Permeability of *Coxiella burnetii* to ribonucleosides

Jeffrey D. Miller† and Herbert A. Thompson†

Author for correspondence: Herbert A. Thompson. Tel: +1 404 639 1083. Fax: +1 404 639 1056.
e-mail: hct2@cdc.gov

Department of Microbiology
and Immunology, Robert E.
Byrd Health Sciences Center,
West Virginia University,
Morgantown, WV 26506,
USA

Knowledge about transport in *Coxiella burnetii*, an obligate phagolysosomal parasite, is incomplete. The authors investigated the capability of isolated, intact, host-free *Coxiella* to transport ribonucleosides while incubated at a pH value typical of lysosomes. Because of the low activities and limitations of obtaining experimental quantities of isolated, purified *Coxiella*, incorporation of substrate into nucleic acid was used as a trap for determination of uptake abilities. Virulent wild-type (phase I) organisms possessed uptake capability for all ribonucleosides. Both phase I and phase II (avirulent) organisms incorporated the purine nucleosides guanosine, adenosine and inosine, and showed a more limited uptake of thymidine and uridine. Both phases were poorly active in cytidine uptake. Neither phase of the organism was capable of transport and incorporation of NTPs, CMP, cytosine or uracil. Water space experiments confirmed that the uptake process concentrated the purine nucleosides within the cytoplasm of both wild-type and phase II *Coxiella* via a low-pH-dependent mechanism. Comparison of uptake rates in *Escherichia coli* versus *Coxiella* verified that the incorporation of ribonucleosides by *Coxiella* is a slow process. It is concluded that *Coxiella* possesses some transport pathways consistent with utilization of pools of nucleosides found within its host cell lysosomal pathway.

Keywords: nucleosides and nucleotides, phase I and phase II activity, cell volume, growth in chick embryos

INTRODUCTION

Intracellular parasites are dependent upon their host cell for metabolic precursors and raw materials for replication. Of importance is how parasites obtain nucleotides for nucleic acid and nucleotide sugar synthesis, cofactor metabolism, and energy supply. Species of *Legionella* show an increased growth rate in medium supplemented with guanine, adenine, cytosine, thymine and uracil (Pine *et al.*, 1986). *Rickettsia prowazekii* uses an ADP/ATP transporter for adenosine nucleotide exchange with the host (Winkler, 1976; Atkinson & Winkler, 1985; Austin & Winkler, 1988). *R. prowazekii* also possesses AMP, UMP and GMP transport mechanisms to obtain nucleotides for metabolism and growth

(Atkinson & Winkler, 1985; Winkler *et al.*, 1999). Early work by Hatch showed that *Chlamydia psittaci* utilized nucleoside triphosphates from the host cell to synthesize parasite RNA (Hatch, 1975); some of this synthesis is parasite stage-specific (Crenshaw *et al.*, 1990). *C. psittaci* also possesses an ATP/ADP exchange transporter similar in function to that seen in *R. prowazekii* (Hatch *et al.*, 1982). *Chlamydia trachomatis* must transport purine nucleotides and uracil from its host cell to compensate for a lack of ability to form deoxyribonucleoside triphosphates (dNTPs) *de novo* (McClarty & Tipples, 1991; McClarty & Qin, 1993), and is believed to have separate nucleotide transport pathways for energy supply and RNA synthesis (Tjaden *et al.*, 1999).

Coxiella burnetii causes Q fever in humans, is a moderate acidophile, and replicates in end-stage lysosomes within phagocytic cells (Hackstadt & Williams, 1981a; Heinzen *et al.*, 1996). Aside from its known acidophilic physiology, little is known about how it interacts with the host while residing within this niche.

† **Present address:** Q Fever Unit, Rickettsial Section, Viral and Rickettsial Zoonoses Branch, Centers for Disease Control and Prevention, 1600 Clifton Road NE, Atlanta, GA 30333, USA.

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

The lysosomal pathway does contain a large quantity of protein and nucleic acid precursors (Barrett, 1984; Pisoni & Thoene, 1989, 1991). *Coxiella* could take advantage of these in its growth process. Despite its moderate genome size (2100 kbp; Willems *et al.*, 1998) *Coxiella* has never been successfully cultured outside of eukaryotic cells. A limited knowledge of its metabolic capabilities has been gained chiefly through two techniques: (a) enzymic studies with cell-free cytoplasmic extracts (for review see Thompson, 1988), and (b) a technique called acid activation in which intact, host-free *Coxiella* in axenic media are subjected to a pH typical of lysosomes, e.g. pH 4.0–5.5 (Hackstadt & Williams, 1981a). *Coxiella* cells are metabolically inactive at neutral pH.

Fragmentary data concerning the uptake of nucleosides and nucleotides by *Coxiella* can be found in previous studies reporting on bacterial transcription and replication processes (Hackstadt & Williams, 1981c; Zuerner & Thompson, 1983; Samuel *et al.*, 1988; Chen *et al.*, 1990). Purified, host-free organisms of the phase I variant of the Nine Mile strain incorporated thymidine, uridine and adenosine into macromolecular material (Hackstadt & Williams, 1981c); this incorporation required an acidic pH and a concomitant energy source (such as glutamate). A considerable portion of the guanosine taken up appeared to be catabolized (Hackstadt & Williams, 1981c). One study (Zuerner & Thompson, 1983) employing two populations of *C. burnetii* harvested from BHK-21 fibroblast cell cultures showed that these differed with respect to incorporation of uridine. Organisms recovered from the tissue culture medium of persistently infected fibroblasts, termed 'naturally released' *C. burnetii*, were deficient in uridine incorporation into RNA during acid activation. Organisms obtained by the mechanical lysis of heavily infected host cells, however, were capable of incorporating uridine into RNA under similar (low pH) conditions. In a similar study on DNA synthesis in acid-activated *C. burnetii*, the inability of naturally released organisms to incorporate thymidine during acid activation was also noted (Chen *et al.*, 1990). This lack of thymidine incorporation was suspected to be due to a nucleoside permeability problem in the extracellular stage of *Coxiella*, since the use of [³²P]orthophosphate as a precursor demonstrated that these cells could synthesize DNA. In the latter study, it was established that a relatively high external concentration of thymidine was necessary to observe DNA synthesis within *Coxiella* cells while incubated free of host cells and that sucrose was also necessary, presumably to ensure osmotic stability.

Recent investigations of the genetics of phase variation in *Coxiella* have focused on the need to study lipopolysaccharide (LPS) pathway steps, particularly those concerned with nucleotide sugar synthesis and metabolism. The present work surveyed nucleic acid precursor uptake in both phase I (wild-type) and phase II (analogous to rough LPS phenotype) organisms released from their host embryo endodermal cell growth sites by

mechanical lysis. The metabolic and transport capabilities of *Coxiella* phase I and phase II organisms had not previously been compared.

In this report, we show that *Coxiella* actively concentrates purine ribonucleosides within its cytoplasm. No evidence was found for transport or diffusion of NTPs, CMP, cytosine or uracil into *Coxiella*. Although phase II organisms were found to be qualitatively similar in many respects, they were generally slower in uptake for most nucleosides tested, and less active in their ability to concentrate inosine and adenosine.

METHODS

Biosafety. All work described on *Coxiella burnetii* Nine Mile phase I organisms was conducted under BSL3 conditions. Since the organism is highly infectious, procedures that could accidentally discharge aerosols, such as tissue disruption, blending and pipetting, were conducted in a biological safety cabinet (BSC). Respirators were used during other procedures that were deemed potentially risky but unable to be carried out in a BSC.

Bacterial strains and culturing. *C. burnetii* Nine Mile clone 7 (phase I) and clone 4 (phase II) were plaque-purified by Mort Peacock at Rocky Mountain Laboratory, Hamilton, MT, USA, and provided by James Samuel and David Waag; seed stocks used were within two egg passages of these clones. They were cultured in the yolk sacs of 7-day-old White Leghorn antibiotic-free embryonated eggs (SPAFAS Inc., Norwich, CT, USA) at a growth temperature of 36 °C. Harvests were performed when a 50% kill was observed, usually at day 8 (phase II) or day 9 (phase I). Yolk sacs were stored at 4 °C in groups of five. After each batch harvest was completed, the purification of *Coxiella* was immediately started. Storage groups were screened for contaminating bacterial growth using sheep blood agar plates.

Escherichia coli ATCC 25922 was grown in Luria–Bertani (LB) broth until the organisms reached an OD₆₀₀ of 0.5 (Beckman model 25 spectrophotometer). *E. coli* were used for incorporation experiments immediately or stored on ice for 21 h before use.

Purification of *Coxiella* strains. The methods used differed significantly from the yolk sac purification methods described previously (Williams *et al.*, 1981; Paretsky *et al.*, 1958). All steps were performed at 4 °C. Yolk sacs were homogenized in batches of 120 g each, with 120 ml SPG buffer (0.7 M sucrose, 3.7 mM KH₂PO₄, 6 mM K₂HPO₄, 0.15 M KCl, 5 mM glutamic acid, pH 7.4) using 400 ml homogenization chambers and Omni Mixer Homogenizer model 17105 (Omni International). Sacs were homogenized for 10 min, alternating every 30 s between 4400 r.p.m. and 10300 r.p.m. while the chamber was bathed in an ice slurry. An additional 60 ml SPG buffer was added and homogenization continued for another 10 s at 4400 r.p.m. The homogenates were centrifuged in 250 ml bottles (Nalgene Nunc International) at 1020 g in a Sorvall GSA rotor (Kendro Laboratory Products) for 15 min. Supernates were centrifuged at 9000 g for 90 min (GSA rotor). Supernates were discarded, and the fat on the bottle walls removed by sterile cotton swabs. Pellets were resuspended in 60 ml SPG buffer, combined, and the differential centrifugation steps repeated. Pellets were suspended in 120 ml SPG buffer, 15 g acid-washed celite added, and the slurry shaken

vigorously. Following centrifugation at 418 *g* for 15 min, the supernates were recentrifuged (GSA rotor, 1280 *g*, 15 min) to remove celite. The resulting *Coxiella*-containing supernates were added to round-bottomed centrifuge bottles and centrifuged at 9000 *g* for 90 min. Each pellet was resuspended in 25 ml SPG buffer and 1 ml trypsin (1 mg ml⁻¹; Life Technologies) then digested at room temperature for 30 min. After the suspension was separated in an SS34 rotor (Kendro Laboratory Products) at 271 *g* for 15 min, the resulting supernates were centrifuged again in an SS34 rotor at 30880 *g* for 30 min. Purified pelleted organisms were resuspended to a total volume of 70 ml of buffer A (22.3 mM K₂HPO₄, 135.7 mM KCl, 13.4 mM NaCl, 89 mM glycine, 10 mM MgCl₂·6H₂O, 1 mM glucose, 1 mM glutamate and 250 mM sucrose, pH 7.0; Zuerner & Thompson, 1983). To remove residual impurities, the organisms were banded through sterile stepped sucrose gradients prepared by layering, in order, 3 ml 2 M sucrose, 2 ml 1.2 M sucrose, and 1 ml each of 1.0, 0.8 and 0.6 M sucrose assembled in 12 ml polycarbonate tubes (Kendro Laboratory Products). After layering 2.5 ml of *Coxiella* in buffer A onto these gradients, they were centrifuged in an HS-4 rotor (Kendro Laboratory Products) at 9430 *g* for 2 h. The purified *Coxiella* banded at the interface between the 1.2 M and 2 M sucrose layers; this zone was removed, combined, diluted with buffer A, and collected by centrifugation (Sorvall SS34 rotor at 30880 *g* for 1 h). The resulting pellet was resuspended in 10 ml buffer A for every 240 g yolk sac starting material and immediately used in acid activation studies. A 0.5 ml sample of this preparation was routinely retained for microbial enumeration (see below).

Enumeration of bacteria. Viable counts for *E. coli* ATCC 25922 were determined by the c.f.u. method. Smooth suspensions of *C. burnetii*, in 0.5 ml buffer A, were transferred to a 1.7 ml microcentrifuge tube and centrifuged at 10000 *g* for 10 min (Beckman Microfuge model 12). The supernate was discarded and the pellet was resuspended in 0.5 ml 0.2 M NaCl. Then 100 µl of this *Coxiella* suspension was added to 1.79 ml 5% formalin and 200 µl 0.2 M NaCl and thoroughly mixed. Equal volumes of the *Coxiella* mixture and a twice-filtered solution of freshly prepared carbolfuchsin stain (Gimenez, 1964) were mixed. *Shigella flexneri* was grown and inactivated as described by Silverman *et al.* (1979), and the standard was prepared by adding 0.1 ml of a well-suspended *Shigella* preparation (2.84 × 10⁹ organisms ml⁻¹) stored in 5% formalin to 4.4 ml H₂O, 0.1 ml 0.2 M NaCl and 0.2 ml twice-filtered Gram's crystal violet stain, followed by thorough mixing. After combining 1 ml of the stained *Coxiella* and 1 ml of the stained *Shigella*, the components were mixed, and 10 µl suspension was applied to a microscope slide and air-dried. The numbers of *Coxiella* (red) and *Shigella* (purple) organisms in 30 fields (2 mm square area on an ocular grid) were determined. The organisms were easily discernible by their size difference. The ratio of *Coxiella*/*Shigella* organisms in the sample was used to calculate the direct *Coxiella* count expressed as organisms ml⁻¹.

Incorporation experiments. Aliquots of *Coxiella* phase I in buffer A (50 µl), phase II in buffer A (200 µl), or *E. coli* ATCC 25922 in LB broth (1.5 ml) were transferred to reaction tubes and the tubes centrifuged for 10 min at 10000 *g* to pellet the bacteria. After careful and complete removal of the supernate fluids, the pellets were resuspended in the appropriate medium [buffered salts (Hackstadt & Williams, 1981a; Zuerner & Thompson, 1983) fortified with 5.5 mM glucose, 0.3 mM thymidine*, 4 µM proline, 8.8 µM isoleucine, 25.4 µM arginine, 6.8 µM serine, 46.4 µM glutamine, 4.0 µM valine, 2.4 µM tyrosine, 3.4 µM aspartate, 2.2 µM histidine, 2.3 µM

methionine, 8.8 µM leucine*, 3.9 µM threonine, 3.5 µM glycine, 9.6 µM cystine, 6.2 µM lysine, 2.0 µM phenylalanine, 8.7 µM asparagine, 0.5 µM tryptophan, 3.96 µM alanine and 2.4 mM glutamate] buffered to either pH 4.5 or pH 7.0. Forty microcurie (1.48 MBq) quantities of ³H-labelled amino acid, nucleoside, nucleotide or nucleobase were added and then the organisms (in pellet form) were suspended in the medium, to a final volume of 0.8 ml. Unlabelled leucine or thymidine (signified by *) was excluded from the axenic medium when radiolabelled leucine or thymidine was added to the solution. The tracers used in the incorporation experiments were: leucine (4,5-³H, 133 Ci mmol⁻¹), inosine (2,8-³H, 44.4 Ci mmol⁻¹), adenosine (2,8-³H, 40.0 Ci mmol⁻¹), ATP (2,8-³H, 22.5 Ci mmol⁻¹), cytosine (5-³H, 22.8 Ci mmol⁻¹), cytidine (5-³H, 17.7 Ci mmol⁻¹), CMP (5-³H, 32.3 Ci mmol⁻¹), CTP (5-³H, 23.0 Ci mmol⁻¹), uracil (5,6-³H, 34.0 Ci mmol⁻¹), uridine (5-³H, 9.3 Ci mmol⁻¹), guanosine (8-³H, 12.5 Ci mmol⁻¹), and thymidine (*methyl*-³H, 2.0 Ci mmol⁻¹) (Amersham Pharmacia Biotech and Moravsek Biochemicals; 1 Ci = 37 GBq). The reaction mixtures were incubated at 37 °C in a gyrotory water bath shaker. Samples (100 µl each) were taken at 0, 1, 2, 4, 8 and 16 or 18 h and pipetted onto Whatman 3MM glass fibre disks. The disks with the bound precipitated material were then prepared for scintillation counting by a slight modification of the method of Mans & Novelli (1960) as previously described (Zuerner & Thompson, 1983; Chen *et al.*, 1990). The filters were counted by liquid scintillation while immersed in 10 ml TT76 [7 parts Triton X-100 (Sigma-Aldrich), 6 parts Econofluor (New England Nuclear)]. Tritium counts were determined in a Wallac model 1410 scintillation counter. Results were plotted in Sigmaplot version 5 (SPSS Inc.), as pmol or fmol substrate incorporated per 10⁹ bacteria versus time (h) and the slopes (from time zero to 4 h) obtained using Microsoft Excel 1997.

Water space assay. Purified phase I and phase II organisms were suspended in 10 ml buffer A. Aliquots of 1 ml (1–4 × 10¹⁰ *Coxiella*) were pipetted into Eppendorf tubes and pelleted for 10 min at 10000 *g*. The pellets were resuspended in a solution of either neutral (pH 7.0) or acidic (pH 4.5) medium (as described for incorporation studies, above) containing 8 µCi ³H₂O (1 mCi mmol⁻¹) ml⁻¹ and 1 µCi ¹⁴C-labelled compound ml⁻¹. The radiochemicals employed, and their specific radioactivities, were: [¹⁴C]sucrose (495 mCi mmol⁻¹), [¹⁴C]inulin carboxylic acid (5.2 mCi mmol⁻¹), [¹⁴C]methoxyinulin (12.1 mCi mmol⁻¹), [¹⁴C]adenosine (50 mCi mmol⁻¹), [¹⁴C]guanosine (53 mCi mmol⁻¹), [¹⁴C]inosine (48 mCi mmol⁻¹), [¹⁴C]thymidine (53 mCi mmol⁻¹), [¹⁴C]cytidine (55 mCi mmol⁻¹) and [¹⁴C]uridine (54 mCi mmol⁻¹) (1 mCi = 37 MBq). To prevent incorporation into RNA, rifampicin at a final concentration of 40 µM was included in the incubation mixtures (Chen *et al.*, 1990; Winkler, 1999). pH 7.0 controls were run for each substrate. To examine guanosine transport, *Coxiella* phase I organisms were resuspended in axenic medium either with or without 40 µM rifampicin at both pH 4.5 and pH 7.0. To further examine the energy dependence and competition for the transporter, the proton ionophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (final concentration 0.05 mM; Sigma-Aldrich) or unlabelled guanosine (final concentration 20 µM; Sigma-Aldrich) were added to the reaction mixtures in the presence of 40 µM rifampicin either at time 0 or at 40 min into the incubation. Finally, 2 mM KOH (10 µl per ml sample) was added to the axenic medium at pH 4.5 at 40 min to neutralize the medium and thus examine the pH dependence of the guanosine transporter during concentrative work.

Mixtures were divided into three equal parts (300 µl reaction

volumes) and incubated for 1 h at 37 °C in a shaking incubator. Upon completion, equal aliquots were layered over perchloric acid/dibutylphthalate/silicone oil step gradients; these were constructed, centrifuged, frozen, fractionated and sampled as described previously (Winkler, 1986; Winkler *et al.*, 1999). Three separate determinations were made from each triplicate reaction. Fractions from the gradients were placed in 20 ml scintillation vials as described with 10 ml TT76 (described above). The beta particle emissions from supernatant and pellet fractions were determined in a Wallac model 1410 scintillation counter using MeV versus pulse height settings, determined empirically, that captured most of the ^3H and ^{14}C energy in separate windows. Count (c.p.m.) data were treated for energy overlap and counting efficiency according to a formula based upon ^3H and ^{14}C standards made within our experimental conditions.

The corrected count data were converted to microlitre volumes as described by Winkler *et al.* (1999). A significant increase in the ^{14}C -labelled substrate/ $^3\text{H}_2\text{O}$ ratio in the pellet compared to the value found in the supernatant suggests concentration of that substrate (Winkler, 1986). ^{14}C substrate values must first be corrected for extracellular water space (^{14}C]inulin) and for extracytoplasmic water space (i.e. the extracellular space plus the periplasmic space as determined by sucrose). The bar graphs depict the corrected ratio of pellet divided by supernatant, with a value of 1 implying complete diffusion into the cell, such that the interior and exterior concentrations of the substrate are equal. A value of 2 indicates a twofold concentration within the cell cytoplasm (Winkler, 1986). The ^3H and ^{14}C values for all of the compounds ($^3\text{H}_2\text{O}$ and ^{14}C -labelled inulin, sucrose, adenosine, inosine, guanosine, cytidine, thymidine and uridine) were determined for phase I and phase II organisms. The values were graphed in Sigmaplot 5.0.

RESULTS

Growth of *C. burnetii* variants in embryonated eggs

C. burnetii Nine Mile phase I (virulent) and phase II (avirulent) organisms were cultured in embryonated eggs for 9 and 8 days, respectively. The yolk sacs were removed and the organisms were purified as described in Methods. The organisms were enumerated compared to a *Shigella flexneri* standard (2.84×10^9 organisms ml^{-1}) and expressed as number of *Coxiella* per g yolk sac. A comparison of four purification batches for each of the variants showed that phase I organisms were recovered at a level almost twice that of phase II organisms [$1.44 \times 10^9 \pm 1.72 \times 10^8$ phase I organisms per g yolk sac compared to $8.52 \times 10^8 \pm 1.54 \times 10^8$ phase II organisms per g yolk sac (means $\pm$ SEM)]. This lower recovery of the phase II organisms in eggs, when compared to the growth and recovery of phase I organisms, is consistent with another report (Waag *et al.*, 1991).

Leucine incorporation in *Coxiella* variants

In studying *Coxiella* growth and transport, certain limitations become apparent. (i) There is difficulty in purifying sufficient quantities of active organisms in a timely manner. (ii) *Coxiella* activities must be distinguished from those contributed by contaminating

host components. Finally, (iii) variations in measured activities between batches of purified organisms make interpretation of results difficult. To meet the constraints of (i), organisms were grown in yolk sacs, and rapidly harvested (see above) and purified. To distinguish activities from those of the host (ii), controls were done under non-activating conditions carried out at pH 7.0. This control detects most neutralophilic activities due to host enzymes, and blood agar plating together with the pH 7.0 control will help rule out non-*Coxiella* bacterial contamination. Dealing with batch variations (iii), especially when doing isotope tracer experiments, is especially problematic. Metabolite pool sizes and enzyme activities are not uniform from batch to batch. However, the rate of incorporation of radioactive leucine into protein during acid activation has proven to be a general indicator of overall metabolic capability in *Coxiella* (see also Chen *et al.*, 1990). Thus, a low leucine activity will usually parallel a lower capacity for DNA synthesis. Some populations of *Coxiella* incorporate leucine normally but completely lack an uptake system for another substrate, such as thymidine. To thus enable better interpretation of results, it was advantageous to include leucine incorporation controls in every acid activation experiment with *Coxiella* (see Table 1; otherwise not shown). Having these data allowed us to assess the relative activity of egg batches (see Table 1). Leucine controls also ensured that the observed lack of uptake of nucleotides and bases was not due to poorly active preparations of cells (see also Table 1).

A total of eight batches of *Coxiella*, four of phase I and four of phase II, were used in this study. All of these were checked for leucine incorporation rates, and all were active. A comparison of phase I vs phase II data was also done. Although a couple of batches showed significant variation, differences between phases were not significant at any timepoint (not shown). Incorporation of leucine into either phase was linear through 4 h at least, and rates varied between 40 and 400 fmol per 10^9 organisms h^{-1} with a mean of about 120.

Comparative uptake activities of *Coxiella* and *E. coli*

An additional challenge in studying *Coxiella* is its low metabolic activity. In a comparative study with *E. coli*, incorporation data were obtained using the same pH 7.0 medium employed for the neutral pH control in *Coxiella* experiments. Aliquots were taken between 0 and 90 min. The slope of the best-fit line was used to determine the rate. Since the time required to obtain purified *Coxiella* from harvested yolk sacs was 21 h, we held the freshly grown *E. coli* cells in LB broth at 4 °C for the same length of time prior to testing. The incorporation of label was complete by 45 min in *E. coli*, suggesting that the available nutrients had been depleted. Ratios of uptake rates, as determined by dividing the *E. coli* rate (mole fraction per 10^9 bacteria h^{-1} , observed at pH 7.0) by the *C. burnetii* rate (mole fraction per 10^9 bacteria h^{-1} , observed at pH 4.5), were determined for four substrates and were as follows: leucine 59; adenosine

Table 1. Comparison of incorporation rates of *Coxiella* phase I and phase II variants

Phase I and phase II organisms were resuspended in axenic medium (pH 4.5 unless indicated otherwise) with ^3H -labelled substrate and incubated for 16 h at 37 °C. Results from four separate experiments are shown. Rates are expressed as fmol substrate per 10^9 *Coxiella* h^{-1} , as determined by the slope of the best-fit line for the early time points (0–4 h). ND, Not done. All batches of either phase I or phase II were metabolically active as determined by the leucine incorporation index.

	Phase I		Phase II	
	Expt 1	Expt 2	Expt 3	Expt 4
Nucleoside				
Inosine	698	2462	315	900
Adenosine	145	ND	45	ND
Guanosine pH 4.5	79	342	112	1388
Guanosine pH 7.0	75	305	53	88
Thymidine	568	ND	215	536
Uridine	101	ND	19	260
Cytidine	39	102	12	32
Amino acid				
Leucine	70	334	42	352
Nucleotides				
ATP	ND	–1	–2	–1
GTP	–2	–1	ND	ND
CTP	–3	ND	ND	–5
CMP	ND	–1	0.4	0.8
UTP	–1	–3	ND	ND
Nucleobases				
Uracil	–0.1	ND	ND	–1
Cytosine	0.8	ND	–3	1

229; inosine 47; cytidine 2088. *C. burnetii* is substantially smaller than *E. coli*, and calculations reveal that an *E. coli* cell possesses approximately five times more surface area. But even when such a surface area correction is applied, differences in nucleoside uptake activities between the two are significant. Thus the low metabolic activities of *Coxiella*, and the requisite difficulty in preparing larger batches of active organisms, precluded transport experiments utilizing classical Millipore filtration assays. We therefore employed incorporation into acid-insoluble material to estimate the ability of organisms to transport and utilize several nucleotides, nucleobases and nucleosides.

Incorporation of nucleosides

To study the transport and incorporation of nucleosides, nucleotides and nucleobases in *Coxiella* variants, *C. burnetii* phase I and phase II organisms were resuspended in axenic medium supplemented with ^3H -labelled substrates (as described in Methods). The data are expressed as incorporation rates (fmol per 10^9 organisms h^{-1}), as determined by the slope of the best-fit line for the early time points 0–4 h (Table 1). Because leucine incorporation rates generally predict the metabolic capability of a *Coxiella* preparation, we have

included those rates for each nucleoside experiment. None of the nucleotides or nucleobases supplemented to the axenic medium were incorporated in an 8 h time period. It was necessary to establish that all batches used in experiments were metabolically fit, even though they may not transport a given substrate (Chen *et al.*, 1990; Zuerner & Thompson, 1983). This assurance was obtained by testing for leucine incorporation into protein (Zuerner & Thompson, 1983; Chen *et al.*, 1990; Redd & Thompson, 1995). All batches used to study nucleotide and nucleobase uptakes were checked, and all possessed leucine incorporation activity. In experiments utilizing radiolabelled ATP, several variations were used to lower the adenylate charge to encourage ADP/ATP exchange at either pH 4.5 or 7.0 (see also Hackstadt & Williams, 1981b), including substrate starvation, ATP synthase inhibition, and various preincubation protocols. In no case did we measure any incorporation of ATP (data not shown). In almost all experiments, purine nucleosides were incorporated to a greater extent than pyrimidine nucleosides. Thymidine was the exception, showing incorporation rates roughly equivalent to the purine nucleosides. In all cases, uridine and cytidine produced low incorporation rates, suggesting that cytidine and uridine are not transported as efficiently as purine nucleosides and

Fig. 1. Incorporation of inosine by *Coxiella* variants and competition with adenosine. Purified *Coxiella* phase I (a) and phase II (b) organisms were resuspended in acidic medium supplemented with 40 μ Ci [³H]inosine and incubated at 37 °C for 8 h. At 0, 1, 2, 4 and 8 h, samples were removed and processed. Rifampicin, when used, was added to a final concentration of 40 μ M. Unlabelled adenosine was added in the competition experiments to a final concentration of 1, 5 or 10 times the amount of [³H]inosine (0.375 μ M) in the medium. Consistent results were obtained in replicate experiments; representative data are shown.

Fig. 2. Incorporation of guanosine by *Coxiella* variants. Purified *Coxiella* phase I (a; rate shown in expt 2, Table 1) and phase II (b; rate shown in expt 3, Table 1) organisms were resuspended in medium buffered to pH 4.5 (●) or pH 7.0 (○) supplemented with 40 μ Ci [³H]guanosine, and incubated at 37 °C. At 0, 1, 2, 4, 8 and 16 or 18 h samples were taken (as described in Methods). Consistent results were obtained in replicate experiments; representative data are shown.

thymidine, or that their internal pool sizes and *de novo* syntheses are by comparison greater.

When inosine incorporation by the two variants was compared, it was found that inosine was incorporated at a faster rate in phase I variants than in phase II variants (Table 1, Fig. 1a, b). In both phases, this incorporation could be eliminated by the addition of 40 μ M rifampicin. Furthermore, inosine was consistently incorporated at a greater rate than adenosine. This is interesting because in the lysosomal pathway, adenosine is quickly converted to inosine (Pisoni & Thoene, 1989, 1991), and the substrates differ in structure only by an amino group on the ring. To examine the specificity of the inosine transporter, unlabelled adenosine at concentrations one,

five and ten times greater than the concentration of radiolabelled inosine (0.375 μ M) was included in the incubations to see if adenosine could compete with the inosine incorporation rate (Fig. 1a). It was found in a single experiment that at any concentration of adenosine, inosine incorporation was not adversely affected. On the contrary, the addition of adenosine appears to have had a slight positive effect upon inosine incorporation, particularly at the 10-fold concentration (Fig. 1a).

Guanosine incorporation in *Coxiella* phase variants was compared (Table 1, Fig. 2). It was found in two separate experiments that purified phase II organisms incorporated radiolabelled guanosine at pH 4.5, but not at

Fig. 3. Water space experiments with *Coxiella* variants. Purified *Coxiella* phase I (a) and phase II (b) organisms ($1-4 \times 10^{10}$ cells) were resuspended in axenic medium at pH 4.5 (■) or pH 7.0 (□) supplemented with 40 μ M rifampicin, 8 μ Ci $^3\text{H}_2\text{O}$ and 1 μ Ci ^{14}C -labelled substrate (sucrose, inulin, inosine, adenosine, guanosine, thymidine, cytidine or uridine). The mixtures were incubated for 1 h at 37 °C and samples were taken and processed. See Methods for details. The results are expressed as cytoplasmic volumes, with 1 equivalent to one cytoplasmic volume. Each bar represents nine individual readings. Error bars represent SEM.

pH 7.0 (Table 1, expts 3 and 4; Fig. 2b). When two batches of purified phase I organisms were compared, it was found that the guanosine incorporation rates for pH 4.5 and pH 7.0 were similar over the 16 h time-course with both experiments (Table 1, expts 1 and 2; Fig. 2a). Metabolic activity had never previously been observed in *Coxiella* at pH 7.0; the organism had been thought to be in an almost static state at neutral pH.

Water space studies

Incorporation assays can be used to indirectly study transport of substrates by treating the accumulation of substrate in an acid insoluble pool as a 'sink' after uptake (Winkler *et al.*, 1999). However, incorporation studies cannot distinguish between active transport processes that concentrate substrate, and passive mechanisms that do not. Furthermore, as we have shown above, some of the pyrimidine nucleosides were weakly incorporated by *Coxiella*. Water space experiments were therefore performed to verify if any of the incorporated nucleosides studied here were concentrated inside the cytoplasm of *Coxiella*. Interpretation of data in water space experiments absolutely depends upon nonconversion of labelled substrate. These experiments were therefore run in the presence of rifampicin to prevent incorporation into RNA.

These water space assays confirmed many of the incorporation results by showing that all nucleoside substrates that were incorporated in the acid precipitation study had diffused into, or were actively concentrated within, phase I and phase II cells. At pH 4.5, both phase I and phase II organisms had significant concentration of inosine (4.2 and 2.3 cytoplasmic volumes, respectively; Fig. 3), but did not concentrate the nucleosides at pH 7.0. Likewise, both phases concentrated guanosine (5.5 cytoplasmic volumes for phase I, 5.2 for phase II) at acidic pH. Guanosine concentration was not observed at pH 7.0 for phase I cells. This is not surprising considering that incorporation assays did not

show significant incorporation of guanosine at neutral pH until 2 h (Fig. 2a) but the water space incorporation assays were carried out for only 1 h. Adenosine was seen to be concentrated at pH 4.5 in phase I cells (cytoplasmic volume 1.8), whereas the concentration effect observed in phase II cells was marginal (1.1 at pH 4.5 and 1.4 at pH 7.0; Fig. 3). Neither phase concentrated cytidine, and in fact the values for this nucleoside, at both pHs and in both phases of organisms, were less than 1. Low values were also found for thymidine and uridine concentration in phase I cells. In no experiment were any of the pyrimidine ribonucleosides seen to be concentrated in either phase I or II organisms. We did not attempt these experiments with the nucleoside triphosphates, or with bases.

Cytoplasmic volume was determined for both phase I and phase II variants from the water space experiments. *Coxiella* phase I variants had a larger cytoplasmic volume ($18.35 \pm 6.29 \mu\text{l}$ per 10^{12} organisms, two experiments) than phase II variants ($9.78 \pm 0.69 \mu\text{l}$ per 10^{12} organisms, three experiments) (means $\pm$ SEM). By using the dimensions $0.25 \mu\text{m} \times 1.0 \mu\text{m}$ for the average organism as measured by light microscopy, and employing the formula for the volume of a perfect cylinder, we calculate a displacement volume of $49 \mu\text{l}$ for 10^{12} *Coxiella* organisms. The large difference between the measured and calculated volumes suggests that much of the 'space' perceived in microscopic measurements of stained *C. burnetii* does not equate to water-based cytoplasm. This calculation ignores the SCV (small cell variant) stage, which is about $0.6 \mu\text{m}$ in length and probably impermeant to sucrose and water (McCaul, 1991).

To gain a greater understanding of the purine transport systems in *Coxiella*, the guanosine transporter was examined more closely using water space techniques to analyse inhibitor effects and efflux of unmodified nucleosides (Fig. 4). Guanosine concentration in *Coxiella* phase I organisms was examined in either the absence (see bars

Fig. 4. Water space examination of guanosine transport. Purified *Coxiella* phase I organisms (2.25×10^{10} cells) were resuspended in axenic medium (pH 4.5 or 7.0) supplemented with $8 \mu\text{Ci } ^3\text{H}_2\text{O ml}^{-1}$ and $1 \mu\text{Ci } [^{14}\text{C}]\text{guanosine ml}^{-1}$. Tubes 3–9 contained $40 \mu\text{M}$ rifampicin. CCCP (final concentration 0.05 mM) was added to tube 5 at time zero, and to tube 7 at time 40 min. Unlabelled guanosine (final concentration $20 \mu\text{M}$) was added to tube 6 at time zero, and to tube 8 at 40 min. Tube 9 received 2 mM KOH ($10 \mu\text{l}$ per ml reaction) at 40 min into the incubation to neutralize the reaction to pH 7.0. The final volume for all reactions was 1 ml. Incubations were carried out for 60 min, and then samples were removed and processed. See Methods for details. The results are expressed as cytoplasmic volumes, with 1 equivalent to one cytoplasmic volume. Each bar represents three separate reactions from which nine determinations were made. Error bars represent SEM.

1 and 2) or the presence (bars 3 and 4) of $40 \mu\text{M}$ rifampicin at both pH 4.5 and 7.0 to determine if guanosine uptake would increase in the absence of rifampicin at acidic pH. Surprisingly, there was no significant difference between the samples incubated in the presence or absence of rifampicin at acidic pH, suggesting that very little of the uptake observed after 1 h (without rifampicin) was due to RNA synthesis. *Coxiella* phase I organisms were also incubated with labelled guanosine and rifampicin in the presence of either CCCP (bar 5) or unlabelled guanosine (bar 6) for 1 h. Because the addition of rifampicin (at the beginning of the experiment) did not significantly affect the quantity of the radiolabelled guanosine transported into the cell after 1 h, it must be concluded that most of the internalized substrate observed after 1 h had not yet been incorporated into RNA. However, its uptake over this period was completely abolished by the protonophore CCCP (bar 5), suggesting that the maintenance of a proton-motive force or, more precisely, the ΔpH , was essential for uptake. Further, the addition of the unlabelled guanosine decreased the amount of labelled guanosine within the incubated *Coxiella* organisms (bar 6), indicating that a specific binding site for guanosine exists.

Rifampicin was used in all water space experiments to block nucleoside incorporation into RNA. This will not block nucleoside incorporation into DNA, or into nucleotide sugars. Water space studies require an unaltered substrate to analyse concentration; otherwise it is another measure of incorporation into macromolecules. To examine substrate efflux from *Coxiella* in the presence of inhibitors and competitors, *Coxiella* phase I organisms were resuspended in axenic medium

at pH 4.5 supplemented with $^3\text{H}_2\text{O}$, $[^{14}\text{C}]\text{guanosine}$ and $40 \mu\text{M}$ rifampicin (as described in Methods). After 40 min incubation at 37°C to preload the cells with radioactive guanosine, either CCCP (final concentration 0.05 mM ; Fig. 4, tube 7), unlabelled guanosine (final concentration $20 \mu\text{M}$, tube 8) or 2 mM KOH (tube 9) was added to the reaction mixture. The samples were then incubated at 37°C for an additional 20 min. After the 40 min period of acid activation, 25–50% of the labelled internalized substrate was diffusible upon addition of excess non-labelled compound, or by the addition of CCCP or KOH (see bars 7, 8 and 9 in Fig. 4). These results suggest that most of the internalized labelled substrates had been metabolized into non-diffusible compounds, perhaps to nucleotides through kinase activity, and into nucleotide sugars, or into DNA, but not into RNA. A portion of the concentrated compound (between 1.7 and 3.9 cytoplasmic volumes; Fig. 4) remains an unaltered part of the nucleoside pool within the cytoplasm and can be diffused from the cell when treated with inhibitors or given a neutral pH environment. The existence of this unaltered and diffusible portion of the nucleoside pool, once incorporated substrates have been taken into account, suggests that the *Coxiella* phase I organisms tested here were capable of the active transport of guanosine.

DISCUSSION

The present study was undertaken to obtain a more comprehensive understanding of *Coxiella*'s capabilities in transport of nucleic acid precursors when studied under conditions that mimic phagolysosomes. The data confirm that *Coxiella* differs substantially from *Chlamy-*

dia and *Rickettsia* in these activities (Hatch *et al.*, 1982; Tjaden *et al.*, 1999; Winkler *et al.*, 1999). This study has revealed no evidence for the transport of ATP, GTP, CTP, UTP, CMP, cytosine or uracil by *C. burnetii*. We have shown that at least one purine ribonucleoside (guanosine) is concentrated, a characteristic that suggests a carrier-mediated, active transport mechanism. Pyrimidine ribonucleosides however were not concentrated under the conditions used, even though these were also incorporated into RNA over long time periods.

In each experimental set, a pH 7.0 control incubation was used to rule out contamination with other bacteria, and to help eliminate the possibility that the observed results were due to host activities that may have co-purified with whole organisms. In all substrates tested except one, this control gave the expected results, i.e. no activity. In the case of guanosine incorporation, no pH dependence was found in phase I organisms. Since there was no evidence of contaminating microbes, and because the activity is rifampicin-sensitive and therefore of microbial origin, we conclude that the phase I organisms do have some capability for long-term guanosine uptake at a neutral pH. This observation was not however verified in water space assays. This may have been due to the relatively shorter incubation times required for the water space experiments. It is possible that the observation may indicate an actual guanosine uptake event that occurs during infection of cells prior to acidification of the *Coxiella* endosome. Further experimentation will be required to elucidate the mechanism of neutral pH uptake of guanosine.

Knowledge of *Coxiella*'s nucleotide biosynthesis pathways (including nucleotide salvage) is incomplete, making it difficult to assess the organism's need for precursors supplied in the phagolysosome. It is known that some enzyme activities, specifically aspartate transcarbamoylase activity and the subsequent reactions through orotate synthesis in the pyrimidine biosynthetic pathway, are present (Mallavia & Paretsky, 1963); the latter observation was confirmed and the *pyrB* gene was cloned and characterized (Hoover & Williams, 1990). Because the organism can incorporate various nucleoside precursors into nucleic acid (Zuerner & Thompson, 1983; Chen *et al.*, 1990), it can be inferred that the required nucleoside diphosphate kinases are present to convert nucleosides to nucleotides. Christian & Paretsky (1977) demonstrated some of these activities within cell-free extracts obtained from purified *Coxiella*. Genome analysis, now in progress for *C. burnetii*, should provide more insight as to which nucleoside biosynthesis and salvage pathways are present.

It is known that *C. burnetii* Nine Mile phase I and phase II organisms differ substantially in their LPS structure. Phase I organisms display non-truncated LPS that possess three rare sugars: L-virenose (6-deoxy-3-C-methylgulose), dihydrohydroxystreptose [(3-C-hydroxymethyl)-lyxose] and galactosaminuronyl- α -(1-6)-glucosamine. Nine Mile phase II LPS structures contain none of these sugars and are severely trun-

cated (Schramek & Mayer, 1982; Schramek *et al.*, 1985; Amano *et al.*, 1987). Phase I and phase II organisms differ in their susceptibility to phagocytosis (Wissemann *et al.*, 1967; Kazar *et al.*, 1975) and in their surface characteristics (Krauss *et al.*, 1977; Vishwanath & Hackstadt, 1988), and therefore can be expected to differ in interactions with various substrates and structures (see discussion by Thompson, 1988). It is well known that rough mutants of *Salmonella typhimurium* and other species are affected in functions including transport, phage attachment, outer-membrane protein assembly and intercellular interactions (Parker *et al.*, 1992; Walsh *et al.*, 2000). These differences in outer-membrane structure may cause the differences in nucleoside incorporation and concentration we observe here between the variants. It may be that surface porins or structures of phase II organisms are perturbed enough to cause a decrease in the ability to transport or diffuse nucleoside substrates across the outer membrane. Another possibility is that the truncation of the LPS in phase II organisms causes charged moieties on the outer membrane surface to become exposed, negatively affecting transport across the outer membrane. Whatever the cause of the decreased transport and concentration of nucleosides, especially purines, across the phase II membrane, the differences are not great enough to suggest that transport mechanisms (such as permease proteins) are genetically absent. The data available do show a lack of pyrimidine ribonucleoside active transport in both *Coxiella* variants.

In these studies, trypsin was used to help eliminate cellular host proteins from the *Coxiella* preparations during purification. It is unlikely that trypsin has significant negative effects on phase I functions (Paretsky *et al.*, 1958; Amano *et al.*, 1984; Redd, 1986; Banerjee-Bhatnagar *et al.*, 1996). We cannot rule out that the use of trypsin in purification may have had some small effect on substrate entry in phase II cells.

It is concluded that both phase I and phase II organisms possess a modest ability to transport purine ribonucleosides when incubated in acidic medium. It is not known to what extent *Coxiella* may modify the environment of its host phagolysosome. If that compartment is unmodified, *Coxiella* may have adapted to this environment by utilizing the readily available concentration gradients of precursors within the host phagolysosome. It may then passively import some nucleosides while utilizing (directly or indirectly) the pH gradient to transport others.

ACKNOWLEDGEMENTS

This work was supported by grant ROI AI34984-03 to H. A. T. from the National Institutes of Health. We gratefully acknowledge a WVU School of Medicine competitive stipend award to J.D.M.

REFERENCES

- Amano, K., Williams, J. C., McCaul, T. F. & Peacock, M. G. (1984). Biochemical and immunological properties of *Coxiella burnetii* cell wall and peptidoglycan-protein complex fractions. *J Bacteriol* 160, 982-988.

- Amano, K., Williams, J. C., Missler, S. R. & Reinhold, V. N. (1987).** Structure and biological relationships of *Coxiella burnetii* lipopolysaccharides. *J Biol Chem* **262**, 4740–4747.
- Atkinson, W. H. & Winkler, H. H. (1985).** Transport of AMP by *Rickettsia prowazekii*. *J Bacteriol* **161**, 32–38.
- Austin, F. A. & Winkler, H. H. (1988).** Relationship of rickettsial physiology and composition to the Rickettsia-host cell interaction. In *Biology of Rickettsial Diseases*, vol. II, pp. 29–49. Edited by D. H. Walker. Boca Raton, FL: CRC Press.
- Banerjee-Bhatnagar, N., Bolt, C. R. & Williams, J. C. (1996).** Pore-forming activity of *Coxiella burnetii* outer membrane protein oligomer comprised of 29·5- and 31-kDa polypeptides. *Ann N Y Acad Sci* **791**, 378–401.
- Barrett, A. J. (1984).** Proteolytic and other metabolic pathways in lysosomes. *Biochem Soc Trans* **12**, 899–902.
- Chen, S.-Y., Vodkin, M., Thompson, H. A. & Williams, J. C. (1990).** Isolated *Coxiella burnetii* synthesizes DNA during acid activation in the absence of host cells. *J Gen Microbiol* **136**, 89–96.
- Christian, R. G. & Paretsky, D. (1977).** Synthesis of ribonucleotides and their participation in ribonucleic acid synthesis by *Coxiella burnetii*. *J Bacteriol* **132**, 841–846.
- Crenshaw, R. W., Fahr, M. J., Wichlan, D. G. & Hatch, T. P. (1990).** Developmental cycle-specific host-free RNA synthesis in *Chlamydia* spp. *Infect Immun* **58**, 3194–3201.
- Gimenez, D. F. (1964).** Staining rickettsiae in yolk-sac cultures. *Stain Technol* **39**, 135–140.
- Hackstadt, T. & Williams, J. C. (1981a).** Biochemical stratagem for obligate parasitism of eukaryotic cells by *Coxiella burnetii*. *Proc Natl Acad Sci USA* **78**, 3240–3244.
- Hackstadt, T. & Williams, J. C. (1981b).** Stability of the adenosine 5'-triphosphate pool in *Coxiella burnetii*: influence of pH and substrate. *J Bacteriol* **148**, 419–425.
- Hackstadt, T. & Williams, J. C. (1981c).** Incorporation of macromolecular precursors by *Coxiella burnetii* in an axenic medium. In *Rickettsiae and Rickettsial Diseases*, pp. 431–440. Edited by W. Burgdorfer & R. L. Anacker. New York: Academic Press.
- Hatch, T. P. (1975).** Utilization of L-cell nucleoside triphosphates by *Chlamydia psittaci* for ribonucleic acid synthesis. *J Bacteriol* **122**, 393–400.
- Hatch, T. P., Al-Hossainy, E. & Silverman, J. A. (1982).** Adenine nucleotide and lysine transport in *Chlamydia psittaci*. *J Bacteriol* **150**, 662–670.
- Heinzen, R., Scidmore, M., Rockey, D. D. & Hackstadt, T. (1996).** Differential interaction with endocytic and exocytic pathways distinguish parasitophorous vacuoles of *Coxiella burnetii* and *Chlamydia trachomatis*. *Infect Immun* **64**, 796–809.
- Hoover, T. A. & Williams, J. C. (1990).** Characterization of *Coxiella burnetii* pyrB. *Ann N Y Acad Sci* **590**, 485–490.
- Kazar, J., Skultetyova, E. & Brezina, R. (1975).** Phagocytosis of *Coxiella burnetii* by macrophages. *Acta Virol* **19**, 426–431.
- Krauss, H., Schiefer, H.-G. & Schmatz, H.-D. (1977).** Ultrastructural investigations on surface structures involved in *Coxiella burnetii* phase variation. *Infect Immun* **15**, 890–896.
- Mallavia, L. & Paretsky, D. (1963).** Studies on the physiology of *Rickettsiae*. Part V. Metabolism of carbamoyl phosphate by *Coxiella burnetii*. *J Bacteriol* **86**, 232–238.
- Mans, R. J. & Novelli, G. D. (1960).** A convenient, rapid and sensitive method of measuring the incorporation of radioactive amino acids into protein. *Biochem Biophys Res Commun* **3**, 540–543.
- McCaul, T. F. (1991).** The developmental cycle of *Coxiella burnetii*. In *Q Fever: the Biology of Coxiella burnetii*, pp. 223–258. Edited by J. C. Williams & H. A. Thompson. Boca Raton, FL: CRC Press.
- McClarty, G. & Qin, B. (1993).** Pyrimidine metabolism by intracellular *Chlamydia psittaci*. *J Bacteriol* **175**, 4652–4661.
- McClarty, G. & Tipples, G. (1991).** In situ studies on incorporation of nucleic acid precursors into *Chlamydia trachomatis* DNA. *J Bacteriol* **173**, 4922–4931.
- Paretsky, D., Downs, C. M., Consigli, R. A. & Joyce, B. K. (1958).** Studies on the physiology of rickettsiae I. Some enzyme systems of *Coxiella burnetii*. *J Infect Dis* **103**, 6–11.
- Parker, C. T., Kloser, A. W., Schnaitman, C. A., Stein, M. A., Gottesman, S. & Gibson, B. W. (1992).** Role of *rfaG* and *rfaP* genes in determining the lipopolysaccharide core structure and outer membrane surface properties of *Escherichia coli* K-12. *J Bacteriol* **174**, 2525–2538.
- Pine, L., Franzus, M. J. & Malcolm, G. B. (1986).** Guanine is a growth factor for *Legionella* species. *J Clin Microbiol* **23**, 163–169.
- Pisoni, R. L. & Thoene, J. G. (1989).** Detection and characterization of a nucleoside transport system in human fibroblast lysosomes. *J Biol Chem* **264**, 4850–4856.
- Pisoni, R. L. & Thoene, J. G. (1991).** The transport system of mammalian lysosomes. *Biochim Biophys Acta* **1071**, 351–373.
- Redd, T. (1986).** *Secreted protein of Coxiella burnetii*. PhD dissertation, West Virginia University.
- Redd, T. & Thompson, H. A. (1995).** Secretion of proteins by *Coxiella burnetii*. *Microbiology* **141**, 363–369.
- Samuel, J. E., Frazier, M. E. & Mallavia, L. P. (1988).** Stability of plasmid sequences in an acute Q-fever strain of *Coxiella burnetii*. *J Gen Microbiol* **134**, 1795–1805.
- Schramek, S. & Mayer, H. (1982).** Different sugar compositions of lipopolysaccharides isolated from phase I and pure phase II cells of *Coxiella burnetii*. *Infect Immun* **38**, 53–57.
- Schramek, S., Radziejewska-Lebrecht, J. & Mayer, H. (1985).** 3-C-branched aldoses in lipopolysaccharide of phase I *Coxiella burnetii* and their role as immunodominant factors. *Eur J Biochem* **148**, 455–461.
- Silverman, D. J., Fiset, P. & Wiseman, C. L., Jr (1979).** Simple, differential staining technique for enumerating Rickettsiae in yolk sac, tissue culture extracts, or purified suspensions. *J Clin Microbiol* **9**, 437–440.
- Thompson, H. A. (1988).** Relationship of the physiology and composition of *Coxiella burnetii* to the *Coxiella*-host cell interaction. In *Biology of Rickettsial Diseases*, vol. II, pp. 51–78. Edited by D. H. Walker.
- Tjaden, J., Winkler, H. H., Schwoppe, C., Van Der Laan, M., Mohlmann, T. & Neuhaus, H. E. (1999).** Two nucleotide transport proteins in *Chlamydia trachomatis*, one for net nucleoside triphosphate uptake and the other for transport of energy. *J Bacteriol* **181**, 1196–1202.
- Vishwanath, S. & Hackstadt, T. (1988).** Lipopolysaccharide phase variation determines the complement-mediated serum susceptibility of *Coxiella burnetii*. *Infect Immun* **56**, 40–44.
- Waag, D. M., Williams, J. C., Peacock, M. G. & Raoult, D. (1991).** Methods of isolation, amplification, and purification of *Coxiella burnetii*. In *Q Fever: the Biology of Coxiella burnetii*, pp. 73–115. Edited by J. C. Williams & H. A. Thompson. Boca Raton, FL: CRC Press.
- Walsh, A. G., Matewish, M. J., Burrows, L. L., Monteiro, M. A., Perry, M. B. & Lam, J. S. (2000).** Lipopolysaccharide core phos-

phates are required for viability and intrinsic drug resistance in *Pseudomonas aeruginosa*. *Mol Microbiol* **35**, 718–727.

Willems, H., Jaeger, C. & Baljer, G. (1998). Physical and genetic map of the obligate intracellular bacterium *Coxiella burnetii*. *J Bacteriol* **180**, 3816–3822.

Williams, J. C., Peacock, M. G. & McCaul, T. F. (1981). Immunological and biological characterization of *Coxiella burnetii*, phases I and II, separated from host components. *Infect Immun* **32**, 840–851.

Winkler, H. H. (1976). Rickettsial permeability: an ADP-ATP transport system. *J Biol Chem* **251**, 389–396.

Winkler, H. H. (1986). Membrane transport in rickettsiae. *Methods Enzymol* **125**, 253–259.

Winkler, H. H., Daugherty, R. & Hu, F. (1999). *Rickettsia prowazekii* transports UMP and GMP, but not CMP, as building blocks for RNA synthesis. *J Bacteriol* **181**, 3238–3241.

Wisseman, C. L., Jr, Fiset, P. & Ormsbee, R. A. (1967). Interaction of rickettsiae and phagocytic host cells. V. Phagocytic and opsonic interactions of phase I and phase II *Coxiella burnetii* with normal and immune human leukocytes and antibodies. *J Immunol* **99**, 669–674.

Zuerner, R. L. & Thompson, H. A. (1983). Protein synthesis by intact *Coxiella burnetii* cells. *J Bacteriol* **156**, 186–191.

Received 31 October 2001; revised 23 March 2002; accepted 15 April 2002.