Reversibility of heat shock in *Chlamydia trachomatis*

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1. SUMMARY

The heat shock effect on chlamydia development was studied. We report here that the reversibility of the heat shock response did not depend on the stage of chlamydial morphogenesis at which transfer to high temperature occurred, and the infectivity of the particles produced was not affected significantly, so long as the heat shock exposure was not prolonged. Exposure to heat shock for more than 9 h resulted in stagnation of the growth cycle, appearance of aberrant reticulate body particles and loss of infectivity. SDS-PAGE analysis of proteins synthesized under prolonged heat shock showed increased relative abundance of heat shock proteins in common with other procaryotic organisms.

2. INTRODUCTION

Heat shock proteins (HSP) are among the most highly conserved and abundant proteins in both procaryotic and eucaryotic cells and are recognized in the immune response to a broad spectrum of pathogens. Chlamydiae are pathogens involved in a wide range of diseases: sexually transmitted diseases, ocular infections, respiratory tract infections, and possibly chronic cardiovascular and arthritic disease. *Chlamydia trachomatis* is a Gram-negative bacterium and an obligate intracellular parasite of eucaryotic cells. *C. trachomatis* replicates by a unique developmental cycle that involves two distinct forms: elementary bodies (EB), a spore-like infective form; and reticulate bodies (RB), the intracellular vegetative form. Recently, investigations of the heat shock response of *C. trachomatis* found that it resembled that of other procaryotes also [1–4]. Chlamydial genes for several heat shock proteins (HSP) have been described, and the sequences of
HSP 60 and HSP 70 have been determined [2,5–8]. However, certain aspects of the C. trachomatis heat shock response have not been sufficiently characterized. The unique developmental cycle of chlamydia prompted us to investigate the reversibility of the heat shock effects.

3. MATERIALS AND METHODS

3.1. Growth of chlamydia and in vivo labelling and analysis of chlamydial proteins

C. trachomatis L2/434/Bu was obtained from Dr. R. Cevenini (Department of Microbiology, University of Bologna, Italy). Chlamydia were grown on BGM cells in RPMI medium without methionine, containing 1 μg ml⁻¹ cycloheximide to block host protein synthesis. Cultures were infected at 37°C with m.o.i. 1-5 and transferred to 42°C at various times after infection for different time periods. Infected cultures were pulse labelled with [³⁵S]-methionine (1–5 μCi ml⁻¹) for 2 h and chased with RPMI medium containing cold methionine. Approximately 40 h post-infection, EB and RB particles were purified by the renografin method [9]. Uninfected cultures labelled and purified under similar conditions showed no cellular protein contamination.

Purified particles were lysed and analysed by 15% acrylamide SDS gel electrophoresis (PAGE) as previously described [10]. In other experiments, particles were lysed for 1 h at 37°C in PBS in the presence of 2% sarcosyl. The sarcosyl soluble fraction was separated from the precipitate by centrifugation for 1 h at 150000 × g. Thiopropyl sepharose affinity chromatography was performed as described by Bavoil et al. [3]. Infectivity assays were performed by the immunoperoxidase method (IPA) [11,12].

3.2. Electron microscopy

Samples were prepared according to the method of Biberfeld [13].

4. RESULTS

Two aspects of the reversibility of the heat shock process were studied: (a) the effect of chlamydial cell cycle when the cells were shifted to high temperature; and (b) how long the culture was kept at high temperature.

Cultures infected at 37°C were transferred to 42°C at various times after infection for different time periods. Infected cultures were pulse labelled with [³⁵S]-methionine (1–5 μCi ml⁻¹) for 2 h and chased with RPMI medium containing cold methionine. Approximately 40 h post-infection, EB and RB particles were purified by the renografin method [9]. Uninfected cultures labelled and purified under similar conditions showed no cellular protein contamination.

![Graph A](image1.png)

![Graph B](image2.png)

Fig. 1. Reversibility of the heat shock effect on chlamydial infectivity. Percent survival was measured as the relative infectivity detected by IPA as compared to control cultures grown continuously at 37°C (total of 1.4 × 10⁷ infective particles/ml). Cultures were transferred to 42°C at various times after infection as indicated, and maintained at 42°C for 2 h (A) or 12 h (B), and subsequently returned to 37°C until 40 h post infection (p.i.) at which time infectivity was measured. Hatched bars: control cultures grown continuously at 37°C; open bars: cultures returned to 37°C after exposure to 42°C; black bars: cultures grown continuously at 42°C.
ALS AND METHODS. Figure 1 (A and B) demonstrates that, with respect to infectivity, the time after infection when cultures were switched to high temperature did not play a significant role with respect to the reversibility of the process. The growth cycle of chlamydia was totally stagnated when cultures were continuously incubated at 42°C and no infectivity was recovered. Infected cultures incubated at 42°C for 2, 5 and 9 h and then returned to 37°C recovered full infectivity (Fig. 1A; 2 h incubation), whereas after 12 h at 42°C, they recovered very low infectivity (Fig. 1B). The heat shock process was irreversible after 20 h at 42°C. Purified EB incubated at 42°C for 24 h or more also lost almost all of their infectivity.

The pattern of chlamydial proteins synthesized after heat shock under growth conditions similar to those in Fig. 1 was analyzed by PAGE electrophoresis (Fig. 2). The polypeptide pattern after 2 h exposure to 42°C did not differ significantly from that of control chlamydia grown continuously at 37°C (Fig. 2A). As expected, cultures pulsed with [35S]L-methionine at various times during the growth cycle of chlamydia showed some variation in the intensity of various polypeptide bands labelled (data not shown). However, no significant difference was observed between cultures that had undergone heat shock as compared with those that had not (Fig. 2, lanes 1, 2, shown for 2 h exposure) when the length of heat shock exposure was less than 9 h (gels not shown). After 20 h at 42°C, some changes were
observed. As shown in Fig. 2 (lanes 3 and 4), the relative intensities of bands of 32 kDa, 40 kDa, 58 kDa, 70 kDa, and 98 kDa were increased. In this experiment each lane was loaded with equal amounts of infected cells. Since 20 h exposure to 42°C leads to death, many polypeptides were labelled less strongly in the heat-shocked bacteria, as expected.

Chlamydia-infected cultures exposed to heat shock at 42°C for 2 h and 20 h were examined by electron microscopy. Cultures exposed for 2 h displayed inclusion bodies with a regular distribution of elementary and reticulate bodies (Fig. 3A). Cultures heat-shocked for 5 h showed some normal inclusion bodies along with some inclusions containing a mosaic-like display of RB (Fig. 3B), whereas after 20 h cultures showed predominantly huge aberrant RB (Fig. 3C).

In order to understand whether morphological changes shown by electron microscopy were due to changes in the outer membrane complex or in the cytoplasmic lysate, we analyzed purified particles by sarcosyl treatment [3]. The sarcosyl insoluble outer membrane complex of chlamydia has been well characterized and contains proteins such as MOMP and 60 kDa that are rich in cysteine residues that undergo oxidation and reduction during various stages of morphogenesis. The EB outer membrane proteins are extensively disulfide bond cross-linked to form a supramacromolecular lattice, whereas outer membranes of RB are deficient in the 60 kDa crp, and the MOMP of RB is not extensively cross-linked [14]. Purified [35S]-labelled chlamydial particles were lysed in the presence of 1% sarcosyl, and the soluble and the insoluble fractions were analyzed by thiopropyl-sepharose affinity chromatography. This method is commonly used for purification of cysteine rich peptides and proteins [15]; it can differentiate between S-S bond containing proteins flowing through the column and polypeptides containing SH residues which attach to the column and are eluted by dithiothreitol (DTT).

In the sarcosyl insoluble fraction of particles purified after prolonged heat shock at 42°C, no crp 60 appeared in the S-S fraction. Also, very little appeared in the SH fraction, as compared to controls where crp 60 did appear in considerable quantity in the S-S containing fraction (Fig. 4). This was expected as the particle population consisted mainly of RB in heat shock treated cells, and of EB in 37°C grown controls (Fig. 3, and see ref. [16]). MOMP appeared as a strong single band in heat-shock-treated cells and as a double band in controls.

Sarcosyl soluble cytoplasmic lysates displayed a polypeptide SDS-PAGE pattern very similar to that described for unfractioned lysate (Fig. 2). No significant qualitative changes in the heat-shocked chlamydia sarcosyl lysate were observed except

![Fig. 4. Effect of heat shock on C. trachomatis polypeptide distribution after extraction with sarcosyl and affinity chromatography on thiopropyl-sepharose. C. trachomatis-infected cultures were transferred to 42°C at 20 h p.i. for 20 h. Both heat shocked cultures and controls were pulse labelled with [35S] methionine for 2 h, and chlamydial particles were purified 40 h p.i. Each purified preparation was extracted with 2% sarcosyl (see MATERIALS AND METHODS) and chromatographed on thiopropyl sepharose; peak fractions were analyzed by SDS-PAGE and autoradiographed. Shown in the sarcosyl insoluble fraction: S-S containing peak fractions (lanes 1, 2); SH containing peak fractions (lanes 3, 4); heat-shocked material (lanes 1, 3); and untreated controls (lanes 2, 4). Arrows indicate the location of 60 kDa crp and MOMP polypeptides.](image-url)
for the increased amounts of heat shock proteins (data not shown).

5. DISCUSSION

In this study we have shown that the reversibility of heat shock effects does not depend on the stage in chlamydial morphogenesis at which the transfer to high temperature is performed. That is, we were not able to discern a certain stage of morphogenesis that was more sensitive to heat shock than any other, as might have been expected, a priori. ‘Prolonged’ exposure to heat (> 9 h) resulted in stagnation of the growth cycle and loss of infectivity, independent of the time elapsed after infection before the heat treatment. It should be noted that since the chlamydial replication cycle is 24-48 h long, a 9-h exposure to heat shock would correspond to a 4–7 min heat shock for \textit{E. coli}, relative to the length of its replication cycle. Furthermore, in some bacteria, much higher temperatures than the 42°C used here (50°C and higher) are necessary to produce irreversible heat shock [17]. Thus, the conditions that resulted in stagnation of the growth cycle in chlamydia were much milder than those often used in investigation of bacterial systems. Of course, the sensitivity to heat shock of chlamydia may also be related to that of the host cell; however, it has been shown that 42°C is not generally lethal to eucaryotic cells in culture, although 45°C is, unless tolerance has been induced. Exposure of eucaryotic cell cultures to non-lethal heat shock treatment followed by a recovery period at 37°C results in acquired thermotolerance which allows increased survival after subsequent and otherwise lethal heat shock [18]. Thus, it is unlikely that the sensitivity of the chlamydia to heat shock was due to cellular sensitivity to 42°C temperatures. As in \textit{E. coli} [17], about 20 heat shock genes have been identified in \textit{C. trachomatis} by Engel et al. [4] following a brief temperature shift from 37–45°C. In this study, we have examined the steady state pattern of chlamydial proteins labelled after heat shock and incorporated into chlamydial particles purified 40 h after infection. As judged by one dimensional PAGE, the polypeptides made in increased amounts after extensive heat shock may correspond to the major heat shock proteins $M_r$ 32, 40, 58, 70, 90 kDa and are in good agreement with the descriptions of Engel et al. [4] and Birkelund et al. [6]. Recent studies have shown that almost all of the stress proteins are present in appreciable levels in the unstressed cell and are involved in a number of very basic and essential biological pathways [19]. The nature of the irreversible damage produced by prolonged heat shock at 42°C is unknown. Our data indicate that prolonged heat shock stagnates chlamydial development in an RB stage, perhaps in an aberrant RB stage. It is possible that, under these conditions, the various functions performed by heat shock proteins, namely, unfolding, translocation and degradation of proteins, as well as folding and/or assembly of oligopeptide protein complexes, are not sufficient to maintain the bacterial metabolic balance, and functions related to DNA replication, transcription, and RB maturation are irreversibly impaired.

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REFERENCES