Ammonia-dependent growth (Adg\textsuperscript{−}) mutants of \textit{Rhodobacter capsulatus} and \textit{Rhodobacter sphaeroides}: comparison of mutant phenotypes and cloning of the wild-type (\textit{adgA}) genes

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Mutants of \textit{Rhodobacter capsulatus} deficient in growth on nitrogen sources other than NH\textsubscript{4}\textsuperscript{+} were compared with mutants of a similar phenotype isolated from \textit{Rhodobacter sphaeroides}. In addition to N\textsubscript{2} and some amino acids (glutamate, alanine, proline, arginine), mutants of \textit{R. sphaeroides} and \textit{R. capsulatus} strain AD2 were unable to utilize NO\textsubscript{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 (\textit{adgA}) genes complementing these mutants were isolated from gene banks of the respective species and localized to a 2-9 kb \textit{BamHI–SalI} fragment in \textit{R. sphaeroides} and to a 1-7 kb \textit{SmaI} fragment in \textit{R. capsulatus}. These two fragments hybridized strongly with each other, showing that they contain homologous sequences. Furthermore, the \textit{adgA} gene from \textit{R. capsulatus} fully restored the wild-type phenotype to Adg\textsuperscript{−} mutants of \textit{R. sphaeroides} and vice versa. Inactivation of the chromosomal \textit{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\textsubscript{4}\textsuperscript{+}. This suggests that the \textit{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\textsubscript{4}\textsuperscript{+}, N\textsubscript{2}, amino acids, and, in some strains, NO\textsubscript{3}. In \textit{Rhodobacter capsulatus}, much is known about the regulation of expression of the genes for N\textsubscript{2} fixation (\textit{nif} genes), and several regulatory \textit{nif} genes have been identified (for reviews, see Haselkorn, 1986; Willison \textit{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 \textit{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 \textit{R. capsulatus}, the regulatory genes \textit{nirR1}, \textit{nirR2} and \textit{nirR4} have been shown to be homologous to the nitrogen regulatory genes \textit{ntrC}, \textit{ntrB} and \textit{ntrA}, respectively (Kranz & Haselkorn, 1985; Jones & Haselkorn, 1989). In enteric bacteria, such as \textit{Escherichia coli} and the N\textsubscript{2}-fixing species \textit{Klebsiella pneumoniae}, the \textit{ntr} gene products are required for the synthesis of several enzymes involved in the utilization of nitrogen compounds, e.g. nitrogenase in \textit{K. pneumoniae}, as well as of various amino acid transport systems (see Magasanik, 1982; Reitzer & Magasanik, 1987). The effects of \textit{ntr} mutations are therefore usually pleiotropic. In contrast, disruption of the \textit{nirR1}, \textit{nirR2} or \textit{nirR4} genes in \textit{R. capsulatus} results in a Nir\textsuperscript{−} phenotype, but not in an Ntr\textsuperscript{−} phenotype, suggesting that the genes involved are specifically involved in controlling nitrogen fixation. However, a \textit{nirR1} mutant, J61, in addition to being unable to synthesize nitrogenase, is also unable to synthesize an inducible methylammonium (ammonium) uptake system (Haselkorn, 1986; Rapp \textit{et al.}, 1986).

Mutants of \textit{R. capsulatus} which are unable to utilize a wide range of compounds, including N\textsubscript{2}, NO\textsubscript{3} and individual amino acids, as sole N source for growth have
been isolated. These mutants have been variously referred to as Nit<sup>-</sup> (Wall et al., 1977), Nut<sup>-</sup> (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<sup>-</sup> mutants of *K. pneumoniae*. An *E. coli* gene which complements the ‘Ntr-like’ mutants of *R. capsulatus* has been isolated (Allibert et al., 1981), 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<sup>-</sup> 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<sup>-</sup> 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-(*NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> as carbon and nitrogen sources, respectively, or in RCV medium (Weaver et al., 1975). For growth tests, (*NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was replaced by specified amino acids at 10 mm final concentration. Diazotrophic cultures were grown under N<sub>2</sub> 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* (Dubenskovsky & Kameneva, 1984), was used as conjugal mobilizing plasmid. In *R. capsulatus*, pPR2013 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).

**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 *BamH*I and the fragments were separated by gel electrophoresis on 0.8% low-melting-point agarose. The fraction of *BamH*I digested 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 *BamH*I. 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 [14C]methylammonium hydrochloride (Amersham) to a final concentration of 7.5 μM (20 μCi ml<sup>-1</sup>; 740 kBq rn<sup>-1</sup>). 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-glutamyltranspeptidase procedure (Shapiro & Stadtman, 1970). In *R. capsulatus*, GS was assayed at pH 7.45, in cells permeabilized with CTAB to stabilize the adenylylation state of the enzyme (Johanson & 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 × SSC (1 × 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

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype or phenotype</th>
<th>Source or reference and comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Escherichia coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HB101</td>
<td>F’ proA2 rpsL20 recA13 hsdS20</td>
<td>Maniatis et al. (1982)</td>
</tr>
<tr>
<td>C600</td>
<td>F’ thi-1 thr-1 leuB6</td>
<td></td>
</tr>
<tr>
<td><strong>Rhodobacter capsulatus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B10</td>
<td>Wild-type</td>
<td>Marrs (1974)</td>
</tr>
<tr>
<td>RC28</td>
<td>adgA28 (ntr-28)</td>
<td></td>
</tr>
<tr>
<td>RC29</td>
<td>adgA29 (ntr-29)</td>
<td></td>
</tr>
<tr>
<td>RC34</td>
<td>adgA34 (ntr-34)</td>
<td></td>
</tr>
<tr>
<td>AD2</td>
<td>Wild-type</td>
<td>Czichos &amp; Klemme (1982)</td>
</tr>
<tr>
<td>C1</td>
<td>Adg’ (Nut-)</td>
<td></td>
</tr>
<tr>
<td>Y262</td>
<td>Prototroph</td>
<td>GTA-overproducing strain (Yen et al., 1979)</td>
</tr>
<tr>
<td><strong>Rhodobacter sphaeroides</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2R</td>
<td>Wild-type</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>NF-8</td>
<td>adgA8 (ntr-8)</td>
<td></td>
</tr>
<tr>
<td>NF-15</td>
<td>adgA15 (ntr-15)</td>
<td>Frolova et al. (1986)</td>
</tr>
<tr>
<td>NF-78</td>
<td>adgA78 (ntr-78)</td>
<td></td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pVZ361</td>
<td>IncQ Su Sm Ic R Mob’</td>
<td>Zinchenko et al. (1985)</td>
</tr>
<tr>
<td>pNF114</td>
<td>IncQ Su Sm Km adipA (R. capsulatus) Mob’</td>
<td>This work; derived from pVZ361 (13.9 kB BamHI insert)</td>
</tr>
<tr>
<td>pNF149</td>
<td>IncQ Su Sm Km adipA (R. capsulatus) Mob’</td>
<td>This work; derived from pVZ361 (3.2 kB BamHI insert)</td>
</tr>
<tr>
<td>pJCW2</td>
<td>IncP Tc Icos adipA (R. capsulatus) Mob’</td>
<td>Magnin et al. (1987)</td>
</tr>
<tr>
<td>pRK290</td>
<td>IncP Tc Mob’’</td>
<td>Ditta et al. (1980)</td>
</tr>
<tr>
<td>pRK2013</td>
<td>Km</td>
<td></td>
</tr>
<tr>
<td>pAP127</td>
<td>IncP Tc adipA (R. capsulatus) Mob’</td>
<td>This work; derived from pRK290 (5.8 kb EcoRI insert)</td>
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<td>pDPT44</td>
<td>Km Ap</td>
<td>Taylor et al. (1983)</td>
</tr>
<tr>
<td>pDPT51</td>
<td>Tp Ap</td>
<td></td>
</tr>
<tr>
<td>pRCN102</td>
<td>IncP Tc nifR1 nifR2 (R. capsulatus) Mob’</td>
<td>Avtges et al. (1985)</td>
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<td>pRCN106</td>
<td>IncP Tc Mob’’</td>
<td></td>
</tr>
<tr>
<td>pRCN200</td>
<td>IncP Tc nifR4 (R. capsulatus) Mob’</td>
<td></td>
</tr>
<tr>
<td>pBF71</td>
<td>Cm Tc ntrA (K. pneumoniae)</td>
<td>de Bruijn &amp; Ausubel (1983)</td>
</tr>
<tr>
<td>pFB514</td>
<td>Cm glnA ntrB ntrC (K. pneumoniae)</td>
<td>de Bruijn &amp; Ausubel (1981)</td>
</tr>
</tbody>
</table>

* 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 BamHI cloning site in topR.

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>[14C]Methylammonium uptake activity* [nmol min⁻¹ (mg protein)⁻¹]</th>
<th>Growth on N₂ and amino acids†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No plasmid</td>
<td>+pNF149</td>
</tr>
<tr>
<td><em>R. capsulatus</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B10 (wild-type)</td>
<td>11.5</td>
<td>11.6</td>
</tr>
<tr>
<td>RC34 (Adg⁻)</td>
<td>0.1</td>
<td>11.3</td>
</tr>
<tr>
<td><em>R. sphaeroides</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2R (wild-type)</td>
<td>11.9</td>
<td>12.0</td>
</tr>
<tr>
<td>NF-8 (Adg⁻)</td>
<td>0.3</td>
<td>11.3</td>
</tr>
</tbody>
</table>

* 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.

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 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).
containing the 2-9 kb EcoRI fragment of R. capsulatus DNA cloned in the plasmid pAP127, and localization of the adgA gene. This plasmid was able to restore the wild-type phenotype to Adg- mutants of R. sphaeroides, cloned in the plasmids pNF114 (a) and pNF149 (b). Both plasmids strain RC34 of R. capsulatus. B, Fig. 2. Physical maps of the chromosomal fragments of R. sphaeroides (Table 2). 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 adgA mutations both in R. capsulatus and in R. sphaeroides (Table 2).

Cloning of adgA 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., 1985). 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 adgA 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 adgA mutations were also complemented by a plasmid constructed by cloning a 1-7 kb SmaI fragment from pAP127 into pDPT51, showing that the adgA gene is located within this fragment. This was confirmed by mapping insertions of Tn5-751 and mini-Mud111734 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 adgA 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 adgA mutations both in R. capsulatus and in R. sphaeroides (Table 2).

Hybridization analysis

The plasmid pNF149 containing the R. sphaeroides adgA 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 adgA gene, and restriction enzyme digestion showed hybridization to be specific for the 1-7 kb SmaI fragment (Fig. 3). This shows that the adgA 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 pRPN102 and pRCN106, which contain the nifR1, nifR2 and nifR4 genes of R. capsulatus. No significant hybridization was observed (data not shown).
Site-directed ‘cassette’ mutagenesis of the \( \textit{R. capsulatus} \) \( \textit{adgA} \) gene

Transposon mutagenesis of the plasmid pRCN106 has shown that the \( \textit{nif} \) 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 \( \textit{Nif}^{-} \) phenotype. In contrast, transposon insertions towards the centre of the fragment, i.e. in the region of the \( \textit{adgA} \) gene, could not be recombined into the chromosome when selection was made on minimal agar medium containing \( \text{NH}_{4}^{+} \) as the nitrogen source (Avtges et al., 1985). This implies that genes in this region might be essential for growth on \( \text{NH}_{4}^{+} \).

In the present study, a \( \text{Km}^{+} \) cassette was inserted in both orientations in the \( \text{BamHI} \) site of pAP127. In one orientation (orientation I, Fig. 1), complementation of \( \textit{Adg}^{-} \) mutants was abolished, whereas in the opposite orientation (II), no effect on complementation was observed. The \( \text{Km}^{+} \) cassette in orientation II was transferred into strain B10 with GTA, and \( \text{Km}^{+} \) recombinants showed a wild-type phenotype with respect to growth. However, with the \( \text{Km}^{+} \) 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, \( \text{Km}^{+} \) colonies were observed after 6 d incubation, compared to 2–3 d for the growth of \( \text{Km}^{+} \) recombinants with the cassette in orientation II. These results suggest that inactivation of the chromosomal \( \textit{adgA} \) 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 \( \textit{R. capsulatus} \) and \( \textit{R. sphaeroides} \) 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 N₂, or individual amino acids, as sole nitrogen source for growth: mutants derived from \( \textit{R. sphaeroides} \) and \( \textit{R. capsulatus} \) strain AD2 are also unable to utilize NO₃. The major difference between mutants of the two species concerns the use of glutamine, which is a good substrate for growth of the \( \textit{R. sphaeroides} \) mutants, and a poor substrate for \( \textit{R. capsulatus} \) 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 (\( \textit{R. sphaeroides} \) only); (iii) appearance of methylammonium (ammonium) transport activity. This suggests that the \( \text{adgA} \) 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 \( \text{adgA} \) mutations are the indirect result of an unspecified metabolic defect. Indeed, in \( \textit{R. capsulatus} \) Adg− mutants, although the \textit{in vivo} activity of nitrogenase is very low (about 1% of the wild-type level), the \textit{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 \textit{et al.}, 1985). This shows that the low \textit{in vivo} activity is in part due to an \textit{in vivo} inhibition of nitrogenase activity.

A high-affinity ammonium transport system is thought to be necessary for cyclic retention of NH₄⁺ during growth on poor nitrogen sources, such as N₂ (Kleiner, 1985). However, the loss of the methylammonium (ammonium) uptake system is not sufficient to explain the phenotype of Adg− mutants, since the \( \text{nitR} \) mutant, J61, also lacks this uptake system (Rapp \textit{et al.}, 1986). In \( \textit{R. capsulatus} \), it has been shown that methylammonium uptake is tightly coupled to its metabolism via GS (Rapp \textit{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 \( \textit{R. sphaeroides} \) grown on glutamine, and was found to be 25–40% of the wild-type activity (Shestakov \textit{et al.}, 1988). During growth in batch culture with a limiting concentration of NH₄⁺, \( \textit{R. capsulatus} \) strain B10 and the mutant strain RC34 showed identical rates of NH₄⁺ uptake, down to external NH₄⁺ concentrations of 1–2 \( \mu \text{M} \) (J. C. Willison, unpublished data), implying the NH₄⁺ 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 \textit{per se}, and not to a deficiency in metabolism.

In addition to the inducible methylammonium uptake system, \( \textit{R. capsulatus} \) and \( \textit{R. sphaeroides} \) both contain a constitutive ammonium uptake system, which is unable to transport methylammonium and shows a \( K_m \) for NH₄⁺ of 1.7 \( \mu \text{M} \) (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 NH₄⁺. Nevertheless, the methylammonium (ammonium) uptake system may be important for growth on NH₄⁺ at sub-micromolar concentrations, since strain RC34 is rapidly outgrown by revertants during growth in NH₄⁺-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 NH₄⁺ 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 NH₄⁺ 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 \( \text{adgA} \) mutations in \( \textit{R. capsulatus} \) 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 \( \textit{R. capsulatus} \) are able to grow at similar rates to the wild-type even at concentrations below 1 mM-NH₄⁺ and a significant difference in growth rate is observed only at the very low steady-state concentrations (<1 \( \mu \text{M} \)) present in NH₄⁺-limited chemostat culture.

The observation that the EcoRI fragment containing the \( \textit{R. capsulatus adgA} \) gene is identical to the fragment cloned in the plasmid pRCN106 by Avtges \textit{et al.} (1985) led us to investigate the effect of insertional inactivation of the \( \text{adgA} \) gene in the \( \textit{R. capsulatus} \) gene, since it has been suggested that pRCN106 may contain a gene or genes essential for growth on NH₄⁺-containing minimal medium (Avtges \textit{et al.}, 1985). An insertion which inactivated the \( \text{adgA} \) gene was recombined into the \( \textit{R. capsulatus} \) 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 \( \text{adgA} \) 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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