The *draTG* gene region of *Rhodobacter capsulatus* is required for post-translational regulation of both the molybdenum and the alternative nitrogenase

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Synthetic oligonucleotides, which were designed according to amino acid sequences conserved between *Rhodospirillum rubrum* and *Azospirillum brasilense* DraT and DraG, respectively, were used to identify the corresponding genes of *Rhodobacter capsulatus*. Sequence analysis of a 1904 bp DNA fragment proved the existence of *R. capsulatus* draT and draG. These two genes were separated by 11 bp only, suggesting that *R. capsulatus* draT and draG were part of one transcriptional unit. In contrast to *R. rubrum*, *A. brasilense* and *Azospirillum lipoferum*, the *R. capsulatus* draTG genes were not located upstream of the structural genes of nitrogenase *nifHDK* but close to the *dctP* gene at a distance of about 1000 kb from the *nifHDK* genes. Deletion mutations in the draTG gene region were constructed and introduced into *R. capsulatus* wild-type and a *nifHDK* deletion strain. The resulting mutant strains were examined for post-translational regulation of the molybdenum and the alternative nitrogenase in response to ammonia and darkness. Under ‘switch-off’ conditions the modified (ADP-ribosylated) and the non-modified forms of component I1 of both the molybdenum and the alternative nitrogenase were detected in a draTG wild-type background by immunoblot analysis, whereas only the non-modified forms were present in the draTG deletion strains. Nitrogenase activity in these strains was followed by the acetylene reduction assay. In contrast to the wild-type, draTG mutants were not affected in nitrogenase activity in response to ammonia or darkness. These results demonstrated that the draTG genes are required for post-translational regulation of both the molybdenum and the heterometal-free nitrogenase in *R. capsulatus*.

Introduction

In the process of biological nitrogen fixation atmospheric dinitrogen is converted into ammonia by the nitrogenase enzyme complex which consists of two components: dinitrogenase (component I or MoFe protein encoded by *nifDK*) and dinitrogenase reductase (component II or Fe protein encoded by *nifH*). Since this process is very energy-demanding, nitrogenase activity is highly regulated. Regulation occurs at the transcriptional level by the *ntr*-system and the regulatory *nifA* gene which is present in all nitrogen fixing bacteria, except cyanobacteria, analysed so far (Gussin *et al.*, 1986; Kranz & Foster-Hartnett, 1990; Hennecke, 1990).

also eliminated nitrogenase activity (Lehman & Roberts, 1991b). Arg102 and adjacent amino acid residues are highly conserved between the NiF proteins from different organisms and a possible role of this residue for ionic interactions between component I and component II has been discussed for Azotobacter vinelandii (Wolle et al., 1992). The enteric nitrogen-fixing bacterium Klebsiella pneumoniae is devoid of a draTG-mediated regulatory mechanism. However, introducing the draTG genes from Azospirillum lipoferum or R. rubrum into K. pneumoniae enabled this organism to respond to ammonia with a reversible regulation of nitrogenase activity (Fu et al., 1990a). The target of ADP-ribosylation is not only conserved in dinitrogenase reductases of the 'conventional' molybdenum nitrogenase but also in the corresponding proteins of alternative nitrogen fixation systems (Robson et al., 1986; Joerger et al., 1989; Schüddekopf et al., 1993) and it has been shown that the alternative nitrogenase of R. rubrum is also modified by DraT (Lehman & Roberts, 1991a).

The photosynthetic non-sulfur purple bacterium Rhodobacter capsulatus also contains two nitrogenase systems: the molybdenum nitrogenase (encoded by nifHDK) and an alternative heterometal-free ('iron-only') nitrogenase (anfHDK; Schneider et al., 1991b; Gollan et al., 1993; Schüddekopf et al., 1993). Only the molybdenum nitrogenase has been analysed for ADP-ribosylation (Jouanneau et al., 1989; Hallenbeck, 1992; Pierrard et al., 1993). Site-directed mutations changing Arg102 into tyrosine or phenylalanine resulted in partially active mutant NiF proteins which were no longer substrates for ADP-ribosylation (Pierrard et al., 1993). However, R. capsulatus strains containing these mutant proteins still showed a 'switch-off' response to ammonia. This result implied a second mechanism of ammonia regulation which might act either on ATP or electron supply of nitrogenase (Pierrard et al., 1993).

In the present study, we describe the identification and characterization of the R. capsulatus draTG genes. R. capsulatus strains carrying mutations in the draTG genes were constructed and analysed for post-translational regulation of both the molybdenum and the alternative nitrogenase.

### Methods

**Bacterial strains and plasmids.** The bacterial strains and plasmids used in this study are listed in Table 1. R. capsulatus strains B10S, W107I and W107II were grown diazotrophically in RCV medium as described previously (Klipp et al., 1988; Masepohl et al., 1988, 1993; Moreno-Vivian et al., 1989a, b). To analyse the alternative nitrogenase, which is repressed by molybdenum, the R. capsulatus nifHDK deletion strains KS36, KS36-W107I and KS36-W107II were grown in molybdenum-deficient medium (Schneider et al., 1991a).

### Hybridization and cloning procedures.** Two degenerate oligonucleotides used as draT: (5'-GG[C/G]G[C/G]T[G/C]T[G/C]A[A/G] [G[J/E]G[TGG[T[G/C]G-3]) or draG-specific probes (5'-GG[C/G]G[C/G]G[A/C/T]G[C/G]G[C/G]A[C/T]A[C/G]G[C/G]-3) were 3' end-labelled (DIG oligonucleotide tailing kit, Boehringer Mannheim) and hybridization experiments were performed using the DIG luminescent detection kit for nucleic acids (Boehringer Mannheim). To isolate the 7-1 kb HindIII fragment carrying the R. capsulatus draTG genes 120 clones of a size-fractionated HindIII gene bank were screened. Two clones containing the 7-1 kb HindIII fragment were identified. A 5-5 kb HindIII–BanHI fragment was subcloned into

### Table 1. Bacterial strains and plasmids

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<th>Designation</th>
<th>Relevant characteristics</th>
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<td><strong>Bacterial strains</strong></td>
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<td>Sp&lt;sup&gt;r&lt;/sup&gt;, Dra&lt;sup&gt;T&lt;/sup&gt; deletion derivative of R. capsulatus</td>
<td>Klipp et al. (1988)</td>
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<td>Rhodobacter capsulatus KS36</td>
<td>Dra&lt;sup&gt;T&lt;/sup&gt; deletion derivatives of R. capsulatus</td>
<td>This study</td>
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<td>Rhodobacter capsulatus W107I/II</td>
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<td>This study</td>
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<td>Rhodobacter capsulatus KS36-W107I/II</td>
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<td>Escherichia coli JM83</td>
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<td>Vieira &amp; Messing (1982)</td>
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<td>Escherichia coli S17-1</td>
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<td>Simon et al. (1983)</td>
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<td><strong>Plasmids</strong></td>
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<td>Ap&lt;sup&gt;r&lt;/sup&gt;, Lac&lt;sup&gt;r&lt;/sup&gt;, Dra&lt;sup&gt;T&lt;/sup&gt;</td>
<td>Vieira &amp; Messing (1982)</td>
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<td>pSVB plasmids</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt;, Lac&lt;sup&gt;r&lt;/sup&gt;, Dra&lt;sup&gt;T&lt;/sup&gt;</td>
<td>Arnold &amp; Pühler (1988)</td>
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Rhodobacter capsulatus draTG

Results and Discussion

Identification and cloning of the R. capsulatus draTG genes

Two amino acid sequences of DraT and DraG, respectively, conserved between *Rhodospirillum rubrum* (Fitzmaurice et al., 1989) and *Azospirillum brasilense* (Zhang et al., 1992) were chosen to design corresponding oligonucleotides on the basis of the *R. capsulatus* codon preference. These conserved regions within the DraT or DraG proteins are marked in Fig. 3. The oligonucleotides (see Methods) were used as hybridization probes to identify *draTG* homologous genes in *R. capsulatus* total DNA. Both probes hybridized with a 7.1 kb HindIII fragment which was isolated from a size-fractionated HindIII gene bank. The physical map of this 7.1 kb HindIII fragment is shown in Fig. 1(a). Detailed Southern hybridization analyses revealed that the *draT*-specific probe hybridized to a 1.1 kb HindIII-EcoRI fragment, whereas the *draG* homologous region was located on a 1.5 kb PstI fragment.

**DNA sequence analysis of the R. capsulatus draTG gene region**

A 1904 bp DNA fragment hybridizing to *draT*- and *draG*-specific probes was subjected to DNA sequence analysis (Fig. 1b) and the complete nucleotide sequence is presented in Fig. 2. Two open reading frames were identified. Assignment of these ORFs as *R. capsulatus* *draT* and *draG* was based on deduced amino acid sequence identity to *dra* gene products from *R. rubrum* (Fitzmaurice et al., 1989) and *A. brasilense* (Zhang et al., 1992). A comparison of the predicted DraT and DraG protein sequences of *R. capsulatus*, *R. rubrum* and *A. brasilense* is given in Fig. 3. The *R. capsulatus* *draT* gene product showed an overall identity of 39% to the *A. brasilense* and 47% to the *R. rubrum* DraT protein. An even higher degree of identity was found for the DraG proteins. About 54% and 62% of the amino acids of *R. capsulatus* DraG are identical to the DraG proteins of *A. brasilense* and *R. rubrum*, respectively. Although both DraT and DraG interact with the same substrates (component II of nitrogenase and adenosine nucleotides), no similarities were found between the primary sequences of DraT and DraG.

![Diagram](image-url)

Fig. 1. Physical and genetic map of the *R. capsulatus* *draTG* gene region. DNA fragments hybridizing to *draT* and *draG*-specific probes are marked by hatched or cross-hatched bars, respectively, above the physical map of the *R. capsulatus* *draTG* gene region (a). The location of *detP* at the right-hand end of the map is based on partial DNA sequence analysis. The sequenced region carrying the *R. capsulatus* *draT* and *draG* genes is emphasized by a thick line (b). A 404 bp internal EcoRI fragment was replaced by an interposon encoding gentamicin resistance resulting in hybrid plasmids pBMW107I and pBMW107II (c). The arrowheads indicate the orientation of the gentamicin resistance gene. The interposon is not drawn to scale. Abbreviations: B (BamHI); E (EcoRI); H (HindIII); P (PstI); S (SalI).
Fig. 2. Complete nucleotide sequence of a DNA fragment containing the \textit{R. capsulatus} \textit{drt} and \textit{drag} genes. The DNA sequence is presented in 5′–3′ direction. The predicted amino acid sequences for two open reading frames (Drt and Drag) are indicated by the single letter code. A putative ribosomal binding site in front of \textit{drag} is marked by dots. The EcoRI sites used for construction of the \textit{drt} deletion mutants (Fig. 1c) are shown below the nucleotide sequence.
Fig. 3. Alignment of predicted amino acid sequences of DraT and DraG from Azospirillum brasilense (Ab), Rhodospirillum rubrum (Rr) and R. capsulatus (Rc). The amino acid sequences are aligned for maximum matching and identical amino acid residues are boxed. Black bars indicate amino acid sequences conserved between A. brasilense and R. rubrum which served as templates to design druT- and draG-specific oligonucleotide probes (see Methods).
The \textit{R. capsulatus} dra\textit{T} and dra\textit{G} genes are separated by 11 bp, implying that they are co-transcribed. The likely initiation codon of the \textit{R. capsulatus} dra\textit{T} and dra\textit{G} genes is ATG. A possible ribosome binding site (Stormo \textit{et al.}, 1982) is located in front of dra\textit{G} whereas no typical Shine–Dalgarno sequence could be identified in front of dra\textit{T}.

The arrangement of the \textit{R. capsulatus} dra\textit{TG} genes is the same as in \textit{R. rubrum} (Fitzmaurice \textit{et al.}, 1989), \textit{A. brasilense} (Zhang \textit{et al.}, 1992) and \textit{Azospirillum lipoferum} (Fu \textit{et al.}, 1990b). However, no open reading frame homologous to the 15 kDa ORF located downstream of \textit{R. rubrum} and \textit{A. brasilense} dra\textit{G} could be identified within the sequenced dra region from \textit{R. capsulatus}. In contrast to \textit{R. rubrum}, \textit{A. brasilense} and \textit{A. lipoferum}, the dra\textit{TG} genes of \textit{R. capsulatus} are not located upstream of the structural genes of nitrogenase (nif\textit{HDK}). However, partial DNA sequence analysis of adjacent DNA fragments (data not shown) and comparison of physical maps revealed that the \textit{R. capsulatus} dra\textit{TG} genes are located close to the dct\textit{P} gene (Fig. 1a; Shaw \textit{et al.}, 1991). The dct\textit{P} gene has previously been mapped at a distance of more than 1000 kb from the nif\textit{HDK} genes (Fonstein \textit{et al.}, 1992).

\textbf{Mutational analysis of the \textit{R. capsulatus} dra\textit{TG} genes}

To inactivate the \textit{R. capsulatus} dra\textit{TG} genes, a 404 bp EcoRI fragment encompassing the 3’ end of dra\textit{T} and the 5’ end of dra\textit{G} was replaced by a gentamicin resistance cassette (Fig. 1c). The gentamicin resistance interposon was previously shown to induce polar or non-polar mutations depending on the orientation (Moreno-Vivian \textit{et al.}, 1989a; Masepohl \textit{et al.}, 1993). The hybrid plasmids pBMW107I and pBMW107II (Fig. 1c) carrying the interposon in both orientations were used to construct \textit{R. capsulatus} dra\textit{TG} mutants. The dra\textit{T} insertions were introduced into \textit{R. capsulatus} wild-type resulting in mutant strains W107I and W107II, and into the nif\textit{HDK} deletion strain KS36, which allows selective expression of the alternative nitrogenase (KS36-W107I and KS36-W107II).

The influence of the dra\textit{TG} mutations on the post-translational regulation of both the molybdenum and the heterometal-free nitrogenase was first examined by immunoblot analysis using sera containing antibodies against AvII or RcII*, respectively (Fig. 4). As expected, a cell-free protein extract of \textit{R. capsulatus} wild-type prepared after activation of the dra\textit{T} gene product by ammonia contained two proteins representing the modified and the non-modified form of component II of the molybdenum nitrogenase. In contrast, the \textit{R. capsulatus} dra\textit{TG} mutant strains contained only the non-modified form of NifH. There was no difference between the \textit{R. capsulatus} dra\textit{TG} strains carrying the non-polar (W107I) or the polar dra\textit{TG} mutation (W107II). To analyse if the alternative nitrogenase of \textit{R. capsulatus} was also subject to ADP-ribosylation by DraT, similar experiments were performed for nif\textit{HDK} deletion strains. In the parental nif\textit{HDK} deletion strain KS36 two forms of component II of the alternative nitrogenase (AnfH) were detected after 'switch-off' by ammonia, whereas the nif\textit{HDK}/dra\textit{TG} double mutants contained only the non-modified form of AnfH (Fig. 4). These results indicated

\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig4}
\caption{Effect of ammonia on the modification of nitrogenase component II. \textit{R. capsulatus} wild-type and mutant strains were grown diazotrophically as described in Methods. Prior to protein extraction ammonia was added to a final concentration of 7.5 mM. Proteins were separated by SDS-PAGE and component II of the molybdenum nitrogenase (NifH) and of the alternative nitrogenase (AnfH) were visualized by Western analysis using specific antisera. The original data from the Western analysis were digitally processed using a Hewlett-Packard Scan Jet I1 P. Lanes 1 and 4, \textit{R. capsulatus} dra\textit{TG}::\text{nifHDK} deletion strain; lane 2, W107I (dra\textit{TG}::\text{> Gm}); lane 3, W107II (dra\textit{TG}::\text{< Gm}); lanes 5 and 8, \textit{R. capsulatus} KS36 (nif\textit{HDK} deletion strain); lane 6, KS36-W107I; lane 7, KS36-W107II.}
\end{figure}
that the draTG genes were required for ADP-riboseylation in response to addition of ammonia of both the molybdenum and the alternative nitrogenase in R. capsulatus.

Expression of the alternative nitrogenase in R. capsulatus is repressed by molybdenum (Schneider et al., 1991a, b). To test the influence of molybdenum on post-translational regulation, the R. capsulatus nifHDK deletion strain KS36 was derepressed for the alternative nitrogenase and molybdenum was added to a final concentration of 3 μM. However, molybdenum did not cause ‘switch-off’ of the alternative nitrogenase (data not shown), implying that the DraT protein was not activated by molybdenum.

Post-translational regulation of nitrogenase activities were followed under ‘switch-off’ conditions in response to ammonia by the acetylene reduction assay. In contrast to the parental strains, neither the molybdenum nor the heterometal-free nitrogenase were inactivated in response to ammonia in a draTG mutant background (Fig. 5a, b). In addition, post-translational regulation of both nitrogenase systems was also analysed in response to darkness (Fig. 5c, d). After 60 min of incubation in the dark the molybdenum nitrogenase activity was reduced to less than 10% in the wild-type, whereas no significant decrease in activity was found for the draTG mutant strain. Compared to R. rubrum (Liang et al., 1991), the inactivation of the molybdenum nitrogenase from R.
capsulatus by darkness was almost complete but slower (40 min versus 60 min). In contrast, recovery of nitrogenase activity in the light was very rapid and corresponded in this aspect to R. rubrum. Inactivation of the alternative nitrogenase in response to darkness was even slower than for the molybdenum nitrogenase. In contrast to the molybdenum nitrogenase, activity of the hetero-metal-free nitrogenase could not be recovered completely by light (Fig. 5d). This result indicated that (i) the modified form of AnH was less stable than the ADP-ribosylated NiF protein, (ii) hydrolysis of the ADP-ribosylated form of AnH could not be achieved completely by DraG, or (iii) component I of the alternative nitrogenase was more labile than the FeMo protein under ‘switch-off’ conditions. However, activity of the alternative nitrogenase was not influenced in the draTG mutant by darkness for at least 70 min making this strain particularly useful for the isolation of the alternative nitrogenase.

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References


