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 II 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 *ntf*-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).

In addition to the transcriptional regulation of *nif* gene expression, some bacteria contain a post-translational regulatory system, which causes rapid inhibition ('switch-off') of nitrogenase component II (Zumft & Castillo, 1978; Pope *et al.*, 1985; Jouanneau *et al.*, 1989; Fu *et al.*, 1990b; Liang *et al.*, 1991; Zhang *et al.*, 1992). Post-translational regulation of nitrogenase activity is best-characterized in *Rhodospirillum rubrum* (Pope *et al.*, 1985; Lowery *et al.*, 1986; Saari *et al.*, 1986; Lowery & Ludden, 1988; Liang *et al.*, 1991). Inactivation of nitrogenase reductase by ADP-ribosylation is catalysed by the *draT* gene product (dinitrogenase reductase ADP-ribosyltransferase) in response to ammonia, oxygen or darkness. The reverse reaction restoring nitrogenase activity is catalysed by the DraG protein (dinitrogenase reductase-activating glycohydrolase).

The target for ADP-ribosylation is the arginine residue at position 101 (with regard to *R. rubrum* NifH; Pope *et al.*, 1985). An Arg-101-Val mutation prevented ADP-ribosylation of the *R. rubrum* mutant NifH protein but

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Table 1. *Bacterial strains and plasmids*

Designation	Relevant characteristics	Reference
<i>Bacterial strains</i>		
<i>Rhodobacter capsulatus</i> B10S	Spontaneous Sm ^r mutant of <i>R. capsulatus</i> B10	Klipp <i>et al.</i> (1988)
<i>Rhodobacter capsulatus</i> KS36	<i>nifHDK</i> deletion derivative of <i>R. capsulatus</i> B10S, Sp ^r	K. Schüddekopf (Bielefeld)
<i>Rhodobacter capsulatus</i> W107I/II	<i>draTG</i> deletion derivatives of <i>R. capsulatus</i> B10S, Gm ^r	This study
<i>Rhodobacter capsulatus</i> KS36-W107I/II	<i>draTG</i> deletion derivatives of <i>R. capsulatus</i> KS36	This study
<i>Escherichia coli</i> JM83	Host for pUC8 and pSVB plasmids	Vieira & Messing (1982)
<i>Escherichia coli</i> S17-1	RP4-2 (Tc::Mu) (Km::Tn7) integrated in the chromosome	Simon <i>et al.</i> (1983)
<i>Plasmids</i>		
pUC8	Ap ^r Lac ⁺	Vieira & Messing (1982)
pSVB plasmids	Ap ^r Lac ⁺	Arnold & Pühler (1988)
pSUP301	Ap ^r Km ^r <i>mob</i>	Simon <i>et al.</i> (1983)
pBMW101	7.1 kb <i>Hind</i> III fragment carrying <i>R. capsulatus draTG</i> cloned into pUC8	This study
pBMW106I	5.5 kb <i>Hind</i> III– <i>Bam</i> HI fragment from pBMW101 cloned into a pSUP301 derivative carrying the <i>aph</i> II gene of Tn5	This study
pBMW107I/II	Gm ^r deletion derivatives of pBMW106I	This study

also eliminated nitrogenase activity (Lehman & Roberts, 1991*b*). Arg₁₀₁ and adjacent amino acid residues are highly conserved between the NifH 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.*, 1990*a*). 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, 1991*a*).

The photosynthetic non-sulphur 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.*, 1991*b*; 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 NifH 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.*, 1989*a, 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.*, 1991*a*).

Hybridization and cloning procedures. Two degenerate oligonucleotides used as *draT*- (5'-GG[C/G]GC[G/C]GT[G/C]CT[G/C]AA[G/A]GG[C/G]TGGGT[G/C]G-3') or *draG*-specific probes (5'-GG[C/G]GG[C/G]GA[C/T]GC[C/G]GA[C/T]AC[C/G]AC[C/G]GG[C/G]GC-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 *Hind*III fragment carrying the *R. capsulatus draTG* genes 120 clones of a size-fractionated *Hind*III gene bank were screened. Two clones containing the 7.1 kb *Hind*III fragment were identified. A 5.5 kb *Hind*III–*Bam*HI fragment was subcloned into

a derivative of the mobilizable vector plasmid pSUP301 by standard methods (Sambrook *et al.*, 1989). A DNA fragment carrying the gentamicin resistance gene (*Gm*) from pPH1JI (Hirsch & Beringer, 1984) was subsequently used to replace an internal 404 bp *EcoRI* fragment (Figs 1 and 2). The resulting hybrid plasmids pBMW107I and pBMW107II were mobilized from *Escherichia coli* S17-1 into *R. capsulatus* B10S and KS36, respectively, by filter matings (Masepohl *et al.*, 1988). Marker rescue was followed by selection for gentamicin resistance and test for loss of the vector-encoded kanamycin resistance as described previously (Masepohl *et al.*, 1988, 1993; Moreno-Vivian *et al.*, 1989a, b).

DNA sequencing. Appropriate restriction fragments of plasmid pBMW101 were subcloned into different pSVB vector plasmids. DNA sequencing by the dideoxy method (Sanger *et al.*, 1977) was performed using a commercial T7 polymerase sequencing kit (Pharmacia). In addition to the universal and reversal primers, two synthetic oligonucleotides (5'-CGCGCTGCTCGGGTTCGATG-3' and 5'-CGCCGGTTTGCCGCCTGGC-3') were used.

Biochemical methods. *R. capsulatus* strains were grown diazotrophically in 3 ml cultures in 15 ml tubes under an atmosphere of pure nitrogen. In late exponential phase cultures the *draT* gene product was activated by addition of ammonia to a final concentration of 7.5 mM. Cell-free protein extracts of these cultures were prepared as described by Masepohl *et al.* (1988). SDS-PAGE was carried out according to Laemmli (1970), followed by immunoblots as described by Sambrook *et al.* (1989). Detection of component II of the molybdenum nitrogenase from *R. capsulatus* was performed using an antiserum against *Azotobacter vinelandii* component II (AvII; Dingler *et al.*, 1988) whereas modification of component II of the alternative nitrogenase from *R. capsulatus* (RcII*) was analysed with an antiserum against partially purified RcII*.

Nitrogenase assays in vivo. Nitrogenase activity was measured in whole cells by the acetylene reduction method in a Hewlett-Packard gas chromatograph model 5890 series II with a Chrompack alumina GC column for the acetylene/ethylene/ethane separation. A 1 ml vol. of cells was withdrawn anaerobically from a 100 ml culture vessel and injected into a 5 ml stoppered serum vial flushed with argon. The vial was placed in an illuminated shaking water bath at 30 °C. The reaction was started by the addition of 0.25 ml acetylene. After 1 or 2 min, depending on the strain analysed, 100 µl samples of the gas phase were taken and analysed by gas chromatographic methods.

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.

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 *dctP* 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 (*Bam*HI); E (*Eco*RI); H (*Hind*III); P (*Pst*I); S (*Sal*I).

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CCGGCTGAGCCGGGCGAGAAACGGCCAGCCCGGGCTGCGCGCAACGTTACCTTCCCAGACCGGGGCAAGACC 75
      M D Q T F S P S A R L G H S T N L V G A P
GCATCATCCGGCCATGGATCAAACCTTTTCTCCTTCCGCACGCCCTTGGCCATTCCACCAATCTGGTCGGGGCGCC 150 DraT
      S A W M A S P G F N D R P V A L H I W G V R E M N
CTCGGCTGGATGGCGAGCCCGGCTTCAACGATCGGCCCTTGGCTGCATATCTGGGGCGTGGCGAAATGAA 225
      P A L F T M L D Q A E T L A E A G E P F W P I W C
CCCGCGCTGTTTACCATGCTTGATCAGGCGGAAACGCTGGCCGAGGCGGCGAGCCTTTCTGGCCTATATGGTG 300
      D V R H R P R A A R R R P G Q R K R F R S S F L R
CGATGTTCCGCATCGACCCGAGCAGCGACGCGCCCGGGCAGCGCAAGCGGTTCCGCTCGTCTCTCTCGG 375
      L I Q G W G F D S N G P E G A V L K G W V E S R F
GCTCATTCAGGGCTGGGGTTTGTACAGCAACGCCCGAGGGCGCGGTGCTGAAGGGCTGGGTGGAAAGCCGCTT 450
      G I L P S F H K E I I P D T Q G P V W Q A Y V A E
CGGCATCTGCCAGCTTTCACAAGGAGATCATCCCGATACCCAGGGCCCGGCTGGCAGGCCTATGTGGCCGA 525
      R M Q S R F H D N A I L V Q L D L L F E F C Q W A
ACGGATGCAAGCCGCTTTCAGCAATGCGATCCTCGTGCGATCGATCTGCTGTTCTGAATTCGCCAATGGGC 600
      V A V S P I R G K A S D A L S R H Q C G R P A W H
GGTGGCCGTTTCCGCTATCCGGGGCAAGCATCTGACGCTCTATCGGGCATCAATGCGGTGACCGGCATGGCA 675
      S G T A K P P R V I R L N N L V S F S S D R E V A
TTCTGGAACAGCAAAGCCCGCGCGTAAATCCGGCTCAACAATCTTGTGCTTTTCTCCGACCGCGAGGTGGC 750
      D C F G D R I I T T R V P L A K V L F F P G L L P
GGACTGCTTTGGCGACCGGATCATCAGACCCGGGTGCGCTGCCAAGGTGCTGTTCTTTCCCGCCGCTGTC 825
      S P L L R G E R E F L V L G G A F R V S I E D I
CTCGCCCTGTTGGGGGCGAGCGGAATTTCTGGTCTTGGCGGCGGTTTCCGGTCAGTATCGAGGATATCTA 900
      M G D V Q D R A M A A F L G F A L G D A L
GGGGGATGCGCGATGGGCGATGTCAGGATCGGGCGATGGCGGCTTTCTCGGCTTTCCTGGGCGATGCGCTG 975 DraG
      G A T V E F L T P R E I A A R H G L H R E I I G G
GGGGCGACGGTTCGAATTCGACCCGCGCGAGATCGCGGCGCGCACGGCTGCACAGGGAGATCATCGGCGGC 1050
      G W L K L K P G A V T D D T Q M A L V L G R S L I
GGCTGCTGAAGCTGAAGCCCGCGCGGTGACCGATGACACGCGATGGCGCTGCTGGGGCGCTCGCTGATC 1125
      A R Q G F D A R D I C A G F A A W L K S G P A D V
GCGCGGCGGGCTTGTATGCAAGGACATCTGCGCGGGTTTGGCGGCTGGCTGAAATCGGGTCCGGCCGATGTC 1200
      G N T C R R G I R R F L E Q G T V E G P Y A E D D
GGCAACACCTGTGCGCGCGGCATCCGGCGGTTTGTGAACAGGGCACGGTCGAGGGCCCTATGCGGAGGATGAC 1275
      A G N G A A M R V L P V A L A T L G H P E R A A A
GCGGGCAATGGCGCGCGATGCGGGTGCTGCCGTGGCGCTGGCGACGCTGGGCCATCCGAAACGCGCCGCGCC 1350
      W T V A Q A H A T H N H P L S D A A C V A L V R M
TGGACCGTGGCGAGGCGCATGCGACGATAACACCCGCTCTCCGATGCGCGCTGTGTCGCGCTGGTGGGATG 1425
      V Q G L V L G G G A A V I A R E A E R L I A Q H R
GTGACGGGGTGTGCTTGGCGGGGGGCGCGGTCTATTGCCGCGAGGCGGAGCGGCTGATCGCGCAACATCGC 1500
      D F R F V P Y R G R S S A F V V E T L Q T V L H Y
GATTCGCTTTCGTGCCCTATCGCGCGGCTCCTCGGCCTTCTGCTGTCGAGACGCTGCAGACGGTGTGATTAC 1575
      T L S S E S F E E V L T G T V N Q G G D A D T T G
ACGCTCTCATCGGAGAGTTTCAGGAGGTGCTGACCGGCACCGTCAATCAGGGCGGCGATGCGGATACGAGGGG 1650
      A L A G M L A G A R G G M A A L P E R W L A A L D
GCGCTGGCGGGATGCTGGCGGGGGCGCGCGGGGATGGCGGCTGCCGGAACGCTGGCTTGGCGCGCTTGAC 1725
      P A V T A E I R A Q V P K L L A L S G I
CCCGCGGTGACGGCCGAGATCCGCGCTCAGGTGCCGAAGCTGCTGGCGCTTTCGGGCATCTGAGTGAGCTGCCGG 1800
      GCCAGATCGGCCTTTCGGGGCAAGAAGCGTCTCGGCCAGCGCGCGCGCGGCGAACCAGGCGCACAAATGCCGG 1875
      ATTGGCCGGCCTCGGGCAGGCCGATACCA 1904

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Fig. 2. Complete nucleotide sequence of a DNA fragment containing the *R. capsulatus draT* and *draG* genes. The DNA sequence is presented in 5'-3' direction. The predicted amino acid sequences for two open reading frames (DraT and DraG) are indicated by the single letter code. A putative ribosomal binding site in front of *draG* is marked by dots. The *EcoRI* sites used for construction of the *draTG* deletion mutants (Fig. 1c) are shown below the nucleotide sequence.

DraT

Ab-T	M A D G S A S G G L R D A R G R L I D P D S P L A L K A H R T N L L N V T A D T L C A E S F N D E P	
Rr-T		M K D M G E D R P G I G H S T N L V G L P T D L L A S A W F N Q A A
Rc-T		M D Q T F S P S A R L G H S T N L V G A P S A W M A S P G F N D R P
		34
Ab-T	R R L R I G G T R S E H A V L F E A L D E S P N S L I A A D I F Q H Y M A P T F G L N P D F S G A E	
Rr-T	P E I H I A G V R E M N R S L F E M L A E A P D L E S A G E A F Y K Y M I A N F G L D P E Q Q D H R	
Rc-T	V A L H I W G V R E M N P A L F T M L D Q A E T L A E A G E P F - W P I W C D V R H R P R A A R R R	83
Ab-T	G T D G - - R R R Y R A S Y L R L L K G W L F D S N N A E G A V L K G W V E S R F G L L P T Y H K E	
Rr-T	P G Q G G A V R R F H A S Y L R L L K G W G Y D T N A K E G A V L K G W V E S R F G L F P T F H R E	
Rc-T	P G Q R - - - K R F R S S F L R L I Q G W G F D S N G P E G A V L K G W V E S R F G I L P S F H K E	130
	[SmaI]	
Ab-T	P I L R F A S D A W R T Y G E E K V A T R F H N N N I N L Q L D L L Y E Y C Q W W M R R G W P D A P	
Rr-T	P I T K F A S K A W I T Y I E E K M T S R F H N N S I Y V Q L D L M Y E F C Q W A L A R - - - F A	
Rc-T	I I P D T Q G P V W Q A Y V A E R M Q S R F H D N A I L V Q L D L L F E F C Q W A V A V - - - S P	176
	[EcoRI]	
Ab-T	M P S R A S H I R L Y R G V N D F E E H H I V A R P D K R T A V L R L N N L S S F S I E R D I A G Q	
Rr-T	A P G E S A L L - L Y R G V N D F T E H Q M I E R I D N R Q V V R M N N L V S F S S D R G V A D C	
Rc-T	I R G K A S D A - L S R - - H Q C G R P A W H S G T A K P P R V I R L N N L V S F S S D R E V A D C	223
	[SalI]	
Ab-T	F G D Y I L E T W V P L T K V V F F R D I L P R Y P F K G E G E Y L V V G G D Y R V G V S L L	
Rr-T	F G D T I L E T R V P V S K I V F F N T L L T S H P L K G E G E Y L V I G G D Y L V K A S Y L	
Rc-T	F G D R I I T T R V P L A K V L F P P G L L P S P L L R G E R E F L V L G G A F R V S I E D I	270
	[SmaI]	

DraG

Ab-G	M T D H S I R S R A L G A Y L G L A C G D A L G A T V E F L T K G E I A H Q Y G V H K H I K G G G W	
Rr-G	M T G P S V H D R A L G A F L G L A V G D A L G A T V E F M T R K E I A Q Q Y G I H R K M T G G G W	
Rc-G	M G D V Q D R A M A A F L G F A L G D A L G A T V E F L T P R E I A A R H G L H R E I I G G G W	48
	[EcoRI]	
Ab-G	L K L P A G Q V T D D T E M S I H L G R A I L A A P E W D A R R A A E E F A V W L K G V P V D V G D	
Rr-G	L R L K P G Q I T D D T E M S L A L G R S L A A K G T L D V A D I C E E F A L W L K S R P V D V G N	
Rc-G	L K L K P G A V T D D T Q M A L V L G R S L I A R Q G F D A R D I C A G F A A W L K S G P A D V G N	98
Ab-G	T T R R G I R R F I M H G T L S E P E S E Y H A G N G A A M R N L P V A L A T L G D D A A P E R W T	
Rr-G	T C R R G I R R F Y M H E G T T A P Y S E G D A G N G A A M R C L P A A L A T L G H P A D L E P W V	
Rc-G	T C R R G I R R F L E Q G T V E G P Y A E D D A G N G A A M R V L P V A L A T L G H P E R A A A W T	148
Ab-G	V E Q A H I T H C N A M S D A A T L T L G H M V R R L V L G G D V R D V R D E S N K L I A K H R Q F	
Rr-G	L A Q A R I T H N H P L S D A A C L T L G R M V H H L I G G R G M K A C R E E A N R L V H Q H R D F	
Rc-G	V A Q A H A T H N H P L S D A A C V A L V R M V Q G L V L G G G A A V I A R E A E R L I A Q H R D F	198
Ab-G	K F Q P Y R G L A T A V I V D T M Q T V M H Y Y F Q T D S V E S C V V E T V N Q G G D A D T T G A I	
Rr-G	H F E P Y K G Q S S A Y I V D T M Q T V L H Y Y F V T D T F K S C L I Q T V N Q G G D A D T T G A L	
Rc-G	R F V P Y R G R S S A F V V E T L Q T V L H Y T L S S E S F E E V L T G T V N Q G G D A D T T G A L	248
	[PstI]	
Ab-G	A G M L A G A T Y G V E T I P P R W L R K L D R D V Y N E I C A Q V D G L L A R A P A V K Q G	
Rr-G	A G M L A G A T Y G V D D I P S G W L S K L D M K V E R E I R Q V D A L L A L A G L D	
Rc-G	A G M L A G A R G G N A A L P E R W L A A L D P A V T A E I R A Q V P K L L A L S G I	291

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 *draT*- and *draG*-specific oligonucleotide probes (see Methods).

Fig. 4. Effect of ammonia on the modification of nitrogenase component II. *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 II P. Lanes 1 and 4, *R. capsulatus* wild-type; lane 2, W107I (*draTG*::[> Gm]), lane 3, W107II (*draTG*::[< Gm]); lanes 5 and 8, *R. capsulatus* KS36 (*nifHDK* deletion strain); lane 6, KS36-W107I; lane 7, KS36-W107II.

The *R. capsulatus draT* and *draG* genes are separated by 11 bp, implying that they are co-transcribed. The likely initiation codon of the *R. capsulatus draT* and *draG* genes is ATG. A possible ribosome binding site (Stormo *et al.*, 1982) is located in front of *draG* whereas no typical Shine-Dalgarno sequence could be identified in front of *draT*.

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

Mutational analysis of the *R. capsulatus draTG* genes

To inactivate the *R. capsulatus draTG* genes, a 404 bp *EcoRI* fragment encompassing the 3' end of *draT* and the 5' end of *draG* 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 *et al.*, 1989a; Masepohl *et al.*, 1993). The hybrid plasmids pBMW107I and pBMW107II (Fig. 1c) carrying the interposon in both orientations were used to construct *R. capsulatus draTG* mutants. The *draTG* insertions were introduced into *R. capsulatus* wild-type resulting in mutant strains W107I and W107II, and into the *nifHDK* deletion strain KS36, which allows selective expression of the alternative nitrogenase (KS36-W107I and KS36-W107II).

The influence of the *draTG* 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 *R. capsulatus* wild-type prepared after activation of the *draT* 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 *R. capsulatus draTG* mutant strains contained only the non-modified form of NifH. There was no difference between the *R. capsulatus* strains carrying the non-polar (W107I) or the polar *draTG* mutation (W107II). To analyse if the alternative nitrogenase of *R. capsulatus* was also subjected to ADP-ribosylation by DraT, similar experiments were performed for *nifHDK* deletion strains. In the parental *nifHDK* deletion strain KS36 two forms of component II of the alternative nitrogenase (AnfH) were detected after 'switch-off' by ammonia, whereas the *nifHDK/draTG* double mutants contained only the non-modified form of AnfH (Fig. 4). These results indicated

Fig. 5. Regulation of nitrogenase activity in response to ammonia and darkness. *R. capsulatus* wild-type and mutant strains were grown in 3 ml cultures to an optical density of 1.5 (OD_{600}) in 15 ml tubes under conditions expressing either the molybdenum (a) or the alternative nitrogenase (b) as described in Methods. At $t = 0$, acetylene was added to a final concentration of 4% (v/v) in the gas phase and nitrogenase activity of these cultures was followed by measuring ethylene accumulation. At the indicated times, ammonia was added to a final concentration of 7.5 mM. Symbols as defined for (c) and (d). To determine nitrogenase activity in response to darkness the same strains expressing the molybdenum (c) or the alternative nitrogenase (d) were grown in 100 ml vessels and 1 ml ($\text{OD}_{600} = 1.5$) aliquots were withdrawn for the acetylene reduction assay. Each sample was incubated at 30 °C in the light and measured for activity after 1 min (for Mo nitrogenase containing strains) or 2 min (for Fe nitrogenase containing strains). 100% activity corresponds to 3240 (wild-type, ●), 3500 (*draTG* deletion strain W1071, ○), 171 (*nifHDK* deletion strain KS36, ■) and 212 (*nifHDK/draTG* double deletion strain KS36-W1071, □) nmol ethylene produced $\text{h}^{-1} \text{ml}^{-1}$. All experiments shown in this figure were repeated twice.

that the *draTG* genes were required for ADP-ribosylation 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 heterometal-free nitrogenase could not be recovered completely by light (Fig. 5d). This result indicated that (i) the modified form of AnFh was less stable than the ADP-ribosylated NifH protein, (ii) hydrolysis of the ADP-ribosylated form of AnFh 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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