The central role of the host cell in symbiotic nitrogen metabolism

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Symbiotic nitrogen recycling enables animals to thrive on nitrogen-poor diets and environments. It traditionally refers to the utilization of animal waste nitrogen by symbiotic micro-organisms to synthesize essential amino acids (EAAs), which are translocated back to the animal host. We applied metabolic modelling and complementary metabolite profiling to investigate nitrogen recycling in the symbiosis between the pea aphid and the intracellular bacterium Buchnera, which synthesizes EAAs. The results differ from traditional notions of nitrogen recycling in two important respects. First, aphid waste ammonia is recycled predominantly by the host cell (bacteriocyte) and not Buchnera. Host cell recycling is mediated by shared biosynthetic pathways for four EAAs, in which aphid transaminases incorporate ammonia-derived nitrogen into carbon skeletons synthesized by Buchnera to generate EAAs. Second, the ammonia substrate for nitrogen recycling is derived from bacteriocyte metabolism, such that the symbiosis is not a sink for nitrogenous waste from other aphid organs. Host cell-mediated nitrogen recycling may be general among insect symbioses with shared EAAs biosynthetic pathways generated by the loss of symbiont genes mediating terminal reactions in EAA synthesis.

Keywords: Acyrthosiphon pisum; Buchnera; flux balance analysis; metabolic modelling; nitrogen recycling; symbiosis

1. INTRODUCTION
Various animal groups exploit nutrient-poor habitats or diets by possessing symbiotic micro-organisms that expand their metabolic repertoire and enhance their nutritional efficiency [1]. In particular, microbial symbionts have been proposed to recycle nitrogen, i.e. to use nitrogenous waste products of the animal (ammonia, urea, uric acid etc.) as a substrate for the synthesis of essential amino acids (EAAs) or other high-value nitrogenous compounds, which are released back to the animal. Nitrogen recycling has been invoked, for example, in reef corals, other alga–invertebrate symbioses and insects feeding on wood, plant sap and other poor diets [2–8].

Our grasp of nutritional interactions in animal–microbial symbioses is being transformed by high throughput sequencing technologies, which enable the metabolic capability of the partners to be deduced from their genome or transcriptome [9]. This is illustrated by recent advances in our understanding of nitrogen recycling in the symbiosis between the pea aphid Acyrthosiphon pisum and its bacterial symbiont Buchnera aphidicola. Buchnera is restricted to the cytoplasm of the ‘host cell’, also known as bacteriocyte, and provides the insect with EAAs [10]. [EAAs are the ten amino acids contributing to protein that the aphid host cannot synthesize. The remaining ten protein amino acids that animals, including the pea aphid, can synthesize are known as non-essential amino acids (nEAAs).] EAA provisioning by Buchnera enables aphids to thrive on their diet of plant phloem sap, which has a low N:C ratio and nitrogen content dominated by nEAAs [11]. Early suggestions that Buchnera may recycle the ammonia waste of aphids to EAAs [12] (figure 1a) are in doubt because Buchnera has a very restricted genetic capacity for ammonia assimilation [13]. An alternative scenario based on transcriptomic and proteomic data [2,14] is that the nitrogen recycling is mediated by the host cell–symbiont complex, with the nEAA substrates for Buchnera-mediated EAA synthesis generated by aphid-mediated ammonia assimilation in the host cell via the enzymes glutamine synthetase/glutamate oxoglutarate aminotransferase (also known as glutamate synthase) (GS/GOGAT) (figure 1b). The GS/GOGAT cycle plays a central role in ammonia assimilation in plants and many bacteria [15,16], and has also been demonstrated to function in animal systems, notably the mosquito Aedes aegypti [17].

This study was motivated by the recognition that metabolic networks constructed from omic data can be too complex to be comprehended by visual inspection alone, but require systematic metabolic modelling [18–21]. We reasoned that the dynamics of nitrogen recycling can best be deciphered by models that provide the context of the multiple, interdependent metabolic connections between the symbiotic partners. This metabolic interdependence arises from the limited metabolic repertoire of Buchnera (a consequence of its small genome size [13]), and includes shared metabolic pathways for the synthesis of four of the ten EAAs [21]. Specifically, Buchnera has the genetic capacity to synthesize the carbon skeleton of the branched chain amino acids (isoleucine, leucine and valine) and phenylalanine, but...
2. MATERIAL AND METHODS

(a) Insect culture

Acyrthosiphon pisum clone CWR09/18 derived from a single parthenogenetic female collected from an alfalfa crop at Freeville, NY in 2009 was maintained on preflowering Vicia faba cv. Windsor at 20°C with photoperiod cycle: 16 h light and 8 h dark. All experiments were conducted on 7-day-old apterous larvae reared from day 2 on a chemically defined diet containing 0.5 M sucrose and 0.15 M amino acids (formulation A of Prosser & Douglas [26]), which contains all proteinamino acids. The aphids were confirmed by diagnostic PCR reactions and microscopy to contain *Buchnera* but no secondary symbionts.

(b) Metabolite analysis

Ammonia and amino acids were quantified, following derivationization with AccQ Tag (Waters), by UPLC (Waters Acquity) with PDA detector (see electronic supplementary material), and normalized to protein determined by DC protein assay (Bio-Rad) with 0–10 μg bovine serum albumin ml⁻¹ as standard. The ammonia content of 15 replicate samples of embryos, fat body, bacteriocytes and guts were dissected into ice-cold PBS, with all tissue types for each replicate from the same 20 aphids. Amino acid analyses used haemolymph (ca 2–3 μl), exuding from amputated legs of 15 replicate sets of 60 aphids immersed in water-saturated mineral oil, collected into a microcapillary tube. Ammonia flux by host cells used host cells dissected with fine pins from 160 7-day-old apterous larvae into 50 μl filter-sterilized medium B comprising 28 mM glucose, 8.6 mM NaCl, 1 mM MgSO₄, 0.1 mM CaCl₂, 0.25 M sucrose, 50 mM phosphate buffer, pH 7.5. The host cells were gently disrupted by aspiration several times into a 200 μl pipette and then centrifuged at 1000 g for 5 min. The preparation was brought to 4 × 10⁶ *Buchnera* cells ml⁻¹, and divided into 5.5 μl aliquots. The experiment was initiated by adding 5.5 μl medium B supplemented with 2 mM glutamic acid, glutamine, aspartic acid, serine and 2-oxobutanoic acid to each aliquot. At 5 min intervals over 40 min, one 11 μl aliquot was centrifuged at 800g for 70 s. The supernatant was flash-frozen in liquid nitrogen and stored at −80°C prior to analysis.

(c) RNAseq

RNA (ca 12 μg) was extracted with the RNeasy Mini kit (Qiagen) from ‘whole bodies’ (WBs), comprising 20 aphids; and host cells, comprising the *Buchnera*-free portion of bacteriocytes dissected from 1000 aphids (see electronic supplementary material, methods). Library preparation and sequencing by Illumina Genome Analyzer II platform were performed by Cornell University Life Sciences Core Laboratories Center (see electronic supplementary material, methods). Bioinformatics programs TopHat [27] and Cufflinks [28] were applied to map sequence reads to reference transcriptome datasets of Hansen & Moran [2] were re-processed using our analysis pipeline, to construct the high confidence set of aphid gene products enriched in host cells.

(d) Metabolic models

*Buchnera* metabolic model iSM199 was founded on model iSM197 [25] (see electronic supplementary material, methods), with EAA stoichiometries in the biomass reaction comprising a ‘selfish’ component (*Buchnera* protein synthesis) and ‘cooperative’ component released to the host at empirically determined rates [15]. The integrated host cell–*Buchnera* model iSM271 comprised iSM199 and host metabolic reactions informed from genes products enriched in three independent datasets (table 1). Aphid gene names were mapped to Enzyme Commission numbers using AphidESTs database (www.aphidests.org), with reactions and reaction names retrieved from BioG database ([29] bigg.ucsd.edu/)
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(Continued.)
and used for a manual assembly of a SBML file. Reactions were assigned to host cell (‘_h_’), Buchnera periplasm (‘_p_’) or Buchnera cytoplasm (‘_c_’), and transport reactions were added for metabolite flux between the three compartments and external space. The models were analysed by FBA in COBRA Toolbox [30], optimizing for maximal flux through the ‘cooperative’ biomass reaction. Flux units were set relative to 100 units of glucose taken up by Buchnera.

3. RESULTS

(a) Ammonia content of host organs

The ammonia content of the host cell (bacteriocyte) was enriched fivefold relative to the WB, and was significantly higher than any other aphid organ (Kruskal–Wallis (4 df): $H = 55.5, p < 0.001$) (figure 2a). These data suggested that the host cell may be either a source or sink for ammonia. To investigate the ammonia relations of the host cell further, we applied metabolic modelling, focusing particularly on ammonia metabolism and the flux of nitrogenous compounds between the host cell and the symbiotic bacterium, Buchnera.

(b) Metabolite exchange between Buchnera and the host cell

The inferred complement of metabolites exchanged between Buchnera and the host cell was computed from the metabolic inputs and outputs to/from Buchnera metabolism, using FBA of the reconstructed Buchnera metabolic network (model iSM199: see electronic supplementary material, file S1). The analysis yielded 33 metabolites transported to the Buchnera cell that are required for metabolic function and growth, and 25 metabolites released from Buchnera to the host cell (see electronic supplementary material, figure S1). Overall, the transfer of nitrogen to Buchnera (243 flux units) was 11 times greater than nitrogen efflux (22 flux units). The principal nitrogenous compounds exported from Buchnera were the six EAAs for which Buchnera codes the complete biosynthetic pathway; and the non-nitrogenous organic compounds released from Buchnera included acetate, the sole by-product of carbohydrate fermentation in this bacterium, and the oxo-acid precursors of the four EAAs with coupled synthesis (see electronic supplementary material, figure S1).

Buchnera possesses one metabolic reaction that consumes ammonia, NAD kinase and the computed flux through this reaction (0.035 flux units) is less than the combined flux through the two reactions that generate ammonia (GMP reductase, 3.43 units; diaminohydroxyphospho-ribosylaminopryrimidine deaminase, 0.01 units) (see electronic supplementary material, file S1). The inferred net ammonia production is incompatible with symbiont-mediated nitrogen recycling (figure 1a), which postulates substantial net uptake of ammonia by the microbial symbiont. The metabolic model also does not support a key prediction of host cell–symbiont-mediated nitrogen recycling (figure 1b): that host-derived glutamate is an important substrate for symbiont EAA synthesis. Just 22 per cent (4.4 flux units) of the total glutamate required for Buchnera EAA biosynthesis (19.8 flux units) is imported from the host cell. The remaining 78 per cent of glutamate is generated endogenously within the Buchnera cell, as a by-product of Buchnera-mediated reactions with glutamine as the nitrogen donor, e.g. via CTP synthetase in pyrimidine
synthesis, and carbamoyl-phosphate synthetase, imidazole glycerol phosphate synthase and anthranilate synthetase in EAA synthesis. In this respect, glutamate differs from the three other nEAA precursors of Buchnera EAAs (aspartate, glutamine and serine), which are derived entirely from the host with no contribution from Buchnera metabolism (see electronic supplementary material, file S1) and have approximately four- to eightfold higher host-to-Buchnera flux than glutamate (see electronic supplementary material, figure S1).

(c) An integrated host cell–symbiont model of the metabolic source and fate of ammonia
To investigate the ammonia and amino acid relations of the symbiosis further, an integrated host cell–symbiont metabolic model was constructed. We first identified the genes with enriched expression in the host cell, using RNAseq to compare the transcriptome of the WB and the Buchnera-free fraction of the host cells (see electronic supplementary material, file S1) and have approximately four- to eightfold higher host-to-Buchnera flux than glutamate (see electronic supplementary material, figure S1).

39 genes have annotated functions, with 90 per cent (35/39) encoding proteins with predicted metabolic roles as either enzymes (32/39) or transporters (3/39) (table 1). Remarkably, the metabolism genes with enriched expression in the host cell are dominated by enzymes predicted from iSM199 to mediate the synthesis of key

Figure 2. Metabolite profiles of 7-day-old pea aphids. (a) Ammonia content of organs. Median values with the same letter superscript do not differ significantly ($p < 0.005$, critical value after Bonferroni correction for 10 comparisons). (b) Amino acid content of haemolymph.
metabolites taken up by Buchnera and process metabolites released from Buchnera. We used the congruence between the Buchnera metabolic model iSM199 and the host metabolism gene products enriched in the host cell to generate an integrated host cell–symbiont model, iSM271, containing 384 reactions and 383 metabolites (see electronic supplementary material, file S3).

The chief nitrogenous compounds in the host cell–symbiont model iSM271 are amino acids. Amino acid metabolite profiling of the aphid haemolymph, which bathes the host cells, provides strong empirical validation of the model. The inferred nitrogen flux from the haemolymph to host cell is mediated principally by four amino acids: asparagine (51.6 flux units), glutamine (16.2 flux units), proline (6.5 flux units) and alanine (4.9 flux units); and these four nEAAs are the most abundant amino acids in aphid haemolymph (figure 2b), collectively accounting for 50 per cent of the total amino acid content.

The complementary prediction that aspartate, glutamate and serine are generated in the host cell and Buchnera, and are not derived from the haemolymph, is supported by their low haemolymph concentrations, representing just 0.25, 0.1 and 2.5 per cent of the haemolymph amino acid content, respectively (figure 2b). These empirical data are fully congruent with the overall predicted structure of the nitrogen relations of the combined host cell–symbiont metabolic network iSM271.

Building on this empirical validation, we used model iSM271 to investigate ammonia relations in the host cell. The sole host reaction inferred to utilize ammonia is GS, and most of the product, glutamine, is consumed to generate glutamate, via GOGAT, confirming the central role of host GS/GOGAT in ammonia assimilation. The ammonia–derived nitrogen is used predominantly in transaminase reactions mediated by the host cell, especially the reactions generating the four EAAs with coupled synthesis. The predicted contribution of ammonia to the glutamine and glutamate pools in Buchnera cells is 2 and 3 flux units, respectively, together representing just 7.8 per cent of the ammonia assimilated (figure 3a). Of the ammonia assimilated via host GS/GOGAT and transaminases, 42 per cent was incorporated into EAAs available for host utilization, of which greater than 95 per cent was the four EAAs with coupled synthesis (group A in figure 3b).

In model iSM271, the ammonia substrate for GS/GOGAT is of endogenous origin (figure 3a), and derived principally from host cell metabolism, which generates ammonia with flux nearly 20 times greater than that of Buchnera. Quantitatively, the most important source of ammonia is as a by-product of asparaginase-mediated production of aspartate, required absolutely for Buchnera EAA synthesis (47 flux units). Additional, but smaller sources of ammonia are cystathionine-γ-lyase (11 units), the candidate route for producing 2-oxobutanoate precursor of isoleucine synthesis by Buchnera [14], and AMP deaminase (5 units), in processing of Buchnera-derived adenine [31]. In summary, model iSM271 predicts that the host cell is enriched in ammonia (figure 2a) because it is a metabolic source, not sink, for ammonia.

**Figure 3.** Ammonia assimilation and nitrogen recycling in the aphid–Buchnera symbiosis. (a) Inferred flux for ammonia assimilation via GS/GOGAT and transaminases, yielding glutamate (glu), glutamine (gln) and amino acids, including metabolic fluxes (solid arrows) and transport fluxes (broken arrows) inferred from host cell–symbiont model iSM271. (b) Inferred flux through host cell transaminase reactions: group A mediates the terminal step in synthesis of the four EAAs with coupled synthesis; group B in synthesis of serine and ornithine, substrates for Buchnera-mediated synthesis of the EAAs tryptophan and arginine, respectively. Asterisk denotes net EAA received by host from recycled ammonia, expressed as per cent of total flux (52 units) through host cell transaminase reactions.

**Figure 4.** Ammonia flux in bacteriocyte preparations. The final experiments tested the key model prediction that the host cell is a metabolic source of ammonia. Intact host...
cells dissected from aphids displayed no detectable uptake of ammonia when incubated with 1 mM NH$_4$Cl, but suspensions of broken host cells produced ammonia at a linear rate of $1.7 \pm 0.15$ nmol mg$^{-1}$ protein min$^{-1}$ (mean ± s.e., three experiments) over periods of 40 min. These data suggest that ammonia is a major product of host cell–symbiont metabolism, and the host cell does not function as a sink for ammonia derived from other tissues.

4. DISCUSSION
The metabolic models were constructed to represent the metabolic networks that support nutrient exchange between the aphid host cell and Buchnera symbiont. Their accuracy is indicated by the remarkable correspondence between the inferred metabolite exchange across the host–Buchnera interface (see electronic supplementary material, figure S1) and enrichment of host cell gene products mediating the synthesis of predicted inputs to and processing of outputs from the Buchnera metabolic model (table 1). This can be attributed to the excellent compatibility between the properties of the Buchnera metabolic network and assumptions of FBA. Specifically, all the metabolism genes are likely expressed constitutively because Buchnera lacks recognizable regulatory sequences [13], and the expression of metabolism genes is barely responsive to variation in metabolic demand [32,33]. Consequently, the Buchnera metabolic network is a realistic representation of its metabolic function, unlike free-living bacteria in which various metabolic capabilities are turned on/off according to need. In addition, the distribution of flux through the Buchnera metabolic network is tightly constrained, with greater than 60 per cent of reactions calculated by the flux variability analysis to be unable to vary at all [25]. This means that there is little opportunity for the empirical network to deviate from the optimal flux distributions obtained by FBA.

Our metabolic models yield a novel scenario for nitrogen recycling (figure 1c): that the host cell bearing the symbionts is primarily responsible for both generating the waste ammonia and recycling it to EAAs. It differs from traditional concepts of symbiotic nitrogen recycling in that the symbionts do not mediate nitrogen recycling, and that the recycled ammonia is not derived from other organs of the host. Three sets of empirical data provide a precise match to the predictions of figure 3: the host cell–Buchnera unit is a net producer of ammonia (this study); the host cell takes up glutamine, but not glutamate [34]; Buchnera preparations (which we now know are contaminated with host cell proteins) incorporate $^{15}$N from $^{15}$N-glutamate into all four EAAs with coupled biosynthesis, but none of the other six EAAs synthesized by Buchnera [34].

The Buchnera and host cells are destroyed when pea aphids are treated with antibiotics, and the resultant aphids have elevated ammonia titres, with two processes invoked to explain this metabolic response: elevated rates of nEAAs degradation, and the loss of the symbiotic sink for ammonia in the antibiotic-treated aphids [35]. This study makes the testable prediction that differences in the pattern of amino acid metabolism between aphids bearing and lacking the symbiosis should be quantitatively sufficient to account for the difference in ammonia titres.

Host-cell-mediated nitrogen recycling is predicted on the shared metabolic pathways for the synthesis of four EAAs, and is dependent on the supply of the carbon skeleton of the EAAs synthesized by the symbiont (figure 1c). Consequently, this mode of nitrogen recycling is likely restricted to the host cells in the aphid, and cannot operate in symbiont-free animals (which lack the capacity to synthesize the carbon skeletons). The evolutionary basis for coupled synthesis of four EAAs is the loss of the symbiont genes coding for the terminal transaminase reactions during the evolutionary transition from the free-living to the symbiotic state, in the context of the pre-existing host capacity for these reactions (but no other reactions in the EAA biosynthetic pathways) [21]. Coupled EAA synthesis gives the host control over both the nitrogen incorporated into these EAAs and the allocation of these EAAs between a host and a symbiont. The four EAAs with coupled synthesis are produced at high rates, collectively accounting for 62 mol% of the total EAA provided to the host (calculated from data in [18]).

Genomic data suggest that phylogenetically distinct bacterial symbionts with much reduced genomes and associated with other insect groups may lack homologous genes to those missing from Buchnera [36], indicative of coupled synthesis of the four EAAs in these symbioses. Further research is required to establish whether the host cell–symbiont metabolic networks in these associations are structured for host cell-mediated nitrogen recycling. Nitrogen recycling has also been invoked in symbioses involving micro-organisms that have not undergone genome reduction, including algae in corals and other invertebrates, and gut bacteria in termites (see Introduction). Unlike Buchnera, these symbionts can generally assimilate host waste nitrogenous compounds and are widely assumed to recycle nitrogen to EAAs [37]. Nevertheless, there are precedents for stringent host controls over symbiont metabolism, especially in relation to nitrogen [38]. Our study generates the hypothesis that nitrogen recycling involving symbionts with a versatile metabolic repertoire could potentially be host-cell mediated, through selective repression of symbiont genes coding the terminal reactions in the synthesis of selected EAAs.

The conclusion that the ammonia substrate for nitrogen recycling in the aphid–Buchnera symbiosis is generated predominantly by the host cell and not by other aphid organs derives initially from the finding that, in model iSM271, the ammonia supply from host cell reactions matches the demand for GS/GOGAT-derived glutamate to meet the empirically determined rates of synthesis and release of EAAs with coupled biosynthesis. The experimental evidence that the host cell is a net producer of ammonia (this study) raises the possibility that our model may underestimate the rate of ammonia production by the host cell, and that nitrogen recycling does not consume all the ammonia generated by the host cell. Consistent with this evidence for limits to the capacity for ammonia assimilation by host-cell-mediated nitrogen recycling in the pea aphid, supplementary dietary ammonia does not promote aphid growth [35].

The more restricted spatial reach of host-cell-mediated nitrogen recycling than the traditional concept of nitrogen recycling (figure 1) raises a broader question: why is ammonia assimilated into glutamine/glutamate not a major
substrate for symbiont-mediated EAA synthesis? One possible explanation is that the direct involvement of symbionts in nitrogen recycling may constrain the capacity of the host to regulate symbiont metabolism. This issue applies especially to symbionts with metabolism structured so that the yield of its products (EAAs) is determined by host demand for those products, and not by the rate at which the host generates nitrogenous waste.

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