A metabolic study of *Buchnera*, the intracellular bacterial symbionts of the pea aphid *Acyrtosiphon pisum*

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Cells of the bacterium *Buchnera* were isolated from embryos of the pea aphid *Acyrtosiphon pisum*, with an intact perisymbiont membrane (the insect membrane which surrounds each bacterial cell inside the aphid). The bacterial preparations respired aerobically, consuming oxygen at an average rate of $24 \text{ nmol (mg protein)}^{-1} \text{ min}^{-1}$. The bacteria took up a range of carboxylic acids and the amino acids glutamate and aspartate from an external concentration of 0.5 mM at rates of $1\text{--}10 \text{ nmol (mg protein)}^{-1} \text{ h}^{-1}$; glucose was taken up at $0.17 \text{ nmol (mg protein)}^{-1} \text{ h}^{-1}$. Glutamate uptake was proportional to its external concentration, at all concentrations tested between $15 \text{ }\mu\text{M}$ and 10 mM . Saturable systems for the uptake of succinate and aspartate were identified. The kinetic constants were: K_m 0.79 mM , V_{max} $12.6 \text{ nmol (mg protein)}^{-1} \text{ min}^{-1}$ for succinate; and K_m 0.22 mM , V_{max} $3.3 \text{ nmol (mg protein)}^{-1} \text{ min}^{-1}$ for aspartate. Succinate uptake was not inhibited by the uncoupler CCCP and was markedly stimulated by ATP, suggesting that its transport is not linked to a proton-motive force but is dependent on an energized membrane and possibly mediated by a co-transport system involving another ion.

Introduction

Buchnera is a member of the γ -Proteobacteria (Munson *et al.*, 1991a), known only in aphids (phloem-feeding insects of the order Homoptera). The bacteria are restricted to a single type of insect cell, known as the mycetocyte, found within the body cavity (haemocoel) of the aphid. Each bacterium is separated from the surrounding insect cell cytoplasm by an insect membrane, known as the perisymbiont membrane. The bacteria are transmitted maternally via the egg or embryo (Hinde, 1971; Brough & Dixon, 1990), and have no free-living stage. As yet, it has not been possible to maintain *Buchnera* in long-term axenic culture, but the bacteria can be isolated from aphids and maintained in a viable condition for several hours (Ishikawa, 1982a; Harrison *et al.*, 1989).

Aphids are dependent on *Buchnera* for normal growth and fecundity (e.g. Mittler, 1971; Ishikawa & Yamaji, 1985; Douglas, 1992). It has been shown that aphids derive essential amino acids from *Buchnera*, supplementing the low essential amino acid content of

their phloem sap diet (Douglas, 1988; Douglas & Prosser, 1992; Sasaki *et al.*, 1991). Beyond their capacity to synthesize essential amino acids, virtually nothing is known about the metabolic capabilities of *Buchnera*.

The aims of this study were twofold: to demonstrate unambiguously that isolated preparations of *Buchnera* are metabolically active; and to identify some carbon sources that *Buchnera* can utilize. Detailed studies were conducted on compounds which are major substrates for other intracellular micro-organisms or were found to be utilized at high rates by *Buchnera*.

Methods

Isolation of *Buchnera*. The bacteria were isolated from embryos of the pea aphid *Acyrtosiphon pisum* (Harris) clone Ox-2 (Prosser & Douglas, 1992). For each experiment 40–50 adult aphids were taken from a parthenogenetic culture of *A. pisum*, maintained on *Vicia faba* var. The Sutton, at 20°C with an 18 h light:6 h dark regime. The embryos were dissected out and homogenized in a glass hand-held tissue grinder with ice-cold Tris/sucrose buffer (50 mM -Tris/HCl pH 7.5 and 0.25 M -sucrose) and 10 mM -dithiothreitol. The homogenate was centrifuged at 3700 g for 30 s. The pellet was washed once with 0.008% (v/v) Nonidet-P40 detergent in Tris/sucrose buffer and three times in detergent-free buffer by centrifugation and resuspension. The final pellet was resuspended in incubation medium comprising 0.5 mM - $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3 mM - NH_4Cl , 0.25 M -sucrose and 50 mM -MOPS/NaOH, pH 7.0. The protein content of the final bacterial preparation was quantified by the method of Bradford (1976) with bovine serum albumin as a standard.

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Abbreviation: CCCP, carbonyl cyanide *m*-chlorophenylhydrazine.

Fig. 1. Transmission electron micrographs of *Buchnera* within a perisymbiont membrane (arrow): (a) isolated from pea aphid embryos, scale bar 0.7 μm ; (b) in the cytoplasm of the aphid mycetocyte, scale bar 0.5 μm .

Table 1. Uptake of carbon compounds by preparations of *Buchnera* over 60 min

Carbon compound (0.5 mM)	Uptake rate [nmol (mg protein) ⁻¹ h ⁻¹ ; (mean $\pm$ SE, <i>n</i> = 3)]	¹⁴ C metabolized to CO ₂ (proportion of radiolabel incorporated by cells)
Glucose	0.17 $\pm$ 0.07	0.59
Succinic acid	1.63 $\pm$ 0.19	0.76
Citric acid	3.70 $\pm$ 0.14	> 0.99
2-Oxoglutaric acid	4.70 $\pm$ 0.30	0.91
Glutamic acid	5.38 $\pm$ 0.04	0.90
Acetic acid	6.70 $\pm$ 0.53	0.96
Aspartic acid	10.3 $\pm$ 0.41	0.92

Oxygen consumption by the bacteria. An oxygen electrode (Rank Bros) connected to a continuous linear recorder was used. To calibrate the readings, the relative solubility of oxygen, liberated from a known amount of hydrogen peroxide by catalase, was determined in both distilled water and incubation medium (Dixon & Kleppe, 1965). Control experiments were performed on bacterial preparations previously incubated at 100 °C for 5 min. The effect of metabolic inhibitors was examined by adding them to the bacterial preparation 5 min after recordings were started.

Transport studies. The following radiochemicals were used: [2,3-¹⁴C]succinate, [1-¹⁴C]acetate, [U-¹⁴C]glucose, [1,5-¹⁴C]citrate, [U-¹⁴C]aspartate and 2-oxo[5-¹⁴C]glutarate from Amersham; and [U-¹⁴C]glutamate from Sigma. Uptake experiments of up to 1 h duration were conducted in 0.5 ml Eppendorf tubes containing 0.4 ml bacterial preparation (0.5 mg protein ml⁻¹). The reaction was started by adding 5 μ l radiolabelled substrate to give a final concentration of 0.5 mM and 12.5 μ Ci ml⁻¹ (462.5 kBq ml⁻¹) and samples were shaken at 100 r.p.m. at room temperature. The reaction was terminated by adding ice-cold buffer and centrifuging immediately for 30 s at 15000 *g*. The pellet was then washed three times by centrifugation and resuspension and assayed for radioactivity. Zero-time values were obtained and subtracted from all values. For the determination of carbon dioxide evolution, bacterial preparations were incubated as above, but in sealed vials with a wick of filter paper impregnated with 10% (w/v) KOH inserted into the lid. After 60 min incubation, 0.1 ml glacial acetic acid was injected into the mixture and the vials were shaken at 800 r.p.m. for a further 60 min. The wick was then air-dried and assayed for radioactivity. For short-term uptake studies, 1.2 ml bacterial suspension (1 mg protein ml⁻¹) was incubated in a 10 ml glass beaker at room temperature with constant stirring. The reaction was started by the addition of radiolabelled substrate, to give a final concentration of between 0.015 and 20 mM and 12.5 μ Ci ml⁻¹. At the desired times, 200 μ l aliquots of the bacteria were removed and transferred onto GF/F filters (Whatman) on a vacuum filter manifold (Amico), and washed with 5 ml incubation medium. The filter was air-dried and assayed for radioactivity. The effect of inhibitors and ATP on the uptake of radiolabelled compounds was examined by adding them to the bacterial suspension 1 min before the radioactive compound.

Assay of radioactivity. All radiolabelled samples were combined with 4 ml scintillation fluid (Pharmacia Optiphase Hisafe II for aqueous samples, and Optiscint Hisafe for dried filters) and counted in an LKB 1219 Rackbeta scintillation counter with preset ¹⁴C windows.

Electron microscopy. Immediately after isolation from aphids, bacterial preparations were fixed for 12 h in 2.5% (w/v) glutaraldehyde in 0.1 M-sodium cacodylate buffer pH 7.4 and 8% (w/v) sucrose. The fixed cells were suspended in 4% (w/v) agarose and post-fixed for 1 h with 1% (w/v) osmium tetroxide in distilled water. Samples were

dehydrated through a graded ethanol series (70–100%, v/v) and propylene oxide before infiltration in Araldite CY212. Ultrathin sections were stained in a saturated solution of uranyl acetate and 3.5% (w/v) lead citrate, and observed in a Philips EM 400 transmission electron microscope.

Results

Condition of the isolated bacteria

Most of the bacteria, freshly isolated from pea aphid embryos and examined by phase-contrast microscopy, were coccoid and 2–3 μ m in diameter. Between 10 and 20% of the cells were dumb-bell shaped and these were identified as bacteria isolated while dividing. The bacteria represented 75–80% of the particles detected in the preparations examined at $\times$ 800 magnification; the remainder included lipid droplets and membrane fragments. The dominant structures in preparations examined by transmission electron microscopy were bacterial cells, with insect mitochondria and amorphous membranous structures observed at very low frequency. Every bacterial cell observed was enclosed within a membrane (Fig. 1*a*). This membrane was interpreted as the perisymbiont membrane which surrounds each cell inside the aphid mycetocyte (Fig. 1*b*).

Oxygen consumption

The bacterial preparations utilized oxygen at a linear rate of 23.7 ± 1.9 nmol (mg protein)⁻¹ min⁻¹ (mean $\pm$ SE, *n* = 26) for at least 30 min. Oxygen consumption was reduced by over 80% by 1 mM-KCN, but it was not affected by 10 μ M-antimycin A, a specific inhibitor of mitochondrial respiration.

Uptake and metabolism of carbon sources

The preparations of bacteria utilized all of the seven carbon sources tested over 60 min (Table 1). The substrates with carboxylate groups (i.e. the carboxylic

Fig. 2. Effect of concentration on the initial uptake rates of: (a) aspartate: $V_{\max} = 3.29 \text{ nmol (mg bacterial protein)}^{-1} \text{ min}^{-1}$, $K_m = 0.22 \text{ mM}$; (b) succinate [inset detail of uptake at concentrations < 1 mM for which $V_{\max} = 12.55 \text{ nmol (mg bacterial protein)}^{-1} \text{ min}^{-1}$, $K_m = 0.785 \text{ mM}$]; (c) glutamate (NB log scales). Each data point is the mean of two independent experiments.

acids and amino acids glutamate and aspartate) were taken up at a rate of $1\text{--}10 \text{ nmol (mg protein)}^{-1} \text{ h}^{-1}$ and between 75 and > 99% of the incorporated substrate was metabolized to carbon dioxide. Glucose was utilized relatively poorly, with an uptake rate of $0.17 \text{ nmol (mg protein)}^{-1} \text{ h}^{-1}$, and 60% recovered as carbon dioxide. α -Cyanohydroxycinnamic acid ($10 \mu\text{M}$), a specific inhibitor of mitochondrial dicarboxylic acid transport, did not influence the uptake of any carbon source (data not shown).

Further experiments were done to study the transport systems of succinate, aspartate and glutamate. The uptake of all three substrates proceeded linearly for at least 3 min. Uptake plots of 90's were used to examine

uptake kinetics (Fig. 2). The uptake of aspartate and concentrations of succinate < 1 mM showed saturation curves consistent with carrier-mediated systems. At higher external concentrations of succinate, uptake was proportional to concentration, indicative of a passive diffusion system. The uptake of glutamate was linear at external concentrations $0.015\text{--}10 \text{ mM}$, with uptake rates ranging from 0.1 to $60 \text{ nmol (mg protein)}^{-1} \text{ min}^{-1}$.

The uptake of succinate (external concentration 0.87 mM) was examined in greater detail. The uncoupler CCCP ($10 \mu\text{M}$) did not depress the initial uptake rate. Cyanide (1 mM) reduced uptake by 25%, while the addition of 3 mM-ATP resulted in a doubling of the initial uptake rate.

Discussion

The isolation procedure used here was suitable for metabolic studies of *Buchnera* because, by both structural and metabolic criteria, the bacterial preparations were not appreciably contaminated with aphid mitochondria or other organelles. The experiments described show that *Buchnera* does not require the complex cellular environment for basic metabolic functions such as respiration and the uptake of organic compounds. This is consistent with previous demonstrations that isolated bacteria can incorporate inorganic sulphate into reduced organic sulphur compounds including the amino acid methionine (Douglas, 1990), and can synthesize protein and DNA (Ishikawa, 1982b).

There are indications that characteristics of *Buchnera* in isolation reflect their condition in association with aphids in two respects. Firstly it is very likely that the bacteria respire aerobically in the symbiosis. This is suggested by sustained oxygen consumption of isolated preparations, at rates comparable to the respiration rate of *Escherichia coli* (Lawford & Haddock, 1973); and by the particularly rich supply of tracheae (structures which deliver oxygen to insect cells) to mycetocytes (C. N. Brough, personal communication). The second issue concerns the supply of nutrients from the insect mycetocyte cytoplasm to *Buchnera*. Both in the intact symbiosis and in isolated preparations, each bacterial cell is enclosed within a perisymbiont membrane. The capacity of the *Buchnera* preparations to take up all seven carbon compounds tested, using each as a respiratory substrate, suggests that *Buchnera* may not be nutritionally fastidious, and a range of compounds can be transported across the perisymbiont membrane in the pea aphid. *Buchnera* has probably been in symbiosis for over 200 million years with no free-living phase (Munson *et al.*, 1991b), and it is very unlikely that these bacteria would have retained the capacity to utilize compounds not available inside the aphid.

This capacity of *Buchnera* to utilize such a wide variety of compounds is in sharp contrast to the condition of the only other intracellular symbiont studied in detail, *Rhizobium* bacteroids from legume root nodules. The principal carbon compounds utilized by the bacteroids are the dicarboxylates malate and succinate [reviewed in McDermott *et al.* (1989) and Day & Copeland (1991)]. Bacteroids of rhizobia can barely utilize tri- or monocarboxylates (Ou Yang *et al.*, 1990) or glucose (Salminen & Streeter, 1987*a*), all of which are utilized by *Buchnera*. Also, the legume perisymbiont membrane, but not the aphid perisymbiont membrane, is essentially impermeable to glutamate and aspartate (Herrada *et al.*, 1989).

Succinate transport by the *Buchnera* preparations was examined in some detail here, because of the importance of dicarboxylates in the nutrition of rhizobial bacteroids. The kinetic constants for the saturable transport system of *Buchnera* within the perisymbiont membrane are similar to the published values for transport across the perisymbiont membrane of the legume-rhizobium association (Udvardi *et al.*, 1988; Herrada *et al.*, 1989). As with rhizobia (Ou Yang *et al.*, 1990), succinate uptake by *Buchnera* was stimulated by ATP. However, unlike the rhizobial system, the carrier in the aphid system is probably not linked to proton-motive force, because uptake is unaffected by the uncoupler CCCP. Uptake may be achieved by co-transport with a different ion, e.g. Na⁺. The slight inhibitory effect of cyanide on succinate uptake suggests that the bacteria are unlikely to provide the major source of energy for the transport system.

Further studies are required to investigate directly the flux of nutrients from the aphid to *Buchnera*, but one compound of considerable potential interest is glutamate. *Buchnera* can take up this amino acid from low external concentrations and at high rates; and, furthermore, it is a major intracellular solute, accounting for 30 mol% of the total free amino acid pool of *Buchnera* (L. F. Whitehead, unpublished). Exogenous glutamate is an important carbon source utilized by a variety of intracellular micro-organisms, including the parasites *Rickettsia*, *Chlamydia* and *Coxiella* (Moulder, 1985), and glutamate, derived from dicarboxylates, is the major endogenous respiratory substrate for rhizobium bacteroids (Salminen & Streeter, 1987*b*). However, with respect to *Buchnera*, the potential significance of glutamate extends beyond its role as a respiratory substrate. Glutamate and other non-essential amino acids (possibly including aspartate) may be a source of nitrogen, utilized in the synthesis of essential amino acids, which are then transferred to the aphid. Indirect evidence for this process, which is known as nitrogen upgrading, has already been obtained for the pea aphid symbiosis (Prosser & Douglas, 1992).

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