JOURNAL OF BACTERIOLOGY, Apr. 1999, p. 2655–2658
0021-9193/99/$04.00+0

Vol. 181, No. 8

Azorhizobium caulinodans P_{II} and GlnK Proteins Control Nitrogen Fixation and Ammonia Assimilation

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Received 15 October 1998/Accepted 5 February 1999

We herein report that *Azorhizobium caulinodans* P_{II} and GlnK are not necessary for glutamine synthetase (GS) adenylylation whereas both proteins are required for complete GS deadenylylation. The disruption of both *glnB* and *glnK* resulted in a high level of GS adenylylation under the condition of nitrogen fixation, leading to ammonium excretion in the free-living state. P_{II} and GlnK also controlled *nif* gene expression because NifA activated *nifH* transcription and nitrogenase activity was derepressed in *glnB glnK* double mutants, but not in wild-type bacteria, grown in the presence of ammonia.

Azorhizobium caulinodans reduces atmospheric nitrogen both in the free-living state and in symbiosis with its host plant, the tropical legume *Sesbania rostrata* (11). In pure culture, this bacterium grows using molecular nitrogen, whereas during symbiosis, fixed nitrogen is exported from the bacteroid to the plant cell and assimilated by the host. Thus, the coupling between nitrogen fixation and ammonia assimilation that exists in the free-living state must be abolished during symbiosis.

Ammonia assimilation proceeds through the glutamine synthetase (GS)-glutamine oxoglutarate aminotransferase pathway. *A. caulinodans* has a single GS (encoded by *glnA*), the activity of which is regulated by adenylylation (10). Two genes with products similar to P_{II} have been characterized in *A. caulinodans*: *glnB*, which is cotranscribed with *glnA* (17); and *glnK*, which is cotranscribed with *amtB*, a gene encoding a protein similar to a known ammonium transporter (18). As in *Escherichia coli*, *glnB* is constitutively transcribed whereas *glnK* expression is regulated by ammonia (17, 22). Neither P_{II} nor GlnK is required for nitrogen fixation in the free-living state. *glnB* mutants are impaired in symbiotic nitrogen fixation (Fix^-), whereas *glnK* mutants are not (Fix^+). P_{II} and GlnK have a minor effect on GS adenylylation (17, 18).

Two proteins similar to P_{II} (P_{II} and GlnK) have been identified in several gram-negative bacteria, including *Herbaspirillum seropedicae*, *Azospirillum brasilense*, and *E. coli* (3, 8, 22). These two proteins are not equivalent in *H. seropedicae* and *A. brasilense* because *glnB* single mutants have impaired nitrogen fixation (3, 9). In contrast, *E. coli* P_{II} and GlnK seem to control GS deadenylylation in the absence of ammonia (2).

We report herein the properties of an *A. caulinodans glnB glnK* double mutant. In contrast to the *glnB* and *glnK* single mutants, GS deadenylylation was strongly impaired during nitrogenase derepression in the double mutant. We also found that the *glnB glnK* double mutant, but not the wild type, derepressed nitrogenase activity in the presence of ammonia, indicating that P_{II} and GlnK are also involved in the regulation of nitrogen fixation.

Characterization of the growth properties of the *glnB glnK* double mutant. A *glnB glnK* double mutant (strain 57625) was constructed by transferring the *glnK* interposon mutation (18) into the *glnB* mutant strain (57620) by conjugation, in order to study the effect of the absence of both proteins.

As previously reported for the *glnB* mutant, the *glnB glnK* mutant (strain 57625) grew less well than the wild type and the *glnK* mutant in liquid minimal medium containing 15 mM ammonia as the sole nitrogen source (17, 18). The generation time of the double mutant strain was 174 min, whereas that of the wild-type strain was 120 min. Maximum optical density (600 nm) for the mutant was 2.4, whereas that for the wild type was 5.5. Both the *glnB* mutant and the *glnB glnK* double mutant grew less well than the wild type on solid nitrogen-free medium containing 15 mM ammonia, 1 mM ammonia, 10 mM nitrate, or 10 mM histidine but grew as well as the wild type on 10 mM glutamine. Unlike the *glnB* or *glnK* single mutants, the *glnB glnK* mutant could not use molecular nitrogen for growth.

P_{II} or GlnK was required for GS deadenylylation. Unadenylylated and total GS activities were measured by the γ-glutamyltransferase assay in the presence and the absence, respectively, of 60 mM Mg^{2+} (Table 1), on whole cells cultured under nitrogenase-derepressing conditions (17) with and without shock by addition of 0.2% NH_4^+. As reported for the *glnB* mutant (57620) (17), total GS activity, which depends on the total amount of enzyme, was higher in the *glnB glnK* mutant (57625) than in the wild type. This may be due to there being more *glnA* transcription under the control of the promoter of the *aphII* gene (which confers kanamycin resistance) inserted in the *glnB* coding sequence. For all strains tested, there was less or equal amount of unadenylylated (or active) GS after ammonia shock than under nitrogenase-derepressing conditions, suggesting that GS adenylylation does not require P_{II} or GlnK.

The percentages of unadenylylated GS were similar in the wild-type strain and the *glnB* or *glnK* single mutants (about 70%) under nitrogenase-derepressing conditions, but the percentage was much lower in the *glnB glnK* double mutant (11%) (Table 1). It must be mentioned that the percentage of unadenylylated GS was probably underestimated since, under these assay conditions, unadenylylated GS may have a specific transferase activity different from that of adenylylated GS, which may account for the increase of the total activities (10). How-

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TABLE 1. Effect of ammonia shock on total GS activity and the percentage of unadenylylated (active) GS in *A. caulinodans* ORS571 and mutant strains

Strain	GS sp act[a,b]		% active GS[b] (unadenylylated)	
	N free[c]	NH_4^+ shock[d]	N free[c]	NH_4^+ shock[d]
ORS571 (wild type)	4.59 ± 0.59	2.80 ± 0.58	70.8 ± 4.1	41.4 ± 6.0
57620 (*glnB* mutant)	32.74 ± 2.02	20.14 ± 3.43	66.4 ± 3.0	12.4 ± 3.2
57621 (*glnK* mutant)	7.43 ± 1.26	5.10 ± 1.29	70.4 ± 11.3	34.2 ± 9.2
57625 (*glnB glnK* mutant)	13.05 ± 2.9	11.68 ± 3.16	11.3 ± 2.8	12.6 ± 4.6
57625 (*glnB glnK* mutant)/pRS1045[e]	21.49 ± 2.26	13.99 ± 2.30	68.2 ± 7.4	26.1 ± 6.2
57625 (*glnB glnK* mutant)/pRS1046[e]	19.77 ± 3.44	13.50 ± 1.89	64.2 ± 13.0	14.6 ± 1.8

[a] Specific activity of GS in pure culture; 1 unit corresponds to 1 μmol of γ-glutamyl hydroxamate · min^{-1} · mg of $protein^{-1}$.

[b] Values are the means ± standard deviations from at least three independent experiments.

[c] Cells were cultured for 3 h in nitrogen-free medium under microaerobic conditions.

[d] Cells were cultured as described in footnote *c* and shocked by incubation with 0.2% NH_4^+.

[e] pRS1045 contains the *glnB* 0.74-kb *Sma*I-*Sal*I fragment of pRS1032 (17) cloned into plasmid pRK415; pRS1046 contains *glnK-amtB* (18).

ever, the low level of active GS present was correlated with the impaired growth of the *glnB glnK* mutant on molecular nitrogen. Both GS activity (Table 1) and growth on molecular dinitrogen were restored in the double mutant strain by expression from plasmids of either *glnB* (from pRS1045) or *glnK* (from pRS1046). Therefore, at least one of the proteins is required for GS deadenylylation under nitrogenase-derepressing conditions.

It is unclear why *A. caulinodans* P_{II} and GlnK are functionally equivalent in GS deadenylylation and not in symbiotic nitrogen fixation. The difference in function may be due to a difference in gene expression during symbiosis. It is also possible that P_{II} and GlnK have activities that differ according to their molecular forms. P_{II} is active as a homotrimer (5, 7), but it is likely that P_{II}/GlnK heterotrimers exist. Thus, P_{II} or GlnK homotrimers may activate GS deadenylylation, and heterotrimers or P_{II} homotrimers may activate symbiotic nitrogen fixation.

The *glnB glnK* double mutant excreted ammonia. *A. caulinodans*, unlike *Bradyrhizobium* species, can grow by consuming molecular nitrogen in pure culture. In the free-living state, only 10% of fixed nitrogen (NH_3) is exported from the cell, the remaining 90% being used for growth (13), whereas *Bradyrhizobium* cultures export all their fixed nitrogen to the medium (4). The absence or inhibition of GS activity blocks ammonium transport in *Klebsiella pneumoniae* (16). We tested whether the low level of unadenylylated GS in the *glnB glnK* double mutant (57625) or the absence of GS in the *glnBA* mutant (57619) affected ammonium excretion during nitrogen fixation in the free-living state by the indophenol method (6). No NH_4^+ was detected in the supernatants of cultures of *glnB* or *glnK* single mutant strains (57620 and 57621), as was also previously reported for the wild-type strain (13). A large amount of NH_4^+ was present in the supernatants of cultures of the *glnBA* mutant and *glnB glnK* double mutant (310 and 362 μM extracellular NH_4^+/optical density unit, respectively). Excretion of NH_4^+ was completely abolished in the *glnB glnK* mutant by expression from plasmids of either *glnB* (57625/pRS1045) or *glnK* (57625/pRS1046). Thus, the absence or inactivation of GS may lead to the accumulation of fixed nitrogen in *A. caulinodans* cells and ultimately to its excretion into the medium.

The *glnB glnK* mutant strain expressed nitrogen fixation genes in the presence of ammonia. The P_{II} and GlnK proteins control ammonium metabolism, in response to ammonia availability, by regulating GS activity. Ammonia negatively regulates nitrogen fixation genes in *A. caulinodans*. In particular it affects *nifA* transcription (15, 21). Thus, we investigated whether P_{II} and GlnK were also involved in the regulation of *nifA* expression. The *A. caulinodans* NifA protein has an estimated molecular mass of 66.8 kDa. It was detected by Western blot analysis in whole-cell extracts from wild-type and mutant strains, using *Bradyrhizobium japonicum* anti-NifA antibodies (19) (Fig. 1). NifA was detected in the wild-type strain and in *glnB* and *glnK* single mutants (57620 and 57621) cultivated under nitrogenase-derepressing conditions (Fig. 1, lanes 1, 4, and 6) but not in the presence of NH_4^+ (lanes 2, 5, and 7) or in the *nifA* mutant (lane 3) (20). NifA was detected in the presence and absence of ammonia in the *glnB glnK* double mutant (lanes 8 and 9) and in the *nifA* mutant containing either the *A. caulinodans nifA* gene (lanes 10 and 11) or the *B. japonicum nifA* gene expressed constitutively in the presence of ammonia (lanes 12 and 13).

nifA was expressed in the presence of ammonia in the *glnB glnK* double mutant strain, indicating that P_{II} and GlnK may inhibit *nifA* transcription and/or regulate *nifA* posttranscriptionally under these conditions. This absence of ammonia regulation could be explained by the very low level of active GS, which could lead to a decrease in the glutamine pool and therefore to an increased α-cetoglutarate/glutamine ratio. This could mimic nitrogen fixation conditions, stimulating expression of *nif* genes, even in the presence of ammonia. However, similar amounts of active GS were found in the *glnB* mutant strain and *glnB glnK* double mutant strain in the presence of ammonia, but only the *glnB glnK* mutant had no ammonia regulation. Thus, P_{II} and GlnK may be involved directly in the repression of NifA synthesis. This control is independent from NtrC, since a translational *glnK-lacZ* fusion (*glnK* is the only gene that is strictly under the control of NtrC to have been characterized for *A. caulinodans*) recombined into the chromosome of either the wild-type strain or the *glnB glnK* mutant is expressed at low levels in the presence of ammonia (1,500 and 2,000 Miller units/mg of protein, respectively, as contrasted with 15,000 Miller units/mg of protein in the wild-type strain under nitrogen-limiting conditions). Thus, the synthesis of a NifA protein by the *glnB glnK* mutant in the presence of ammonia cannot be accounted for by a constitutive NtrC activity.

We assessed NifA activity by integrating a translational *nifH-lacZY* fusion (15) into the chromosomes of the same strains. The activation of *nifH* transcription correlated with the detection of the NifA polypeptide in all but one case (Table 2). The *A. caulinodans* NifA protein was detected in the presence of ammonia if it was produced constitutively (Fig. 1). It did not activate *A. caulinodans nifH* expression, whereas the *B. japoni-*

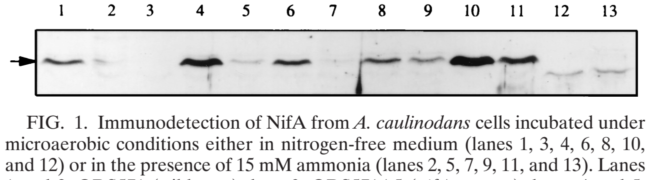

FIG. 1. Immunodetection of NifA from *A. caulinodans* cells incubated under microaerobic conditions either in nitrogen-free medium (lanes 1, 3, 4, 6, 8, 10, and 12) or in the presence of 15 mM ammonia (lanes 2, 5, 7, 9, 11, and 13). Lanes 1 and 2, ORS571 (wild type); lane 3, ORS571A5 (*nifA* mutant); lanes 4 and 5, 57620 (*glnB* mutant); lanes 6 and 7, 57621 (*glnK* mutant); lanes 8 and 9, 57625 (*glnB glnK* mutant); lanes 10 and 11, ORS571A5/pRS1022 (containing *A. caulinodans* constitutive *nifA* [15]); and lanes 12 and 13, ORS571A5/pRJ7556 (containing *B. japonicum* constitutive *nifA* [12]).

TABLE 2. β-Galactosidase activities of the translational *nifH-lacZY* fusion recombined into the chromosomes of the wild-type and mutant strains of *A. caulinodans* carrying or not carrying the constitutively expressed *nifA* from *A. caulinodans* or *B. japonicum*

Strain	β-Galactosidase sp act[a]		
	N free, 3% O_2[b]	NH_4^+, 3% O_2[c]	NH_4^+, air[d]
57721 (wild type)	14,229.0 ± 3,876.5	34.6 ± 32.5	10.5 ± 0.81
57820 (*glnB* mutant)	20,123.0 ± 5,480.3	22.6 ± 18.3	14.6 ± 11.1
57821 (*glnK* mutant)	9,804.6 ± 953.8	6.2 ± 5.5	4.0 ± 0.3
57720 (*nifA* mutant)	11.5 ± 9.3	3.3 ± 2.3	9.9 ± 9.9
57825 (*glnB glnK* mutant)	7,661.7 ± 478.8	6,535.4 ± 532.1	10.7 ± 8.3
57720 (*nifA* mutant)/ pRS1022[e]	17,169.6 ± 3,463.2	91.0 ± 27.3	5.5 ± 2.7
57820 (*glnB* mutant)/ pRS1022[e]	28,319.8 ± 4,652.6	305.5 ± 221.8	9.7 ± 0.6
57821 (*glnK* mutant)/ pRS1022[e]	15,791.0 ± 3,111.6	798.6 ± 205.1	13.4 ± 7.3
57720 (*nifA* mutant)/ pRJ7556[e]	31,650.4 ± 6,762.7	19,878.6 ± 6,333.1	175.75 ± 71.5

[a] Specific activity of β-galactosidase in pure culture expressed in Miller units · mg of protein^{-1}. Values are the means ± standard deviations from at least three independent experiments.

[b] Cells were cultured for 4 h in nitrogen-free medium under microaerobic conditions.

[c] Cells were cultured for 4 h in minimal medium containing 20 mM NH_4^+ under microaerobic conditions.

[d] Cells were cultured for 4 h in minimal medium containing 20 mM NH_4^+ in the presence of air.

[e] pRS1022 contains the constitutively expressed *nifA* from *A. caulinodans* (15), and pRJ7556 contains that from *B. japonicum* (12).

cum NifA protein, which is active in the presence of ammonia, did. This may be due to regulation of the activity or differences in the stability of the *A. caulinodans* NifA protein, in the presence of ammonia.

P_{II} or GlnK may be required in any case because *nifH* transcription, under nitrogenase-derepressing conditions, in the *glnB glnK* mutant (strain 57825) is half that in the wild type (strain 57721), suggesting that P_{II} and GlnK might also have a positive role in *nifH* transcription in the absence of ammonia. However, transcription levels were similar in the absence and presence of ammonia in the double mutant, suggesting that P_{II} and GlnK are required for *nif* gene repression by ammonia (Table 2). The absence of either P_{II} (strain 57820) or GlnK (strain 57821) did not lead to activation of *nifH* transcription by the constitutively expressed NifA, in the presence of ammonia (Table 2).

Two mechanisms have been put forward to account for the regulation of NifA activity in response to ammonia. Arsène et al. suggested that the N-terminal part of the *A. brasilense* NifA negatively regulates the activating domain, whereas P_{II} maintains NifA in an active form under nitrogenase-derepressing conditions (1). This model is not applicable to *A. caulinodans* because (i) P_{II} and GlnK are not required for NifA activity under nitrogen-derepressing conditions and (ii) NifA proteins from which the N terminus has been deleted are inactive (data not shown). NifA activity in *K. pneumoniae* is inhibited by NifL in the presence of excess ammonia. GlnK, whether uridylylated or not, is required to abolish the inhibition of NifA activity by NifL under nitrogen-limiting conditions, but this inhibition was restored in the presence of ammonia, suggesting the existence of another mechanism (14). This model could be applied to *A. caulinodans* if one postulates the existence of a repressor of NifA activity in the presence of ammonia.

Nitrogenase was active in the presence of ammonia in the *glnB glnK* mutant strain. As *nifH* was expressed in the presence of ammonia in the *glnB glnK* mutant strain, we investigated whether the nitrogenase was active (17). Nitrogenase activities were similar in the wild-type strain and the *glnB glnK* mutant strain under nitrogenase-derepressing conditions (Fig. 2). No nitrogenase activity was detected in the wild-type strain in the presence of 10 mM NH_4^+, whereas nitrogenase activity was detected in the *glnB glnK* mutant strain (Fig. 2), suggesting that P_{II} and GlnK may also be required for the regulation of nitrogenase activity.

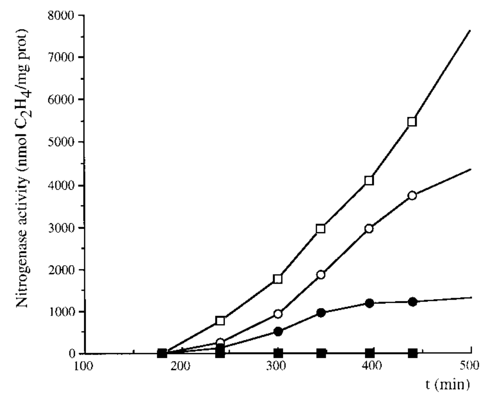

FIG. 2. Kinetics of nitrogenase activities in ORS571 (wild type) (open squares) or the 57625 strain (*glnB glnK* mutant) (open circles) under microaerobic conditions in nitrogen-free medium or of the same strains in medium with 10 mM ammonia added to the medium at time 0 (closed squares and closed circles, respectively).

In summary, P_{II} and GlnK are the key elements controlling nitrogen fixation and ammonia assimilation in *A. caulinodans*. In the presence of ammonia, either protein is involved in the repression of nitrogen fixation, whereas under nitrogen-fixing conditions they stimulate GS deadenylylation. Future work should focus on determining the mechanisms by which these two proteins regulate both processes.

N. M.-R. is a recipient of a predoctoral fellowship from the Ministère de l'Enseignement Supérieur et de la Recherche.

We thank C. Elmerich for critical reading of the manuscript, M. de Zamaroczy for discussion, and N. Desnoues for skillful technical help. We also thank H.-M. Fischer for kindly providing a NifA antiserum and plasmid pRJ7556, which constitutively expresses the *B. japonicum nifA* gene.

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