

## Correction

### MICROBIOLOGY

Correction for “Developmental stage-specific metabolic and transcriptional activity of *Chlamydia trachomatis* in an axenic medium,” by Anders Omsland, Janet Sager, Vinod Nair, Daniel E. Sturdevant, and Ted Hackstadt, which appeared in issue 48, November 27, 2012, of *Proc Natl Acad Sci USA* (109: 19781–19785; first published November 5, 2012; 10.1073/pnas.1212831109).

The authors note that on page 19781, left column, second full paragraph, lines 7–8, “Thus, EBs are frequently characterized as metabolically dormant (8–10)” should instead appear as “Thus, EBs are frequently characterized as metabolically dormant (8, 9).” Ref. 10 was inadvertently cited in this sentence; however, it is appropriately cited throughout the remainder of the manuscript. This error does not affect the conclusions of the article.

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# Developmental stage-specific metabolic and transcriptional activity of *Chlamydia trachomatis* in an axenic medium

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*Chlamydia trachomatis* is among the most clinically significant human pathogens, yet their obligate intracellular nature places severe restrictions upon research. Chlamydiae undergo a biphasic developmental cycle characterized by an infectious cell type known as an elementary body (EB) and an intracellular replicative form called a reticulate body (RB). EBs have historically been described as metabolically dormant. A cell-free (axenic) culture system was developed, which showed high levels of metabolic and biosynthetic activity from both EBs and RBs, although the requirements differed for each. EBs preferentially used glucose-6-phosphate as an energy source, whereas RBs required ATP. Both developmental forms showed increased activity when incubated under microaerobic conditions. Incorporation of isotopically labeled amino acids into proteins from both developmental forms indicated unique expression profiles, which were confirmed by genome-wide transcriptional analysis. The described axenic culture system will greatly enhance biochemical and physiological analyses of chlamydiae.

metabolism | transcription | intracellular parasite

The bacterial obligate intracellular pathogen, *Chlamydia trachomatis*, comprises several different serological variants or serovars that cause distinctly different diseases in humans. Trachoma, the leading cause of infectious blindness, remains a serious problem in developing countries (1). *C. trachomatis* is also among the most common sexually transmitted pathogens (2). A rise in the number of infections in recent years and emergence of new clinical variants (3) illustrates the need for improved understanding of *C. trachomatis* physiology and virulence.

*C. trachomatis* undergoes an intracellular developmental cycle that involves a transition from the infectious elementary body (EB) to a vegetative reticulate body (RB) (4). Following replication, reversion to progeny EBs occurs asynchronously before release and reinfection. Although axenic metabolic activity and protein synthesis have been established for RBs (5–7), only limited activity has been described for EBs. Thus, EBs are frequently characterized as metabolically dormant (8–10).

With a genome of only 1.04 Mb (8), *C. trachomatis* metabolic activity is dependent on scavenging of many metabolic intermediates from the host cell. Indeed, analysis of the *C. trachomatis* genomic sequence has revealed that the organism is devoid of several enzymes and even entire metabolic pathways (8), underscoring the adaptations of *C. trachomatis* to intracellular parasitism. During infection of cultured host cells, *C. trachomatis* scavenges nutrients, including nucleotides (11), amino acids (12), and lipids (13–19). Here, we describe a host cell-free (axenic) medium for *C. trachomatis* and compare directly the energy source requirements and biosynthetic capacity of EBs and RBs.

## Results

**Characterization of *C. trachomatis* EB and RB Populations.** *C. trachomatis* EBs and RBs were purified from infected HeLa cells by density gradient centrifugation (20). Bacterial stocks were screened by comparison of inclusion-forming unit (IFU) titer

and direct particle count (Table 1). Only preparations with an IFU/particle ratio of 0.9 or higher, indicating >90% viability, were used in experiments of EB metabolic activity. Transmission electron microscopy (TEM) was used to verify the ultrastructural characteristics of enriched EBs and RBs (Fig. S1A). TEM showed a uniform population of typical EBs (21), characterized by small size (~0.3- $\mu$ m diameter) and condensed chromatin. Conversely, the enriched RB population comprised larger cells (~1.0- $\mu$ m diameter) with relaxed chromatin (21). Consistent with the ultrastructural analysis, silver staining of bacterial lysates separated by SDS/PAGE revealed distinct protein profiles (Fig. S1B).

Because EBs and RBs differ in their ability to withstand osmotic stress (21), their structural integrity was compared by exposing enriched EBs and RBs to brief incubation in isotonic buffer or deionized water (dH<sub>2</sub>O) (Fig. S1C). Incubation of EBs and RBs in dH<sub>2</sub>O showed significantly more lysis of RBs than EBs.

Correlations between direct particle counts, optical densities, and protein content were established (Fig. S2). EB and RB suspensions normalized to an equivalent OD<sub>600</sub> contained fourfold to fivefold more EBs than RBs, consistent with the smaller size of the EB (Fig. S2A). Populations of EBs and RBs were also normalized according to total protein. The RB protein density (total protein/particle) was found to be ~10-fold higher than that of EBs (Fig. S2B).

**Design of a Metabolically Permissive Nutrient Medium.** The chlamydial inclusion membrane is permeable to ions but not reporters >500 Da (22). Accordingly, the concentrations of potassium (K<sup>+</sup>) and sodium (Na<sup>+</sup>) in the inclusion lumen mimic that of the host cytosol (23). Therefore, a buffer referred to as intracellular phosphate buffer (IPB) (5 mM KH<sub>2</sub>PO<sub>4</sub>, 10 mM Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O, 109.6 mM K-gluconate, 8 mM KCl, 1 mM MgCl<sub>2</sub>·6H<sub>2</sub>O) was designed that contains K<sup>+</sup>, Na<sup>+</sup>, and chloride (Cl<sup>-</sup>) concentrations reflecting those found in the eukaryotic cytoplasm.

[<sup>35</sup>S]Cys-Met incorporation by scintillation counting and autoradiography during incubation of enriched EBs in IPB supplemented with different nutrients was used as the basis for medium development. Dose-response analyses were carried out for individual nutrients. The additive effects of medium components with or without a carbon or energy source are shown in Fig. S3. Incubation of bacteria in IPB alone or supplemented with 1% FBS and 25  $\mu$ M amino acids (except cysteine and methionine, which were present at 1  $\mu$ M) resulted in minimal incorporation. However,

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The authors declare no conflict of interest.

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Data deposition: The microarray data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, [www.ncbi.nlm.nih.gov/geo](http://www.ncbi.nlm.nih.gov/geo) (accession no. GSE39530).

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**Table 1. Infectivity per particle of enriched *C. trachomatis* EB preparations**

| Stock no. | DPC  | IFU titer                                  | Infectivity, % |
|-----------|--|--|----------------|
| 1         | $3.78 \times 10^{10} \pm 1.81 \times 10^9$ | $3.47 \times 10^{10} \pm 6.98 \times 10^8$ | 91.8           |
| 2         | $2.53 \times 10^{10} \pm 1.09 \times 10^9$ | $1.63 \times 10^{10} \pm 6.21 \times 10^8$ | 64.4           |
| 3         | $3.35 \times 10^{10} \pm 1.16 \times 10^9$ | $2.76 \times 10^{10} \pm 1.82 \times 10^9$ | 82.4           |
| 4         | $1.82 \times 10^{10} \pm 7.45 \times 10^8$ | $1.74 \times 10^{10} \pm 1.39 \times 10^9$ | 95.6           |

Numbers represent the mean  $\pm$  SEM of at least two independent experiments performed in duplicate. Percentage infectivity was calculated from the mean. DPC, direct particle count; IFU, inclusion-forming unit.

addition of 0.5 mM glucose 6-phosphate (G6P), a predicted chlamydial primary carbon and energy source (24), produced a 16-fold increase in protein synthesis.

The structural integrity of EBs is conferred by a disulfide cross-linked outer membrane protein complex that can be relaxed by reducing agents such as DTT (21). Reduction of EB disulfide bonds promotes axenic metabolic activity (21, 25). EBs were incubated in IPB containing amino acids, FBS, and G6P, and supplemented with increasing concentrations of DTT (Fig. S3 C and D). A dose-dependent increase in incorporation was observed with a maximal 5.6-fold increase over controls.

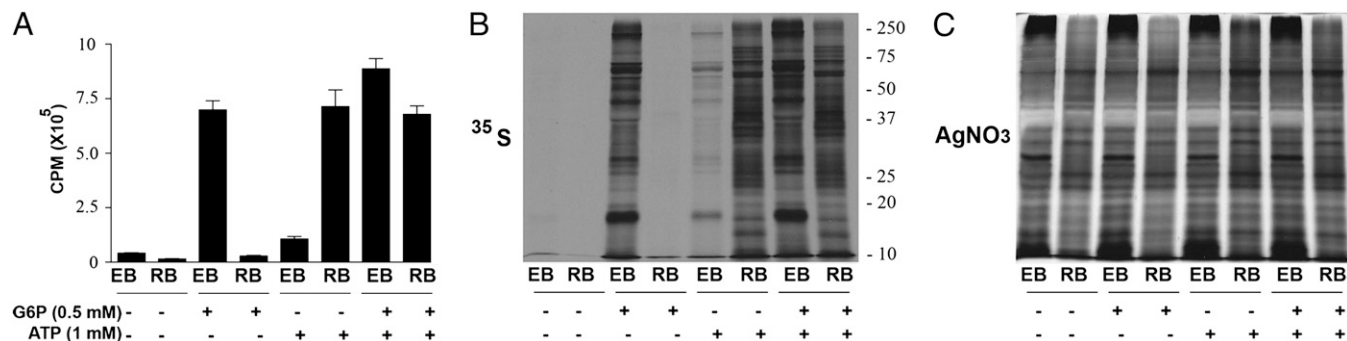
**Carbon and Energy Source Utilization by *C. trachomatis* in Axenic Medium.** *C. trachomatis* EBs were incubated in medium containing 0, 0.1, 0.5, and 1 mM G6P, and ATP pools were measured (Fig. S4). Dose-dependent increases in bacterial [ $^{35}$ S]Cys-Met incorporation and ATP pools indicate that EBs use G6P for production of ATP to drive bacterial de novo protein synthesis. However, RBs did not incorporate [ $^{35}$ S]Cys-Met in the presence of G6P. Based on the acquisition of ATP by *Chlamydia psittaci* RBs, but not EBs (5), the possibility that EBs and RBs rely on different energy sources was assessed. Indeed, incubation of RBs in medium containing a concentration gradient of ATP showed dose-dependent [ $^{35}$ S]Cys-Met incorporation (Fig. S4). Direct comparisons of EB and RB protein synthesis in media supplemented with optimal concentrations of G6P, ATP, or both, revealed differential energy source utilization between the cell forms (Fig. 1). EBs responded principally to G6P, but RBs responded exclusively to ATP and showed no additive effect with both substrates present.

**Comparison of *C. trachomatis* EB and RB Protein Synthesis in Axenic Media.** IPB supplemented with 1% FBS, 25  $\mu$ M amino acids, 0.5 mM G6P, 1.0 mM ATP, 0.5 mM DTT, and 50  $\mu$ M GTP, UTP, and CTP is referred to as *Chlamydia* intracellular phosphate-1 (CIP-1) medium (Table S1). Hatch et al. (6) demonstrated chlamydial axenic metabolic activity in a Tris-HCl-based medium containing amino acids, molecular ATP, as well as an ATP-regenerating

system. Recently, Haider et al. (10) reported metabolic activity by *C. trachomatis* in DGM-21A medium. The ability of CIP-1 to support *C. trachomatis* protein synthesis was compared with that of Hatch medium and DGM-21A (Fig. 2). As reported (6), Hatch medium supported RB, but negligible EB protein synthesis. DGM-21A supported marginal incorporation by either RBs or EBs. Overall, *C. trachomatis* EB and RB protein synthesis was higher in CIP-1 compared with alternative media used previously. This protein synthetic activity in CIP-1 plateaued after 6 h for RBs but remained active for up to 12 h for EBs (Fig. S5).

***C. trachomatis* Protein Synthesis in CIP-1 Is Sensitive to Translational and Transcriptional Inhibitors.** *C. trachomatis* EBs are known to contain mRNA transcripts, or carry over RNA, entrapped during differentiation of RBs back to EBs (4, 26). To assess the requirements for protein synthesis, EBs were incubated in the presence or absence of chloramphenicol or rifamycin, and [ $^{35}$ S]Cys-Met incorporation was determined (Fig. 3). Only background levels of incorporation were observed in the presence of either antibiotic, indicating that axenic protein synthesis by *C. trachomatis* EBs is dependent on de novo transcription. Because transcriptional activity is necessary for chlamydial protein synthesis, we examined the effects of supplementation with the ribonucleotides GTP, UTP, and CTP (Fig. S6). EBs and RBs responded to NTP supplementation in a dose-dependent manner.

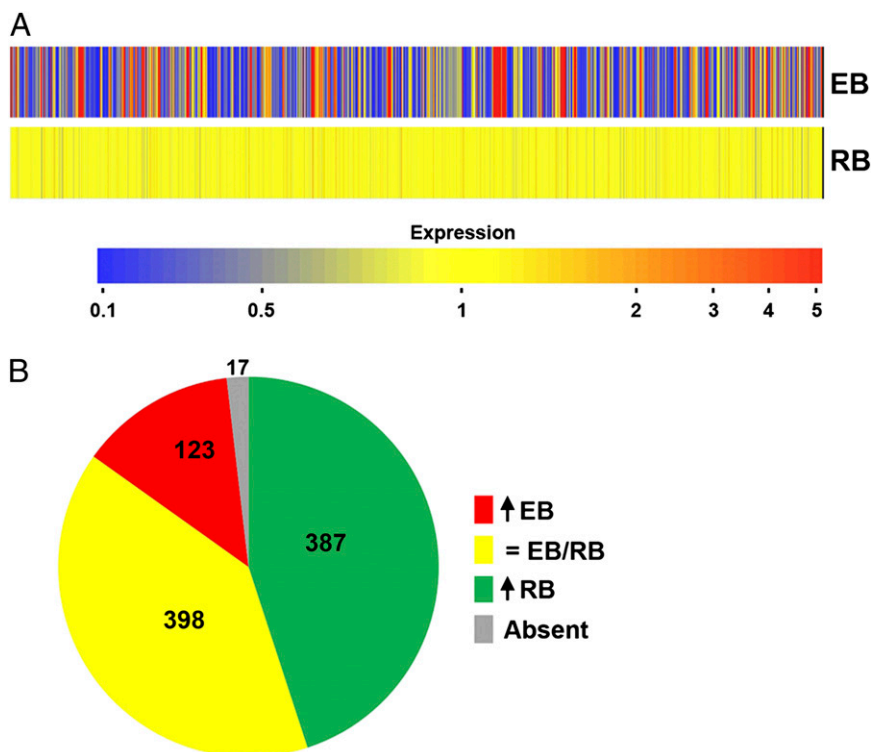
**Microaerobic Conditions Enhance *C. trachomatis* Metabolic Activity in Axenic Medium.** Genome sequence analysis indicates that cytochrome *bd*, a respiratory chain terminal oxidase with high affinity for oxygen, is encoded by *C. trachomatis*. Because cytochrome *bd* is associated with bacterial microaerobic metabolism, the effect of reduced oxygen tension on *C. trachomatis* metabolic activity was tested. EBs were preincubated for 24 h in CIP-1 under 20, 10, 2.5% oxygen tensions or under anaerobic conditions. Anaerobic conditions for 24 h resulted in loss of EB metabolic activity. However, increases of 1.6- and 2.3-fold in [ $^{35}$ S]Cys-Met incorporation were observed for EBs incubated in 10 or 2.5%



**Fig. 1.** Differential energy source utilization by EBs and RBs. Purified EBs and RBs were incubated in medium containing G6P, ATP, or both. (A) Scintillation counting and (B) autoradiography of bacterial lysates show differential energy source utilization by the two cell forms. (C) Silver staining shows equal loading of samples with distinct protein profiles. Data are expressed as the mean ( $n = 4$ )  $\pm$  SEM. Background signal from heat-inactivated organisms was subtracted from all samples. An autoradiograph from a representative experiment is shown. Molecular weight markers are in kilodaltons.







**Fig. 4.** Developmental stage-specific transcription of purified EBs and RBs. EBs and RBs were incubated in CIP-1 for 3 h under 2.5% oxygen and RNA extracted for microarray analysis. (A) Heat map showing axenic EB gene expression relative to axenic RBs. (B) Number of genes up-regulated more than threefold in EBs, up-regulated more than threefold in RBs, or expressed at relatively equivalent levels in both. Seventeen transcripts were not detected.

appropriate conditions. It is unclear what specific signal(s) trigger EB germination but the availability of glucose-6-phosphate in CIP-1 stimulates both ATP production and protein synthesis and is accompanied by a transcriptional up-regulation of many previously identified early transcripts. Whether EBs can metabolize to maintain ATP stores and viability in more natural extracellular environments, such as mucosal surfaces, remains to be determined.

Chlamydiae have been proposed to undergo a temporal program of gene expression consisting of early, midcycle, and late genes (4). Among the early genes are many of those associated with inclusion development to create an appropriate intracellular niche. Late genes include those involved in the secondary differentiation of RBs back to EBs such as the histone-like proteins involved in DNA condensation and the cysteine-rich proteins that stabilize the cell wall outer membrane complex. Host-free RNA synthesis has been observed from freshly purified chlamydial developmental forms isolated at 1 or 24 h postinfection (29). The transcripts from 1- and 24-h specimens hybridized to unique genomic profiles, although with the exception of the gene encoding MOMP, the genes transcribed were not identified. Genes expressed by EBs in CIP-1 include many expected during early transcription, such as those encoding inclusion membrane proteins (Incs). A few classical late genes such as those encoding the cysteine-rich outer membrane complex proteins, OmcA and B and the histone-like protein, Hc2, were also up-regulated in EBs. Another prominent late gene, encoding Hc1, was not up-regulated. Thus, although the transcriptional profiles of EBs and RBs were unique, they did not entirely reflect the in vivo situation. The potential for recognizing additional developmental regulatory signals is enhanced by the availability of an axenic medium supporting transcription and translation. Whether shifts in developmental stage-specific gene expression profiles occur or can be induced during axenic culture will be a prominent goal of future studies.

The findings presented herein will facilitate the study of chlamydial metabolism and physiology under controlled nutritional and physiochemical conditions. Moreover, robust axenic metabolic activity will complement current research tools used to study chlamydiae, including the recent development of basic genetic tools (30, 31). The identification of metabolic activity by *C. trachomatis* EBs challenges the categorization of the chlamydial EB as metabolically inert/dormant. As such, EBs may be best characterized as conditionally active. CIP-1 can be used as a foundation for further studies on *C. trachomatis* axenic metabolic activity and serves as a basis for exploration of additional factors that may stimulate differentiation and eventually replication.

## Materials and Methods

**Propagation and Purification of *C. trachomatis*.** *C. trachomatis* serovar L2 (LGV 434) was propagated in HeLa 229 cells and purified by Renografin density gradient centrifugation as described (20). RBs were isolated 14–16 h postinfection, whereas EBs were isolated 43–45 h postinfection. IFUs were determined as previously described (32) using indirect immunofluorescence with a rabbit polyclonal anti-*C. trachomatis* L2 EB antibody followed by an anti-rabbit IgG Alexa Fluor 488-conjugated secondary antibody (Invitrogen).

**TEM.** Ultrastructural analysis of *C. trachomatis* EBs and RBs were analyzed by TEM as described (33). Micrographs were captured using a Hitachi H-7500 electron microscope (Hitachi) equipped with a Hamamatsu XR-100 digital camera system (AMT).

**Normalization of EB and RB Populations.** EBs and RBs were fixed [2.5% (vol/vol) Glut/0.05 M sucrose in 0.1 M NaCl, pH 6.8], and cellular densities were adjusted to OD<sub>600</sub> of 0.5, 0.25, and 0.1. Direct particle counts were determined as previously described (34). For measurement of bacterial total protein content, bacterial suspensions were lysed in 1% (wt/vol) SDS for 5 min at 100 °C, and total protein was determined using a Bio-Rad D<sub>c</sub> Protein Assay kit (Bio-Rad).

**C. trachomatis Osmotic Stability.** Differential sensitivity of enriched *C. trachomatis* EBs and RBs to hypoosmotic stress was determined as previously described (21) by suspension in K-36 buffer (0.05 M  $K_2HPO_4/KH_2PO_4$ , 0.1 M KCl, 0.15 M NaCl, pH 7.0) or  $dH_2O$  for 5 min. The  $OD_{600}$  was then determined.

**Protein Synthesis by [ $^{35}S$ ]Cysteine–Methionine Incorporation.** *C. trachomatis* protein synthesis was measured by incorporation of [ $^{35}S$ ]Cys-Met (MP Bio-medicals). Chlamydiae were incubated for 6 h under the indicated conditions in microcentrifuge tubes containing 500  $\mu$ L of medium supplemented with 50–100  $\mu$ Ci of [ $^{35}S$ ]Cys-Met. Organisms were then pelleted, washed with K-36, and lysed in Laemmli sample buffer at 100 °C for 5 min. Equal volumes of lysate were used for quantification of [ $^{35}S$ ]Cys-Met incorporation by scintillation counting. For qualitative analysis of radiolabel incorporation into bacterial proteins, equal volumes of sample lysate were separated by SDS/PAGE and proteins were visualized by autoradiography by standard methods as described (35). Silver staining of SDS/PAGE gels was done using a SilverQuest kit (Invitrogen) according to the manufacturer's recommendations. For direct comparison of EB and RB metabolic activity, bacterial inocula were normalized according to total protein content. Incubations of bacteria in microaerobic or anaerobic environments were performed using Innova CO-48 (New Brunswick Scientific) and MCO-5M (Sanyo) incubators, or a GasPak jar system (BD Biosciences), respectively.

**Measurement of Chlamydial ATP Pool.** ATP pools were measured using an ATP Determination Kit (Invitrogen) according to the manufacturer's instructions. Luminescence was measured using a Synergy-4 Multi-Mode Microplate Reader (BioTek Instruments). ATP content was determined by comparison with a standard curve.

**Transcription Microarray Analysis.** Density gradient-purified EBs and RBs were incubated in quadruplicate in four-well plates containing 500  $\mu$ L of CIP-1 for 3 h under 2.5% oxygen in the presence or absence of rifamycin (10  $\mu$ g/mL). Following incubation, bacteria were collected by centrifugation. Bacterial RNA was stabilized immediately by addition of 500  $\mu$ L of RLT lysis buffer (Qiagen) and samples were frozen at  $-80$  °C. RNA was extracted using a RNeasy mini kit according to manufacturer's recommendations (Qiagen). DNase treatment of bacterial RNA was performed in 100- $\mu$ L reactions using 20 U of RNAase-free DNase I (New England Biolabs) per reaction. cDNA synthesis and fragmentation, and 3'-terminal labeling were performed according to the Affymetrix instructions for "Prokaryotic Target Preparation" (Affymetrix). The quality of the RNA starting material, cDNA, and the fragmentation efficiency were monitored using a 2100 Bioanalyzer (Agilent Technologies). Labeled cDNA target was hybridized to custom Affymetrix GeneChip microarrays containing 926 *C. trachomatis* probe sets according to standard procedures essentially as described (36). Chips were scanned using a 7Gplus GeneChip scanner (Affymetrix), and data were analyzed using GeneSpring GX 7.3 (Agilent Technologies) and Partek Genomics Suite software (Partek). Additional experimental details are provided at [www.ncbi.nlm.nih.gov/geo](http://www.ncbi.nlm.nih.gov/geo) (accession no. GSE39530).

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