wide range of compounds as nitrogen source for growth, including NH_4^+ , N_2 , amino acids, and, in some strains, NO_3^- . In *Rhodobacter capsulatus*, much is known about the regulation of expression of the genes for N_2 fixation (*nif* genes), and several regulatory *nif* genes have been identified (for reviews, see Haselkorn, 1986; Willison *et al.*, 1990). However, little is known about the regulation of the utilization of other nitrogen sources, either at the genetic or at the physiological level (cf. Caballero *et al.*, 1989).

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Abbreviations: CTAB, cetyltrimethylammonium bromide; GOGAT, glutamate synthase (glutamine:oxoglutarate aminotransferase); GS, glutamine synthetase; GTA, gene transfer agent.

In *R. capsulatus*, the regulatory genes *nifR1*, *nifR2* and *nifR4* have been shown to be homologous to the nitrogen regulatory genes *ntrC*, *ntrB* and *ntrA*, respectively (Kranz & Haselkorn, 1985; Jones & Haselkorn, 1989). In enteric bacteria, such as *Escherichia coli* and the N_2 -fixing species *Klebsiella pneumoniae*, the *ntr* gene products are required for the synthesis of several enzymes involved in the utilization of nitrogen compounds, e.g. nitrogenase in *K. pneumoniae*, as well as of various amino acid transport systems (see Magasanik, 1982; Reitzer & Magasanik, 1987). The effects of *ntr* mutations are therefore usually pleiotropic. In contrast, disruption of the *nifR1*, *nifR2* or *nifR4* genes in *R. capsulatus* results in a Nif^- phenotype, but not in an Ntr^- phenotype, suggesting that the genes involved are specifically involved in controlling nitrogen fixation. However, a *nifR1* mutant, J61, in addition to being unable to synthesize nitrogenase, is also unable to synthesize an inducible methylammonium (ammonium) uptake system (Haselkorn, 1986; Rapp *et al.*, 1986).

Mutants of *R. capsulatus* which are unable to utilize a wide range of compounds, including N_2 , NO_3^- and individual amino acids, as sole N source for growth have

been isolated. These mutants have been variously referred to as Nit^- (Wall *et al.*, 1977), Nut^- (Czichos & Klemme, 1980) and 'Ntr-like' (Willison *et al.*, 1985), the latter on the basis of the similarity of their phenotype to that of the Ntr^- mutants of *K. pneumoniae*. An *E. coli* gene which complements the 'Ntr-like' mutants of *R. capsulatus* has been isolated (Allibert *et al.*, 1987) and nucleotide sequencing of the gene has shown it to be unrelated to the known *ntr* genes of *E. coli*. The approximate map position of the gene (34–39 min) was determined, and no gene involved in nitrogen metabolism has so far been mapped to this region.

Mutants with similar phenotypes to those of 'Ntr-like' mutants of *R. capsulatus* have also been described in *Rhodobacter sphaeroides* (Frolova *et al.*, 1986; Shestakov *et al.*, 1988). In the present article, we compare the phenotypic characteristics of 'Ntr-like' mutants from the two species, and describe the cloning of the wild-type genes which complement the respective mutations. Some differences were observed between mutants of the two species. However, the wild-type genes from the two species cross-hybridized, and were able to complement mutations in the heterologous gene, implying that a similar gene is involved in both cases. Since the *ntr* terminology has not been universally adopted (cf. Reitzer & Magasanik, 1987), and a regulatory role for the gene has not been unequivocally demonstrated, we propose the name *adgA* (ammonia-dependent growth) for the gene, and Adg^- for the corresponding mutant phenotype.

Methods

Bacterial strains and plasmids. These are listed in Table 1. *R. sphaeroides* strain 2R was obtained from the collection of the Department of Genetics, Moscow State University. Adg^- mutants derived from *R. capsulatus* strain B10 and *R. sphaeroides* 2R were isolated after chemical mutagenesis followed by penicillin selection, and reverted to the wild-type at frequencies consistent with the presence of single point mutations (Willison *et al.*, 1985; Frolova *et al.*, 1986).

Media and growth conditions. *E. coli* strains HB101 and C600, used as plasmid hosts, were grown either in complex LB medium or in minimal A medium (Miller, 1972) with appropriate supplements. *Rhodobacter* strains were grown either in Ormerod medium (Ormerod *et al.*, 1961) containing 30 mM-sodium DL-malate and 10 mM- $(\text{NH}_4)_2\text{SO}_4$ as carbon and nitrogen sources, respectively, or in RCV medium (Weaver *et al.*, 1975). For growth tests, $(\text{NH}_4)_2\text{SO}_4$ was replaced by specified amino acids at 10 mM final concentration. Diazotrophic cultures were grown under N_2 in Ormerod medium without added combined nitrogen source and illuminated with a bank of three 60 W incandescent lamps. Complex (YPS) medium was formulated as described by Weaver *et al.* (1975).

Bacterial matings and transformations. These were performed as described earlier (Saano & Zinchenko, 1987). The plasmid pAS8-121Δ3, which is not able to replicate in *R. sphaeroides* (Dubeikovsky &

Kameneva, 1984), was used as conjugative mobilizing plasmid. In *R. capsulatus*, pRK2013 was used as the mobilizing plasmid for pRK290-derived plasmids (Ditta *et al.*, 1980) and pDPT51 was used to mobilize pDPT44-derived plasmids (Taylor *et al.*, 1983).

Isolation and purification of plasmid DNAs. For characterization of recombinant DNAs, plasmid DNAs were isolated from bacteria by the boiling method (Holmes & Quigley, 1981). DNAs required for plasmid construction experiments were purified by centrifugation in three-layer CsCl /ethidium bromide gradients for 6 h (Babykin & Zinchenko, 1984).

Analysis of plasmid DNAs. Standard techniques of molecular cloning were used (Maniatis *et al.*, 1982). Restriction enzymes and T4 DNA ligase were purchased from commercial sources and used according to the manufacturer's instructions.

Construction of gene bank. *R. sphaeroides* chromosomal DNA was partially digested with *Bam*HI and the fragments were separated by gel electrophoresis on 0.8% low-melting-point agarose. The fraction of *Bam*HI fragments with an average size of 9–15 kb was isolated from the gel and ligated to the vector of direct selection pVZ361 digested with *Bam*HI. The ligation mixture was used to transform *E. coli* C600 cells and transformant clones were selected for streptomycin resistance. The resulting gene bank consisted of approximately 8000 clones. The bank was then divided into 40 fractions (about 200 clones per fraction) and stored in 15% (v/v) glycerol at -70°C .

Methylammonium uptake. Methylammonium uptake was assayed essentially as described by Rapp *et al.* (1986). Cells grown for transport studies (4 ml of a culture grown to mid-exponential phase in Ormerod medium) were harvested by centrifugation. After two washes with an equal volume of Ormerod medium lacking ammonium (pH 6.8), the cells were suspended in 0.6 ml of the same medium and incubated at 35°C for 2 h. (Preliminary experiments showed that derepression of the methylammonium transport system was maximal after this time.) Uptake was then initiated by adding [^{14}C]methylammonium hydrochloride (Amersham) to a final concentration of $7.5\ \mu\text{M}$ ($20\ \mu\text{Ci ml}^{-1}$; $740\ \text{kBq ml}^{-1}$). At various times (30 s to 10 min after the addition of methylammonium), 0.1 ml samples were filtered through 0.45 μm pore-size nitrocellulose filters (Millipore). The filters were placed in vials with 5 ml Bray's scintillation cocktail and radioactivity was determined by scintillation counting. The rate of uptake was determined from the initial linear portion of the curve.

Enzyme assays. Nitrogenase activity was determined by measurement of acetylene reduction as described previously (Willison & Vignais, 1982). Glutamine synthetase (GS) was assayed by the γ -glutamyltransferase procedure (Shapiro & Stadtman, 1970). In *R. capsulatus*, GS was assayed at pH 7.45, in cells permeabilized with CTAB to stabilize the adenylation state of the enzyme (Johansson & Gest, 1977). Glutamate synthase (GOGAT) was assayed as described by Yelton & Yoch (1981). The protein content of whole cells was measured by the method of Lowry after digestion with 0.2 M-NaOH for 1 min in a boiling water bath.

DNA-DNA hybridization. DNA fragments were separated in 0.7% agarose gels. The Southern blotting technique (Southern, 1975) was used to transfer the DNA onto the surface of Hybond-N nylon membranes (Amersham). The DNA probes were labelled with biotin-11-dUTP (BRL) using a nick-translation kit (N5000; Amersham). Hybridization was carried out at 42°C in the presence of 45% (v/v) formamide and $5\times\text{SSC}$ (1 $\times\text{SSC}$ contains 0.15 M-NaCl, 15 mM-sodium citrate, pH 7.0). Hybridization reactions of biotinylated probes were detected with a streptavidin-alkaline phosphatase kit from BRL.

Transposon and 'cassette' mutagenesis. Plasmid pAP127 was mutagenized *in vivo* either with Tn5-751 (Rella *et al.*, 1985) or with mini-

Table 1. *Bacterial strains and plasmids*

Strain or plasmid	Relevant genotype or phenotype*	Source or reference and comments
<i>Escherichia coli</i>		
HB101	F ⁻ <i>proA2 rpsL20 recA13</i> <i>hsdS20</i> (r _B m _B)	} Maniatis <i>et al.</i> (1982)
C600	F ⁻ <i>thi-1 thr-1 leuB6</i>	
<i>Rhodobacter capsulatus</i>		
B10	Wild-type	Marrs (1974)
RC27	<i>adgA27</i> (<i>ntr-27</i>)	} Willison <i>et al.</i> (1985)
RC28	<i>adgA28</i> (<i>ntr-28</i>)	
RC29	<i>adgA29</i> (<i>ntr-29</i>)	
RC34	<i>adgA34</i> (<i>ntr-34</i>)	} Czichos & Klemme (1982)
AD2	Wild-type	
C1	Adg ⁻ (Nut ⁻)	} GTA-overproducing strain (Yen <i>et al.</i> , 1979)
Y262	Prototroph	
<i>Rhodobacter sphaeroides</i>		
2R	Wild-type	Laboratory collection
NF-8	<i>adgA8</i> (<i>ntr-8</i>)	} Frolova <i>et al.</i> (1986)
NF-15	<i>adgA15</i> (<i>ntr-15</i>)	
NF-78	<i>adgA78</i> (<i>ntr-78</i>)	
Plasmids		
pAS8-121Δ3	IncP Ap Sm Icel Tra ⁺	Dubeikovsky & Kameneva (1984)
pVZ361	IncQ Su (Sm)† Km <i>tnpR</i> Mob ⁺	Zinchenko <i>et al.</i> (1985)
pNF114	IncQ Su Sm Km <i>adgA</i> (<i>R. sphaeroides</i>) Mob ⁺	This work; derived from pVZ361 (13.9 kb <i>Bam</i> HI insert)
pNF149	IncQ Su Sm Km <i>adgA</i> (<i>R. sphaeroides</i>) Mob ⁺	This work; derived from pVZ361 (3.2 kb <i>Bam</i> HI insert)
pJCW2	IncP Tc <i>λcos adgA</i> (<i>R. capsulatus</i>) Mob ⁺	Magnin <i>et al.</i> (1987)
pRK290	IncP Tc Mob ⁺	} Ditta <i>et al.</i> (1980)
pRK2013	Km	
pAP127	IncP Tc <i>adgA</i> (<i>R. capsulatus</i>) Mob ⁺	This work; derived from pRK290 (5.8 kb <i>Eco</i> RI insert)
pDPT44	Km Ap	} Taylor <i>et al.</i> (1983)
pDPT51	Tp Ap	
pRCN102	IncP Tc <i>nifR1 nifR2</i> (<i>R. capsulatus</i>) Mob ⁺	} Avtges <i>et al.</i> (1985)
pRCN106	IncP Tc Mob ⁺	
pRCN200	IncP Tc <i>nifR4</i> (<i>R. capsulatus</i>) Mob ⁺	
pBF71	Cm Tc <i>ntrA</i> (<i>K. pneumoniae</i>)	de Bruijn & Ausubel (1983)
pFB514	Cm <i>glnA ntrB ntrC</i> (<i>K. pneumoniae</i>)	de Bruijn & Ausubel (1981)

* Abbreviations: *adg*, ammonia-dependent growth; *glnA*, structural gene for glutamine synthetase (GS); *nif*, nitrogen fixation; *ntr*, nitrogen regulation; GTA, gene transfer agent; Icel, immunity to colicin E1; Inc, plasmid incompatibility group; Ap, ampicillin resistance; Km, kanamycin resistance; Sm, streptomycin resistance; Su, sulphonamide resistance; Tc, tetracycline resistance; Tp, trimethoprim resistance; Tra⁺, encodes transfer functions; Mob⁺, mobilizable plasmid. In brackets is shown either the original designation of *adgA* alleles, or the species origin of cloned genes.

† The streptomycin resistance marker of pVZ361 is only expressed when DNA fragments are inserted into the unique *Bam*HI cloning site in *tnpR*.

MudIII734 (Castilho *et al.*, 1984). Plasmids containing transposon insertions within the 5.8 kb insert were then tested for their ability to complement *R. capsulatus* strain RC34. Site-directed 'cassette' mutagenesis was carried out as described by Scolnik & Haselkorn (1984), using gene transfer agent (GTA). A Km^r cassette from pUC4-KIXX (Pharmacia) was cloned into pAP127, and the resulting plasmid was transferred by conjugation into the GTA-overproducing *R. capsulatus* strain, Y262. A cell-free filtrate from this strain was then used to transfer Km^r to the wild-type strain B10.

Results

Growth characteristics

The *R. sphaeroides* mutant strains NF-8, NF-15 and NF-78 grew at similar rates to the wild-type on NH₄⁺ and glutamine, but failed to grow with N₂, NO₃⁻, glutamate, alanine, proline or arginine as sole nitrogen source

Table 2. Methylammonium uptake activities in wild-type and *Adg*⁻ mutant strains of *R. sphaeroides* and *R. capsulatus*, and restoration of the wild-type phenotype by plasmids containing the cloned *adgA* genes

Strain	[¹⁴ C]Methylammonium uptake activity* [nmol min ⁻¹ (mg protein) ⁻¹]				Growth on N ₂ and amino acids†			
	No plasmid	+pNF149	+pAP127	+pRCN106	No plasmid	+pNF149	+pAP127	+pRCN106
<i>R. capsulatus</i>								
B10 (wild-type)	11.5	11.6	11.3	11.6	+	+	+	+
RC34 (<i>Adg</i> ⁻)	0.1	11.3	11.0	11.9	-	+	+	+
<i>R. sphaeroides</i>								
2R (wild-type)	11.9	12.0	11.4	12.1	+	+	+	+
NF-8 (<i>Adg</i> ⁻)	0.3	11.3	10.9	11.5	-	+	+	+

* Methylammonium uptake was measured as described in Methods using cells grown with excess NH₄⁺, which were then washed free of NH₄⁺ and starved for 2 h in NH₄⁺-free medium. Uptake activity was measured in the control strains without recombinant plasmids, and in strains containing each of the plasmids shown. The values shown are the means of duplicate determinations.

† Glutamate, alanine, proline or arginine.

(Frolova *et al.*, 1986). The *R. capsulatus* mutants RC27, RC28, RC29 and RC34, derived from the wild-type strain B10, showed similar growth properties, except that growth on glutamine was very slow (Willison *et al.*, 1985; Allibert *et al.*, 1987). Strain B10 is unable to utilize NO₃⁻ as sole nitrogen source, so no conclusion can be drawn from these mutants as to the effects of *adgA* mutations on NO₃⁻ assimilation in *R. capsulatus*. In contrast, the wild-type *R. capsulatus* strain AD2 is able to assimilate NO₃⁻, and a mutant derivative, C1, has been isolated which is unable to utilize a range of nitrogen sources, including NO₃⁻ (Czichos & Klemme, 1982). The plasmid pAP127, which contains the cloned *adgA* from strain B10 (see below) was transferred to strain C1 by conjugation, and was found to restore the wild-type phenotype to this strain. This indicates that the mutation in strain C1 is also in the *adgA* gene, and that *adgA* mutations affect growth on NO₃⁻, as in *R. sphaeroides*.

Enzyme activities

No significant difference was observed between the GS activities of *R. capsulatus* strains B10 and RC34 grown in the presence of either an excess or a limiting concentration of NH₄⁺: the measured activities were between 400 and 500 nmol min⁻¹ (mg protein)⁻¹ in all cases. In contrast, in *R. sphaeroides* strain 2R, GS activity was fivefold higher under nitrogen limitation than in the presence of excess of NH₄⁺, and only 1.5- to 2-fold higher in the *Adg*⁻ mutants NF-8, NF-15 and NF-78 (Frolova *et al.*, 1986). Some differences between strains of *R. capsulatus* may occur, however, since in *R. capsulatus* strain E1F1, the GS activity of cells grown in malate medium was fourfold higher with NO₃⁻ or glutamate as nitrogen source with NH₄⁺ (Caballero *et al.*, 1989), whereas in the present study, the GS activity of strain B10 grown on glutamate was only 60% higher than in cells grown on NH₄⁺ (data not shown).

As for GS, no significant difference was observed between the GOGAT activities of *R. capsulatus* strains B10 and RC34, and in both strains, the activity was higher in nitrogen-limited cells than in cells grown with excess NH₄⁺. In contrast, in *R. sphaeroides* 2R, the GOGAT activity was threefold lower in NH₄⁺-starved than in NH₄⁺-sufficient cells, whereas in the *Adg*⁻ mutants, no effect of NH₄⁺ status on GOGAT activity was observed (Frolova *et al.*, 1986).

Methylammonium uptake activity

R. capsulatus and *R. sphaeroides* both contain a methylammonium transport system, which is present in glutamate-grown or N₂-grown cells, but not in cells grown on NH₄⁺ (Alef & Kleiner, 1982; Rapp *et al.*, 1986; Cordts & Gibson, 1987). Methylammonium uptake is inhibited by low concentrations of NH₄⁺ ions, implying that uptake of methylammonium is due to an NH₄⁺ transport system. NH₄⁺-grown cells of *R. capsulatus* and *R. sphaeroides* also contain ammonium uptake systems, which are unable to transport methylammonium (Genthner & Wall, 1985; Cordts & Gibson, 1987).

Since *Adg*⁻ mutants of *R. capsulatus* and *R. sphaeroides* are unable to grow on N₂ or glutamate, methylammonium uptake activity was assayed in cells which were grown with an excess of NH₄⁺, then washed and resuspended in NH₄⁺-free medium. In the respective wild-type strains, maximum derepression of uptake activity was observed after 2 h incubation in this medium; in the *Adg*⁻ mutants, however, very little uptake activity was detected after this time (Table 2). Wild-type levels of uptake activity were restored to the mutants by the presence of plasmids containing the cloned *adgA* genes from either *R. capsulatus* or *R. sphaeroides* (Table 2; see below).

Fig. 1. Physical map of the 5.8 kb *EcoRI* fragment of *R. capsulatus* DNA cloned in the plasmid pAP127, and localization of the *adhA* gene. This plasmid was able to restore the wild-type phenotype to *Adg*⁻ mutants of *R. capsulatus*, and the positions of mini-MudII 1734 insertions (▼) and Tn5-751 insertions (▽) which destroyed this ability are shown. All these insertions were located within a 1.7 kb *SmaI* fragment, and a plasmid containing this fragment was able to complement all *adhA* mutations. This fragment hybridized specifically with plasmid pNF149 containing the *R. sphaeroides adhA* gene (see Fig. 3) and the plasmid pAP127 was able to complement the *adhA* mutations of *R. sphaeroides Adg*⁻ mutants. Also shown are the two possible orientations of a *Km*^r cassette which was cloned into the unique *BamHI* site of pAP127 (see Methods). The arrows show the direction of transcription of the *Km*^r gene in each orientation. B, *BamHI*; E, *EcoRI*; H, *HpaI*; S, *SmaI*.

Fig. 2. Physical maps of the chromosomal fragments of *R. sphaeroides* cloned in the plasmids pNF114 (a) and pNF149 (b). Both plasmids were able to restore the wild-type phenotype to *Adg*⁻ mutants of *R. sphaeroides*, and complementation was also observed with a plasmid containing the 2.9 kb *BamHI*-*SalI* fragment from pNF149. The plasmid pNF149 was also able to complement the *adhA34* mutation of strain RC34 of *R. capsulatus*. B, *BamHI*; Bg, *BglII*; E, *EcoRV*; S, *SalI*.

Cloning of *adhA* genes

A cosmid clone, pJCW2, which restores the wild-type phenotype to *Adg*⁻ mutants of *R. capsulatus* has been isolated from an *R. capsulatus* gene bank (Colbeau *et al.*, 1986; Magnin *et al.*, 1987). This cosmid contains approximately 20 kb of *R. capsulatus* DNA. A 5.8 kb *EcoRI* fragment from this cosmid was subcloned in pRK290, to give plasmid pAP127, which complements *adhA* mutations both in *R. capsulatus* and in *R. sphaeroides* (Table 2). A restriction map of the 5.8 kb *EcoRI* fragment is shown in Fig. 1. The *adhA* mutations

were also complemented by a plasmid constructed by cloning a 1.7 kb *SmaI* fragment from pAP127 into pDPT51, showing that the *adhA* gene is located within this fragment. This was confirmed by mapping insertions of Tn5-751 and mini-MudII 1734 in pAP127 which abolished complementation of the *Adg*⁻ mutants. All such insertions were found to be located within the 1.7 kb *SmaI* fragment (Fig. 1).

To isolate the *R. sphaeroides adhA* gene, a gene bank was constructed in the plasmid vector pVZ361, and a clone was isolated which restored the wild-type phenotype to the *Adg*⁻ mutants NF-8, NF-15 and NF-78. A restriction map of the 13.9 kb *BamHI* fragment in this clone is shown in Fig. 2. The plasmid (pNF114) was digested with *BamHI* and fragments were subcloned in pVZ361. A 3.2 kb *BamHI* fragment was found to contain the gene, and digestion with *SalI* yielded a smaller complementing fragment of 2.9 kb (Fig. 2). Like pAP127, the plasmid containing the 3.2 kb *BamHI* fragment (pNF149) was able to complement *adhA* mutations both in *R. capsulatus* and in *R. sphaeroides* (Table 2).

Hybridization analysis

The plasmid pNF149 containing the *R. sphaeroides adhA* gene was labelled by nick-translation and used as a probe in hybridization experiments. The probe hybridized with the 5.8 kb *EcoRI* fragment of pAP127 containing the *R. capsulatus adhA* gene, and restriction enzyme digestion showed hybridization to be specific for the 1.7 kb *SmaI* fragment (Fig. 3). This shows that the *adhA* genes from *R. capsulatus* and *R. sphaeroides* are structurally as well as functionally homologous. The *R. capsulatus* probe also hybridized with pRCN106, a plasmid isolated by Avtges *et al.* (1985). This plasmid was able to complement the mutation in a *Nif*⁻ mutant of *R. capsulatus*, strain PA3, and was reported to contain a 6.0 kb *EcoRI* insert of *R. capsulatus* DNA (Avtges *et al.*, 1985). Further hybridization experiments (Fig. 3) showed that the 5.8 kb *EcoRI* insert of pAP127 and the '6.0 kb' insert of pRCN106 are identical. Consistent with this, pRCN106 was able fully to restore the wild-type phenotype to *Adg*⁻ mutants of *R. capsulatus* and *R. sphaeroides* (Table 2).

Plasmids containing the 5.8 kb *EcoRI* fragment from *R. capsulatus* and the 3.2 kb *BamHI* fragment from *R. sphaeroides* were tested for hybridization with DNA fragments from plasmids pBF71 and pFB514, which contain the *ntrA* and the *ntrBC* genes of *K. pneumoniae* respectively, and with DNA fragments from plasmids pRCN102 and pRCN106, which contain the *nifR1*, *nifR2* and *nifR4* genes of *R. capsulatus*. No significant hybridization was observed (data not shown).

Fig. 3. Agarose gel (0.7%) electrophoresis (a) and Southern blot hybridization (b) of: plasmid pNF114 digested with *Bam*HI (1); phage λ DNA digested with *Hind*III (2); plasmid pAP127 digested with *Eco*RI (3), *Sma*I (4) and *Eco*RI + *Sma*I (5); and plasmid pRCN106 digested with *Eco*RI (6), *Sma*I (7) and *Eco*RI + *Sma*I (8). Biotinylated plasmid pNF149 was used as a probe. The arrows and numbers on the left-hand side indicate the positions and sizes of the *Bam*HI fragments derived from pNF114. The arrows and numbers on the right-hand side show the positions and sizes of fragments of *R. capsulatus* DNA (derived from pAP127 and pRCN106) which showed significant hybridization with the probe. The origin of the strongly hybridizing band at 8–9 kb in *Eco*RI digests of pAP127 and pRCN106 (panel b, lanes 3, 5, 6 and 8), which showed up as faint band in the ethidium-bromide-stained agarose gel (panel a), is obscure. However, since whole plasmid DNA was used as a probe, it might have been due to a contaminating plasmid. We have no explanation for the difference in restriction endonuclease digestion patterns between pAP127 and pRCN106, other than the presence of an insertion or rearrangement in the vector portion of pRCN106. It should be noted that those fragments internal to the 5.8 kb *Eco*RI insert are identical in the two plasmids.

Site-directed 'cassette' mutagenesis of the *R. capsulatus* *adgA* gene

Transposon mutagenesis of the plasmid pRCN106 has shown that the *nif* gene which complements the mutation in strain PA3 is located near one end of the cloned fragment (Avtges *et al.*, 1985). Recombination with the chromosome of transposon insertions near this end of the fragment resulted in a *Nif*[−] phenotype. In contrast, transposon insertions towards the centre of the fragment, i.e. in the region of the *adgA* gene, could not be recombined into the chromosome when selection was made on minimal agar medium containing NH₄⁺ as the nitrogen source (Avtges *et al.*, 1985). This implies that genes in this region might be essential for growth on NH₄⁺.

In the present study, a Km^r cassette was inserted in both orientations in the *Bam*HI site of pAP127. In one orientation (orientation I, Fig. 1), complementation of *Adg*[−] mutants was abolished, whereas in the opposite orientation (II), no effect on complementation was

observed. The Km^r cassette in orientation II was transferred into strain B10 with GTA, and Km^r recombinants showed a wild-type phenotype with respect to growth. However, with the Km^r cassette in orientation I, recombinants were observed in only one experiment out of three when selected for on a minimal medium, and in all three experiments when selected for on complex (YPS) medium. In both cases, Km^r colonies were observed after 6 d incubation, compared to 2–3 d for the growth of Km^r recombinants with the cassette in orientation II. These results suggest that inactivation of the chromosomal *adgA* gene severely decreases the growth rate of cells on both minimal medium and rich medium and affects (in a non-reproducible way) the ability of cells to form isolated colonies on minimal medium.

Discussion

In the present work we have shown that mutants of *R. capsulatus* and *R. sphaeroides* with similar pleiotropic

defects in nitrogen metabolism are complemented by DNA fragments that show significant sequence homology (in DNA-DNA hybridization experiments) and therefore presumably contain related genes. These mutants (termed Adg^-) are characterized by an inability to use N_2 , or individual amino acids, as sole nitrogen source for growth: mutants derived from *R. sphaeroides* and *R. capsulatus* strain AD2 are also unable to utilize NO_3^- . The major difference between mutants of the two species concerns the use of glutamine, which is a good substrate for growth of the *R. sphaeroides* mutants, and a poor substrate for *R. capsulatus* mutants.

A number of regulatory responses shown by the wild-type strains in response to nitrogen starvation were not shown in the Adg^- mutants, namely: (i) development of high levels of nitrogenase activity; (ii) increase in GS activity (*R. sphaeroides* only); (iii) appearance of methylammonium (ammonium) transport activity. This suggests that the *adgA* gene may encode a regulatory protein which is necessary for increasing the expression of certain genes under conditions of nitrogen starvation. On the other hand, it is also possible that the pleiotropic effects of *adgA* mutations are the indirect result of an unspecified metabolic defect. Indeed, in *R. capsulatus* Adg^- mutants, although the *in vivo* activity of nitrogenase is very low (about 1% of the wild-type level), the *in vitro* nitrogenase activity (measured with dithionite as electron donor) and the content of nitrogenase protein are about 20% of the wild-type level (Willison & Vignais, 1982; Willison *et al.*, 1985). This shows that the low *in vivo* activity is in part due to an *in vivo* inhibition of nitrogenase activity.

A high-affinity ammonium transport system is thought to be necessary for cyclic retention of NH_4^+ during growth on poor nitrogen sources, such as N_2 (Kleiner, 1985). However, the loss of the methylammonium (ammonium) uptake system is not sufficient to explain the phenotype of Adg^- mutants, since the *nifR1* mutant, J61, also lacks this uptake system (Rapp *et al.*, 1986). In *R. capsulatus*, it has been shown that methylammonium uptake is tightly coupled to its metabolism via GS (Rapp *et al.*, 1986), so the loss of uptake activity in the Adg^- mutants might have been due to an inability to metabolize methylamine. The biosynthetic activity of GS has been measured in Adg^- mutants of *R. sphaeroides* grown on glutamine, and was found to be 25–40% of the wild-type activity (Shestakov *et al.*, 1988). During growth in batch culture with a limiting concentration of NH_4^+ , *R. capsulatus* strain B10 and the mutant strain RC34 showed identical rates of NH_4^+ uptake, down to external NH_4^+ concentrations of 1–2 μM (J. C. Willison, unpublished data), implying the NH_4^+ assimilation via GS is unaffected in the mutants. It seems likely, therefore, that the low methylammonium

uptake activity measured in Adg^- mutants is due to a loss of transport activity *per se*, and not to a deficiency in metabolism.

In addition to the inducible methylammonium uptake system, *R. capsulatus* and *R. sphaeroides* both contain a constitutive ammonium uptake system, which is unable to transport methylammonium and shows a K_m for NH_4^+ of 1.7 μM (Genthner & Wall, 1985; Cordts & Gibson, 1987). This transport system is presumably present in the Adg^- mutants, since these are able to take up and utilize low concentrations of NH_4^+ . Nevertheless, the methylammonium (ammonium) uptake system may be important for growth on NH_4^+ at sub-micromolar concentrations, since strain RC34 is rapidly outgrown by revertants during growth in NH_4^+ -limited continuous culture (Willison & Lac, 1988).

In the enteric bacteria, several biosynthetic reactions are catalysed by glutamine aminotransferases, which in some cases are able to utilize NH_4^+ in place of glutamine when the former is present in high concentrations (>1 mM). Mutants which have lost the glutamine aminotransferase function of one of these enzymes may therefore be able to grow on minimal medium if high concentrations of NH_4^+ are present (Reitzer & Magasanik, 1987). It seems unlikely that the Adg^- mutants are of this type, however, since: (i) the *E. coli* gene which complements *adgA* mutations in *R. capsulatus* is located between 34 and 39 min on the *E. coli* chromosome, whereas none of the genes coding for the known glutamine aminotransferases is located in this region; and (ii) the Adg^- mutants of *R. capsulatus* are able to grow at similar rates to the wild-type even at concentrations below 1 mM- NH_4^+ and a significant difference in growth rate is observed only at the very low steady-state concentrations (<1 μM) present in NH_4^+ -limited chemostat culture.

The observation that the *EcoRI* fragment containing the *R. capsulatus adgA* gene is identical to the fragment cloned in the plasmid pRCN106 by Avtges *et al.* (1985) led us to investigate the effect of insertional inactivation of the *adgA* gene in the *R. capsulatus* gene, since it has been suggested that pRCN106 may contain a gene or genes essential for growth on NH_4^+ -containing minimal medium (Avtges *et al.*, 1985). An insertion which inactivated the *adgA* gene was recombined into the *R. capsulatus* chromosome and was found to severely decrease the growth rate of cells both on minimal medium and on complex, YPS medium. This suggests that the *adgA* gene plays an important role in cell growth, and that the Adg^- phenotype may arise only from point mutations, which either decrease the expression of the gene, or alter the function of the gene product.

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