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# Impact of Host Developmental Age on the Transcriptome of the Symbiotic Bacterium *Buchnera aphidicola* in the Pea Aphid (*Acyrtosiphon pisum*)<sup>∇†</sup>

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**Of the 617 genes from *Buchnera aphidicola*, the obligate bacterial symbiont of the pea aphid, 23% were differentially expressed in embryos compared to adults. Genes involved in flagellar apparatus and riboflavin synthesis exhibited particularly robust upregulation in embryos, suggesting functional differences between the symbiosis in the adult and embryo insect.**

An animal is a habitat for microorganisms. The conditions and resources encountered by microorganisms vary spatially across different organs (gut, skin, internal organs, etc.) and cell types and also temporally with the developmental age and physiological condition of the animal host. The greatest developmental range is encountered by microorganisms that persist throughout the host life cycle and are transmitted vertically via the egg, as occurs for certain symbioses in insects (3, 5, 9). This study focuses on the impact of the host developmental age on resident microorganisms with the core hypothesis that the pattern of gene expression and function of symbiotic microorganisms varies with the developmental age of the host.

The symbiosis between aphids and their symbiotic gamma-proteobacterium *Buchnera aphidicola* is particularly amenable to developmental studies because of special reproductive features of the aphid. Aphids are parthenogenetic and viviparous and display telescoping of generations (the female initiates embryogenesis as an embryo or larva). As a consequence, a newly emerged adult contains multiple embryos of different developmental ages, each bearing its own complement of vertically acquired *B. aphidicola* as well as *B. aphidicola* in bacteriocytes in the maternal body cavity (2).

In common with most intracellular bacteria, *B. aphidicola* has a reduced genome (12, 15). Most research has been conducted on *B. aphidicola* in the pea aphid (*Acyrtosiphon pisum*). For this system, transcription of *B. aphidicola* genes is highly regulated in the sense that expression levels vary reliably among different genes by more than 10-fold under normal conditions (19), but changes in expression in response to environmental perturbation (e.g., diet composition and temperature) are small and involve few genes (13, 23). These data are

consistent with the apparent absence of most recognized signature sequences controlling expression levels of individual genes or operons in the *B. aphidicola* genome (12, 15, 17). Many functions of *B. aphidicola* may therefore be controlled by a small number of global transcriptional regulators (e.g., *hns*, *ihf*, and *fis*) and possibly by processes independent of transcriptional controls, such as nutrient supply from the aphid host (18). Even so, indirect evidence that the conditions and resources encountered by *B. aphidicola* might vary substantially with developmental age follows from the observation that *B. aphidicola* proliferation rates are particularly high in very young embryos and particularly low in the maternal bacteriocytes of adult aphids (21, 22). *B. aphidicola* contributes to the nutrition of its host by the synthesis and release of essential amino acids (1) and vitamins, notably riboflavin (11), traits that enable the insect to utilize its plant phloem sap, which is deficient in these nutrients (1). The amount of these nutrients released may vary between the *B. aphidicola* populations in the maternal and embryo bacteriocytes. The purpose of this study was to investigate specifically whether the transcriptional expression profile of *B. aphidicola* in embryos differs from that in the adult of the pea aphid *Acyrtosiphon pisum*.

**Tissue preparation.** Embryos and maternal bacteriocytes were removed from prereproductive adult *A. pisum* in ice-cold RNase-free buffer B (25 mmol liter<sup>-1</sup> KCl, 21 mmol liter<sup>-1</sup> MgCl<sub>2</sub>, 250 mmol liter<sup>-1</sup> sucrose, 35 mmol liter<sup>-1</sup> Tris-HCl, in 0.1% diethyl pyrocarbonate-water; pH 7.05). The embryos were classified by length into three groups: early (<0.4 mm), intermediate (0.4 to 0.8 mm), and late (>0.8 mm), equivalent to developmental stages 0 to 15, 16 to 18, and 19 and 20, respectively (8). Total RNA (i.e., both aphid and bacterial RNA) was extracted with the RNeasy mini kit (Qiagen), and genomic DNA was removed by treatment with RQ1 RNase-free DNase (1 unit μg<sup>-1</sup> RNA; Promega, USA). To obtain sufficient bacterial RNA for microarray analysis, 500 ng RNA was amplified with the MessageAmp II-bacteria kit (Ambion) (20), and 30 μg of amino-allyl amplified RNA was coupled with either Cy3 or Cy5 monoreactive dye (Amersham, United Kingdom).

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**Microarray experiments.** Microarray slides containing the full *B. aphidicola* gene complement (617 genes) were prepared as previously described (4, 13). Six slides were hybridized in a dye swap reference design experiment, using maternal bacteriocytes as the reference. Normalization and statistical analysis of microarray data were performed using the R software (<http://www.r-project.org/>) (13). Overall, the false-positive detection rate was estimated at 3%, based on a mixture of control and simulated slides using the procedure described in reference 13. Two criteria were adopted for differential expression: (i) a significant difference ( $P < 0.05$ ) in a global analysis of variance and at least one between-group comparison and (ii) a mean difference in expression of 1.50-fold for upregulated genes and 0.66-fold for downregulated genes. Microarray data were deposited in the ArrayExpress database under accession number E-MEXP-2057.

**Validation of microarray data by qRT-PCR.** Expression of 14 *B. aphidicola* genes in three independent isolations of the sample groups was quantified by quantitative reverse transcription-PCR (qRT-PCR). cDNA was prepared from template RNA prior to amplification. The direction of differential expression did not differ from the microarray results for 12 of the 14 genes validated for early and intermediate embryo groups and for 11 of the 14 genes validated for late embryos (see Fig. S1 in the supplemental material), and the gradient of the regression equation describing the relationship between the microarray and qRT-PCR data (at  $y = 0.86x + 0.13$ ,  $r^2 = 71.6\%$ ) did not differ significantly from unity (by *t* test,  $t = 3.59$ , 36 df;  $P > 0.05$ ), giving confidence in the microarray data using amplified RNA.

**Global patterns of differential gene expression.** The expression of 140 (23%) of 617 *B. aphidicola* genes differed significantly between the embryos and maternal tissues as determined by the statistical criteria outlined above. Seventy-four genes were upregulated, 55 genes were downregulated, and 11 genes exhibited a mixed response between the embryo developmental groups (Fig. 1). These included 33 upregulated and 21 downregulated genes with an expression difference consistently greater than twofold. The high incidence of differential expression of *B. aphidicola* genes suggests that the functional role of the bacteria varies significantly with developmental age of the host. This variation has been masked in previous studies of the *B. aphidicola* transcriptome (13, 23) because *B. aphidicola* bacteria from embryos and maternal tissues were pooled.

**Pattern of gene expression in embryos at different stages.** The number of *B. aphidicola* genes differentially expressed between embryos and maternal tissues varied significantly among the early, intermediate, and late embryos (79, 81, and 38 genes, respectively) ( $\chi^2 = 19.98$ , 2 df;  $P < 0.001$ ), indicating that the expression profile of *B. aphidicola* in the developmentally advanced embryos is more similar to the profile of *B. aphidicola* in adult tissues than in intermediate and early embryos. The null hypothesis of equal frequency of upregulated and downregulated genes was supported for intermediate and late embryos, but early embryos had a significant excess of upregulated genes (49 upregulated and 30 downregulated genes) ( $\chi^2 = 4.6$ , 1 df;  $P = 0.033$ ). The broad implication of these data is that gene expression by *B. aphidicola* is not uniform in embryos and varies with embryo development.

#### Functional characterization of differentially expressed genes.

Nearly all *B. aphidicola* genes, including all differentially expressed genes reported here, have *Escherichia coli* orthologs (15). Therefore, the function of differentially expressed genes was investigated using the MultiFun classification of *E. coli* (14). The results of this analysis revealed patterns with respect to only two categories: cell structure (classification group 6) and metabolism (classification group 1). Of 175 *B. aphidicola* genes with functions in cell structure, differential expression was observed for 20 genes from early embryos, 33 genes from intermediate embryos, and 12 genes from late embryos ( $\chi^2 = 11.83$ , 2 df;  $P = 0.003$ ). Inspection of the data revealed that flagellar genes contributed disproportionately to the differentially expressed cell structure genes ( $\chi^2 = 6.66$ , 1 df;  $P = 0.009$ ), particularly to the upregulated group (binomial test;  $P < 0.05$ ). Despite its lifestyle as an obligate intracellular symbiont with no free-living stage, *B. aphidicola* retains genes for all flagellar proteins except those involved in filament formation and motor function (6, 15). The remnant flagellar apparatus comprising the hook and basal bodies lacks the components required for propulsion and may mediate the export of proteins in a fashion analogous to type three secretion systems of various other bacteria (6, 7, 10, 13). The significant upregulation of *flagA*, *flagD*, and *flagH* genes suggests that the flagellar apparatus may be more abundant in *B. aphidicola* from embryos than adult tissues, with the possibility that protein secretion from *B. aphidicola* may be more important in the rapidly growing intermediate embryos than in other developmental stages, especially adult tissues.

The expression of *B. aphidicola* genes involved in metabolism varied significantly across the embryo groups with higher frequencies of differential *B. aphidicola* gene expression in early and intermediate embryos (at 33 and 30 of 282 genes, respectively) than late embryos (12 of 282 genes) ( $\chi^2 = 11.32$ , 2 df;  $P = 0.003$ ). Of the *B. aphidicola* genes in the early embryos, 62% were upregulated, suggesting that *B. aphidicola* in these embryos may be metabolically more active than those in adult tissues.

Genes involved in the syntheses of essential amino acids and riboflavin were investigated in detail because these nutrients are released to the aphid host. Among the 55 *B. aphidicola* genes with a function in essential amino acid synthesis, 12 (22%) were differentially expressed in the embryos, comprising 9 upregulated genes (including two genes [each] involved in tryptophan synthesis and isoleucine synthesis and three genes involved in histidine synthesis) and 3 downregulated genes.

*ribF* is the sole gene with metabolic function that was significantly downregulated in all embryos relative to the adult tissues. This gene codes for riboflavin kinase/flavin mononucleotide adenyltransferase, which mediates the metabolism of riboflavin to flavin adenine dinucleotide and reduced flavin mononucleotide, i.e., consuming riboflavin. Among the six genes coding for reactions in riboflavin synthesis, three were significantly upregulated, *ribA* and *ribD2* in early embryos and *ribH* in intermediate embryos. Taken together with the dietary evidence that the pea aphid derives riboflavin from *B. aphidicola* (11), these data raise the possibility that *B. aphidicola* in embryos release more riboflavin than *B. aphidicola* in adult bacteriocytes.

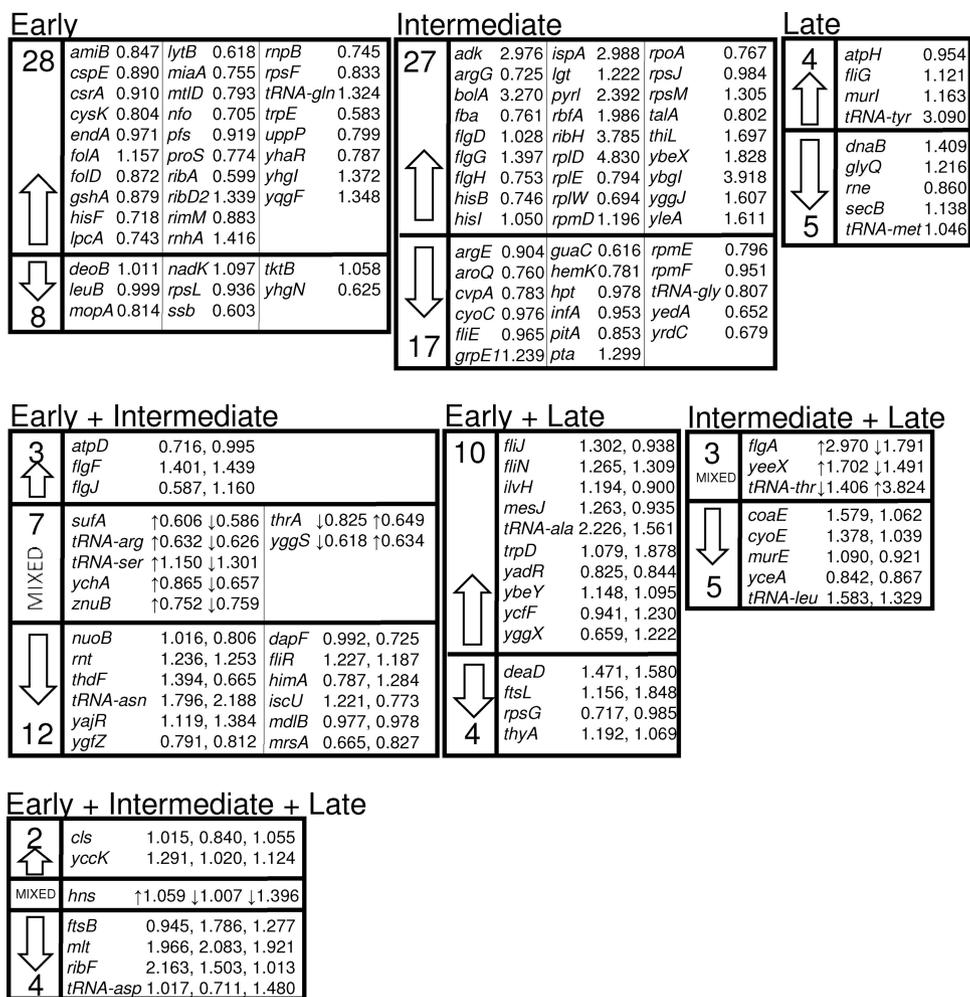


FIG. 1. Differential expression of *Buchnera aphidicola* genes in pea aphid embryos. Each box represents individual embryo groups (early, intermediate, or late; see text for full definition) or combinations of embryo groups in which *B. aphidicola* genes were differentially expressed compared to *B. aphidicola* from the maternal bacteriocytes. Gene names are followed by the  $\log_2$  change in expression ( $n$ -fold values) and are grouped into upregulated genes ( $\uparrow$ ), downregulated genes ( $\downarrow$ ), and those genes expressed in two or more embryo groups with mixed response (MIXED). The values for the change in expression in genes differentially expressed in two or more embryo groups follow the sequence in the group heading. As an example, the *sufA* gene is differentially expressed in both early and intermediate embryos but is upregulated in early embryos (with a  $\log_2$  change of 0.606-fold) and downregulated in intermediate embryos (with a  $\log_2$  change of 0.586-fold). The numbers accompanying the arrows in single or mixed groups indicate the number of genes in each category.

In summary, two key conclusions arise from this study. (i) The transcriptome of *B. aphidicola* varies significantly according to the developmental age of the symbiosis, with the greatest number of significantly differentially expressed genes apparent between *B. aphidicola* in early embryos and adult bacteriocytes. (ii) Genes that function in the flagellar apparatus, with a predicted role in protein export, and genes involved in riboflavin synthesis are particularly upregulated in embryos. These results imply that the symbiotic interactions between the developing host and bacterial partner are dynamic, changing in response to the developmental stage of the host. Developmental variation in the transcriptome has also been reported recently for *Blochmannia floridanus*, the primary bacterial endosymbiont of carpenter ants (16), raising the possibility that this variation might be general among bacteria with massively reduced genomes in intimate symbioses with animals.

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