Secretion of proteins by *Coxiella burnetii*

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Viable *Coxiella burnetii* organisms were isolated from the culture medium of persistently infected Baby Hamster Kidney (BHK-21) fibroblasts. When these organisms were incubated in host-cell-free medium at low pH, some of the *de novo*-synthesized protein made by the bacteria was translocated to the exterior of the cell. The exported protein was detectable after 2–7 h incubation at 37 °C. No evidence was found to suggest that protein accumulation in the medium was due to leakiness caused by cell damage. Both DCCD (dicyclohexylcarbodiimide) and CCCP (carbonyl cyanide *m*-chlorophenylhydrazine) inhibited the process to some extent. Exported protein was represented largely by three polypeptides with molecular masses of 34, 24 and 12 kDa. *De novo*-synthesized proteins corresponding to these molecular masses were not detected in cytoplasmic fractions, but a membrane fraction might possess a similar form. It was concluded that a physiological process of protein translocation occurred in *C. burnetii* during acid activation in a defined medium. Organisms that were extracted directly from the cytoplasm of infected fibroblasts by a mechanical disruption procedure were also active in *de novo* protein synthesis; however they exported much less of the protein.

Keywords: *Coxiella burnetii*, protein secretion, intracellular bacteria

INTRODUCTION

Coxiella burnetii is an obligate intracellular prokaryotic parasite and the aetiological agent of Q fever in humans. The organism grows within the phagolysosomal compartment in eukaryotic hosts (Burton *et al.*, 1971; Hackstadt & Williams, 1981a; Akporiaye *et al.*, 1983). Metabolic activity, but not growth, can be detected when the organism is incubated in defined media that contain oxidizable substrates and a sufficient hydrogen ion concentration (Hackstadt & Williams, 1981a; Zuerner & Thompson, 1983). This is called acid activation of metabolism. During acid activation in media containing low concentrations of glucose, glutamate and amino acids, *C. burnetii* synthesizes RNA and at least 30 polypeptide species (Zuerner & Thompson, 1983; Chen *et al.*, 1990).

The functions and activities of proteins on the *C. burnetii* cell surface are likely to be important for understanding aspects of its intracellular survival and growth (Williams, 1981; Williams & Waag, 1991). Basic knowledge of the

synthesis and export of proteins to the outer membrane, or into the organism's exterior growth environment, is therefore of interest. It seemed reasonable to assume that some of the polypeptides synthesized during acid activation of *C. burnetii* *in vitro* may be transported to membranous and extracellular compartments. If so, study of the synthesis and translocation of proteins in *C. burnetii* during acid activation in a host-cell-free, defined medium could provide a convenient alternative to the more difficult approach with studies of protein translocation performed *in vivo*, i.e. during residence in eukaryotic hosts.

The present communication identifies a population of *C. burnetii* that translocates protein during acid activation of metabolism *in vitro*. Some of the proteins synthesized *de novo* are found in the membrane fraction. A few polypeptides are exported from the cell by an active process.

METHODS

Organisms and hosts. *Coxiella burnetii*, Nine Mile strain, phase I, was originally obtained from Dr David Paretsky, University of Kansas, Lawrence, KS, USA. Baby Hamster Kidney cells (BHK-21) were obtained from the American Type Culture Collection (CCL10). The fibroblasts were grown in Dulbecco's Modified Eagle's Medium (DMEM) which was amended to provide a high glucose concentration (4.5 g l⁻¹; the use of

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazine; DCCD, dicyclohexylcarbodiimide; IBHK cells, persistently infected Baby Hamster Kidney cells.

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standard Dulbecco's containing 1.0 g glucose l^{-1} will not produce the results described herein). The DMEM was supplemented with 3.7% (w/v) sodium bicarbonate and 10% (v/v) newborn calf serum, and fibroblasts were then grown under a 15% (v/v) CO_2 atmosphere. *C. burnetii* phase I organisms were passaged in guinea pigs, and spleen homogenates were used to establish persistent infections in fibroblast cultures as described previously (Zuerner & Thompson, 1983).

Growth, harvest and preparation of *C. burnetii*. Six days prior to harvest of organisms, confluent persistently infected fibroblasts (referred to as IBHK cells) were trypsinized, washed, diluted in medium, and then reseeded in new flasks such that a 1:3 dilution (1/3 split) of fibroblast density occurred. Seventy-two hours later, the medium on the IBHK monolayers was changed. After another 48 h, the medium was again changed, and two populations of *C. burnetii* were harvested 12–16 h later. This timing (of the culture feeding and splitting schedule) was important for obtaining high yields of active organisms, as was the maintenance of a 15% CO_2 atmosphere during growth.

Naturally released *C. burnetii* organisms were harvested from spent tissue culture medium by methods described previously (Zuerner & Thompson, 1983; Chen *et al.*, 1990). The resulting pellets were washed twice in buffer A (22.3 mM K_2HPO_4 , 136.7 mM KCl, 13.4 mM NaCl, 89 mM glycine, 10 mM $MgCl_2 \cdot 6H_2O$, 1.0 mM glucose, 1.0 mM glutamate, 250 mM sucrose, pH 7.0). Organisms prepared in this manner appear to be morphologically and structurally intact (McCaul *et al.*, 1991a).

Mechanically released *C. burnetii* were obtained from the remaining adherent fibroblast population as described by Zuerner & Thompson (1983). The efficiency of fibroblast lysis was 87–93%, as estimated by the trypan blue exclusion method applied to fibroblast preparations before and after vortex lysis. Unlysed cells showed little or no evidence of intracellular *C. burnetii* when assessed by Gimenez staining.

The numbers of physical particles of *C. burnetii* resulting from these harvest methods were estimated by the Silverman method (Silverman *et al.*, 1979). An 80 cm^2 monolayer culture of IBHK fibroblasts (3×10^7 host cells) yielded approximately 1×10^9 organisms in the medium (naturally released *C. burnetii*), and $3-4 \times 10^9$ organisms from disrupted host cells (mechanically released *C. burnetii*).

Labelling of *C. burnetii* proteins during growth in tissue culture. Medium was removed from IBHK cells 12 h prior to harvest. Fresh DMEM (13.5 ml, containing all 20 amino acids) plus newborn calf serum (1.5 ml) was supplemented with 0.43 ml L-[4,5- ^3H]leucine [1.0 mCi (37 MBq) ml^{-1} , 100 Ci mol^{-1}], 0.78 ml sterile distilled water and 0.15 ml of a 1 mg ml^{-1} solution of cycloheximide, and was added to each 75–80 cm^2 monolayer of IBHK cells. The IBHK cells were then incubated for 12 h at 37 °C in a 15% CO_2 atmosphere. Naturally released and mechanically released *C. burnetii* organisms were then harvested and purified as described above. These organisms, and proteins released from them, were used for protein release studies as indicated in Results.

Radiolabelling of *C. burnetii* proteins during acid activation of metabolism: basic assay. Naturally released or mechanically released *C. burnetii* organisms were suspended (separately) in cold buffer B (49 mM KH_2PO_4 , 169 mM KCl, 16 mM NaCl, 120 mM glycine, 135 mM KCl, 13.4 mM NaCl, 20 mM $MgCl_2$, 250 mM sucrose, 0.1 mM glutamate, 0.1 mM glucose, and the remaining 18 naturally occurring amino acids, each at 0.06 mM; final pH 4.5). Either L-[4,5- ^3H]leucine [1.67 Ci (61.8 GBq) $mmol^{-1}$] or L-[5- ^3H]proline [1.67 Ci (61.8 GBq) $mmol^{-1}$] was included in the 1.0 ml incubation mixtures. Alternatively, some

experiments utilized L-[U- ^{14}C]leucine [0.208 (7.7 GBq) Ci $mmol^{-1}$]. Incubations were carried out in sterile capped tubes at 37 °C under an air atmosphere, with constant shaking, for either 4, 7 or 16 h depending upon the experimental protocol. Time-course studies were performed by the removal of 50–100 μl samples at the designated times. Samples were either (1) applied directly to Whatman no. 3 filter paper discs, dried, and then processed for trichloroacetic acid (TCA)-insoluble radioactivity by the method of Mans & Novelli (1960) or (2) centrifuged, with cell pellets and supernates assayed separately for TCA-precipitable protein radioactivity as specified below. Counting efficiency for ^3H was 23–25%, and for ^{14}C , 65–70%.

Fractionation and analysis of labelled proteins. Samples (100 μl) were removed from incubation mixtures and centrifuged for 7–10 min (at 6–10 °C) with a Fisher model 235B microcentrifuge. Supernatant solutions were removed, applied to Whatman no. 3 discs and hot TCA-precipitable radioactivity analysed by liquid scintillation spectrometry (Mans & Novelli, 1960). Cell pellets containing cell-associated radioactivity were suspended in buffer A. The suspension (100 μl) was applied to discs, and TCA-precipitable radioactivity determined by the same method.

In some experiments, the *C. burnetii* cell pellets were further fractionated by a NaOH technique (Russel & Model, 1982). Cell pellets were suspended in 1 ml 0.1 M NaOH (made fresh from a 10 M stock). After vigorous mixing, the preparations were placed in an ice bath for 1 h, and were then centrifuged in a microfuge for 15 min. Supernatants were acidified by addition of 0.1 ml 100% (w/v) TCA; 1 ml 5% (w/v) TCA was added to the pellet fractions. After incubation in an ice bath for 15 min, both fractions were centrifuged for 15 min and the supernatants discarded. The pellets from each fraction (membrane or cytoplasm) were finally suspended in distilled water, applied to Whatman no. 3 discs, dried, treated with TCA, dehydrated, and radioassayed (Zuerner & Thompson, 1983; Chen *et al.*, 1990). Results were expressed as c.p.m. in a 1 ml incubation reaction, or as pmol amino acid incorporated per 10^9 *C. burnetii* organisms.

Alternatively, some samples were resuspended in electrophoresis sample buffer (lacking bromophenyl blue) and were radioassayed in a water-miscible scintillation cocktail prior to application to electrophoresis gels.

PAGE of proteins. Four different sample types were subjected to SDS-PAGE. (1) The cell supernatants containing radiolabelled polypeptides were concentrated by use of Minicon CS15 macrosolute concentrators (Amicon, 15 kDa molecular mass cutoff). Wells of concentrators were first rinsed with a 1 mg ml^{-1} solution of bovine serum albumin. Supernatant solutions (0.6 ml volumes) were added and concentrated to a final volume of approximately 50 μl . Each concentrated sample was added to an equal volume of electrophoresis sample buffer (0.16 M Tris/HCl, pH 6.8, 3.2% SDS, 32.3% glycerol, 0.45 M 2-mercaptoethanol, and 1–2 drops of 0.01% bromophenyl blue) and boiled for 5 min. (2) Cell pellets were extracted for protein by a freeze-lysis method. They were suspended in 10 mM Tris/HCl, pH 7.4, containing 1 mM $MgCl_2$, 1 mg egg white lysozyme ml^{-1} and 12 μg DNase ml^{-1} . Organisms were then lysed by five cycles of freeze-thawing in a dry ice/acetone bath and a 37 °C water bath, respectively. Nonidet P-40 was added to a final concentration of 0.6%, followed by incubation for 1 h at room temperature. Extracted protein was added to an equal volume of electrophoresis sample buffer and boiled for 5 min prior to electrophoresis. (3) The membrane (NaOH-insoluble) and (4) cytoplasmic fractions prepared as described (above) were washed once in Hanks' balanced salts solution and protein

pellets were resuspended directly in electrophoresis sample buffer and boiled for 5 min.

After cooling, boiled samples (40 µl) were electrophoresed in 6–15% linear gradient polyacrylamide slab gels at 150 V and 15 °C. After staining and destaining to locate standard markers, gels were fixed in 50% methanol/30% acetic acid for 10 min, impregnated with En³Hance (New England Nuclear) for 1 h and then washed in 3% (v/v) glycerol for 1 h. Gels were dried under vacuum and exposed to Kodak Royal X-Omat AR film at –70 °C for the times indicated.

Sources of materials. Radioisotopes were purchased from Amersham. Dulbecco's Modified Eagle's medium and newborn calf serum were obtained from GIBCO. *N,N'*-Dicyclohexylcarbodiimide (DCCD), bovine serum albumin (fraction V, no. A-7906), egg white lysozyme and DNase were purchased from Sigma. Carbonyl cyanide *m*-chlorophenylhydrazine (CCCP) was from Calbiochem. All electrophoresis reagents were obtained from Bio-Rad. En³Hance and other liquid scintillation reagents were purchased from New England Nuclear.

RESULTS

Characterization of the protein translocation process

When either naturally or mechanically released *C. burnetii* cells are incubated with oxidizable substrate and radio-labelled amino acid in buffered salts solution at pH 4.5, a significant amount of amino acid is incorporated into protein throughout a 16 h incubation period (Zuerner & Thompson, 1983; Thompson *et al.*, 1984, 1990; Chen *et al.*, 1990). In the present work, the distribution of this *de novo*-synthesized protein was determined. Secretion, release or leakage of protein synthesized *de novo* during acid activation by naturally released organisms was a consistent observation. Over a period of 16 h of activation in the presence of radiolabelled amino acid, naturally released organisms incorporated a total of between 800 and 1494 pmol leucine (per 10⁹ organisms) into total protein (sum of structural plus secreted) in five different experimental trials (results not shown; the five trials

comprised organisms harvested from five different batches of IBHK cells of various passage histories on five different dates over a period of six months). In those same trials, mechanically released *C. burnetii* were not as active, incorporating 118–390 pmol per 10⁹ organisms. The secretion process was observed regardless of the concentrations of organisms activated and the amino acid label used ([³⁵S]methionine, [³H]- or [¹⁴C]leucine, or [³H]proline are all effective in tracing secreted protein); the patterns of peptide and polypeptide species detected after electrophoresis and fluorography differed somewhat in minor details depending upon which of these precursors was used (not shown), and the quantity of protein synthesized and secreted varied proportionately with the concentration of organisms being activated (not shown). The mechanically released organisms consistently secreted little if any *de novo*-synthesized protein. In contrast, after 16 h of acid activation the naturally released organisms had secreted 13–54% (five trials) of the total *de novo*-synthesized protein.

Typical compartmentalization of incorporated leucine after this period of synthesis, in both naturally and mechanically released cells, is shown in Table 1. Approximately 28% of the total TCA-precipitable radioactivity recovered had accumulated in the medium during incubation of naturally released *C. burnetii*. In contrast, mechanically released *C. burnetii* distributed only 2.4% of the labelled protein into the medium. When activation studies were carried out with [³H]proline, very similar distributions were found: naturally released organisms secreted 26.9% and mechanically released, 6.0% (not shown). Both cell populations compartmentalized a large amount of the *de novo*-synthesized protein into a membranous, alkali-insoluble fraction (Table 1). Both retained a significant amount (usually 40–50% of the total incorporated counts) in the alkali-soluble cytoplasmic fraction.

Table 1. Distribution of proteins labelled with L-[³H]leucine during acid activation of *C. burnetii* cells

Cell type	Total incorporation*		Incorporated into proteins secreted into medium‡		Incorporated into cell-associated proteins§				Recovery (% of total)
	10 ⁻³ × C.p.m.†	% of total			NaOH-insoluble		NaOH soluble		
			10 ⁻³ × C.p.m.	% of total	10 ⁻³ × C.p.m.	% of total	10 ⁻³ × C.p.m.	% of total	
Naturally released	2054	100	569	27.7	366	17.8	837	40.7	86.2
Mechanically released	782	100	19	2.4	205	26.2	337	43.1	71.7

* Total incorporation represents hot TCA-precipitable radioactivity measured at the end of the 16 h incubation, prior to separation of cells from the reaction medium.

† Uncorrected counts are shown. One picomole of leucine is equivalent to 545 c.p.m. Counting efficiency was 25% and did not vary significantly from sample to sample.

‡ Hot TCA-precipitable counts found in the supernatant fraction after centrifugation of reaction medium.

§ Pelleted cells were fractionated as described by Russel & Model (1982). By this method, the alkali-insoluble fraction contains membrane (inner plus outer) proteins and the soluble fraction presumably contains cytoplasmic and periplasmic proteins.

Fig. 1. Long-term time study of the secretion of radiolabelled protein into the medium by naturally-released *C. burnetii* during acid activation. Proteins were labelled with L-[^3H]leucine, and supernatant protein radioactivity determined as described in Methods. In experiment 2 (filled symbols), phenylmethylsulfonyl fluoride (PMSF; 2 mM) was added to aliquots after they were removed (from acid activation mixtures) and chilled, to reduce protease activity prior to radioassay. The presence of 2.0 mM PMSF during activation severely inhibited leucine incorporation. ●, ○, Acid activation at pH 4.5; ■, □, control incubations at pH 7.0.

A time-course of protein translocation to the medium during acid activation of naturally released organisms was established (Fig. 1). Except in experiments designed specifically to study the kinetics and energetics of secretion (see below), the extracellular accumulation of protein was usually detectable only after 2 h or more of incubation. The rate of protein accumulation in the medium thereafter increased, especially between 2 and 4 h of incubation. From these results, which were also reasonably consistent, it was suspected that protein secretion competence in these organisms developed only after 2 h or more of metabolic activation (acid activation) within the defined medium.

The fate of protein synthesized (and radiolabelled *in situ*) by *C. burnetii* during a normal growth cycle in tissue culture fibroblasts was also investigated. IBHK cells in monolayer culture were labelled for 12 h with [^3H]leucine in the presence of cycloheximide (to reduce host-cell protein synthesis). Radiolabelled, naturally released *C. burnetii* cells were allowed to accumulate in the medium, were harvested by the usual protocol, and were then acid-activated under precisely the same conditions as described above, except that these activations contained no radiolabelled amino acids. The experiment also included measurement of prelabelled protein lost from mechanically released *C. burnetii*; the latter served as a negative control. For both cell types (naturally and mechanically released), only small quantities (2.1% and 4.0%, respectively) of prelabelled cellular protein were recovered

Table 2. Effect of inhibitors on the distribution of L-[^{14}C]leucine into TCA-precipitable radioactivity

Conditions used	[^{14}C]Leucine incorporated*	
	Cell-associated	In medium
Complete system		
pH 7.0	2.08	9.3
pH 4.5	41.81	29.6
Amino acids omitted†	2.5	9.3
Glucose omitted	26.9	18.1
High glucose (2 mM)	105.5	39.4
Plus chloramphenicol	4.2	10.7
Plus DCCD	5.56	8.1
Plus CCCP	1.67	6.6

* Values are expressed as pmol [^{14}C]leucine incorporated per 10^9 cells, during a 7 h acid-activation incubation using standard conditions (see text).

† Only radiolabelled leucine was added; no other amino acids were included, labelled or otherwise.

from the medium following a 4 h incubation. The results were similar when non-radiolabelled protein synthesis was inhibited using chloramphenicol during the 4 h incubation (not shown). These results indicate that cellular integrity was maintained during the harvesting and incubation procedures and that large amounts of prelabelled protein were not lost from either *C. burnetii* cell type.

Effect of metabolic inhibitors on protein accumulation

The data presented thus far are consistent with a physiological protein secretion mechanism in naturally released *C. burnetii*. But it was possible that an extracellular or cell-surface-located reaction converted ^3H in [^3H]leucine or [^3H]proline from a TCA-soluble to TCA-precipitable form, or that some other conversion, such as the extremely tight chelation of radiolabelled amino acid by a periplasmic binding protein, might explain some of these results. To test this possibility, the effect of inhibitors, amino acids and glucose upon the distribution of L-[U- ^{14}C]leucine into insoluble fractions was investigated. Naturally released *C. burnetii* were incubated in defined medium for 7 h under the various conditions shown (Table 2), and cell pellets recovered. All variables tested had profound effects upon the amount of TCA-insoluble radioactivity that was recovered in both the medium and the cell pellet (Table 2), but in all cases the quantities of radioactive protein found in *C. burnetii* supernates changed in parallel with those found in the pellet (cellular) fractions. Thus, appearance of [^{14}C]leucine in the extracellular, TCA-precipitable fraction was dependent upon cytoplasmic protein synthesis (it was inhibited by chloramphenicol) and energy metabolism (it

Fig. 2. PAGE analysis of *de novo*-synthesized proteins found in extracellular, membrane and cytoplasmic compartments after acid activation of naturally-released *C. burnetii*. Lane A, NaOH-insoluble (membrane) proteins; lane B, NaOH-soluble (periplasmic and cytoplasmic) proteins; lane C, supernatant proteins secreted by naturally released *C. burnetii*; lane D, supernatant proteins secreted by mechanically released *C. burnetii*.

was inhibited by the membrane ATPase inhibitor DCCD and by the proton ionophore CCCP), and also required the presence of metabolic substrates (amino acids including glutamate, plus glucose). The data, obtained from an experiment where [^{14}C]leucine rather than [^3H]leucine was used, thus argue against an extracellular process being responsible for the ^3H incorporation observed in other experiments. Rather, *de novo* protein synthesis was required prior to the appearance of TCA-precipitable radioactivity in the medium.

Characterization of translocated proteins

Proteins which had accumulated in the medium during a 16 h period of acid activation were concentrated and examined by SDS-PAGE and fluorography. Only a few major labelled bands, corresponding to molecular masses of 34, 24 and 12 kDa, were detected (Fig. 2, lane C). The presence of low-molecular-mass bands cannot be precisely explained. The procedure used to concentrate supernatant samples should have excluded fragments smaller than 15 kDa. These smaller fragments may represent residual quantities of a protein initially present in much larger

amounts or, alternatively, components of a larger protein that dissociates during sample preparation for electrophoresis. The mechanically released organisms secreted much less protein (see also Fig. 1), but produced a qualitatively similar pattern of proteins (Fig. 2, lane D), except that proportionally less of the components of lowest molecular mass were evident.

In order to determine if there were correlations in molecular mass between secreted proteins and those found in either membrane or cytoplasmic fractions, cellular pellets were extracted by the NaOH fractionation method. Alkali-insoluble and soluble fractions were boiled in SDS/2-mercaptoethanol, electrophoresed, and fluorographed. The membrane fraction (alkali-insoluble, Fig. 2, lane A) contained two major labelled species of approximately 26 and 20 kDa; several minor labelled bands, ranging from about 90 to 10 kDa, were also evident. Cytoplasmic fraction proteins are shown in Fig. 2, lane B. It is clear that the heavily labelled, 26 kDa protein associated with the membrane (lane A) has no obvious size counterpart in the cytoplasm (lane B). Overall, there was little size correlation between labelled species found in the cytoplasmic fraction when compared with the membrane fractions. Further, except for the 24 kDa protein, there was little size similarity between extracellular, secreted proteins and those found in the cytoplasmic compartment.

DISCUSSION

It was originally hypothesized that protein release by *C. burnetii* represented nonspecific leakage. The present experiments were designed to test this hypothesis, according to which it would be predicted that (i) energy poisons or proton ionophores should not affect protein release; (ii) proteins labelled *in situ* during tissue culture growth should be released during subsequent acid-activation incubation; (iii) electrophoresis patterns of released proteins should shadow or resemble those of the cytoplasmic proteins; (iv) the leakage of radiolabelled protein to the exterior should be detectable as soon as cytoplasmic protein becomes radiolabelled by *de novo* synthesis. The data that have been obtained do not accord with the hypothesis. Instead, they indicate specific secretion of selected proteins synthesized *de novo* during acid activation. Mechanically released cells secrete almost no protein. Development of competence for secretion in naturally released cells appears to be time dependent, occurring only after 2–4 h of acid activation. Activation by low pH does not appear to damage organisms, since prelabelled protein does not leak from cells. Also, the secreted protein pattern on gels does not mirror that of the cytoplasmic proteins, eliminating a general leakage phenomenon.

The naturally released population of *C. burnetii* has been previously examined by electron microscopy (McCaul *et al.*, 1991a; Redd, 1986). Evidence for damaged organisms was not found in those studies; however, naturally released organisms in various stages of differentiation,

from large to small cell variants, were obvious. A connection between a differentiation stage and protein secretion therefore cannot be ruled out.

It was previously reported that the 58 kDa *htpB* gene product, which is a heat-shock protein in *C. burnetii* and is analogous to the *E. coli groEL* protein, dissociates from naturally released *C. burnetii* following induction of its synthesis *in vitro* (Thompson *et al.*, 1990). Those studies also showed that a predominant, non-heat-shock protein of approximately 28 kDa is synthesized and secreted during incubations of naturally released *C. burnetii* at 21 °C and, to a lesser extent, at 42 °C. Thus the data in this report, as well as those reported previously from this laboratory, are consistent in the observation that naturally released *C. burnetii* organisms are protein secretors (Thompson *et al.*, 1984, 1990). A specific and small subset of proteins, rather than a random group, seems to participate in this translocation. Most notably, the synthesis, translocation and secretion of a protein (or a set of polypeptides) variously estimated on gels to be 24–29 kDa in size has been consistently observed during acid activation of *C. burnetii* organisms harvested from tissue culture media. It has been suggested that the protein may be the *com1* gene product, which is an outer-membrane protein in *C. burnetii* (Hendrix *et al.*, 1993). *com1* has a motif that resembles the active site of a protein disulfide oxidoreductase (Hendrix *et al.*, 1993). Alternatively, the discovery of a similar-sized *C. burnetii* surface protein, termed simply the 29.5 kDa protein, may instead be related to the present observations; the 29.5 kDa protein is probably not *com1* (McCaul *et al.*, 1991b).

Organisms obtained by intentional shear lysis (mechanically released) of infected host fibroblasts do not secrete significant amounts of protein. If this is indicative of the culture stage from which they were obtained, i.e. phagolysosome-bound organisms, and if gene expression and cellular processes observed during acid activation are an accurate reflection of the stage from which the organisms were previously obtained, then a possible conclusion is that the intracellular organisms in these long-term persistent infections are not protein secretors. Thus phagocytosed *C. burnetii* might secrete stage-specific proteins primarily after the initial pH shock of phagosome–lysosome fusion and only after traversing an extracellular stage. Organisms may not secrete, or may do so less actively, during later stages of growth and development within the phagolysosome. Alternatively, it is possible that the protein secreted represents a subset of gene products not made *in vivo*, but unique to acid activation *in vitro*.

Invasive bacteria might be expected to express and secrete proteins primarily as a consequence of their interactions with hosts. Some of these proteins may modify host function (Thompson, 1988; Small *et al.*, 1994). Furthermore, *Coxiella* might synthesize and secrete proteins specific for its infectious cycle or developmental cycle (McCaul *et al.*, 1991a). How these potential protein functions relate to the present observations remains

uncertain. However, a means by which to study and obtain secreted protein has been established, and this may benefit studies of pathogenic functions and of host interaction.

ACKNOWLEDGEMENTS

Initial phases of this work were supported by grant PCM-7903282 from the National Science Foundation. We are grateful for support from the Biomedical Research Support Committee, WVU, to finish the project. We thank Christopher Kolanko for technical assistance.

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Received 9 June 1994; revised 15 September 1994; accepted 24 October 1994.