Isolated *Coxiella burnetii* synthesizes DNA during acid activation in the absence of host cells

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Two populations of *Coxiella burnetii* were isolated from fibroblast tissue cultures and examined for their ability to synthesize DNA when incubated in a defined medium. Both the populations released by mechanical lysis of heavily infected host cells, as well as those recovered from the tissue culture medium, incorporated $H_3^{32}PO_4$ into DNA. Incorporation occurred at pH 4.5 but not at pH 7.0, and proceeded for 12–15 h. When incorporation of [3H]thymidine was studied, only the organisms obtained by mechanical lysis of host cells were active. Those which had been released by natural means into the tissue culture medium, and then recovered for study, did not incorporate precursor thymidine but were extremely active in protein biosynthesis. In mechanically released organisms, thymidine incorporation was inhibited immediately by rifamycin (40 μM) and hydroxyurea (10 mM), but it was not affected by chloramphenicol (310 μM) until 4 h after addition of the drug. Incorporation of $H_3^{32}PO_4$ by both populations of organisms was also inhibited by rifamycin, chloramphenicol and hydroxyurea, but the time sequence of inhibition differed. Southern hybridization utilizing ^{32}P -labelled DNA suggested that both populations synthesized authentic chromosomal DNA sequences, as well as QpH1 plasmid DNA, during acid activation of metabolism.

Introduction

Coxiella burnetii is an obligate intracellular bacterium and the agent of Q fever in humans. The organism is a moderate acidophile (Hackstadt, 1983; Hackstadt & Williams, 1981a), and parasitizes the phagolysosomal compartments of animal-host cells (Akporiaye *et al.*, 1983; Burton *et al.*, 1971, 1978; Hackstadt & Williams, 1981a). Although *C. burnetii* cannot be cultured in axenic media, both catabolic and anabolic reactions can be measured outside host cells in defined media, provided that the pH is in the range 4.0–5.5 (Hackstadt, 1983; Hackstadt & Williams 1981a, b, c, 1983; Hendrix & Mallavia, 1984; Thompson *et al.*, 1984; Zuerner & Thompson, 1983). Success of the latter procedure, termed 'acid activation' of metabolism, is explained at least in part by energization of *C. burnetii* membranes

and of substrate transporters (proton symport: Hackstadt & Williams, 1983; Hendrix & Mallavia, 1984). Thus, when the organism is maintained *in vitro* at pH 4.5, in the presence of an oxidizable substrate such as glutamate, the substrate is transported and metabolized, ATP is synthesized, and the energy charge approaches that presumably required for bacterial growth [Hackstadt & Williams, 1981b, 1983; see Thompson (1988) for review]. Indeed, rapid isolation of *C. burnetii* from fibroblast cultures, followed by acid activation *in vitro* in media containing glucose, glutamate and the other amino acids, results in sustained protein biosynthesis by the organism (Zuerner & Thompson, 1983). Under these conditions, approximately 24 polypeptide species are synthesized *de novo* by *C. burnetii* (Thompson *et al.*, 1984; Zuerner & Thompson, 1983); over 100 are seen if resolved on two-dimensional gels (R. L. Zuerner & H. A. Thompson, unpublished). The translation measured during acid activation required *de novo* RNA synthesis. This suggests that *C. burnetii* mRNAs do not survive

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even a rapid isolation procedure (Zuerner & Thompson, 1983). Transcripts synthesized during acid activation hybridize to both chromosomal and plasmid DNA (Frazier *et al.*, 1985).

It is clear, then, that both gene transcription and translation occur in *C. burnetii* during artificial acid activation outside host cells. Since these biosyntheses are generally agreed to be the most energy-demanding reactions carried out by bacteria (Ingraham *et al.*, 1983), it can be tentatively concluded that at least some of the cells in the *C. burnetii* populations studied are not energy-limited. It is also apparent that ATP production and protein synthesis are not equivalent to growth. Growth of *C. burnetii* during acid activation has not been detected. At most, a 15–30% increase in *C. burnetii* protein biomass is observed over a 24 h incubation period (Zuerner & Thompson, 1983). Our current concept of the *C. burnetii* cell under these conditions is that of an organism locked into a physiological maintenance state: few if any cells divide, and most cells probably do not traverse a significant portion of the cell cycle. Previous reports showed that acid activated organisms could incorporate nucleic acid precursors, including thymidine (Hackstadt & Williams, 1981c). In an attempt to further elucidate the nature of the clamp on cell division in *C. burnetii* outside the host, DNA synthesis in the organism was investigated.

Two populations of *C. burnetii* can be harvested from persistently infected BHK-21 fibroblasts grown in monolayer culture (Zuerner & Thompson, 1983). One population arises by either natural lytic events of heavily infected hosts or by exocytosis from infected cells. Organisms are allowed to accumulate in the medium over a 12–24 h period and then are harvested directly. The other population is obtained by mechanical (shear) lysis of infected host cells (Zuerner & Thompson, 1983). As shown here, the two populations differ somewhat in their suitability for specific biochemical investigations. The present report gives evidence that only one *C. burnetii* population incorporates [³H]thymidine into chromosomal DNA during acid activation of metabolism. However, both populations incorporate H₃³²PO₄ into chromosomal and plasmid DNA. The data suggest that DNA metabolism comprising either chromosomal replication or repair, or both, can occur in *C. burnetii* outside (parasitized) host cells.

Methods

Growth of fibroblasts. Baby hamster kidney cells (BHK-21) were obtained from the American Type Culture Collection (ATCC CL10). The fibroblasts were grown in monolayer cultures in Nunc 80 cm² flasks with Dulbecco's Modified Eagle's medium (DME) containing 4 g glucose l⁻¹ and supplemented with 0.37% sodium bicarbonate and

10% (v/v) heat-inactivated newborn calf serum (Gibco). Cells were adapted to growth in a 15% (v/v) CO₂ atmosphere at 37 °C prior to infection.

Infection of fibroblasts and growth of *C. burnetii*. Nine Mile strain phase I clone 7 [CB9MIC7; 307 guinea pig passages, tissue-culture-cloned, followed by three egg passages (Williams *et al.*, 1981)], was suspended in phosphate-buffered saline (PBS; 50 mM-potassium phosphate, pH 7.4, 0.9%, w/v, NaCl) containing 5 mM-glutamate and 250 mM-sucrose. Approximately 1 × 10⁸ organisms in 0.5 ml were used to infect each 80 cm² fibroblast monolayer. A second source of *C. burnetii* Nine Mile strain phase I (uncloned), obtained originally from Dr D. Paretsky (University of Kansas, USA), was also used in some of these experiments. All ensuing procedures used to establish persistent infections with either source of organism were done as described previously (Zuerner & Thompson, 1983). Infected BHK fibroblasts were cultivated for approximately 2 weeks to establish persistent infections. Thereafter, cultures of infected fibroblasts could be carried continuously through 10–15 passages without addition of either *C. burnetii* or fibroblasts. *C. burnetii* organisms were counted as described previously (Silverman *et al.*, 1979).

C. burnetii Nine Mile strain phase II clone 4 (CB9MIIC4) was cultured and isolated as described before (Vodkin *et al.*, 1986).

Harvesting populations of *C. burnetii*. Six days prior to harvest, confluent cultures of infected BHK-21 fibroblasts were trypsinized, diluted 1:3 with medium and reseeded into new 80 cm² flasks (1:3 split). After 72 h, the medium (12 ml DME with 10% serum) was changed on the newly confluent monolayers. After an additional 48 h (and 24 h prior to harvest of *C. burnetii*) the tissue culture medium was changed a second time. This timing, and the maintenance of a 15% CO₂ atmosphere over cultures of infected fibroblasts, were important for obtaining consistently high yields of active organisms.

C. burnetii organisms released by natural events (either host cell lysis or exocytosis) were harvested directly from the tissue culture medium after the 24 h accumulation period and washed by centrifugation (Zuerner & Thompson, 1983). Rinsed monolayers were then trypsinized, and mechanically lysed to release intracellular *C. burnetii*, which were then partially purified by differential centrifugation (Zuerner & Thompson, 1983). Organisms, obtained either from the medium or from host cells, were finally suspended in ice-cold glycine/phosphate buffer (120 mM-glycine, 169 mM-KCl, 16 mM-NaCl, 250 mM-sucrose, and 49 mM-KH₂PO₄ for pH 4.5 or 49 mM-K₂HPO₄ for pH 7.0) at a concentration of approximately 1–2 × 10¹¹ organisms ml⁻¹.

Measurement of DNA synthesis in a defined medium. Suspensions of *C. burnetii* were added such that they represented one-tenth the final volume of acid activation incubation mixtures. The final concentrations of chemical components were 250 mM-sucrose, 5 mM-glucose, 5 mM-glutamate, 77 mM-glycine, 0.1 mM of the 18 other amino acids, 118 mM-KCl, 11.9 mM-NaCl, 20 mM-MgCl₂, 17.4 mM-KH₂PO₄ (pH 4.5) or 17.4 mM-K₂HPO₄ (pH 7.0) and 0.6 mM-thymidine, containing 100 µCi (3.67 MBq) [methyl-³H]thymidine (Amersham or ICN) per ml⁻¹ of incubation mixture. The final specific radioactivity of [³H]thymidine was 166 Ci mol⁻¹ (6.14 TBq mol⁻¹). Reactants were incubated with moderate shaking at 37 °C under an atmosphere of air. Samples (50 or 100 µl) were withdrawn at the times indicated and applied to Whatman no. 3 filter paper discs. The radioactivity incorporated into nucleic acids precipitated with 10% (w/v) TCA was determined by standard liquid scintillation procedures. The efficiency of counting was approximately 25%. Results of representative experiments expressed as c.p.m. per sample are shown. Repeat experiments resulted in less than ±15% error from those shown in Figs. 1–3.

The chemical homogeneity of [methyl-³H]thymidine used was verified by paper chromatography (Gasparic & Characek, 1978).

Extraction of *C. burnetii* DNA. To extract DNA probes for hybridization procedures, $1-2 \times 10^{11}$ organisms were first labelled by incubation with 1–2 mCi (37–64 MBq) $H_3^{32}PO_4$ under conditions as described for acid activation of organisms (above), except that the phosphate content of the incubation media was decreased to one-tenth that used for 3H -labelling. After incubation for 12 h, *C. burnetii* organisms were pelleted by centrifugation at 30000 r.p.m. in a Spinco 40 rotor (Beckman), and DNA was extracted by an alkaline phenol method described previously (Vodkin *et al.*, 1986). DNA was dissolved in 10 mM-Tris/HCl (pH 8.0) containing 1 mM- Na_2EDTA and stored at 4 °C until used.

Blot hybridizations. Chromosomal DNAs from phase I organisms (CB9MIC7) and from phase II organisms (CB9MIIC4) were obtained by alkaline phenol extraction (Vodkin *et al.*, 1986). The entire DNA sequence found in plasmid QpH1 (obtained from CB9MIC7 and designated pCB9MIC7) was cloned in the cosmid pH79 to yield pQpH201, while a plasmid found in CBKAVI, a *C. burnetii* heart valve strain, was also cloned completely into pH79 to yield pQAV102 (M. H. Vodkin & J. C. Williams, unpublished). The procedures used to construct these recombinants were similar to those described for making a *C. burnetii* genomic library in the cosmid vector pH79, as reported previously (Vodkin & Williams, 1986). Chromosomal DNAs were restricted with *Hae*III, whereas plasmid DNAs were restricted with *Eco*RI. Restriction digestions were done according to the specifications of the supplier (BRL). Electrophoresis was done in 0.9% agarose at 60 V for 2.5 h. DNA blotting was done by the procedure of Southern (1975). DNA probes used were ^{32}P -labelled DNA obtained by alkaline phenol extraction of DNA from mechanically released and naturally released *C. burnetii* after their acid activation in the presence of $H_3^{32}PO_4$ (see above).

Results

Thymidine incorporation by mechanically derived *C. burnetii*

C. burnetii organisms extracted from the intracellular compartments of infected host fibroblasts (by mechanical shear lysis) actively incorporated $[^3H]$ thymidine into the TCA-precipitable fraction when incubated in the defined medium at pH 4.5. This incorporation of $[^3H]$ thymidine typically proceeded for 12–15 h before cessation and did not occur at pH 7.0 (Fig. 1). The naturally released cells were not active in $[^3H]$ thymidine incorporation under the conditions tested (Fig. 1). Potential inhibitory effects of the naturally released cells, or of some inhibitor in that preparation of cells, was investigated by combining the two cell types. The presence of the naturally released cells had little effect, if any, on thymidine incorporation by mechanically released cells (Fig. 1). The naturally released *C. burnetii* did, however, incorporate $[^3H]$ leucine into protein (Fig. 2). Hence, the naturally released organisms used were biosynthetically active (Zuerner & Thompson, 1983) but were unable to incorporate $[^3H]$ thymidine into DNA.

The isolation procedure used to obtain mechanically released organisms from the fibroblast host cells did not result in pure organisms free of host material (Zuerner &

Fig. 1. Time-course of $[methyl-^3H]$ thymidine incorporation by *C. burnetii* during acid activation. Organisms were propagated and prepared for host-cell-free incubations as described in Methods. Incubations were done at 37 °C in 1 ml vols, from which 50 μ l samples were withdrawn at the indicated times; each sample contained approximately 5.5×10^8 organisms. One pmol of $[^3H]$ thymidine was equivalent to 92 c.p.m. Incorporation by mechanically released organisms (○, ●), by naturally released organisms (△, ▲), and by mixtures of the two cell types (△, ▲) was measured at pH 4.5 (open symbols) and at pH 7.0 (filled symbols).

Fig. 2. Incorporation of L-(4,5- 3H)leucine by naturally released *C. burnetii*. Organisms (CB9MIC7), propagated as described in Methods, were suspended evenly in pH 4.5 buffer and the suspension added to an incubation mixture without isotope. The mixed suspension of cells was then divided into two equal portions, one of which received $[methyl-^3H]$ thymidine (0.6 mM; 166 Ci mol $^{-1}$ (6.14 TBq mol $^{-1}$)) and the other received L-(4,5- 3H)leucine (0.1 mM; 992 Ci mol $^{-1}$ (36.7 TBq mol $^{-1}$)). Incubation was done under standard conditions. Samples (50 μ l, each containing 7×10^8 organisms) were withdrawn at the indicated times and assayed for incorporated radioactivity, by either the hot-TCA (leucine) or cold-acid (thymidine) extraction technique. One pmol of $[^3H]$ leucine was equivalent to 545 c.p.m. ---, Leucine incorporation; —, thymidine incorporation. Incubations at pH 4.5 are represented by open symbols; identically prepared suspensions incubated at pH 7.0 are indicated by filled symbols.

Table 1. [^3H]Thymidine incorporation by *C. burnetii*

Incorporation of [^3H]thymidine by mechanically released *C. burnetii* and by a sham preparation of BHK-21 cells was determined during incubation in a defined medium (see Methods). Equivalent numbers of host BHK-21 cells were used for sham preparations and for the preparation of mechanically released *C. burnetii* organisms.

Time (h)	Relative [^3H]thymidine incorporation (c.p.m.)			
	Sham preparation		<i>C. burnetii</i> (Mechanically-released)	
	pH 7.0	pH 4.5	pH 7.0	pH 4.5
0	1073	683	1078	788
12	1683	1594	1482	15963

Thompson, 1983). Rather, the procedure was based upon the development of assay conditions for *C. burnetii* activity exclusive of host nuclear or mitochondrial DNA synthesis. To ascertain the contribution (if any) of contaminating host cell components to the total incorporation observed, sham preparations extracted from uninfected BHK-21 fibroblasts were compared with those obtained from infected cells (Table 1). Material obtained by applying identical extraction procedures to uninfected cells was also incubated and found to have only limited capability for [^3H]thymidine incorporation at pH 4.5. Only a small increment of incorporation (above time-zero backgrounds) was observed in the sham preparations incubated at pH 7.0; incorporation by *C. burnetii* (after extraction from an equal number of infected host cells) was ten times higher than in the sham preparations (Table 1). In other trials, purified and heat-killed *C. burnetii* organisms were added to uninfected fibroblasts and then re-isolated. These preparations also showed little or no activity (data not shown).

Effects of inhibitors on DNA synthesis

The incorporation of [^3H]thymidine by mechanically released organisms was inhibited by hydroxyurea, a drug which affects the ribonucleotide reductase step of DNA precursor formation (Sinha & Snustad, 1972). Other inhibitors conventionally used to inhibit bacterial DNA synthesis, such as nalidixic and oxolinic acids, could not be effectively used because of their insolubility at low pH. [^3H]thymidine incorporation was also sensitive to rifamycin, which inhibits bacterial RNA polymerase. The inclusion of rifamycin in incubation mixtures at pH 4.5 resulted in an early effect on thymidine incorporation, with little thymidine uptake observed after 2 h of incubation (Fig. 3). Inclusion of chloramphenicol in the

Fig. 3. Effects of inhibitors on [$\text{methyl-}^3\text{H}$]thymidine incorporation by mechanically released *C. burnetii* CB9MIC7. All inhibitors were added at time-zero. Conditions of incubation, sampling and assay were otherwise as described in Fig. 1. Each sample contained 1×10^9 *C. burnetii*. $\circ$, pH 4.5, control without inhibitors; $\triangle$, 0.31 mM-chloramphenicol; $\triangle$, 10 mM-hydroxyurea; $\blacksquare$, 40 μM -rifamycin; $\bullet$, pH 7.0, without inhibitors.

incubation mixtures had no effect upon thymidine incorporation until the fourth hour of incubation, when thymidine incorporation ceased abruptly (Fig. 3). Chloramphenicol at this concentration (0.3 mM) effectively inhibited 95–99% of leucine incorporation by mechanically released organisms (not shown: see Zuerner & Thompson, 1983). Thus the initial 4 h period of DNA synthesis by *C. burnetii* during acid activation was dependent upon an active RNA polymerase, but not upon protein synthesis. Further experiments showed that mechanically released *C. burnetii* cells were similarly sensitive to inhibition by rifamycin regardless of when the drug was added (results from additions of rifamycin made at 2, 4, 6 and 8 h are not shown).

Previous observations (S. Y. Chen, H. A. Thompson & R. L. Zuerner, unpublished; Zuerner & Thompson, 1983) suggested that naturally released organisms did not readily incorporate uridine into RNA, even though these organisms required *de novo* RNA synthesis in order to simultaneously synthesize protein. Likewise, the present observations led to the suspicion that the inability of naturally released organisms to incorporate thymidine was indicative of a thymidine transport (or diffusion) deficiency in this cell population, rather than a lack of DNA synthesis. This hypothesis was tested by doing studies utilizing a different DNA precursor, $\text{H}_3^{32}\text{PO}_4$. The results of these experiments are summarized in Table 2. Both mechanically released and naturally

Table 2. $H_3^{32}PO_4$ incorporation by *C. burnetii*

pH	Inhibitor	$H_3^{32}PO_4$ incorporation*							
		Mechanically released				Naturally released			
		Time (h) ... 0	2	4	12	0	2	4	12
4.5	None	5.9	35.3	81.0	100	22.9	22.6	64.9	100
4.5	Rifamycin	4.6	23.1	39.1	43.0	5.7	8.0	10.5	10.9
4.5	Chloramphenicol	22.9	25.6	56.7	73.8	4.6	5.8	9.5	23.4
4.5	Hydroxyurea	10.1	10.7	6.8	5.8	4.6	8.6	7.9	18.2
7.0	None	7.5	8.6	7.5	5.9	5.8	13.9	9.5	37.6

*Percentage of control without inhibitor at pH 4.5 after 12 h.

released organisms incorporated ^{32}P into TCA-precipitable, alkali-stable material, and this uptake was dependent upon a low pH. In both populations, hydroxyurea inhibited incorporation and activity was diminished in the presence of rifamycin, especially in the naturally released population. Incorporation by both populations was sensitive to chloramphenicol. Hence, a great deal of the ^{32}P incorporation by both populations depended upon both RNA and protein synthesis.

Characteristics of labelling DNA with $H_3^{32}PO_4$ and Southern blot hybridizations

Inclusion of $H_3^{32}PO_4$ in the media during acid activation resulted in a time-dependent, pH-dependent incorporation of ^{32}P by both mechanically released and naturally released organisms (Table 2). The ^{32}P -labelled DNA was extracted as described previously (Vodkin & Williams, 1986). *C. burnetii* phase I and phase II chromosomal DNA was digested with *Hae*III, and plasmid DNA was digested with *Eco*RI. The restricted DNA was then electrophoresed (Fig. 4). DNA fragments were transferred to nylon membranes (Hybond-N; Amersham), hybridized and autoradiographed. In Fig. 4B, DNA labelled during acid activation of mechanically released organisms was used as a hybridization probe. The probe hybridized to the chromosomal DNA of either phase I or phase II *C. burnetii*, such that a pattern resembling the ethidium-bromide-stained gels was evident (compare lanes 2 and 3 in Fig. 4B with those lanes in Fig. 4A). Very similar restriction patterns were observed on the autoradiograph of the *Hae*III-digested ^{32}P -labelled DNA also (not shown). The synthesized DNA also contained sequences that hybridized to fragments of the *C. burnetii* plasmid pCB9MIC7 (the QpH1 plasmid from *C. burnetii* 9MIC7 strain, lane 4) and to plasmid pCBKAVI (lane 5), although the latter hybridization was very weak. The

digestion of pQpH201 with *Eco*RI yielded 11 pieces of DNA and only the third largest piece (about 7 kb) contained vector DNA. The probe (Fig. 4B, lane 4) hybridized to three or more bands. Thus the low signal strength in this hybridization is likely to be due to the overall low amount of plasmid DNA in the probe DNA, and not to nonspecific hybridization to vector or other DNA. *Eco*RI-digested pQAV102 also yielded only one DNA fragment that contained vector DNA, and yet there were two faint hybridization regions (lane 5). The probe did not hybridize to *Eco*RI-restricted BHK-21 host-cell DNA (lane 6). Similarly, the ^{32}P -labelled DNA from naturally released organisms likewise hybridized to the same restricted DNAs after Southern transfer (not shown).

Discussion

Incorporation of $H_3^{32}PO_4$ into both populations of *C. burnetii*, and of [3H]thymidine into DNA by mechanically released *C. burnetii* only, occurred at pH 4.5 but not at pH 7.0, proceeded for approximately 12 h, and resulted in labelling of both QpH1 (pCB9MIC7) plasmid DNA and chromosomal DNA. Incorporated material was RNAase resistant and DNAase sensitive (not shown). The pH stimulation index, taken as the incorporation at pH 4.5 divided by that at pH 7.0, varied from 3 to 10. The energization of the organism, or transport of the precursors, or both, is thus dependent upon a low-pH environment. It was also established that the presence of sucrose was important for DNA synthesis (data not shown). Without sucrose in the medium, DNA synthesis was delayed for several hours and the DNA that was synthesized was unstable. This latter observation suggests that osmotically fragile organisms, likely the LCV (large cell variant) forms (McCaul & Williams, 1981), were involved in the DNA synthesis described here.

Fig. 4. Homology of DNA synthesized by mechanically released organisms during acid activation. A, ethidium bromide staining of *C. burnetii* DNAs. Digestions were done according to the specifications of enzyme suppliers. Lane 1, λ DNA digested with *Hind*III; lane 2, CB9MIC7 DNA digested with *Hae*III; lane 3, CB9MIC4 DNA digested with *Hae*III; lane 4, pQpH201 (cloned plasmid QpHI from strain CB9MIC7) digested with *Eco*RI; lane 5, pQAV102 (cloned plasmid from strain CBKAVI) digested with *Eco*RI; lane 6, uninfected fibroblast DNA digested with *Eco*RI. B, Southern blot hybridization to the agarose gel (panel A) using ³²P-labelled DNA from mechanically released *C. burnetii* as probe. DNA was labelled by incubating mechanically released *C. burnetii* organisms with $\text{H}_3^{32}\text{PO}_4$ at pH 4.5 (acid activation) and was extracted by the alkaline phenol technique. Digested, electrophoresed DNAs were transferred to Hybond nylon filters and prehybridized. Hybridization was done using 1×10^5 c.p.m. of probe, in the presence of sheared salmon sperm DNA, for 20 h at 65 °C under high stringency conditions. Film was exposed for 5 d prior development. Arrowheads indicate three visible hybridization bands in lane 4 and three barely perceptible bands in lane 5.

[³H]Thymidine incorporation by mechanically released organisms occurred only when exogenously added thymidine concentrations were high (300–600 μM). With high specific radioactivity thymidine (1–2 μM), significant labelling was not observed (not shown). This

suggests that higher concentrations of substrate are necessary in order that the organism may transport or use thymidine. During labelling with $\text{H}_3^{32}\text{PO}_4$, it was also necessary to include 600 μM -thymidine. We do not know the reason for the inability of naturally released

organisms to incorporate thymidine. Since they do synthesize DNA, it seems likely that either transport of the substrate or some early step in thymidine conversion to dTTP may be defective. We had noted before that this population is similarly poorly active in uridine incorporation (Zuerner & Thompson, 1983). The explanation for these observations may be trivial. When mechanically released organisms were incubated for 24 h in the tissue culture medium (pH 7.4) prior to acid activation, they lost most of their ability to incorporate thymidine (data not shown). We therefore believe that enzymes responsible for thymidine transport and incorporation in *C. burnetii* are unstable. It is known that other genera of obligate intracellular parasites lack some enzymes for nucleoside and nucleotide metabolism. For example, *Chlamydia psittaci* lacks thymidine kinase (Tribby & Moulder, 1966) and *Rickettsia typhi* lacks both uridine kinase and thymidine kinase (Williams & Peterson, 1976).

The genome size of *C. burnetii* Nine Mile strain phase I was found to be 1.04×10^9 Da (Myers *et al.*, 1980). The Nine Mile strain also possesses a plasmid, QpH1 (pCB9MIC7 from clone 7), that is 36 kb (2.4×10^7 Da) in size and has a copy number of three (Mallavia *et al.*, 1984; Samuel *et al.*, 1983; Vodkin *et al.*, 1986). A different plasmid, QpRS (pQKAVI), is found in *C. burnetii* isolates obtained from endocarditis patients (Samuel *et al.*, 1985; Vodkin *et al.*, 1986). In experiments not shown here, both Nine Mile sublines used replicated the QpH1 plasmid during propagation in BHK-21 fibroblasts in the presence of aphidicolin. This was determined by restriction analysis of the DNA banding at a density of 1.62 g cm^{-3} ; such DNA is readily extractable from freshly isolated, mechanically released organisms. We therefore know that organisms used for DNA synthesis experiments in the present study contain plasmid DNA (results not shown). Thus, it was not surprising that DNA labelled with ^{32}P during acid activation hybridized to at least some QpH1 (pCBMIC7) plasmid fragments in Southern blotting procedures (Fig. 4). It is therefore likely that both naturally released and mechanically released *C. burnetii* synthesize plasmid and chromosomal DNA during these incubations outside host cells.

The DNA synthesis observed depended upon a rifamycin-sensitive step. Transcription is essential for initiation of DNA replication, but not repair, in many systems. It may function for (i) synthesis of an RNA primer, e.g. replication of ColE1-type plasmids (Minden & Marians, 1985), or (ii) transcriptional activation of DNA replication, e.g. replication of *E. coli* chromosome and phage λ DNA (Baker & Kornberg, 1988; Furth & Wickner, 1983). An alternative possibility, that transcription and translation of replication protein genes is

necessary, is less satisfying because chloramphenicol did not inhibit [^3H]thymidine incorporation until after 4 h of DNA synthesis (Fig. 3). Therefore the present studies indicate that processes with properties characteristic of DNA initiation were operative in *C. burnetii* as studied here, and suggest that this synthesis is not solely DNA repair. We have calculated that the quantity of DNA made during acid activation of mechanically released organisms represented an increase of 23% after a 12 h period. The calculation assumes no dilution of isotope by intracellular pools, and therefore should be conservative.

The long time-period during which DNA is made in *C. burnetii* in the presence of chloramphenicol (4 h) does not appear to be typical of other bacterial species under normal growth conditions. If chromosome replication is indeed occurring in the present case, and if re-initiation events only, and not elongation, require protein synthesis, then the results suggest that chromosome elongation during acid activation is extremely slow. On the other hand, this long-term refractility of *C. burnetii* DNA synthesis to chloramphenicol in the medium used here might suggest that we are studying the organism under poor nutrient conditions, such that an alternative pathway of DNA synthesis is being used. Significant DNA synthesis in *E. coli* can occur in the absence of protein synthesis, in mutants that lack RNAase H, producing constitutive Stable DNA Replication (cSDR). This alternative pathway in *E. coli* may operate under certain growth conditions in wild-type cells (Von Meyenberg *et al.*, 1987). Whatever the mechanism, it is clear that *C. burnetii* isolated from their hosts do not immediately require synthesis of new proteins in order to synthesize DNA. However, RNA polymerase activity is required almost immediately upon acid activation.

We have shown, for the first time, that *C. burnetii* can synthesize DNA in the absence of host cells. However, the data do not permit us to ascertain why *C. burnetii* does not grow in the acidic, host-free medium. By definition, growth implies at least a doubling of both mass and numbers within a bacterial culture. It thus appears that *C. burnetii* replicates its DNA and yet does not finish a division cycle to form daughter cells. It is possible that this DNA synthesis observed during acid activation represents only partial chromosome replication. Alternatively, that portion of the organisms undergoing DNA synthesis in these studies may represent only a small subset of the population. Such a subset might be traversing one or more complete, but undetected, division cycles. These points require further investigation.

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