

Identification of alanine dehydrogenase and its role in mixed secretion of ammonium and alanine by pea bacteroids

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Summary

N₂-fixation by *Rhizobium*-legume symbionts is of major ecological and agricultural importance, responsible for producing a substantial fraction of the biosphere's nitrogen. On the basis of ¹⁵N-labelling studies, it had been generally accepted that ammonium is the sole secretion product of N₂-fixation by the bacteroid and that the plant is responsible for assimilating it into amino acids. However, this paradigm has been challenged in a recent ¹⁵N-labelling study showing that soybean bacteroids only secrete alanine. Hitherto, nitrogen secretion has only been assessed from *in vitro* ¹⁵N-labelling studies of isolated bacteroids. We show that both ammonium and alanine are secreted by pea bacteroids. The *in vitro* partitioning between them will depend on whether the system is open or closed, as well as the ammonium concentration and bacteroid density. To overcome these limitations we identified and mutated the gene for alanine dehydrogenase (*aldA*) and demonstrate that *AldA* is the primary route for alanine synthesis in isolated bacteroids. Bacteroids of the *aldA* mutant fix nitrogen but only secrete ammonium at a significant rate, resulting in lower total nitrogen secretion. Peas inoculated with the *aldA* mutant are green and healthy, demonstrating that ammonium secretion by bacteroids can provide sufficient nitrogen for plant growth. However, plants inoculated with the mutant are reduced in biomass compared with those inoculated with the wild type. The labelling and

plant growth studies suggest that alanine synthesis and secretion contributes to the efficiency of N₂-fixation and therefore biomass accumulation.

Introduction

The *Rhizobium*-legume symbioses have been extensively examined because of their major contribution to global nitrogen input and interest in a complex eukaryote–prokaryote interaction. N₂-fixation by the bacterial micro-symbiont (bacteroid) is driven by the provision of C₄-dicarboxylates by the plant and, in return, the bacteroid secretes reduced nitrogen. Early studies on symbiotic N₂-fixation suggested that fixed N₂ is secreted from bacteroids exclusively as ammonium and all assimilation into amino acids occurs in the plant cytosol. This was based on the observation that in ¹⁵N-labelling studies the secretion product of N₂-reducing soybean or serradela bacteroids is ammonium (Kennedy, 1966; Bergersen and Turner, 1967; Bergersen and Turner, 1990). Similarly, free-living *Bradyrhizobium japonicum*, induced to fix nitrogen *ex planta*, also secretes ammonium (O'Gara and Shanmugam, 1976). More recently an ammonium channel has been identified on the peribacteroid membrane of soybean with appropriate properties for export from the bacteroid to the plant (Tyerman *et al.*, 1995). In addition, bacteroids apparently have low activities of the appropriate enzymes required for assimilation of ammonium into glutamate, while the plant cytosol has high activities of GOGAT and glutamine synthetase (Brown and Dilworth, 1975; Kurz *et al.*, 1975; Cullimore and Bennett, 1991; Temple *et al.*, 1998).

Labelling studies have been conducted in both closed and open systems. In the most sophisticated open-system study used to model the nodule environment, with continuous monitoring of respiration and N₂-fixation, secretion of amino acids was undetectable (Bergersen and Turner, 1990). In closed studies, alanine secretion from both isolated bacteroids and intact symbiosomes has been observed (Kretovich *et al.*, 1986; Appels and Haaker, 1991; Rosendahl *et al.*, 1992). The possible significance of this was not fully appreciated until a closed ¹⁵N-labelling study with purified soybean bacteroids showed that alanine was the sole secretion product of N₂-fixation (Waters *et al.*, 1998).

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We show here that, although labelling studies are essential to determine the secretion products of N_2 -fixation, the proportions can vary dramatically depending on the assay conditions. To determine whether alanine is a significant, or indeed the only, secretion product of N_2 -fixation by bacteroids requires the molecular identification and mutation of the pathway for alanine synthesis. Any effect of nitrogen partitioning between alanine and ammonium can then be determined *in planta*.

Results

Secretion of nitrogen products by pea bacteroids

As it has been suggested that ammonium secretion by bacteroids is due to plant contamination (Waters *et al.*, 1998), it is essential to obtain pure samples. Bacteroids of *Rhizobium leguminosarum* bv. *viciae* strain 3841 were therefore rapidly isolated through a prespun Percoll gradient, which has been shown to produce bacteroids, free of significant contamination with plant enzymes (Reibach *et al.*, 1981; Rosendahl *et al.*, 1992). With special regard to this study the bacteroid suspensions were tested by incubation in alanine (1 or 2 mM) and there was no loss of alanine or accumulation of excess ammonium. Therefore, this purification technique provides bacteroids suitable for assessing the products of N_2 -fixation.

Secretion of total amino acids and ammonium was tested at low bacteroid densities ($\approx 2 \text{ mg ml}^{-1}$ bacteroid protein) at O_2 tensions from 1% (0.05 atm) to 0.1% (0.005 atm) and only ammonium was detected in significant amounts (Fig. 1). Total amino acid analysis showed that no significant peaks of amino acids were secreted at any O_2 tension, including 0.1% (0.005 atm), where it has been suggested that electron partitioning in soybean bacteroids may favour alanine synthesis (Waters *et al.*, 1998). The only detectable change was a drop in total ammonium release over the range 0.1–0.5% O_2 to $\approx 40\%$ of the rate at 1%. The absence of any detectable alanine synthesis was also confirmed by the use of the coupled alanine dehydrogenase assay. Over a large number of independent assays the mean rate of ammonium synthesis at 1% O_2 was $21.2 \text{ nmol h}^{-1} \text{ mg}^{-1}$ protein (SE = 2.2, $n = 14$). The O_2 tension was also raised to 5% (0.24 atm), which caused a total loss of ammonium secretion, consistent with its synthesis being dependent on O_2 -sensitive nitrogenase activity. It was confirmed by the use of $^{15}N_2$ that the ammonium comes from N_2 -fixation (see below). Altering the pH over the range 6.5–9.5 did not significantly alter ammonium secretion. The optimized conditions employed in *in vitro* bacteroid secretion assays were 1% $O_2/99\%$ N_2 , pH 7.4, and 2 mM L-malate. These rates are consistent with those previously obtained for isolated soybean bacteroids

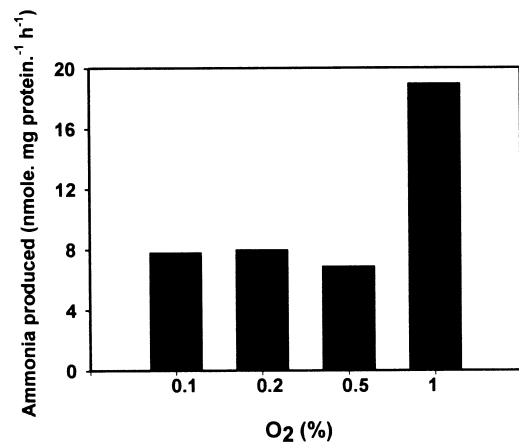


Fig. 1. Effect of O_2 tension on the rate of secretion of ammonium by low-density pea bacteroids (3.1 mg ml^{-1}). No amino acids were detected either by total amino acid analysis or with the coupled alanine dehydrogenase assay.

(Bergersen and Turner, 1967; Bergersen and Turner, 1990; Waters *et al.*, 1998).

At higher bacteroid densities ($\approx 10 \text{ mg ml}^{-1}$ bacteroid protein), both ammonium and alanine were detected (Fig. 2). At 1% O_2 the average rates of ammonium and alanine production were $19.7 \text{ nmol h}^{-1} \text{ mg}^{-1}$ protein (SE = 2.0, $n = 5$) and $7.8 \text{ nmol h}^{-1} \text{ mg}^{-1}$ protein (SE = 0.6, $n = 8$) respectively. Rates were linear for at least 4 h. Because the higher bacteroid density might alter the O_2 demand and electron partitioning (Waters *et al.*, 1998), the experiment was repeated at 0.1% O_2 . Alanine and ammonium were secreted at $2.9 \text{ nmol h}^{-1} \text{ mg}^{-1}$

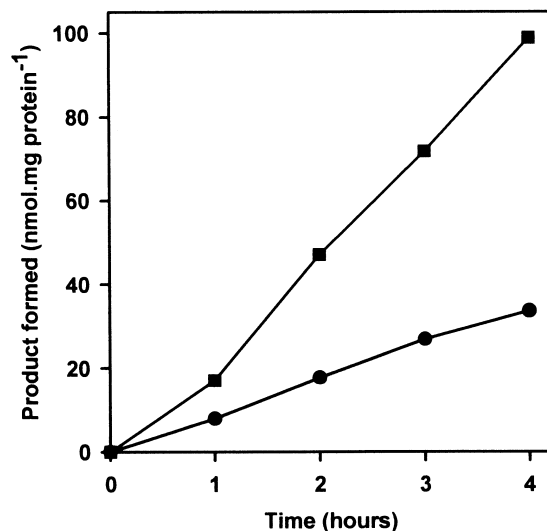


Fig. 2. A representative bacteroid secretion assay showing that both ammonium and alanine were produced at linear rates by a high-density bacteroid preparation (9.6 mg ml^{-1}). Ammonium (■) and alanine (●) were produced at rates of 24.3 and $8.6 \text{ nmol h}^{-1} \text{ mg}^{-1}$ protein respectively. The initial ammonium concentration was 0.26 mM .

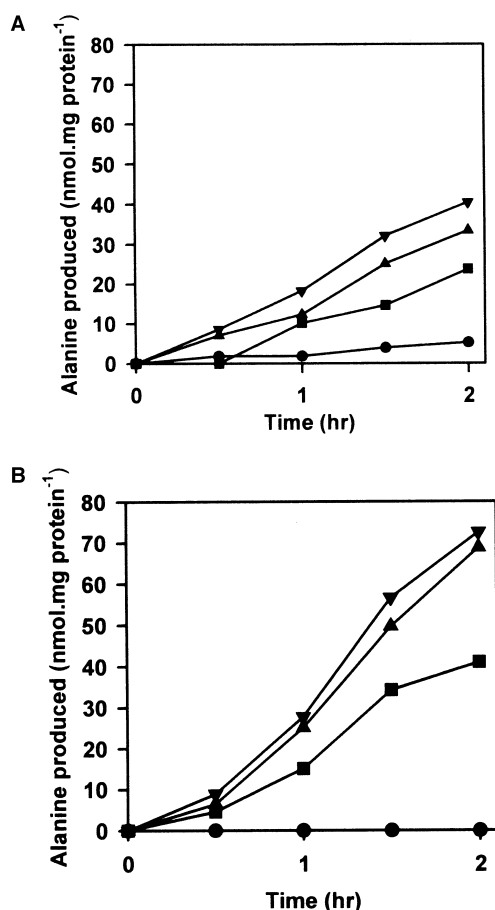


Fig. 3. Production of alanine from low-density bacteroid preparations (3.0 mg ml^{-1}) supplemented with ammonium (no added ammonium ●; 2 mM ■; 5 mM ▲; 10 mM ▼) and malate (1 mM in A and 10 mM in B). Note that alanine production increases with increasing ammonium and malate concentrations.

protein and $7.7 \text{ nmol h}^{-1} \text{ mg}^{-1}$ protein respectively; approximately one-third of that at 1% O_2 . Total amino acid analysis of the secretion products of bacteroids incubated at 0.1% or 1% O_2 showed that alanine was the only amino acid secreted in significant amounts. It is particularly notable that there is an increase in the total specific rate of nitrogen secreted (ammonium plus alanine) by high- versus low-density bacteroid preparations. Activating alanine synthesis does not decrease ammonium secretion.

At a constant specific activity of N_2 -fixation the ammonium concentration should rise more rapidly in dense bacteroid preparations and alanine synthesis may result from the increased ammonium concentration. To test this, ammonium was titrated into low-density bacteroid preparations (Fig. 3A and B). With ammonium at 10 mM, alanine was secreted at 20.9 and $47.5 \text{ nmol h}^{-1} \text{ mg}^{-1}$ protein at 1 and 10 mM L-malate respectively. Therefore, added ammonium stimulates alanine production and higher malate concentrations enhanced alanine

synthesis, presumably by increased provision of carbon skeletons for alanine synthesis.

Kinetic analysis showed alanine to be secreted with a whole cell V_{max} of $95.8 \text{ nmol h}^{-1} \text{ mg}^{-1}$ protein and an apparent K_m of 3.2 mM for ammonium (Fig. 4). The concentration of ammonium in soybean bacteroids in the nodule has been estimated to be 12 mM (Streeter, 1989). Assuming pea nodules are similar, alanine should be secreted from pea bacteroids at significant amounts *in planta*. However, as ammonium was also secreted, there ought to be a mixture of ammonium and alanine supplied to the plant by the bacteroid (Fig. 2).

Bacteroids isolated under air did not fix nitrogen as measured by ammonium release. However, when incubated in 10 mM ammonium and 10 mM malate, alanine was secreted at $25.4 \text{ nmol h}^{-1} \text{ mg}^{-1}$ protein. Thus, alanine is an assimilation product of N_2 -fixation but its synthesis is not dependent upon nitrogenase activity.

In all of the above experiments, N_2 with natural abundance ^{14}N and ^{15}N was used. To confirm that the ammonium produced is the direct product of nitrogenase action, a high-density preparation of pea bacteroids was incubated at 1% O_2 , 2 mM L-malate with 99 atom percentage $^{15}\text{N}_2$. Supernatant samples were removed at 0, and 1, 2, 3 and 4 h and the ^{15}N enrichment of ammonium analysed. After subtraction of the ammonium present at t_0 the newly released ammonium had an ^{15}N enrichment of 90.4 (SE 9.8, $n = 4$) atom percentage excess, consistent with it being almost entirely the product of N_2 -fixation. The t_0 ammonium concentration was 0.66 mM and the t_4 was 0.99 mM, thus the ^{15}N -labelling of the total ammonium pool was 29.6 atom percent

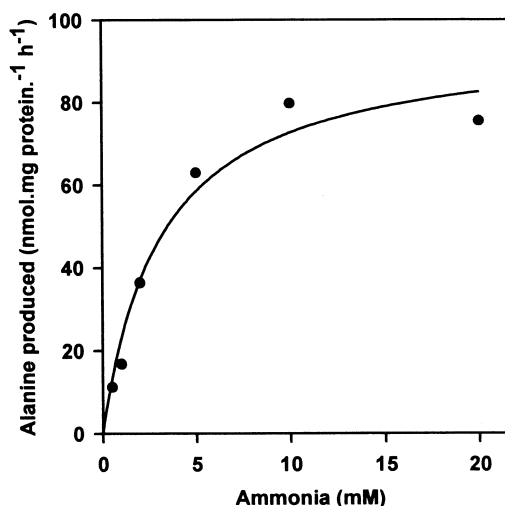


Fig. 4. Alanine secretion from low-density bacteroid preparations (1.7 mg ml^{-1}) incubated for 1 h at various ammonium concentrations with malate at 2 mM. The production of ammonium due to N_2 reduction is calculated to increase all ammonium concentrations by 0.036 mM. The apparent K_m for ammonium was 3.2 mM and the V_{max} was $95.8 \text{ nmol h}^{-1} \text{ mg}^{-1}$ protein.

excess. At t_4 the labelling of the alanine pool with ^{15}N was 21.5 atom percentage excess. Considering that initially the ammonium pool was only labelled at background ^{15}N , this is a very high enrichment of the alanine pool, consistent with it coming from the ammonium pool. To confirm this, low-density bacteroids were incubated in either 10 or 20 mM $^{15}\text{NH}_4\text{Cl}$ (10 atom percentage excess) and supernatant samples were removed after 1 h. The alanine formed was 9.6 and 10.1 atom percentage excess ^{15}N for 10 and 20 mM respectively. Alanine must be the product of *de novo* synthesis, a result that is confirmed below by mutational analysis.

Alanine secretion may have been overlooked in previous labelling studies owing to deamination by plant material (Waters *et al.*, 1998), but even if bacteroid suspensions were pure, sufficient ammonium may not have accumulated to enable alanine synthesis. This may be a problem where a low-moderate bacteroid density was used or in any open system where ammonium is removed before it can accumulate (Bergersen and Turner, 1990). Conversely, very high bacteroid densities in a closed system may result in alanine secretion being predominant. Because the secretion products of N_2 -fixation by isolated bacteroids depend on factors including the density of cells, as well as the malate and ammonium concentrations, it is not possible to extrapolate directly to what will happen in the plant. Instead, it is necessary to identify and mutate the pathway of alanine synthesis to determine what occurs *in planta*.

Identification of the pathways of alanine catabolism and synthesis

To identify the pathways of alanine synthesis and catabolism, $\approx 15\,000$ Tn5 mutants of *R. leguminosarum* strain 3841 were tested for growth on alanine as the sole carbon source. One strain, RU1275, failed to grow on alanine. The transposon was cloned and the flanking DNA sequenced. Tn5 is located in a gene with high identity to *lrp* (leucine responsive regulatory protein) from a number of organisms. To identify what it might regulate in *R. leguminosarum* strain 3841 the entire region was sequenced. Divergent from the *lrp*-like gene, which we have designated *dadR* (EMBL accession number AJ249196), are *dadX* and *dadA*. The transposon in strain RU1275 is located in the N-terminus of *dadR* with a 9 bp repeat of CATCAAGAT. The *dad* operon is the principal alanine catabolic operon in several bacteria and consists of an alanine racemase (*DadX*) and the small subunit of the electron transport chain linked D-alanine dehydrogenase (*DadA*), not to be confused with NAD^+ -dependent L-alanine dehydrogenase (*AldA*) (Wild and Klotkowski, 1981; Mathew *et al.*, 1996). *DadX* isomerizes L-alanine to D-alanine, which is then oxidized to pyruvate by *DadA*.

The complete loss of growth on alanine suggests that the products of the *dad* operon constitute the principal pathway for alanine catabolism in *R. leguminosarum* strain 3841. Strain RU1275 has wild-type levels of L-alanine dehydrogenase (*AldA*) activity, suggesting that *AldA* is not primarily a catabolic enzyme. This may explain our failure to isolate an *aldA* mutant by this screening strategy.

Identification of multicopy *aldA* as a suppressor of strain RU1275

The mutant strain RU1275 was complemented for growth on alanine by the cosmid pRU3131, which was confirmed to contain the *dad* genes by direct DNA sequencing from the cosmid. However, a second unrelated cosmid, pRU3135, was obtained that rescued growth of strain RU1275 on alanine as the sole carbon source. To identify the gene responsible, transposon Tn5-*lacZ* mutagenesis of pRU3135 was conducted. A cosmid, pRU3138, unable to suppress growth of RU1275 on alanine as the sole carbon source, was identified. Sequencing from the transposon identified *aldA* as the gene mutated and therefore responsible for suppressing the *dad* mutation (EMBL accession number aj238118). The Tn5-*lacZ* insertion in pRU3138 is oriented so that *LacZ* is inactive and the 9 bp repeat is CCTCGTGGC. The *aldA* gene encodes a putative protein of 377 amino acids with a relative molecular mass of 39 537. It has 36–63% amino acid identity to a wide range of *AldA* sequences over its whole length. An *aldA* mutant of 3841, strain RU1327, was constructed by recombination of pRU3138 into the chromosome. The mutant lacked any detectable *AldA* activity and was confirmed genetically by Southern blotting, while the wild-type *AldA* activity was $0.07\ \mu\text{mol min}^{-1}$ (mg protein^{-1}) (SE 0.01, $n = 3$). Furthermore, *AldA* activity was restored to RU1327 [$0.262\ \mu\text{mol min}^{-1}$ (mg protein^{-1}) SE 0.027, $n = 3$] by pRU708, which encodes a cloned PCR product of the *aldA* sequence.

As stated above, wild-type levels of *AldA* are present in RU1275, but are insufficient for catabolism of alanine as the sole carbon source. Consistent with this the *aldA* mutant, strain RU1327, grew as well as the wild type on glucose, succinate, L-malate, pyruvate, *myo*-inositol, glutamate and alanine. Thus, apart from the absence of *AldA* activity there was no detectable difference from the wild type. Suppression of strain RU1275 only occurred when *aldA* was present in multiple copies on pRU3135.

Effect of mutation of *aldA* on plant symbiotic performance

If *AldA* is the biosynthetic route for alanine synthesis in bacteroids then its affinity for ammonium might be predicted to be close to that of alanine synthesis by

bacteroids. In crude extracts the apparent K_m of AldA for ammonium was determined as 5.1 mM, similar to that obtained for alanine secretion by isolated bacteroids (3.2 mM) (Fig. 4). To test whether AldA is the primary route for alanine synthesis in the bacteroid and whether alanine is the sole nitrogen export product, or whether a more complex partitioning process exists, strain RU1327 was inoculated onto pea plants. Strain RU1327 nodulated pea plants forming normal healthy pink nodules. There were no significant differences in nodule number or mass between plants inoculated with the wild type and strain RU1327 (data not shown). Isolated bacteroids of strain RU1327 secreted ammonium at normal rates ($21.1 \text{ nmol h}^{-1} \text{ mg}^{-1} \text{ protein}$), while alanine secretion was undetectable using the coupled alanine dehydrogenase assay, even at a high-bacteroid density with exogenous ammonium present (10 mM). The absence of alanine secretion was not compensated for by a rise in ammonium secretion, leading to a lower total nitrogen secretion. AldA activity was undetectable in mutant bacteroid extracts, whereas wild-type AldA activity was $0.09 \text{ } \mu\text{mol min}^{-1} \text{ mg protein}^{-1}$ (SE = 0.01, $n = 3$). These data confirm that alanine synthesis is predominantly via AldA in the bacteroid. Total amino acid analysis showed that under the physiologically relevant concentrations of malate (10 mM) and ammonium (10 mM), alanine was the only significant amino acid secreted by the wild-type strain, 3841. Under the same conditions, RU1327 bacteroids produced a barely detectable peak of alanine, 10-fold less than 3841 bacteroids, and there was no significant secretion of any other amino acid. These data support the proposal that significant secretion of other amino acids does not occur in either 3841 or RU1327. Furthermore, normal rates of ammonium secretion were detected in RU1327, showing that ammonium diffuses rapidly from the bacteroid *per se* and not as the result of alanine deamination.

The peak of N_2 -fixation in pea occurs at flowering or during early pod-filling, which for this pea variety, grown at an average temperature of 20°C , is 4 weeks after sowing (Sprent, 1982). Plants were harvested at 4 weeks and showed no significant difference in acetylene reduction between the mutant and wild type (11.1 , SE = 1.0 and 9.4 , SE = $1.4 \text{ } \mu\text{mol g wet weight nodule}^{-1}$, respectively, $n = 4$, three plants per replicate). It is only after about 6 weeks that pea plants show signs of nitrogen deficiency. Plants inoculated with strain RU1327 harvested at this time were green, which is expected for a fix-plus phenotype. However, these plants had 20% less total shoot dry weight ($2.70 \text{ g dry weight plant}^{-1}$ SE = 0.13, $n = 56$) compared with plants inoculated with strain 3841 ($3.28 \text{ g dry weight plant}^{-1}$ SE = 0.14, $n = 54$; $P < 0.004$). While the decrease in dry weight is highly statistically significant, a 20% decrease in dry weight is

less dramatic than mutations that prevent N_2 -fixation, which have around a 50% reduction in dry weight at 6 weeks and are yellow. There was no change in relative dry weight of the leaves and stems versus pods and seeds because the latter constituted 46% and 45% of the total shoot dry weight in strains RU1327 and 3841 respectively. Overall these data suggest that alanine synthesis by AldA has a significant effect on the efficiency of nitrogen export, but cannot be the sole nitrogen secretion product. If alanine were the sole secreted nitrogen product, *aldA* mutants would resemble uninoculated pea plants, or those inoculated with fix-minus strains. The dominant observation must be that ammonium secretion can meet the plants nitrogen requirement but that cosecretion of ammonium and alanine may affect the efficiency of nitrogen assimilation and total export to the plant.

Discussion

For over 30 years it has been accepted that bacteroids secrete ammonium as the product of N_2 -fixation and do not assimilate it into amino acids (Bergersen and Turner, 1967; Bergersen and Turner, 1990). The recent demonstration that isolated soybean bacteroids only secrete alanine is a direct challenge to this paradigm (Waters *et al.*, 1998). These previous studies appear incompatible, but we show here that while alanine is a major secretion product in isolated pea bacteroids, ammonium is also secreted at high rates. The major factor determining the partitioning of nitrogen between ammonium and alanine is the absolute ammonium concentration. At saturating levels of ammonium the rate of alanine synthesis would be $95.8 \text{ nmol h}^{-1} \text{ mg}^{-1} \text{ protein}$, 4.8-fold higher than the measured rate of ammonium release. However, at very low concentrations of ammonium, alanine synthesis is undetectable.

When soybean bacteroids were incubated in $^{15}\text{N}_2$, the alanine pool, but not the ammonium pool was highly labelled (Waters *et al.*, 1998). Because ammonium is the product of nitrogenase, this indicates a tight coupling between N_2 reduction to ammonium and its assimilation into alanine, such that ammonium is never freely released. We find that ammonium is secreted by pea bacteroids and is highly labelled with ^{15}N . This demonstrates that the ammonium pool is separate from alanine and it diffuses rapidly from the cell. It is not simply the breakdown product of alanine.

An *aldA* mutant, strain RU1327, was inoculated onto peas and the isolated bacteroids fixed nitrogen and secreted ammonium at normal rates, although alanine release was barely detectable by total amino acid analysis (10-fold reduction). There was however, a 20% decrease in dry weight, consistent with alanine secretion affecting

the efficiency of nitrogen transfer to the plant. These results indicate that alanine cannot be the sole *in planta* nitrogen secretion product from pea bacteroids. Instead, the labelling and mutational data for peas are consistent with a plastic partitioning between direct export of ammonium and its assimilation and secretion as alanine. It would however, be simplistic to try and correlate directly the percentage drop in plant yield to the percentage of total nitrogen that is exported as alanine.

Alanine synthesis is a pivotal connection between carbon and nitrogen metabolism in bacteroids. The rate of ammonium secretion by low- and high-density bacteroids is not significantly different, yet high-density bacteroids also secrete alanine, leading to higher total nitrogen secretion. This enhancement is not due to intrinsic differences in density, because high-density preparations of RU1327 have the same rate of ammonium secretion as low or high density wild-type bacteroids. These data are consistent with the enhancement of nitrogen secretion being due to synthesis of alanine by AldA. One of the most important aspects of this is that the size of the ammonium pool will alter the rate of alanine synthesis and hence carbon metabolism. Blocking alanine synthesis lowers the total nitrogen secreted and may have profound effects on carbon metabolism. Thus, carbon and nitrogen metabolism are cross-regulated. This homeostatic model is different from previous models that consider only ammonium or alanine as the secretion product.

Alanine and aspartate have been detected in earlier studies as secretion products of bacteroids (Kretovich *et al.*, 1986; Appels and Haaker, 1991; Rosendahl *et al.*, 1992). They have been thought of as transamination products because they often required the addition of exogenous amino acids to stimulate alanine secretion (Appels and Haaker, 1991). When isolated pea peribacteroid units were fed ^{14}C -malate 12% of the label appeared as amino acids in the incubation medium in 30 min (Rosendahl *et al.*, 1992), indicating not only rapid synthesis of alanine but high rates of secretion across both the bacteroid and peribacteroid membrane. Isolated N_2 -fixing peribacteroid units also secreted aspartate, but at lower rates than alanine (Rosendahl *et al.*, 1992). The full significance of this study was not fully appreciated, perhaps because absolute rates of alanine synthesis were not given and no measurements of ammonium secretion were made. The variability in much of the literature as to whether alanine is secreted or not can now be explained by the demonstration that AldA is responsible for ammonium assimilation in bacteroids. Differences in local conditions, particularly bacteroid density, endogenous ammonium and bacteroid contamination with plant extract will drastically alter the measured alanine.

Given that the apparent K_m for ammonium by AldA is

5.1 mM in *R. leguminosarum* and 8.9 mM (Smith and Emerich, 1993) in *B. japonicum*, it is important to consider how active the enzyme will be in the nodule. The estimated concentration of ammonium in soybean bacteroids is 12 mM, well above the K_m of the enzyme from either pea or soybean bacteroids (Streeter, 1989). In addition, the ammonium channel in the soybean peribacteroid membrane has a high K_m for ammonium (37.5 mM) (Tyerman *et al.*, 1995), indicating that it may maintain a high ammonium concentration in the bacteroid, promoting alanine synthesis by AldA. Modulation of the activity of the ammonium channel may alter the partitioning between ammonium and alanine export from bacteroids. The absolute activity of AldA in different rhizobia may also alter the partitioning to alanine (Stripf and Werner, 1978; Smith and Emerich, 1993).

Although the peribacteroid membrane is highly impermeable to amino acid uptake into the bacteroid, alanine is rapidly secreted out of the pea peribacteroid unit towards the plant cytosol (Udvardi *et al.*, 1990; Rosendahl *et al.*, 1992; Hernandez *et al.*, 1996). Such directionality is consistent with a mechanism to prevent the reimport of a primary nitrogen secretion product into the symbiosome. Alanine is a suitable vector for nitrogen transport, as only two enzymes are required to link malate and succinate, the probable carbon sources for the bacteroid to the assimilation of ammonium produced by N_2 -fixation. This explains why in intact pea nodules fed $^{14}\text{CO}_2$, alanine is the second most rapidly labelled compound in bacteroids, after malate (Salminen and Streeter, 1992).

In summary, these data are consistent with AldA being the main route for alanine synthesis in pea bacteroids and that ammonium and alanine are likely to be the primary nitrogen secretion products *in planta*. Mutating *aldA* does not prevent N_2 -fixation but reduces both nitrogen secretion by bacteroids and plant biomass. The partitioning between ammonium and alanine may vary between different species of legume and be affected by regulation of ammonium export by the peribacteroid membrane. Mixed secretion of ammonium and alanine is consistent with the current understanding of ammonium assimilation by the plant fraction of nodules but there is an outstanding question regarding the fate of exported alanine.

Experimental procedures

Bacterial strains and culture conditions

R. leguminosarum strain 3841 is a spontaneous streptomycin-resistant derivative of strain 300 (Glenn *et al.*, 1980). All strains were grown at 28°C on either Tryptone Yeast extract (Beringer, 1974) or acid minimal salts medium (Poole *et al.*, 1994). Antibiotics were used at the following concentrations

($\mu\text{g ml}^{-1}$): kanamycin, 40; streptomycin, 500; and gentamycin, 20.

Plant growth and bacteroid isolation

Rhizobium leguminosarum bv. *viciae* strains 3841 and RU1327 were used to inoculate surface-sterilized pea seeds (*Pisum sativum* L. cv. Avola) at the time of sowing. Plants were grown in pots filled with a sand–gravel–vermiculite mixture in a glasshouse, watered with a zero nitrogen nutrient solution and harvested at flowering (generally 4–5 weeks). Nodules were picked from the plant roots and the gross wet weight noted. Bacteroids were isolated under argon within an AtmosBag (Aldrich) with argon-purged isolation buffer (100 mM phosphate pH 7.4, 300 mM sucrose, 2 mM MgCl_2) (Bergersen and Turner, 1990) and using a Percoll density gradient method (Reibach *et al.*, 1981). Bacteroid suspension was loaded onto a prepared Percoll gradient (centrifuged for 45 min at 36 900 *g*, Percoll 55% in isolation buffer) and subjected to centrifugation (15 min at 36 900 *g*). The bacteroid fraction was carefully isolated and washed twice to remove Percoll. Bacteroids were resuspended to ≈ 2 mg or ≈ 10 mg protein ml^{-1} (low/high-density preparations respectively) in isolation buffer with the phosphate lowered to 50 mM.

Secretion assays

Anaerobically isolated bacteroids were placed in Schott bottles (100 ml) that were sealed with neoprene. The lid contained a port for insertion of hypodermic needles. Bottles were sparged, usually with 1% O_2 :99% N_2 prior to use. Prepared bacteroids were injected, incubated at 26°C with gentle agitation and allowed to equilibrate for 20 min prior to any experiment. Samples were taken from the bottle using a syringe and needle and centrifuged to pellet the bacteroids. The supernatant was assayed for total amino acids, alanine and ammonium.

Analytical techniques

The ammonium concentration of an acidified distillate of supernatant was determined by the phenol hypochlorite assay (Dilworth and Glenn, 1982). The alanine concentration was determined with the coupled alanine dehydrogenase assay (Walshaw *et al.*, 1997). To measure total amino acids released, bacteroid supernatant was run through a Sephadex SP-25 (Pharmacia) column and the amino acid eluate freeze-dried (Redgwell, 1980). Reconstituted samples were analysed for individual free amino acids using a Biochrom 20 amino acid analyser (Biochrom). Labelling with $^{15}\text{N}_2$ was carried out with high-density bacteroids as described above, except that samples were incubated in 1% O_2 , 20% $^{15}\text{N}_2$ (99 atom percentage, Isotec), 79% argon. The ammonium released was distilled, acidified and dried slowly over a period of 36 h at 25°C, and analysed for total nitrogen and $^{15}\text{NH}_4^+$ using a Europa Scientific Roboprep combustion analyser interfaced to a VG 622 isotope ratio mass spectrometer referenced against IAEA quality standard 305. To analyse alanine for ^{15}N , samples were purified using an SP-25

column as for total amino acid analysis and freeze dried. Dried samples were redissolved in 20 ml pyridine and derivitized using an equivalent volume of *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (MTBSTFA), mixed and heated in locking eppendorf tubes for 30 min at 70°C. Samples (1.5 ml) were injected into a VG Masslab GC-MS MD800 using an AS800 autosampler, separated on a BPX5 column and analysed, initially in scan mode, using the mass spectrometer (Mawhinney *et al.*, 1986). Subsequently, the mass spectrometer was programmed in single ion monitoring (SIM) mode and a second injection used to monitor the ion count for the largest significant fragment (for alanine; mass 260), mass + 1 (for alanine; mass 261), and mass + 2 (for alanine; mass 262). The amount of ^{15}N incorporated into alanine was assessed by measuring the ratio of the M peak to the M+1 peak and the atom percentage excess (APE) calculated as:

$$\text{APE} = (Re - Rc) / [1 + (Re - Rc)] \times 100$$

where *Re* is M+1/M for enriched sample and *Rc* is M+1/M for control samples (Robinson *et al.*, 1991). Analysis of norleucine as an internal standard revealed no significant enrichment. Alanine synthesis by low-density bacteroids was also measured using the GC-mass spectrometer, where either 10 or 20 mM NH_4Cl (Sigma 10% atom percentage excess) was included. Protein was determined using the Lowry–Folin method. Acetylene reduction was measured on whole plants as previously described (Trinick *et al.*, 1976).

Enzyme assays

Alanine dehydrogenase was assayed from *Rhizobium* cultures grown in acid minimal salts liquid culture with succinate and ammonium chloride at 10 mM to an $\text{OD}_{600\text{nm}}$ of ≈ 1.0 . Cells were centrifuged, washed in 10 mM HEPES (pH 7.4) and resuspended in 12 ml of ice-cold 40 mM HEPES (pH 7.4), 20% glycerol and 2 mM DTT. Cells were lysed using a French press at 69 MPa and cell debris removed by centrifugation at 28 000 *g* and 4°C for 30 min. Alanine dehydrogenase activity was assayed at 28°C by measuring the change in absorbance at 340 nm due to oxidation of NADH with 50 mM Tris-HCl buffer (pH 8.5); 0.2 mM NADH; 5 mM pyruvate and 100 mM NH_4Cl .

Genetic analysis

All DNA and genetic analysis was carried out as described previously (Walshaw *et al.*, 1997). The *aldA* gene was PCR amplified with primers p199 (atacaaaagaaggcggcatcc) and p200 (agctcggcgttggtgatgc), cloned as a 1.45 kb fragment into pCR2.1[®]-TOPO[®] (Invitrogen) and transferred as a *SacI*-*KpnI* fragment into pTR101 (Weinstein *et al.*, 1992) producing pRU708.

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aldA and *dadRXA* have been deposited in the EMBL database accession nos AJ238118 and AJ249196.

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