JOURNAL OF BACTERIOLOGY, Aug. 2005, p. 5493–5495 Vol. 187, No. 15
0021-9193/05/$08.00+0 doi:10.1128/JB.187.15.5493–5495.2005

De Novo Alanine Synthesis by Bacteroids of *Mesorhizobium loti* Is Not Required for Nitrogen Transfer in the Determinate Nodules of *Lotus corniculatus*

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Received 24 November 2004/Accepted 26 April 2005

Deletion of both alanine dehydrogenase genes (*aldA*) in *Mesorhizobium loti* resulted in the loss of AldA enzyme activity from cultured bacteria and bacteroids but had no effect on the symbiotic performance of *Lotus corniculatus* plants. Thus, neither indeterminate pea nodules nor determinate *L. corniculatus* nodules export alanine as the sole nitrogen secretion product.

Nitrogen fixation by *Rhizobium*-legume symbioses results in the production of ammonium as the primary stable product of nitrogen fixation (3), and it has been generally accepted that bacteroids secrete this product directly to the plant (4, 9), where it is assimilated by glutamine synthetase-glutamate synthase (5, 20). However, the recent demonstration that only alanine is secreted by soybean bacteroids suggests a different mechanism of nitrogen transport from bacteroid to plant (21). This has been disputed by others who could not demonstrate alanine secretion by soybean bacteroids (10). Using the *Rhizobium leguminosarum*-pea symbiosis as a tractable genetic and biochemical system, we demonstrated that both alanine and ammonia are secreted by isolated bacteroids (1). The de novo synthesis of alanine by pea bacteroids was shown by mutagenesis and $^{15}N_2$-labeling studies to be due to alanine dehydrogenase (AldA) (1). However, AldA activity was not essential for symbiotic nitrogen fixation in pea nodules, providing further evidence that alanine was not the sole nitrogen secretion product. Under all tested conditions, ammonium remained the principal secretion product, but the proportions of alanine secreted varied. The rate of synthesis of alanine depended on a number of factors, but the key factors were the concentration of ammonium and the activity of AldA. While the affinity of pea and soybean AldA for ammonium is modest (K_m of 5 to 9 mM [1, 16]), the ammonium concentration in soybean bacteroids has been estimated by extrapolation from leakage rates to be 12 mM (18).

It has also been known for some time that isolated pea bacteroids secrete alanine and aspartate and that this is stimulated by the addition of glutamate (2, 15). This suggests that transamination, rather than de novo synthesis by AldA, is an important route for amino acid synthesis and secretion. Recently, the Aap and Bra amino acid transport systems of *R. leguminosarum* were mutated, and this was shown to prevent effective nitrogen fixation by peas, even though nitrogenase was still active in bacteroids (12). This observation together with the requirement for AatA (aspartate aminotransferase) activity led to the proposal that an amino acid cycle based on transamination is essential for nitrogen fixation and assimilation in pea nodules. It is crucial to appreciate that a transamination cycle is not a method to assimilate or export fixed nitrogen, since the nitrogen donor would be an amino acid supplied by the plant. In an amino acid cycle, AldA might have a secondary role of balancing alanine and pyruvate levels in the cell but it would not be the main pathway for assimilation of ammonium (11).

It appears that bacteroids from pea plants, which form indeterminate nodules, have an amino acid cycle operating but do not use alanine as the primary export product of N_2 assimilation. However, the situation for legumes that form determinate nodules is less clear. The availability of several rhizobial genome sequences reveals that *R. leguminosarum* and *Sinorhizobium meliloti*, which infect plants that form indeterminate nodules, have one *aldA* gene, while *Bradyrhizobium japonicum* and *Mesorhizobium loti*, which infect plants that form determinate nodules, have two copies of *aldA*. Furthermore, while the activity of AldA in pea bacteroids is only 90 nmol min^{-1} mg protein^{-1}, in soybean bacteroids, it is 300 to 1,000 nmol min^{-1} mg protein^{-1} (17, 19), which could shift nitrogen export from ammonium to alanine.

This possibility immediately poses the question of whether the two copies of *aldA* lead to very high enzyme activity, resulting in the obligatory secretion of alanine as the sole nitrogen secretion product in determinate nodules. To answer this focused but extremely important question, we mutated both copies of *aldA* in the sequenced *M. loti* strain MAFF 303099 (8) and determined their symbiotic phenotype.

Construction of deletion mutants. There are two *aldA* genes in *M. loti*, one in the chromosome (*aldA$_c$*; locus tag mll0362) and the other on plasmid pMla (*aldA$_p$*; locus tag mll9089). These genes are 94% identical at the DNA level and consequently have only one amino acid substitution (A37T). Due to the very high sequence identity, both genes were removed by deletion of the entire reading frame without attempting to insert markers, which might have been confounded by their high sequence identity, into either gene. An overlap PCR (7) was used with primers mlcaldA1 (AAAAAGAGCTCGCCAA

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TABLE 1. Activity of AldA in strains of *M. loti* grown on various carbon and nitrogen sources

Strain	AldA activity (μmol min^{-1} mg $protein^{-1}$)[a] on:				
	Succinate-NH_4^+	Alanine	Glucose-NH_4^+	Glucose-alanine	Bacteroids
MAFF 303099	2.8201 ± 0.92	3.0977 ± 0.63	0.3810 ± 0.1	1.6210 ± 0.43	0.382
RU1731 (Δ$aldA_c$)	0.6895 ± 0.006	2.7066 ± 0.46	0.04862 ± 0.01	0.8879 ± 0.25	—
RU1732 (Δ$aldA_p$)	1.3152 ± 0.28	3.5496 ± 0.82	0.3536 ± 0.14	0.7871 ± 0.17	—
RU1810 (Δ$aldA_c$ Δ$aldA_p$)	ND	ND	ND	ND	ND

[a] Values shown are means from at least three independent replicates ± SEMs. Cultures were grown on acid minimal salts (13) with the carbon and nitrogen sources indicated in the table at concentrations of 10 mM. AldA activity on bacteroids is the average for two independent *L. corniculatus* nodule harvests and was determined on cultures harvested and assayed as previously described (1). ND, no detectable activity; —, not determined.

AGCATCGAAGCCATA), mlcaldA2 (TATCGACAAAGCT TATCGTTAAGCCGAAAGCAGTTCTCGCG), mlcaldA3 (TAACGATAAGCTTTGTCGATAACATATTCGCGGAC CGAGCCTG), and mlcaldA4 (AAAAAGAGCTCATCGGA GCCCGCCTTGCTGTTT) for $aldA_c$ and with primers mlpaldA1 (AAAAATCTAGACCGCGCAGACTTTGTCTGTT), mlpaldA2 (TATCGACAAAGCTTATCGTTACGAGCCGA AAGCTGTTCTGG), mlpaldA3 (TAACGATAAGCTTTGT CGATACGCGATATTCGTGGTTCTTG), and mlpaldA4 (A AAATCTAGAATCATCGCAAATATGGCCGG) for $aldA_p$. PCR primers 1 and 2 were used to amplify a product on one side of *aldA*, and primers 3 and 4 were used to amplify the other side. The products shared a 21-bp overlap and a HindIII site (contained in primers 2 and 3). Finally, the products of the two primer pairs were mixed and reamplified with primers 1 and 4 to yield the combined regions on either side of *aldA*. The procedure was repeated for both $aldA_c$ and $aldA_p$, and the final PCR products were cloned into pCR2.1 TOPO, yielding pRU945 and pRU946, respectively. The overlap primers containing a HindIII site and omega tetracycline and kanamycin cassettes (6) were cloned into this site in the chromosomal and plasmid regions, respectively. These were transferred into pJQ200SK (14) as SacI fragments and conjugated via *Escherichia coli* S17-1 into *M. loti* strain MAFF 303099, and recombinants resistant to sucrose were isolated. Strain RU1731 (tetracycline resistant) has $aldA_c$ deleted, strain RU1732 (neomycin resistant) has $aldA_p$ deleted, and strain RU1810 (tetracycline resistant and neomycin resistant) has both copies of *aldA* deleted. The deletions were confirmed by PCR mapping of the strains with primers p410 (GCCAAAAAAAGCCCGCCG GA), p411 (GCGCACCGATTCGTGGTTCA), p412 (CCGA GTAACCAGGCTTGTCG), and p413 (TCCGACAATACA GGGAGTGA). All strains grew at similar rates on minimal medium containing succinate and ammonia, glucose and ammonium, glucose and alanine, or alanine alone as the carbon and nitrogen sources (data not shown). Therefore, as was found for *R. leguminosarum* (1), AldA is not required for the use of alanine as a nitrogen or carbon source by *M. loti*. Since the DadXA system appears to be present in *M. loti*, this is the likely route of alanine catabolism, while alanine can be synthesized by transamination from amino acids such as glutamate.

The various strains were grown on acid minimal salts (13) with different carbon and nitrogen sources, and the activities of AldA were measured as previously described (1) (Table 1). Activity in the wild type was high (0.38 μmol min^{-1} mg $protein^{-1}$) in cultures grown on glucose, a condition where activity cannot be detected in *R. leguminosarum*. This activity was induced seven- to eightfold (to 2.8 to 3 μmol min^{-1} mg $protein^{-1}$) when cultures were grown on either succinate-ammonium or alanine (Table 1). The absolute activities of cultures grown on succinate-ammonium or alanine were 38- and 4-fold higher, respectively, than those of the corresponding cultures of *R. leguminosarum* (11). In the glucose-grown culture, most of the activity was contributed by the chromosomally encoded protein, whereas in the succinate- or glucose-alanine-grown cultures, the plasmid-encoded protein contributed around one-third to one-half of the total activity. In alanine-grown cultures, mutating either gene did not alter the activity significantly, and unlike under the other growth conditions, the enzyme activity in the two single mutants did not add up to the activity seen in the wild type. This suggests that there may be some compensation at a regulatory level for the loss of one gene when cultures are grown on alanine. However, the double mutant lacked any detectable alanine dehydrogenase activity. Bacteroids from *Lotus corniculatus* had high AldA activity compared to that of pea bacteroids (385 versus 90 nmol min^{-1} mg $protein^{-1}$), although the activity is at the lower end seen in uninduced laboratory cultures (Table 1). As expected, bacteroids of the double mutant (RU1810) isolated from *L. corniculatus* nodules had no detectable AldA activity.

Plant growth. The wild type and double mutant strain (RU1810) were inoculated onto *L. corniculatus* seedlings that were grown in 250 ml of sterile vermiculite and gravel (2:1) and watered with nitrogen-free rooting solution. After 7 weeks of growth, the uninoculated plants were stunted and yellow, while plants inoculated with the wild type or RU1810 were healthy and green. The uninoculated plants lacked nodules, while the nodules of wild-type- and RU1810-inoculated plants contained only bacteria with the expected antibiotic resistance markers. Plants were dried at 80°C for 48 h, and wild-type-inoculated, RU1810-inoculated, and uninoculated mean plant weights were 13.7 mg ± 0.47 mg (mean ± standard error of the mean [SEM]) ($n = 300$), 13.9 mg ± 0.36 mg ($n = 260$), and 4.8 mg ± 0.11 mg ($n = 300$), respectively. While *t* tests confirmed that the results with the uninoculated plants were significantly different from those with both the wild-type- and the RU1810-inoculated plants ($P < 0.001$), results with the wild-type- and RU1810-inoculated plants were not significantly different ($P = 0.96$). Thus, while *M. loti* bacteroids have high alanine dehydrogenase activity, mutating both genes had no significant effect on symbiotic performance and plant growth under nitrogen starvation. This shows that de novo alanine synthesis cannot be essential for the transfer of nitrogen in the determinate nodules of *L. corniculatus*. Such a result is consistent

with the mutational and labeling data from peas indicating that ammonium is the main form of transferred nitrogen between the bacteroid and the plant.

We have recently proposed that amino acid cycling is essential for productive nitrogen fixation in peas (12). In this model, amino acids such as aspartate and alanine are made by transamination and not by de novo synthesis from ammonium and a keto acid. However, some de novo amino acid synthesis by AldA could help regulate amino acid cycling by either increasing or decreasing alanine levels, independently of transamination. The data presented here make it clear that such a role for AldA can only be a secondary one and that AldA cannot provide alanine as the sole nitrogen secretion product from the bacteroid to the plant. Thus, there does not appear to be a fundamental difference between determinate and indeterminate nodules in products of nitrogen secretion. This reinforces the classical model that ammonia is not assimilated by bacteroids but in the plant by the glutamine synthetase-glutamate synthase pathway.

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