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Chlamydia Trachomatis hijacks energy stores from the host and accumulates glycogen in the inclusion lumen through a dual pathway

Lena Gehre

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Université Pierre et Marie Curie

Ecole doctorale Complexité du Vivant

Unité de Biologie cellulaire de l'infection microbienne

Chlamydia trachomatis hijacks energy stores from the host and accumulates glycogen in the inclusion lumen through a dual pathway

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Thèse de doctorat de Microbiologie Cellulaire

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ABBREVIATIONS

2DG	2-deoxyglucose
AB	Aberrant bodie
ADP-Glc	ADP-glucose
AGL	Glycogen debranching enzyme (human)
BSA	Bovine serum albumin
BV	Bacterial vaginosis
cDNA	Complementary DNA
CERT	Ceramide transfer protein
COMC	Chlamydial outer membrane complex
EB	Elementary body
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
gDNA	Genomic DNA
Glc1P	Glucose-1-phosphate
Glc6P	Glucose-6-phosphate
GlgA	Glycogen synthase (bacterial)
GlgB	Glycogen branching enzyme (bacterial)
GlgC	ADP-glucose pyrophosphorylase (bacterial)
GlgP	Glycogen phosphorylase
GlgX	Glycogen debranching enzyme
Gsp	General secretory pathway
Gys1	Glycogen synthase 1 (human)
HSGAG	Heparan-sulfate glycosaminoglycans
IFNγ	Interferon gamma
Inc protein	Inclusion membrane protein
L2	<i>Chlamydia trachomatis</i> LGV 2
LDL	Low-density lipoprotein
LGV	Lymphogranuloma venereum
LPS	Lipopolysaccharide
MalQ	Amylomaltase (bacterial)
MCS	Multiple cloning site
MEF	Mouse embryonic fibroblast
MEP	Methylerythritol phosphate
MOI	Multiplicity of infection
MOMP	Major outer membrane protein
MTOC	Microtubule organizing centre
MVB	Multivesicular body
Omc	Outer membrane complex
ORF	Open reading frame
PAS	Periodic acid Schiff
PATAg	Periodic acid-thiocarbohydrazide-silver proteinate reaction
PDI	Protein disulfide isomerase
PFA	Paraformaldehyde
PGM	Phosphoglucomutase (bacterial)
PH	Pleckstrin homology
Pmp	Polymorphic membrane protein

PTS	Phosphotransferase system
qPCR	Quantitative polymerase chain reaction
RB	Reticulate body
RT-PCR	Reverse transcription polymerase chain reaction
SL	Sphingolipids
SLC35D2	Solute carrier family D2
STI	Sexually transmitted infection
T2S	Type 2 secretion
T3S	Type 3 secretion
T3SS	Type 3 secretion system
T5S	Type 5 secretion
TCA	Tricarboxylic acid
Tf	Transferrin
TF	Transcription factor
UDP-Glc	UDP-glucose
UGP2	UDP-glucose pyrophosphorylase 2 (human)
WHO	World Health Organization

ABSTRACT

The human pathogen *Chlamydia trachomatis* is an obligate intracellular bacterium, which develops in a parasitophorous compartment called inclusion. The inclusion membrane serves as a barrier to host defense mechanisms, but limits access to nutrients. One essential nutrient for *C. trachomatis* is glucose, and its polymer, glycogen, is highly abundant in the inclusion lumen. This work aimed to reconstitute the glucose flow in *C. trachomatis* infected cells and to understand the mechanisms for glycogen accumulation.

The common view is that glycogen is produced in the bacteria, and that bacterial lysis accounts for the free glycogen observed in the inclusion lumen. We showed, however, that glycogen is detected within the inclusion lumen first, and appears in the bacteria only later. Host glycogen synthase is translocated into the inclusion lumen, and glycogen-filled vesicles are often seen in the inclusion lumen, suggesting that vesicular import of host glycogen takes place. However, knocking-down host glycogen synthase by siRNA, and thereby strongly depleting cytoplasmic glycogen, did not significantly alter its accumulation in the inclusion lumen, indicating that an alternative pathway took place. We provided evidence that bacterial glycogen synthesis and degradation enzymes were secreted into the inclusion lumen, through a type 3 secretion process. These findings suggested an alternative scenario, i.e. *de novo* glycogen synthesis inside the inclusion lumen, controlled by bacterial enzymes. Depletion of host UDP-glucose pyrophosphorylase (the enzyme synthesizing UDP-glucose, a building block of glycogen) led to a strong decrease in intraluminal glycogen stores. Similar results were obtained when we silenced the expression of the UDP-glucose transporter, SLC35D2. When expressed by transfection an HA-tagged version of this transporter was recruited to the inclusion membrane. Furthermore, we found that the chlamydial enzyme that initiates glycogen synthesis was very unusual in that it was able to use UDP-glucose, a substrate produced by eukaryotic cells and not by bacteria. All these results strongly indicate that SLC35D2 is recruited to the inclusion membrane, and that UDP-glucose is imported into the lumen. The presence of glycogen enzymes in the inclusion lumen now fuels glycogen synthesis.

In summary, our work demonstrates that glycogen storage in *C. trachomatis* inclusions is the result of two different strategies, bulk acquisition of host glycogen and *de novo* synthesis. The latter mechanism implicates the import of host UDP-glucose, and the secretion of bacterial enzymes into the inclusion lumen. These processes allow the bacteria to build an energy store, out of reach for the host.

RESUME (UNE PAGE)

Chlamydia trachomatis est une bactérie intracellulaire obligatoire pathogène pour l'homme, qui se développe dans un compartiment appelé inclusion. La membrane de l'inclusion constitue une protection contre les défenses de l'hôte, mais limite l'accès aux nutriments. Un élément essentiel pour *C. trachomatis* est le glucose. Son polymère, le glycogène, est abondant dans le lumen de l'inclusion. Ce travail a eu pour objectif de reconstituer le flux de glucose dans des cellules infectées et d'expliquer l'accumulation du glycogène.

L'opinion dominante est que le glycogène est produit par les bactéries, et que le polymère présent dans la lumière de l'inclusion est issu de la lyse d'une fraction des bactéries. Cependant nous démontrons ici que l'apparition du glycogène luminal précède celui dans les bactéries. La glycogène synthase de l'hôte est transloquée dans la lumière de l'inclusion, où l'on observe fréquemment des vésicules remplies de glycogène, suggérant l'existence d'un mécanisme vésiculaire d'import de glycogène de l'hôte. Cependant, alors que l'extinction de l'expression de la glycogène synthase par RNA interférence conduit à une forte diminution du glycogène cytoplasmique, ce traitement n'inhibe pas l'apparition de glycogène dans l'inclusion, ce qui indique l'existence d'une voie alternative. Nous avons mis en évidence que les enzymes bactériennes de synthèse et de dégradation du glycogène sont sécrétées dans la lumière de l'inclusion par un mécanisme de sécrétion de type 3. Ces résultats suggèrent un scénario alternatif, à savoir la synthèse de glycogène *de novo* dans l'inclusion. L'extinction de l'expression de la UDP-glucose pyrophosphorylase (l'enzyme que synthétise de l'UDP-glucose, substrat de synthèse du glycogène), ou de celle du transporteur d'UDP-glucose SLC35D2, conduisent à une réduction importante du glycogène luminal. Exprimé par transfection, une version étiquetée de ce transporteur se localise au niveau de la membrane de l'inclusion. De plus, l'enzyme bactérienne qui initie la synthèse de glycogène fonctionne sur l'UDP-glucose, un substrat produit par ces cellules eucaryotes et non par les bactéries. Tous ces résultats indiquent très fortement que le transporteur SLC35D2 est recruté à la membrane de l'inclusion, et que l'UDP-glucose est transloqué vers le lumen, puis pris en charge par les enzymes bactériennes pour une synthèse de glycogène *de novo*.

En résumé, notre travail démontre que l'accumulation de glycogène dans la lumière de l'inclusion est le résultat de deux processus, l'import de glycogène « brut » de l'hôte, et la synthèse *de novo*. Ce dernier implique l'import d'UDP-glucose et la sécrétion d'enzymes bactériennes dans le lumen de l'inclusion. Ces mécanismes permettent aux bactéries de stocker des molécules énergétique, inaccessibles à l'hôte.

RESUME (VERSION LONGUE)

Introduction

Les *Chlamydiae* sont des bactéries intracellulaires obligatoires à Gram négatif qui présentent un cycle de développement unique. Leurs hôtes sont très variés, allant des protozoaires (comme les amibes) pour des espèces probablement symbiotiques, aux animaux (mammifères, insectes, poissons) pour les *Chlamydiae* pathogènes (*Chlamydiaceae*). Dans certains cas, l'infection est apparemment sans conséquence pour l'hôte. Chez les mammifères, l'infection peut déclencher des manifestations cliniques qui varient en fonction de la souche bactérienne incriminée, et de la localisation de l'infection (oculaire, génitale ou voies respiratoires). Quatre espèces différentes de *Chlamydiaceae* ont été isolées chez l'homme : *C. trachomatis*, *C. pneumoniae*, *C. psittaci* and *C. abortus*. Alors que *C. psittaci* et *C. abortus* sont des pathogènes d'origine animale (transmis respectivement par les oiseaux et le bétail), pour lesquels des cas d'infections chez l'humain surviennent seulement lorsque celui-ci est exposé à des animaux infectés, *C. trachomatis* est pathogène uniquement pour l'homme. Elle est responsable du trachome, première cause infectieuse de cécité dans le monde avec aujourd'hui 2,2 millions de personnes infectées, ainsi que d'infections des voies génito-urinaires. Ces dernières sont considérées comme des Infections Sexuellement Transmissibles (IST) et sont, avec plus de 100 millions de nouveaux cas recensés annuellement, la première cause d'IST d'origine bactérienne. Enfin l'infection humaine par *C. pneumoniae* est très répandue, et cette espèce est aussi trouvée chez l'animal (chevaux, marsupiaux, amphibiens). Cette espèce est responsable de maladies de l'appareil respiratoire : pneumonie, pharyngite, laryngite, sinusite et bronchite.

Toutes les *Chlamydiaceae* sont des pathogènes intracellulaires obligatoires qui partagent un cycle de développement biphasique. La bactérie se présente sous deux formes: le corps élémentaire (EB), infectieux, et le corps réticulé (RB), répliatif. Les EBs s'attachent puis entrent dans la cellule-hôte où ils restent dans une vacuole parasitophore, appelée inclusion, durant tout leur cycle de développement. Après infection, les EBs se transforment en RBs qui se multiplient par fission binaire. Puis les RBs commencent à se transformer en EBs de façon asynchrone. Après 48 h d'infection, la plupart des RBs se sont différenciés en EBs et sont maintenant prêts à quitter la cellule-hôte, soit par lyse, soit par extrusion de l'inclusion. Un nouveau cycle de l'infection peut alors commencer. Le mode de développement intracellulaire obligatoire des *Chlamydiae* a fortement entravé les progrès de la recherche, en

particulier dans le domaine de la manipulation génétique de ces bactéries, ce qui a contribué à fragmenter notre compréhension de leur biologie. En adéquation avec leur habitat, les *Chlamydiae* ont perdu la capacité de synthétiser de nombreux métabolites vitaux. Elles dépendent de la cellule-hôte pour les nutriments essentiels tels que les acides aminés, les nucléotides et les lipides. L'acquisition de ces nutriments se produit donc à travers une interaction étroite avec l'hôte et ses organites. Les systèmes de sécrétion spécialisés, tels que le système de sécrétion de type 3 (SST3), permettent à des protéines bactériennes d'atteindre le cytoplasme de l'hôte, où elles jouent un rôle essentiel dans la régulation des interactions hôte-pathogène.

On sait, depuis les années trente, que les inclusions de cellules infectées de *C. trachomatis* testent positives à la coloration à l'iode, ce qui signe l'une accumulation de glycogène. Le glycogène est un polysaccharide de stockage qui peut être facilement décomposé en molécules de glucose (Glc), et est présent chez les animaux, les champignons et les bactéries. Il est intéressant de noter que les *Chlamydiae* encodent toutes les enzymes (de synthèse : GlgC, GlgA, GlgB et de dégradation : GlgP, GlgX, MalQ) nécessaires au bon fonctionnement du métabolisme du glycogène. Une précédente étude, réalisée grâce à la microscopie électronique, a permis de détecter du glycogène, non seulement dans la lumière d'inclusion de *C. trachomatis*, mais aussi dans les EBs. Il a été proposé que le glycogène intraluminal résulte de la lyse bactérienne, une hypothèse largement acceptée par la communauté scientifique. On sait également, depuis plus de dix ans, que la consommation de Glc est essentielle pour *C. trachomatis*, car aucune forme infectieuse n'est produite en l'absence de Glc. Ceci démontre l'importance de la substance nutritive, et probablement de son glycogène polysaccharide, pour l'agent pathogène. Nous avons été intrigués par le manque de preuves solides soutenant l'hypothèse que la lyse bactérienne est la source du glycogène intraluminal. Ce travail cherche à reconstituer le flux de Glc dans les cellules infectées de *C. trachomatis*, et à comprendre les mécanismes de l'accumulation intraluminale et bactérienne du glycogène.

Résultats et discussion

Afin de déterminer la localisation subcellulaire du glycogène dans les cellules infectées de *C. trachomatis*, nous avons utilisé la microscopie électronique en transmission (MET). Le glycogène a été observé dans le lumen de l'inclusion ainsi que dans les EBs, mais les RBs en étaient dépourvus. Compte tenu de l'abondance du glycogène intraluminal, nous avons pensé que la lyse bactérienne était une source improbable. En privant les cellules de Glc, puis en l'introduisant à nouveau brièvement, nous avons démontré que l'accumulation de glycogène

se produit d'abord dans l'inclusion, avant d'apparaître dans la bactérie, ce qui prouve sans équivoque que l'accumulation de glycogène intraluminal n'est pas due à la lyse bactérienne. Pour étudier l'évolution dans le temps de l'accumulation du glycogène, nous avons examiné des cellules infectées par *C. trachomatis* sur des périodes de plus en plus longues en microscopie électronique à transmission. Aucune trace de glycogène n'a été détectée dans l'inclusion naissante ni dans les bactéries jusqu'à 16 heures après infection. La première manifestation d'accumulation de glycogène a eu lieu entre 16 et 20 heures après infection, et le glycogène a continué à s'accumuler par la suite au fur et à mesure que l'inclusion grandissait. Par ailleurs, avant 20 heures d'infection, l'inclusion contenait principalement des RBs, ce qui indique que l'accumulation de glycogène est déclenchée par les RBs, plus actif d'un point de vue métabolique. Nous avons entrepris une analyse transcriptionnelle par qRT-PCR afin de déterminer les profils d'expression des enzymes impliquées dans le métabolisme du glycogène. Nous avons pensé que les enzymes catalysant la synthèse du glycogène pourraient ne pas s'exprimer en même temps que les enzymes catalysant la dégradation du glycogène, étant donné que ces deux activités se neutraliseraient mutuellement. La plupart des gènes liés au glycogène ont montré une tendance très similaire: *glgC*, *glgX*, *malQ*, *pgm* (phosphoglucomutase) et *uhpC* (transporteur des hexoses phosphates) ont été détectés dès 3 heures après infection (hpi), la plupart ont culminé à 16 hpi et diminué à 40 hpi, ce qui indique que l'expression de ces gènes suit l'augmentation du nombre de RBs entre 8 et 24 hpi. Seuls *glgA* (le gène glycogène synthase) et *glgB* semblent suivre une règle différente. *glgB* a obtenu son plus haut niveau à 1 et 3 hpi, puis a considérablement diminué par la suite. La première manifestation de l'expression de *glgA* est intervenue vers 16 hpi, suivie d'une forte augmentation. Ce modèle d'expression coïncide parfaitement avec la cinétique d'accumulation du glycogène, ce qui indique que *glgA* pourrait être contrôler les réserves de glycogène dans l'inclusion. Curieusement il ne semble pas y avoir une règle stricte concernant la transcription des autres enzymes glycogène, puisqu'on a observé une expression simultanée des gènes de la synthèse et de la dégradation du glycogène à tous les stades du cycle de développement. Nos observations ont soulevé une question incontournable : comment le glycogène s'accumule-t-il dans le lumen de l'inclusion? Nous avons envisagé deux scénarios. Dans le premier, un dérivé du Glc (tel que le phosphate d'hexose ou le sucre nucléotidique) est importé dans l'inclusion par un transporteur. Une synthèse *de novo* de glycogène pourrait alors se mettre en place grâce à l'activité d'enzymes présentes dans le lumen. Un scénario alternatif consiste en l'import de glycogène « brut » depuis le cytoplasme de l'hôte, par invagination de la membrane de l'inclusion. Nous avons fréquemment observé des vésicules

remplies de glycogène dans la lumière de l'inclusion, argumentant pour ce second scénario. De plus, lorsque nous avons marqué la glycogène synthase de l'hôte, Gys1, nous avons détecté cette enzyme non seulement dans le cytoplasme, mais aussi dans la lumière de l'inclusion. L'extinction de l'expression de Gys1 par siRNA a aboli ce signal, confirmant sa spécificité. Comme il est connu que Gys1 s'associe au glycogène, l'import de Gys1 dans l'inclusion procède probablement du même mécanisme que celui de glycogène de l'hôte. Cependant une expérience a révélé que le premier scénario envisagé ci-dessus était probablement aussi à l'œuvre. En effet, l'extinction de l'expression de Gys1, qui entraîne la disparition presque totale de glycogène cytoplasmique, n'a pas d'effet sur l'accumulation de glycogène dans l'inclusion, indiquant qu'une autre source de glycogène existe. L'extinction de l'expression de la UDP-Glc pyrophosphorylase (l'enzyme qui synthétise de l'UDP-Glc, substrat de synthèse du glycogène), ou de celle du transporteur d'UDP-Glc SLC35D2, conduisent à une réduction importante du glycogène luminal. Exprimée par transfection, une version étiquetée de ce transporteur se localise au niveau de la membrane de l'inclusion. De plus, l'enzyme bactérienne qui initie la synthèse de glycogène fonctionne sur l'UDP-Glc, un substrat produit par ces cellules eucaryotes et non par les bactéries. Nous avons démontré cette propriété de deux manières. D'une part, l'expression de l'enzyme bactérienne dans une cellule humaine, où seul l'UDP-Glc est disponible, conduit à une accumulation importante de glycogène, indiquant que l'enzyme est capable de métaboliser ce substrat. D'autre part, des zymogrammes faits à partir d'extraits d'*E. coli* exprimant l'enzyme chlamydial ont démontré la capacité de cette dernière à polymériser à la fois l'ADP-Glc et l'UDP-Glc.

Tous ces résultats indiquent très fortement que le transporteur SLC35D2 est recruté à la membrane de l'inclusion, et que l'UDP-Glc est transloqué vers le lumen, puis pris en charge par les enzymes bactériennes pour une synthèse de glycogène *de novo*. Ce scénario implique que des enzymes du métabolisme du glycogène sont présentes dans la lumière de l'inclusion. Nous avons utilisé un essai hétérologue de sécrétion, chez *Shigella flexneri*, pour tester la présence, dans les enzymes chlamydiales, de signaux de sécrétion. Nous avons montré que GlgA, GlgB, GlgX, GlgP et MalQ mais pas GlgC contenaient un signal de sécrétion de type 3 N-terminal. D'autre part, nous avons obtenus des anticorps spécifiques et démontré la sécrétion de GlgX, dans la lumière de l'inclusion ainsi qu'au niveau de la membrane de l'inclusion. Comme GlgC synthétise de l'ADP-Glc, et que nous avons montré que c'est de l'UDP-Glc qui est importé dans l'inclusion, nos résultats sont cohérents : GlgA peut prendre en charge l'UDP-Glc directement dans la lumière de l'inclusion, et la sécrétion de GlgC serait superflue.

Nos résultats montrent que les bactéries synthétisent du glycogène dans la lumière de l'inclusion. Ceci n'a de sens que si elles sont capables ensuite de le dégrader pour importer des monomères de sucre, et notre démonstration de la sécrétion de GlgX va dans ce sens. Cependant, la dégradation de glycogène produit du Glc-1-phosphate (Glc1P). Les Chlamydiae ont un transporteur d'hexose phosphate, UhpC, annoté comme transporteur de Glc-6-phosphate (Glc6P). Nous avons testé la capacité des bactéries à importer différentes formes de Glc radioactif, et démontré qu'elles importent du Glc6P, et pas de Glc1P ni de Glc. Ce paradoxe a été résolu lorsque nous mis en évidence la présence d'un signal de sécrétion de type 3 dans la phosphoglucomutase de *C. trachomatis*. Ainsi, nous proposons que le Glc1P issu de la dégradation du glycogène est converti en Glc6P par cette enzyme avant import dans les bactéries.

Forts de ces résultats, nous proposons donc que *C. trachomatis* accumule le glycogène dans l'inclusion par deux stratégies indépendantes. Rapidement au cours du cycle infectieux SLC35D2, et éventuellement d'autres transporteurs, sont recrutés par la membrane de l'inclusion et de l'UDP-Glc est importé dans l'inclusion. L'activité des enzymes de glycogène sécrétées par les RBs dans l'inclusion mène à l'apparition de la synthèse du glycogène luminal entre 16 et 20 heures après infection. En outre, du glycogène cytoplasmique est importé dans l'inclusion par invagination de la membrane de l'inclusion. Dans les cellules de culture, cette seconde voie n'apporte qu'une contribution mineure à la teneur globale en glycogène luminal. A ce stade de l'infection, l'inclusion contient principalement des RBs, qui eux-mêmes n'accumulent pas de glycogène. Plus tard, les EBs apparaissent et synthétisent du glycogène. Cela se produit lors de la dégradation du glycogène intraluminal en Glc1P, qui est par la suite converti en Glc6P, un substrat importable par UhpC.

Conclusion

Plusieurs agents pathogènes intracellulaires se développent à l'intérieur d'un compartiment vacuolaire. L'avantage reconnu de cette localisation est d'offrir une protection contre les mécanismes de défense de l'hôte présents dans le cytosol. Notre travail révèle un atout supplémentaire à ce lieu de résidence : la possibilité de séquestrer des molécules à l'intérieur de la vacuole, qui sont ainsi hors de portée de l'hôte. Nous décrivons ici la séquestration d'une molécule de stockage d'énergie, ainsi que la transformation de la vacuole en compartiment métaboliquement actif. Pour d'autres bactéries ou parasites intracellulaires, la vacuole pourrait également servir d'espace de « stockage » de molécules de l'hôte, non seulement pour leur utilisation par le parasite, mais aussi pour en priver l'hôte, perturbant ainsi

les voies de signalisation, ou d'autres fonctions cellulaires importantes pour sa défense contre ces parasites.

INTRODUCTION

1. *Chlamydiae*

Chlamydiae are obligate intracellular gram-negative bacteria sharing a unique developmental cycle. Their hosts are very diverse, from protozoa (like amoeba) for symbiotic *Chlamydiae* to animals (mammals, insects, fish) for pathogenic *Chlamydiae*. The infection is in some cases apparently harmless for the host. In mammals infections can trigger clinical manifestations, which vary with the bacterial strain involved and with the site of infection (ocular, genital or respiratory tract). The obligate intracellular lifestyle of *Chlamydiae* has strongly hampered advances in research, especially in genetic manipulation of these bacteria, making our understanding of their biology very fragmented. However, progress was made in the last couple of years leading to the first transformation of *Chlamydiae* (Wang et al., 2011), random mutagenesis strategies (Kari et al., 2011; Nguyen and Valdivia, 2012) and recently even a first description of targeted mutagenesis (Johnson and Fisher, 2013). Omsland developed an axenic medium in which the bacteria stay viable and metabolically active to a certain extent for a couple of hours (Omsland et al., 2012). These valuable tools will surely spike *Chlamydiae* research in the future and lead to the unravelling of many black holes.

1.1 Phylogeny

The phylum *Chlamydiae* consists of only one Class and one Order, called the *Chlamydiales*, which contains several Families. rRNA-based phylogenetic trees indicate the ancient divergence of the Phylum about 2 billion years ago, and the further division between “pathogenic” (Family *Chlamydiaceae*) and “environmental” (Families *Parachlamydiaceae*, *Waddliaceae*, *Simkaniaceae* etc) *Chlamydiae* about 700 million years ago (Horn et al., 2004). The two sister groups have main features in common such as their obligate and biphasic intracellular developmental cycle (see chapter 3), but have different metabolic abilities. The genome of “environmental” *Chlamydiae* is larger, likely reflecting the more fluctuating environmental conditions that these bacteria are exposed to. Figure 1 depicts a phylogenetic tree proposed by Bush and Everett (Bush and Everett, 2001) with some modifications, e.g. integration of the newly identified *Protochlamydia amoebophila* and *Neochlamydia hartmannellae* (Collingro et al., 2005; Corsaro and Venditti, 2006; Horn et al., 2000).

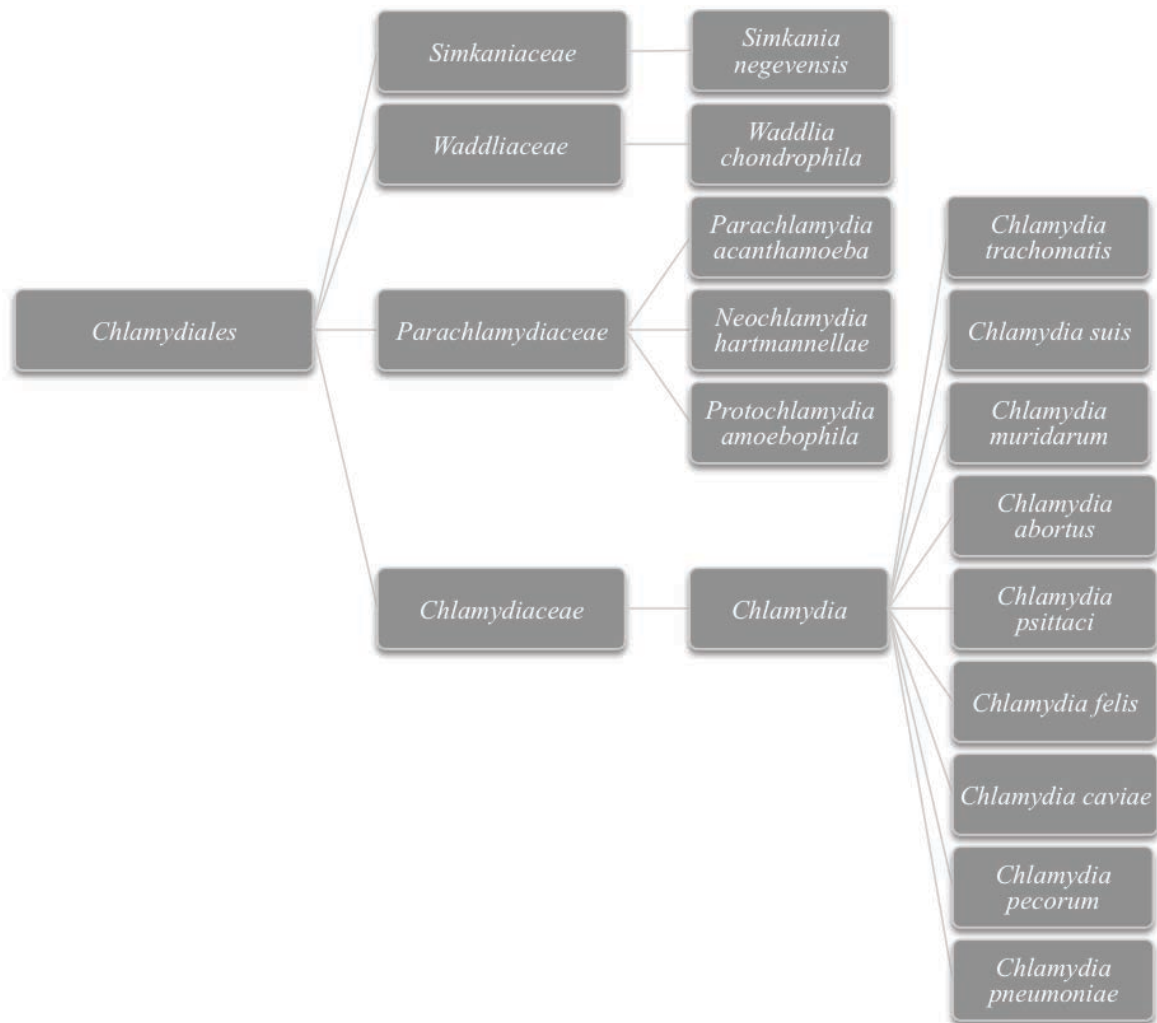


Figure 1: Phylogenetic structure of the order *Chlamydiales*. Distances are not proportional. Measured by rRNA sequence data and DNA-DNA hybridization. Adapted from (Bush and Everett, 2001).

2. Human pathogens

Four different species of the *Chlamydiaceae* have been isolated in humans: *C. trachomatis*, *C. pneumoniae*, *C. psittaci* and *C. abortus*. While *C. psittaci* and *C. abortus* are animal pathogens (birds and cattle, respectively) with infections of humans only occurring when exposed to an infected animal reservoir, *C. trachomatis* and *C. pneumoniae* are human pathogens, with the latter one also having animal hosts besides man (e.g. horses, marsupials, amphibians). Finally, while *Parachlamydiaceae* are mainly believed to be symbionts of amoeba, evidence of parachlamydial infections in fish, birds, mammals and even humans is growing (Greub, 2009; Greub and Raoult, 2002). As this thesis will focus on *C. trachomatis*,

the diseases associated with this strain will be described in more detail, but Figure 2 gives a brief overview over the common hosts of most of the *Chlamydiaceae*.

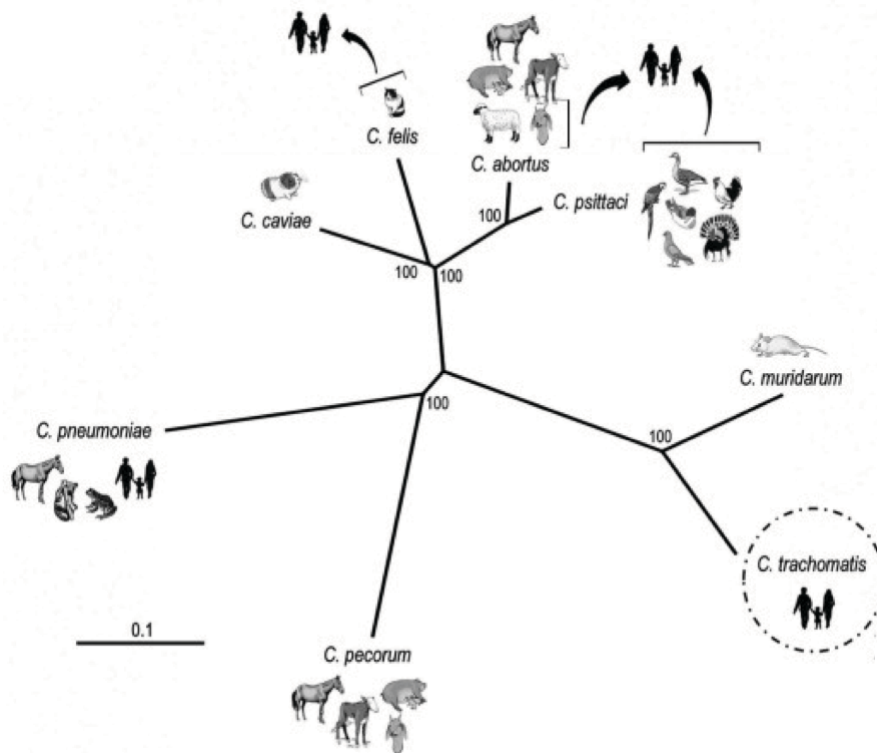


Figure 2: Phylogenetic reconstruction of the *Chlamydia* genus. The natural hosts are shown for each species except *C. suis* (due to a lack of data). (Nunes and Gomes, 2014).

2.1 *C. trachomatis*

C. trachomatis includes 15 different serovars responsible for different diseases in the human population. Serovars A, B, Ba and C are the causative agents of trachoma, while serovars D-K provoke different urogenital tract infections. L1, L2 and L3 are responsible for lymphogranuloma venereum.

2.1.1 Trachoma, disease and history

The origins of the word "trachoma" lie in the Greek word for "roughness". The eye disease trachoma has been known for thousands of years as a blinding disease. Signs of trachoma reach back to the Egyptian empire 3500 years ago, followed by the Roman and Greek Empires (al-Rifai, 1988). Returning crusaders are believed to be (at least partially) responsible for its introduction to Europe. French surgeons became aware of this blinding agent during the Napoleonic campaigns in Egypt, but failed to see its contagious character and attributed the symptoms to sand or noxious night vapours. The British, however, believed it to be a virus and took measures, lowering their incidence of blindness. Trachoma happened to become responsible for more than half of the detentions of would-be U.S. immigrants on Ellis' Island, New York, and the reason for many deportations back (Solomon et al., 2004). Its causative agent was first visualized by Halberstaedter and von Prowazek by Giemsa staining in 1907 (Halberstädter and von Prowazek, 1907). Even though its transmissibility had already been established in the minds of the public, it was not demonstrated till 1957, when Tang et al. succeeded in its first isolation using chicken embryos whose yolk sac had been inoculated with material from the eyes of infected humans. Transmission of the infection to the eyes of monkeys confirmed the presence of a pathogenic agent (Tang et al., 1957). For a long time the trachoma agent was considered to be a virus, or a transitional remnant of the evolution from bacteria to viruses, a hypothesis that had been suggested by Green in 1935 (Green, 1935). It was not till 1966 that *Chlamydia* was unequivocally shown to belong to the domain of bacteria (Moulder, 1966). The possibility of growing *Chlamydia* in cell culture led to a leap forward in *Chlamydia* biology (Gordon et al., 1960). Its first genome was sequenced at the end of the millennium rendering in depth studies of this pathogen possible (Stephens et al., 1998). Recently, the field of genetic manipulations has slowly opened up for this organism (Wang et al., 2011) and progress is made yearly, sparking the hope that genetic manipulation of *Chlamydia* will soon be a standard procedure on the way to elucidate its biology in details. Trachoma belongs to the neglected tropical diseases. According to the World Health Organization (WHO) it is the leading cause of infectious blindness, with currently 2.2 million infected people, of whom 1.2 are irreversibly blind (Pascolini and Mariotti, 2012). Figure 3 is taken from a report of the WHO about the spread of trachoma, illustrating its distribution mainly in areas of countries in which hygiene conditions are not satisfactory. The infections spread through personal contact or by flies that had been in contact with eye or nose discharge from an infected person. The rate of prevalence in preschool children in endemic regions

might reach up to 60 - 90 %. The bacterium develops in the conjunctiva of the eye, leading to conjunctival inflammation, which can be accompanied with discomfort and pain. Recurrent infections can lead to scarring of the eyelid causing it to eventually turn inwards, where constant rubbing of the eye-lashes on the eyeball are not only painful, but also provoke an abrasion of the cornea, which will ultimately lead to irreversible blindness (Thylefors et al., 1987).

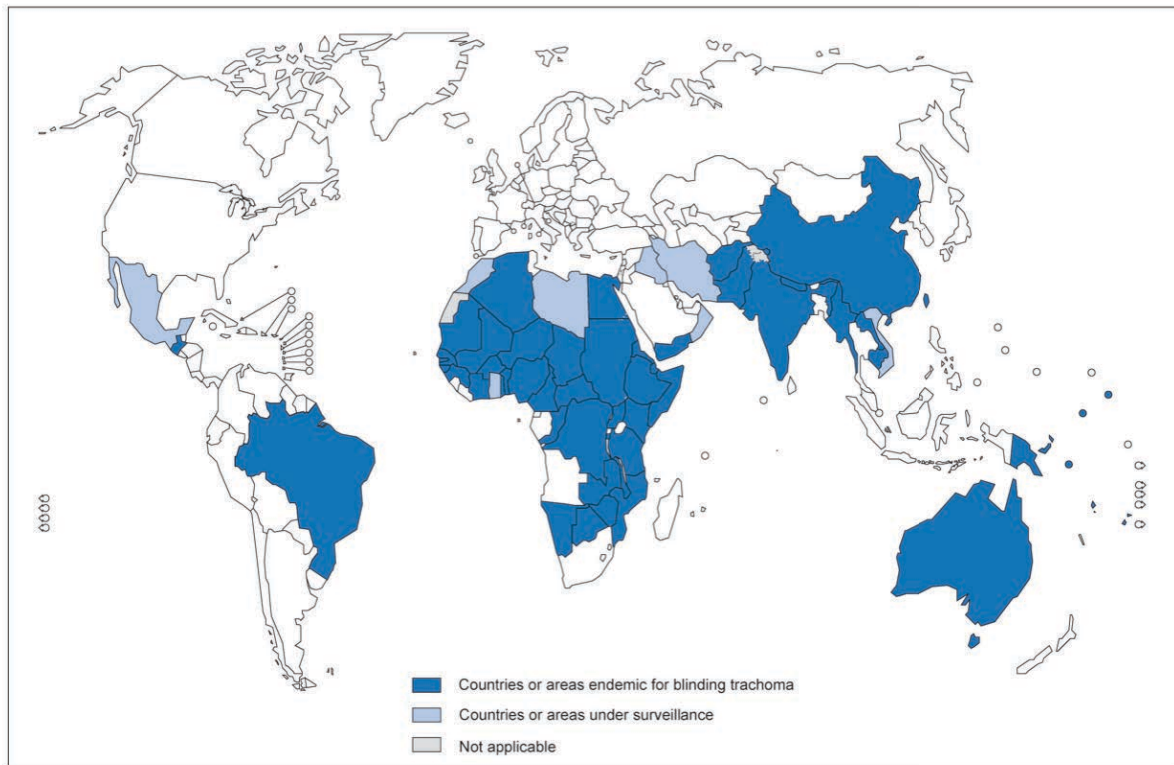


Figure 3: Distribution of trachoma, worldwide. Depicted are the 53 endemic countries. WHO report on trachoma, 2010.

2.1.2 Urogenital tract infections

With over 105.7 million new cases worldwide in 2008 (WHO) *C. trachomatis* is the first cause of a sexually transmitted infection (STI) with bacterial origin. Figure 4 depicts the 2008 incidence cases as surveyed by the WHO. Note that the highest incidence rates lie within industrialised nations. Infections are common in both men and women, and can stay limited to the lower parts of the genital tract or migrate to upper parts, causing cervicitis (inflammation of uterine cervix), salpingitis (inflammation of fallopian tubes) or endometritis (inflammation of the inner lining of the uterus) in women, and epididymitis (inflammation of

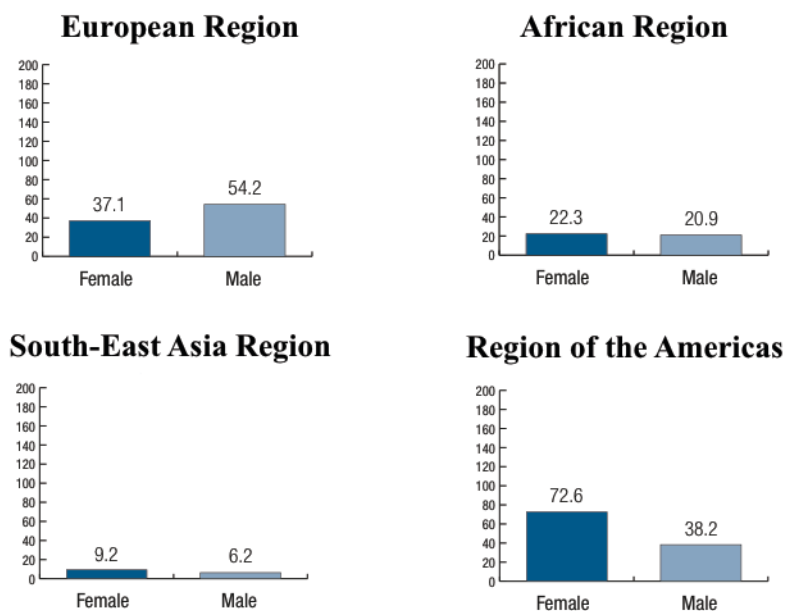


Figure 4: STI *C. trachomatis* incidence per 1000 in the European region, African region, South-East Asia region and in the region of the Americas. Taken from « Global incidence and prevalence of selected curable sexually transmitted infections - 2008 », WHO.

the coiled tube at the back of the testicles) and urethritis (inflammation of urethra) in men (Paavonen and Eggert-Kruse, 1999). However, the infection stays often asymptomatic and thus untreated, with the possible outcome of ectopic pregnancies and infertility in women.

2.1.3 Lymphogranuloma venereum

The serovars causing lymphogranuloma venereum (LGV) differ from other serovars in that they are much more invasive. Similar to serovar D-K, their transmission is through sexual contact, but they spread to subepithelial tissues, infect monocytes and disseminate to the regional lymph nodes, causing lymphadenitis (inflammation of lymph nodes) and in some cases necrosis with pus formation (Mabey and Peeling, 2002). LGV had been rare in industrialised countries before LGV proctitis started to emerge in populations of men who have sex with men. It is noteworthy that co-infections with HIV occur in the majority of LGV proctitis cases (Van der Bij et al., 2006). The ulcerative nature of LGV can in general facilitate the acquisition and transmission of STIs.

2.1.4 Diagnosis and treatment

Diagnosis and treatment vary depending on the disease elicited by *C. trachomatis*. The diagnosis of trachoma is generally made on clinical grounds. Considering the regions of

highest prevalence, a careful examination of the eye with a binocular is a quick and affordable method compared to laboratory diagnosis. Different grading systems of the progress of the disease help to standardise field surveys and research studies. The gold standard for laboratory diagnosis has long been cell culture, because of its near perfect specificity (Solomon et al., 2004). It has been replaced by nucleic acid amplification tests, which are highly specific too, and have the advantage of a higher sensitivity than cell culture (Johnson et al., 2000). Treatments of the infected eye vary from surgical reorientation of the eye-lid to antibiotic administration. Typically, tetracycline or azithromycin are locally administered for a duration of 6 weeks. A suitable strategy also implies environmental changes and a focus on hygiene to reduce the spread and reoccurrence of the infection (Hu et al., 2010).

Chlamydial urogenital tract and LGV infections are generally detected via nucleic acid amplification tests on urine samples or vaginal swabs. Recommended treatments for non-LGV *Chlamydiae* are a single dose of azithromycin, or doxycycline twice a day for 7 days (www.cdc.gov), with cure rates of 97 % and 98 %, respectively.

The typical treatment for LGV is 3 weeks of doxycycline administration, twice a day. The different recommendations between LGV and non-LGV strains require genotyping of the infective agent, which is not widely accessible for standard laboratory methods (McLean et al., 2007).

A vaccine against *C. trachomatis* has yet to be developed. The vaccine-development process is challenged by the nature of the bacterium as well as by other aspects, e.g. the pathogenesis of a chlamydial infection, which is partially induced by the immune response of the patient (Brunham and Rey-Ladino, 2005).

The best strategy to avoid an infection with *C. trachomatis* to date is the use of sexual protection and careful facial hygiene.

2.2 *C. pneumoniae*

C. pneumoniae is a causative agent of community-acquired pneumonia, pharyngitis, laryngitis, sinusitis and bronchitis. The severity of the disease can range from asymptomatic, in most cases to severe, in rare cases. There is also evidence suggesting that *C. pneumoniae* could play a role in atherosclerosis, as the bacterium gains access to the vasculature during local inflammation of the respiratory tract (Watson and Alp, 2008). Isolation of the pathogen from a patient remains difficult due to its inaccessibility, so antibody tests using paired acute- and convalescent-phase sera have been used for diagnosis. If the diagnosis is clear and if the

symptoms require therapy, then antibiotics such as doxycycline can be administered (www.CDC.gov).

3. Biphasic developmental cycle

All *Chlamydiaceae* are obligate intracellular pathogens sharing a biphasic developmental cycle. The gram negative bacteria exist in two different forms, the infectious elementary body (EB) and the replicative reticulate body (RB). They are easily distinguished based on their shapes, the EB being around 0.3 μm and the RB being around 1 μm . The infectious EBs attach and enter the host cell, where they stay in a parasitophorous vacuole, called inclusion, throughout their whole developmental cycle. Four to eight hours post infection (hpi) the EB converts into the RB, which is metabolically highly active and proliferates through binary fission. At around 20 hpi RBs start to convert into EBs in an asynchronous manner. By around 48 hpi most RBs have differentiated to EBs, now prepared to leave the host cell, either through lysis of the host cell or extrusion of the inclusion (Figure 5). A new cycle of infection starts (AbdelRahman and Belland, 2005).

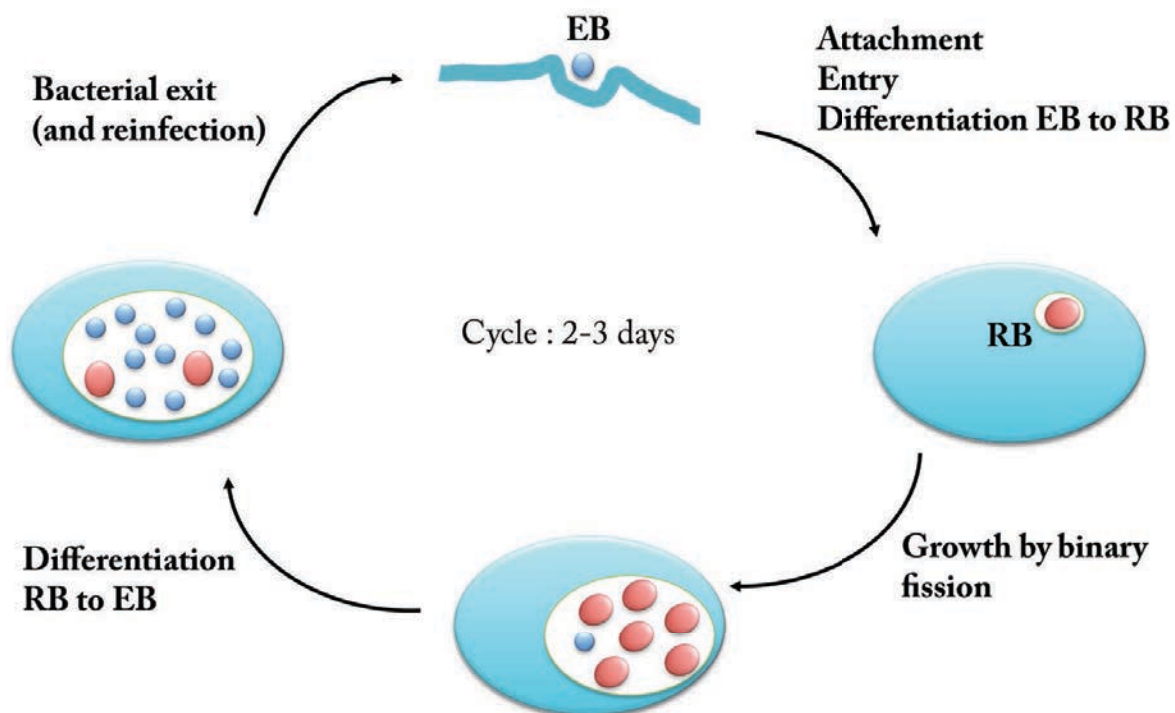


Figure 5: Biphasic developmental cycle of *Chlamydia*. The infectious elementary body (EB) attaches to the host cell, enters and converts to the reticulate body (RB) within a parasitophorous vacuole called inclusion. Replication takes place by binary fission of RBs and RBs eventually convert into EBs. *Chlamydiae* exit the cell by either lysis of the host cell or extrusion of the whole inclusion.

3.1 Entry to Exit

3.1.1 Attachment and Entry

The main targets of most of *C. trachomatis* infections are epithelial cells, even though a variety of cell types can be successfully infected *in vitro*. The first step is the adhesion of the EB to a host cell, which appears to be a two-step mechanism (Figure 6 A&B). Through elegant experiments Zhang and Stephens could show that *C. trachomatis* binds to heparan-sulfate glycosaminoglycans (HSGAG) on the host cell surface as well as in the extracellular space (Zhang and Stephens, 1992). Extracellular HS-GAG can be bound by a host HS-receptor. The chlamydial outer membrane complex B (OmcB) and EB-associated HS-GAGs have also been implicated in the attachment process (Tan and Bavoil, 2012; Zhang and Stephens, 1992). The major outer membrane protein (MOMP), a chlamydial surface protein, probably undergoes interaction with host bound GAGs, while further interactions might occur via chlamydial mannose-rich moieties and host cell mannose receptors (Su et al., 1996). All these interactions are reversible. The second stage involves more specific and irreversible adhesions of chlamydial polymorphic membrane proteins (Pmps) to so far, unknown host cell receptors (Becker and Hegemann, 2014).

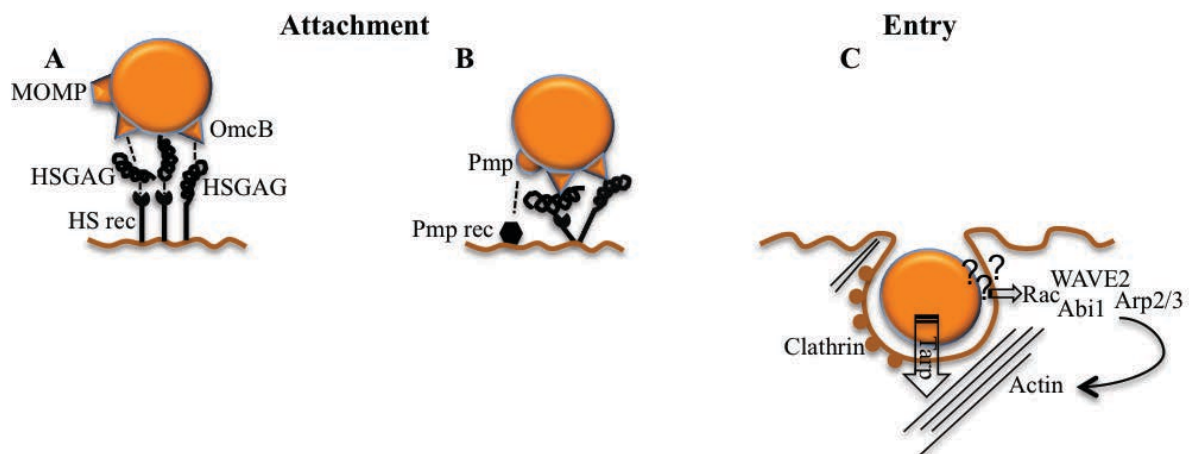


Figure 6: Generalized model for attachment and entry of *C. trachomatis*. Attachment of *Chlamydia* is thought to be a two-step mechanism. (A) Initial binding of the EB to the host cell is reversible and happens via binding of the OmcB protein to HS and/or HSGAG in soluble form or associated with the host cell surface. Chlamydial surface bound HSGAGs and mannose-rich moieties and host cell mannose receptors may be involved, too. (B) Pmp proteins (and probably other adhesins) bind in the next, irreversible step to as yet unidentified host cell receptors. (C) These tight interactions might then allow injection of Tarp. Tarp and Arp2/3 are proposed to function in concert to promote the actin cytoskeletal rearrangements leading to the internalization of *Chlamydia*.

One candidate is the protein disulfide isomerase (PDI)/oestrogen receptor complex (Hall et al., 2011). These tight interactions might now allow the injection of chlamydial effector proteins into the cytosol of the host cell promoting the entry (Figure 6 C). EBs have a preformed type 3 secretion (T3S) apparatus (Fields et al., 2003), which they use to translocate Tarp (translocated actin recruiting phosphoprotein) into the cytoplasm. This protein is able to recruit actin and nucleate its assembly (Clifton et al., 2004). The Rac/WAVE2/Abi1/Arp2/3 pathway functions in concert with Tarp to rearrange actin at the entry site, although the exact mechanism has not been elucidated yet (Carabeo et al., 2007; Carabeo et al., 2004; Subtil et al., 2004). It seems that not only chlamydial effector proteins provoke the bacterium's entry, but that additionally host receptor mediated uptake is happening. The details of how the attachment and entry take place are not fully understood yet. Species-dependent differences have been noticed in many steps, challenging the research community. For example, while the EGF receptor seems to be important in *C. pneumoniae* entry, it does not serve *C. trachomatis* entry (Mölleken et al., 2013). The involvement of clathrin in the entry process remains controversial (Boleti et al., 1999; Hybiske and Stephens, 2007).

3.1.2 Building the inclusion and survival

Chlamydiae are endocytosed into a membrane-bound vacuole known as the inclusion, which grows throughout the developmental cycle to harbour the increasing number of progeny (Figure 7). Interestingly, the inclusion membrane does not seem to display markers of the endocytic or lysosomal pathway (Scidmore et al., 2003). Bacterial activity is required to inhibit fusion with the lysosomal pathway, as inhibition of bacterial protein synthesis results in degradation of the bacteria in lysosomes (Scidmore et al., 1996). Early gene expression is first detected within an hour after entry, and chlamydial proteins called Inc proteins are produced, that are inserted into the inclusion membrane. Inc proteins probably play a central role in controlling cellular interactions of the nascent inclusion with the host cytoplasm, but the function of only a handful of them (out of more than 50 Inc proteins) has been investigated. One of them is probably responsible for the interaction with host dynein, resulting in the transport of the inclusion along microtubules to the microtubule organizing centre (MTOC) (Clausen et al., 1997; Grieshaber et al., 2003). While establishing the appropriate intracellular niche, the EB converts to an RB within the first few hpi (Shaw et al., 2000), which will be explained in more detail in chapter 3.2. A different set of genes, the mid-

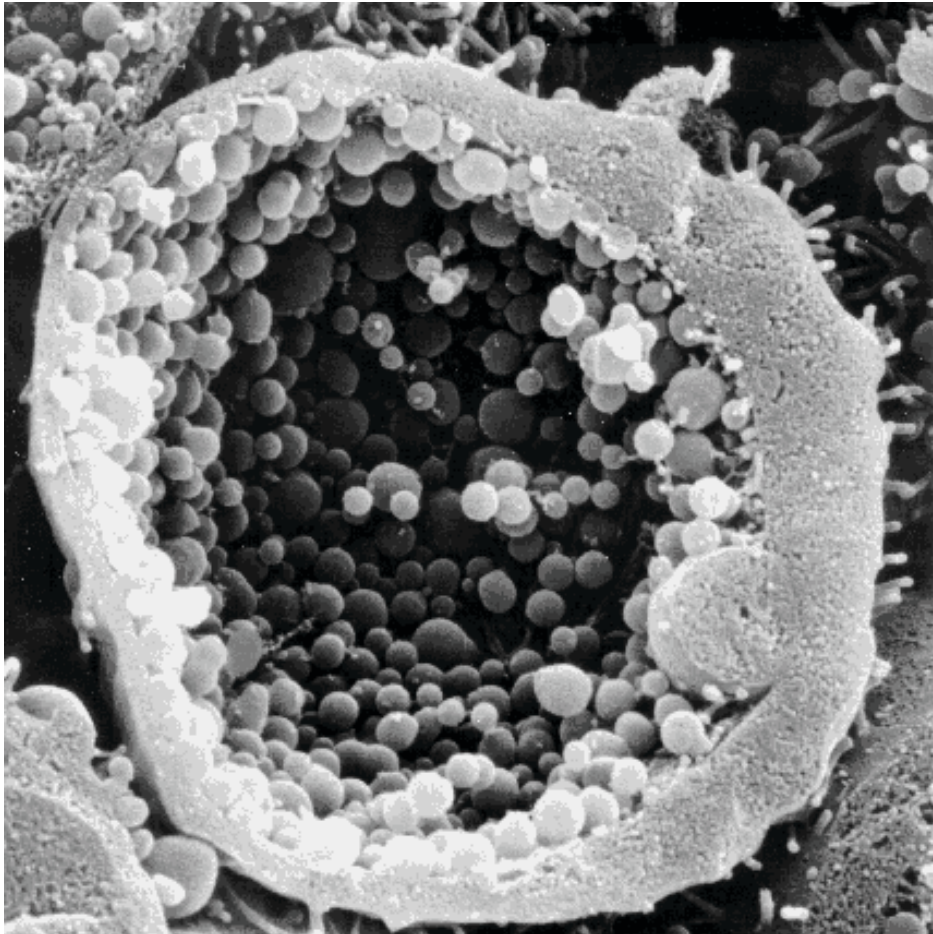


Figure 7: Electron micrograph of a freeze-fractured inclusion of *C. abortus* at 30 hpi. Unpublished image of M. Ward and C. Inman, Southampton.

cycle genes are expressed, controlling bacterial metabolism, replication and interactions with the host (Belland et al., 2003). RBs repeatedly divide by binary fission, yielding up to 1000 bacteria per infected cell (Shaw et al., 2000). At 18 to 24 hpi some RBs start to undergo conversion into EBs in an asynchronous manner, strongly linked to the switch from the transcription of mid-cycle genes to late genes (see chapter 3.2). At around 48 hpi *Chlamydia* exit the cell through pathogen-driven lysis of the inclusion and the host plasma membrane or through extrusion of the intact inclusion. However, the latter has only been observed in cell culture and not in vivo (Rank et al., 2011).

3.2 Characteristics of EBs and RBs

3.2.1 Ultrastructure of the chlamydial surface

Different methods for purification and fractionation of EBs were pioneered around 50 years ago, giving rise to the first descriptions of chlamydial ultrastructure (Manire, 1966; Moulder, 1962). It became clear that *Chlamydiae* infected cells contained two morphologically different forms: the EBs, an electron-dense coccoid structure with a diameter of about 0.3 μm , and the RBs being around 1 μm in size (Miyashita and Matsumoto, 1992). RBs also displayed a high fragility compared to EBs (Manire and Tamura, 1967). This fragility challenged (and still does) purification of RBs, shifting emphasis towards EB surface structures in subsequent studies. Manire and colleagues noticed regularly spaced hexagonal lattices covering the surface of EBs (Manire, 1966), the chlamydial outer membrane complex (COMC). It was demonstrated later on that subunits of this complex were heavily intra- and interconnected through disulfide cross-links (Hackstadt and Caldwell, 1985). The COMC is mainly composed of the major outer membrane protein (MOMP) and the cysteine rich proteins OmcA and OmcB. MOMP is also present on the surface of RBs, but in a reduced state, probably accounting for the higher susceptibility of RBs to osmotic shock. It has also been suggested that this change in the redox status contributes to EB-to-RB conversion (Hackstadt et al., 1985). The family of polymorphic outer membrane proteins (Pmps) is another abundant group of proteins on the chlamydial surface. They were first discovered when genome sequencing revealed a surprising number of autotransporters, proteins that insert their β -barrel domain in a pore like fashion into the outer membrane in order to secrete the functional passenger domain (details can be found in chapter 4.1) (Grimwood and Stephens, 1999). *C. trachomatis* encodes for 9 different Pmps with a low degree of conservation. They have been proposed to be a potential source of diversity in adhesion of the bacterium to the host cell and to also play a role in chlamydial immune evasion (Becker and Hegemann, 2014). Some of these Pmps are transcribed throughout the developmental cycle, some of them are only expressed in EBs. Generally, the composition of the envelopes of extracellular EB and intracellular RB strongly differ and reflect their distinct requirements for the survival in two environmental surroundings. Whereas EBs need osmotic protection, to mask immunodominant epitopes on their surface and to express adhesion molecules, RBs require pores for easy acquisition of nutrients and for secretion systems to communicate with the host cell.

3.2.2 Transcriptional regulation

EBs had long been thought to be metabolically inert, which had recently been proven wrong (Haider et al., 2010; Omsland et al., 2012). Even though they possess a reduced metabolic activity compared to RBs, they are still able to undergo protein synthesis. However, their chromatin is highly condensed, which can be attributed to two histone H1 homologs, Hc1 and Hc2 (Brickman et al., 1993; Hackstadt et al., 1991; Wagar and Stephens, 1988). The respective genes *hctA* and *hctB* are transcribed late in the developmental cycle concomitant with RB-to-EB differentiation and nucleoid condensation. Two loci negatively regulate Hc1. One encodes for IspE, an intermediate enzyme of the nonmevalonate methylerythritol 4-phosphate (MEP) pathway. Experiments with Hc1-expressing *E. coli* suggest that a metabolite of this MEP pathway is responsible for the dissociation of Hc1 from chlamydial chromatin (Grieshaber et al., 2004). The second locus encodes for the small regulatory RNA (smRNA) IhtA acting as an additional checkpoint to negatively regulate Hc1 synthesis (Grieshaber et al., 2006). RB-to-EB conversion is the signal for the transcription of the approximately 500 mid-cycle genes (Belland et al., 2003). Transcription of chlamydial genes is proposed to be partially regulated through different levels of DNA supercoiling. Intriguingly, high stages of supercoiling *in vivo* in the mid-cycle of *Chlamydia* correlate with upregulation of the overall expression (Niehus et al., 2008). Gene regulation through smRNAs and transcription factors (TF; both activators and repressors) are two more active mechanisms used by the bacterium (Koo and Stephens, 2003; Wilson and Tan, 2002). RBs mainly express housekeeping genes or genes needed for growth and replication. At approximately 20 hpi single RBs start to convert to EBs. Two specific sigma-factors, regulated by TFs, lead to the transcription of late genes, which induce the transcription of *hctA* and *hctB* and other genes (Rao et al., 2009; Yu and Tan, 2003). Whether expression of Hc1 and Hc2 triggers the RB-to-EB conversion via chromatin condensation or whether another force drives the conversion is yet to be understood.

3.3 Persistence

The general definition of persistence is when a subpopulation of bacteria becomes "invisible" to the host, in a way that the host's immune response is silenced, with no symptoms of the presence of the bacteria. Recurrent *C. trachomatis* infections in humans have frequently been observed. Whether these recurrent infections are indeed a matter of

persistence or simple reinfection is not clear. Early *in vitro* studies revealed that *C. trachomatis* developed an abnormal morphology in the presence of interferon gamma (IFN γ) (Shemer and Sarov, 1985), which was later described as "morphologically enlarged, aberrant, non-dividing, viable but non cultivable" aberrant bodies (ABs) (Wyrick, 2010). Over the decades more elicitors for such an *in vitro* persistent state have been described: amino acid starvation, iron deprivation, viral or protozoan co-infections, chlamydiophages or exposure to certain antibiotics. Interestingly, IFN γ exposure copies the effect of amino acid starvation, as it exerts its activity through tryptophan depletion. The common denominator that most of these stressors possess is nutrient deprivation. Upon removal of the stressors, ABs can convert back to normal developmental forms giving rise to infectious EBs (Bavoil, 2014; Wyrick, 2010). Figure 8 depicts chlamydial inclusions after IFN γ exposure. The stage of persistence might enable *Chlamydia* to survive unfavourable conditions for a certain time. However, the question whether these *in vitro* studies reflect an *in vivo* infection remains to be solved.

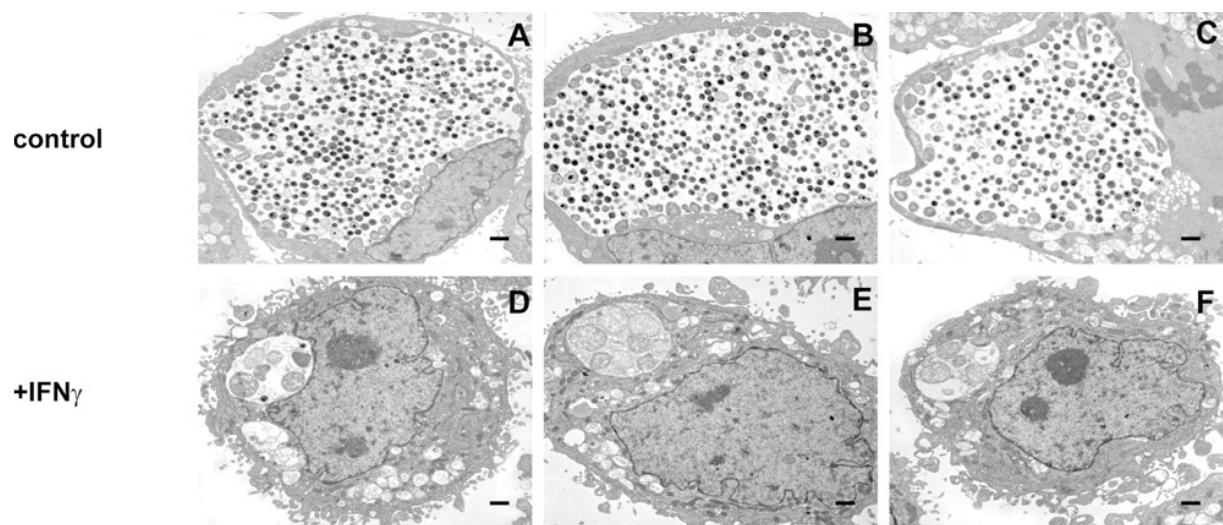


Figure 8: Representative electron micrographs of inclusions of *C. trachomatis* grown in medium alone (A-C) and in the presence of 10 ng/ml IFN γ (D-F). Scale bars correspond to 0.5 μ m. Image taken from (Ibana et al., 2011).

4. Host-pathogen interactions

As an obligate intracellular pathogen with a highly reduced genome of around 900 genes *C. trachomatis* depends strongly on the host cell (Stephens et al., 1998). Interactions with the host are indispensable for the establishment and maintenance of the niche, and for the acquisition of nutrients. They are aimed at subverting the host defence systems. Specialised

secretion systems enable the delivery of bacteria-derived exoproteins that play a pivotal role in regulating host-pathogen interactions, thereby contributing to the virulence of the pathogens.

4.1 Chlamydial secretions systems

A wide range of secretion systems have been discovered in bacteria in the last decades enabling them to translocate proteins across several membranes. *Chlamydiae* possess three different secretion systems: Type II (T2S), type III (T3S) and type V (T5S) (Figure 9). The T2S system, a ubiquitous secretion system in Gram-negative bacteria, secretes folded proteins from the periplasm into the extracellular space. Initially, the effector is synthesized with an N-terminal signal peptide targeting it for translocation to the periplasm through a pore formed by the Sec complex. After cleavage of the signal peptide the already folded protein resides in the periplasmic space, and will subsequently be secreted through a complex of pore forming proteins, the general secretory pathway (Gsp) (Korotkov et al., 2012). The only known chlamydial T2S effector is CPAF, which can be found in the host cell cytosol (Chen et al., 2010). T2S alone does not explain translocation through the inclusion membrane, since it merely provides a release into the extrabacterial space. Small outer membrane vesicles (OMV) have been suggested to deliver T2S proteins into the host cytosol, a mechanism that had been shown to function in other bacterial systems (Ellis and Kuehn, 2010; Giles et al., 2006). *Chlamydia* also exploits the T5S, or autotransporter, mechanism. Similar to the T2S system, it requires the help of the Sec pathway to deliver the exoproteins to the periplasm. Once there, a C-terminal β -barrel domain of the effector protein inserts into the outer membrane. The functional passenger domain is subsequently exposed on the bacterial surface and can be cleaved off and released, even though this is not always the case (Saier, 2006). An example of autotransporters is the family of polymorphic outer membrane proteins (Pmp), which cover the surface of EBs, playing a role in chlamydial attachment and niche adaptation (Tanzer et al., 2001). The widest spread mechanism for protein secretion into a eukaryotic cell in Gram-negative bacteria is the T3S system, which has first been shown to exist in *Chlamydia* in 1997 (Hsia et al., 1997). Secretion occurs through a complex secretory apparatus referred to as "injectisome", which has a very conserved structure. It includes a basal secretory apparatus spanning the inner membrane, the periplasmic space and the outer membrane. The needle complex bridges the space between the bacterial surface and the target membrane, where the tip complex is inserted. Target membranes for translocation are the host

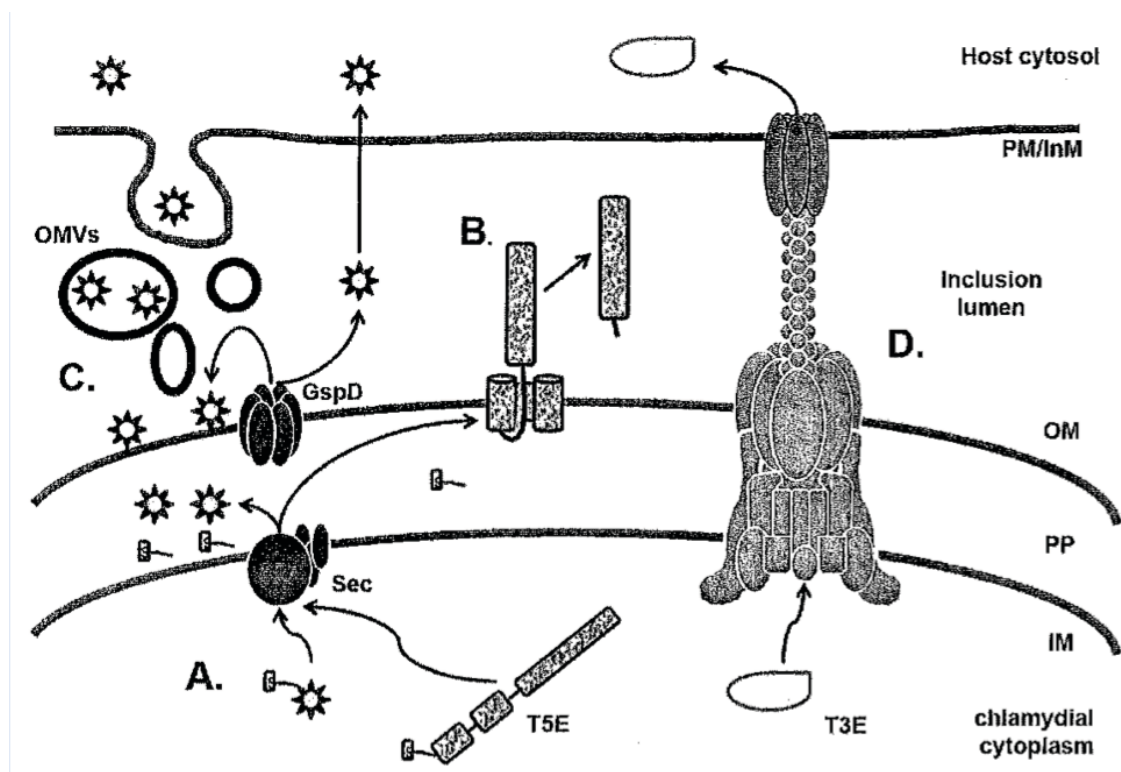


Figure 9: *Chlamydia* employs T2S, T3S and T5S. (A) Export of proteins mediated by the T2S system requires the Sec pathway to cross the inner membrane (IM). Once the substrate is in the periplasm (PP), the signal peptide is cleaved and the protein is secreted across the chlamydial outer membrane (OM) through the GspD secretin. (B) T5S effectors equally use the Sec pathway. Hydrophobic domains insert into the OM to form a pore, which guides the passenger domain into the extrabacterial space. Passenger domains can either be cleaved and released or stay bound to the bacterial surface. (C) Translocation of T2S or cleaved T5S substrates into the host cytoplasm might be achieved by transport through OMVs that fuse with the inclusion (InM) or plasma membrane (PM). (D) Single-step secretion of effectors into the host cytoplasm or the inclusion lumen is mediated by T3S. Image taken from (Tan and Bavoil, 2012).

cell plasma membrane or the inclusion membrane (Betts-Hampikian and Fields, 2010). T3S systems are active in the early steps of chlamydial invasion, when they inject Tarp and probably other effectors into the host cell, and stay essential throughout the whole developmental cycle. TepP ("translocated early phosphoprotein") is a recently identified early T3 effector protein that was proposed to act downstream of Tarp and to amplify signalling cascades (Chen et al., 2014). It has equally been shown that CT694 is a chlamydial early T3 effector, which might act upon invasion (Hower et al., 2009). Chaperones are likely to pilot substrates to the injectisome, prevent premature folding or association of proteins participating in interactions, which are to take place outside the bacteria. Few chlamydial chaperones have been discovered so far, and it is likely that not all T3S effectors require one.

In general, T3S systems are supposed to be regulated and stimulated through contact with the host cell membrane or host molecules (Hueck, 1998). While contact with the host cell membrane might trigger the translocation of early effectors such as Tarp and TepP, it is not known how the secretion of effectors from within the inclusion is regulated. Many effector proteins remain to be identified, for which computational prediction of secretion signals depicts a very useful tool. While T2S and T5S signals are well characterised, the T3S signal has not been fully elucidated yet. It could be shown that the T3S signal lies in the N-terminus or 5' end of an effector or its mRNA (Anderson and Schneewind, 1997; Lloyd et al., 2001). Several teams have developed tools for computational predictions of T3 effectors, but in *Chlamydia* they are only of limited use because they fail to find T3S signals in many Inc proteins, which are all T3S substrates (Dehoux et al., 2011). Therefore, the best strategy to predict that a given protein might be a T3S substrate remains to test for the presence of a T3S signal directly in vivo. Due to given difficulties to do so in *Chlamydia*, heterologous secretion systems were largely used (Fields and Hackstadt, 2000; Pais et al., 2013; Subtil et al., 2005; Subtil et al., 2001).

Chlamydia might engage all these different secretion systems as each of them could be adapted to achieve physiologically distinctive goals. T2S could be responsible for bulk transport of effectors, whereas T5S is obviously a straightforward mechanism to anchor proteins to the surface. T3S effectors can be highly localized and regulated, making it the perfect mechanism for fine-tuning of host-*Chlamydia* interaction.

4.2 Recruitment and uptake of host organelles and lipids

Consistent with the obligate intracellular lifestyle, *Chlamydiae* have lost the ability to synthesize many vital metabolites. They rely on the host cell for essential nutrients, such as amino acids, nucleotides and lipids (Stephens et al., 1998). The acquisition of nutrients occurs hence through a tight interaction with the host and its organelles.

4.2.1 Recruitment of host lipids

C. trachomatis encodes for the genes required for lipid synthesis, but still acquires preferentially host-derived lipids. Its lipid composition, which consists out of sphingolipids (SLs), glycerophospholipids (such as phosphatidylethanolamines or phosphatidylcholines) and cholesterol consequently resembles strongly to the one of the host (Wylie et al., 1997).

The SL precursor, ceramide, is synthesized within the host in the ER, processed in the Golgi apparatus and accumulates at the plasma membrane (Perry and Ridgway, 2005; Tan and Bavoil, 2012). Inhibition of SL synthesis leads to loss of inclusion membrane integrity and a subsequent disruption of the normal inclusion development, accompanied by premature RB-to-EB differentiation and early release of EBs (Robertson et al., 2009). Hackstadt could show that fluorescently labelled ceramide analogues trafficked in live cells to the inclusion in a manner that resembles Golgi apparatus-to-plasma membrane vesicular transport (Hackstadt et al., 1995). On the other hand, plasma membrane SLs are not directed towards the inclusion, concordant with the segregation of the inclusion from the endocytic traffic (Hackstadt et al., 1996). Cholesterol, a lipid not normally identified in prokaryotes, can be found in EBs and in the inclusion membrane. Carabeo and colleagues demonstrated that this cholesterol stems from two different sources: *de novo* synthesised by the host cell, or derived from the extracellular medium via the low-density lipoproteins (LDL) pathway. It was also suggested that the host cell cholesterol might be co-transported to the inclusion with SL in redirected Golgi-vesicles, as acquisition of both is sensitive to addition of Brefeldin A causing the interruption of normal Golgi apparatus vesicular traffic (Carabeo et al., 2003). In addition to this pathway *Chlamydia* also acquires host ceramides through a non-vesicular pathway. The host's interorganelle lipid transfer highly relies on membrane contact sites between organelles, notably with the ER (Levine and Loewen, 2006). The bulk of ceramide ER-to-Golgi transport is coordinated through the ceramide transfer protein (CERT), which directly transfers the precursor from one organelle to the other at the membrane contact sites. Early ultrastructural studies had already reported ER tubules closely apposed to *C. trachomatis* inclusions and another study revealed the enrichment of certain ER proteins on the inclusion membrane (Majeed et al., 1999; Peterson and de la Maza, 1988). CERT is equally localised to and enriched at the inclusion membrane. These structures resemble to membrane contact sites between the ER and the inclusion membrane (Derre et al., 2011; Dumoux et al., 2012). An attractive candidate for the recruitment of CERT to the inclusion membrane is the inclusion membrane protein IncD (CT115), as it colocalises in patches with CERT on the membrane and interacts with its Pleckstrin homology (PH) domain (Agaisse and Derre, 2014; Derre et al., 2011). Additionally, *C. caviae* lacks IncD and is unable to recruit CERT. Intriguingly, CERT depletion causes reduced inclusion size and less infectious progeny, a different phenotype to what is observed for Brefeldin A treatment leading to a decrease in Golgi-to-inclusion traffic. It has consequently been hypothesised that the ceramides obtained via these

two different pathways are subsequently used in different ways by *Chlamydia* (Elwell et al., 2011).

4.2.2 Interaction with host organelles

Chlamydiae exploit many of the host organelles to ensure their survival (Figure 10). For instance, the Golgi apparatus is disrupted and fragmented into mini-stacks during *Chlamydia* infection surrounding the inclusion, which possibly facilitates SL delivery to the bacterial compartment by increasing Golgi proximity (Heuer et al., 2009).

A multivesicular body (MVB) is an endocytic compartment in which the peripheral membrane is invaginated, leading to the formation of small intraluminal vesicles. MVBs can either fuse with the plasma membrane to release so-called exosomes, or fuse with the lysosomal pathway for further degradation of the incorporated proteins (Gruenberg and Stenmark, 2004). MVB markers CD63 and LBPA were detected on vesicular structures within the inclusion lumen, and CD63 was found on the inclusion membrane (Beatty, 2006; Beatty, 2008). The small intraluminal vesicles in MVBs are enriched in cholesterol and SLs, and addition of a drug inhibiting cholesterol transport from the late endosomes appeared to

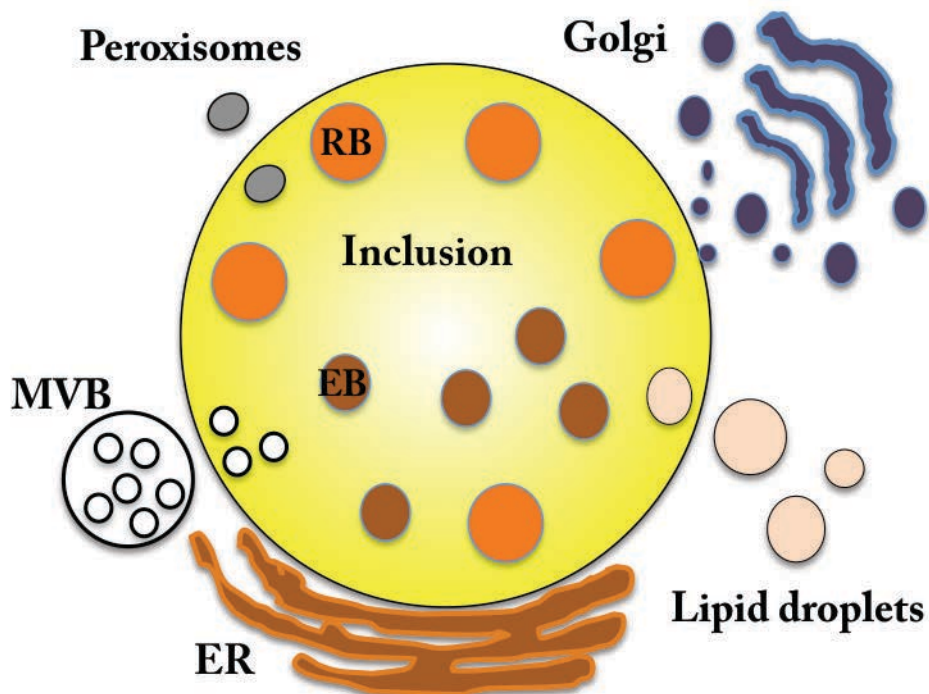


Figure 10: *Chlamydiae* interact with the ER and the Golgi apparatus and take up lipid droplets, peroxisomes and MVBs.

decrease acquisition of cholesterol and SL by *Chlamydia* (Beatty, 2006; Beatty, 2008). However, RNAi screens suggest that the MVB pathway is not required for *C. trachomatis* replication (Derre et al., 2007). This could be due to redundant systems of lipid acquisition. Further investigations are necessary to confirm the observations made by Beatty, as other studies could neither verify CD63 within the inclusion lumen nor the effect of the drug used to inhibit MVB cholesterol transport (Ouellette and Carabeo, 2010).

Lipid droplets (LDs) are organelles derived from the ER consisting out of a neutral lipid core surrounded by a phospholipid monolayer (Guo et al., 2009). It has been shown that these organelles are recruited to the periphery of the chlamydial inclusion. Three different chlamydial proteins (Lda 1 to 3) are translocated through the inclusion membrane and localise to LDs (Kumar et al., 2006). Interestingly, the translocation of entire LDs into the inclusion lumen has equally been observed (Cocchiari et al., 2008). One hypothesis for the function of LDs in the inclusion lumen is obviously subversion of the host's lipid storage, and nutrient acquisition. Alternatively, because LDs have been implicated in signalling, inflammatory response and trafficking, it has also been suggested that co-option of these pathways might serve the pathogen beyond nutrient uptake (Tan and Bavoil, 2012). Figure 11 gives a more detailed overview over the previously mentioned pathways.

Early electron microscopy studies revealed a close association of mitochondria with the *C. psittaci* inclusion (Matsumoto et al., 1991). siRNA-mediated depletion of the essential mitochondrial Tom components resulted in a reduction of progeny during an infection with *C. caviae*, a close relative of *C. psittaci* (Derre et al., 2007). However, the same interactions with the mitochondria could not be shown for *C. trachomatis*, indicating that *C. psittaci* and *C. caviae* are particularly susceptible to mitochondrial alterations.

Transferrin (Tf) is a host iron-binding protein that binds to its receptor localised at the plasma membrane, delivering its ligand to the cell through receptor-mediated endocytosis. Tf-positive endosomes accumulate around the inclusion. Rab4 and Rab11, which are required for Tf receptor recycling, are enriched at the inclusion membrane (Rzomp et al., 2003; Scidmore et al., 2003; Scidmore et al., 1996). Rab4 and Rab11 disruption results in retention of Tf around the inclusion and in a decrease of the inclusion size, reflecting the necessity of iron for normal completion of the chlamydial cycle (Ouellette and Carabeo, 2010).

A recent study revealed that peroxisomes are equally found in the inclusion lumen (Boncompain et al., 2014). Plasmalogens, a type of ether phospholipids typically not found in aerobic bacteria, were identified in *C. trachomatis* by lipidomics. Plasmalogen synthesis occurs in peroxisomes and the plasmalogens were only detected in bacteria grown in cells

with intact peroxisomes. Identification of the side chains of these bacterial plasmalogens through lipidomic analysis revealed that they result from a metabolic collaboration between host peroxisomal enzymes and chlamydial enzymes for acyl chain synthesis.

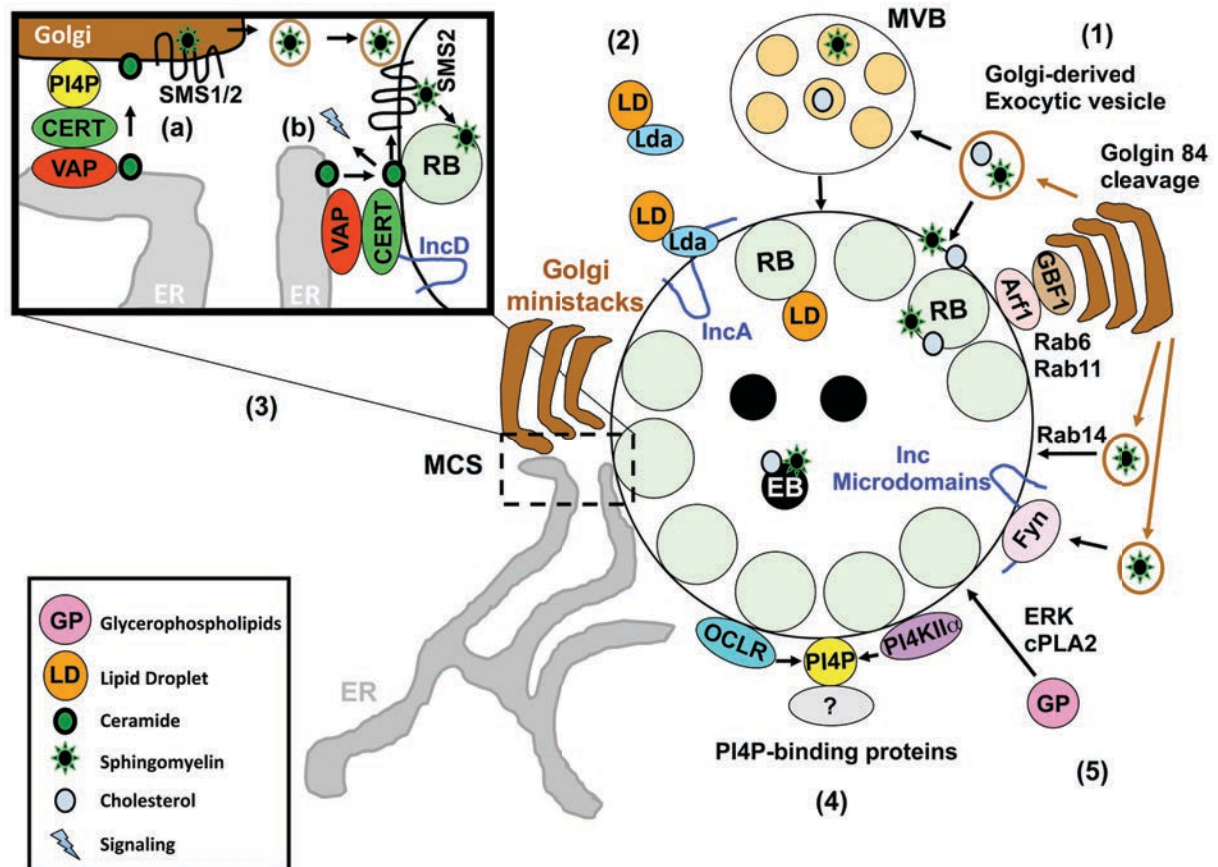


Figure 11: Lipid acquisition occurs through host-derived non-vesicular and vesicular trafficking from the ER, Golgi, MVBs and lipid droplets. (1) *Chlamydiae* induce Golgi fragmentation into mini-stacks surrounding the inclusion through a Rab6 and Rab11 dependent mechanism. Golgin-84 implication proved to be an artefact. SM and cholesterol are acquired through the interception of MVBs and Golgi-derived exocytic vesicles (via a process mediated by GBF1-dependent activation of Arf1, Rab14 and/or Fyn signalling from Inc microdomains). (2) Lipid droplets are the target of chlamydial Ldas and are translocated into the inclusion lumen at IncA sites. (3) CERT is found at the membrane contact sites between the ER (VAP) and Golgi (PI4P) (a) and between the ER (VAP) and the inclusion through its interaction with IncD (b). Golgi- or inclusion-localized SMS1/2 converts the precursor ceramide to SM. (4) PI4P-binding proteins might be recruited via the generation of PI4P pools at the inclusion through OCLR and PI4KII α . (5) Host glycerophospholipids (PI and PC) are first modified by host enzymes via ERK and cPLA2 activation and then undergo a second, *Chlamydia*-dependent enzymatical modification before they get incorporated into the bacterial membrane. Figure taken from (Elwell and Engel, 2012).

4.3 Interface of interaction: the inclusion membrane

The inclusion membrane represents the main interface between the bacteria and the host. It does not only serve as a barrier to protect *Chlamydia* from the host's defence strategies, but also as a means for close interaction with the host cell. Several studies tried to elucidate its composition. Early studies showed that it is mostly devoid of plasma membrane or endosome markers (Scidmore et al., 2003) but rich in chlamydial Inc proteins (Bannantine et al., 1998; Scidmore-Carlson et al., 1999). Even though they possess only minimal amino acid conservation, these Inc proteins share one characteristic: a large bilobed hydrophobic domain of approximately 60 amino acids. Based on this feature bioinformatic analysis estimated that *C. trachomatis* encodes for more than 50 Inc proteins, accounting for around 6 % of the whole genome (Bannantine et al., 2000; Dehoux et al., 2011). In an attempt to validate these Inc candidates, specific antibodies were produced and detected 22 of these endogenous proteins on the inclusion membrane (Li et al., 2008). Interestingly, many of the Inc proteins follow specific transcriptional patterns and contain T3S signals, indicating temporally orchestrated functions during chlamydial development (Moore and Ouellette, 2014). Only one Inc protein has been well characterized, IncA. IncA contains two SNARE-like motifs, which most likely promote homodimeric interactions necessary for the fusion of inclusions in cells infected with multiple EBs, as a natural IncA deficient strain fails to do so (Delevoye et al., 2008). As mentioned above, recruitment of CERT to the inclusion membrane depends on IncD (Agaisse and Derre, 2014; Derre et al., 2011). While topological analysis indicate that both the C- and N-termini reach into the host cell cytoplasm (Agaisse and Derre, 2014; Bauler and Hackstadt, 2014), some of the putative Inc proteins possess only very short cytoplasmic domains, suggesting that interaction with host proteins might not occur. This has led to the idea that many of the Inc proteins may function by providing structure and by organizing the inclusion membrane (Figure 12) (Moore and Ouellette, 2014). It has equally been demonstrated that some of the Inc proteins recruit and interact with different Rab GTPases, playing multiple and often still unknown roles in different aspects of *Chlamydia* development like mediating the interaction with various host cell organelles (Scidmore, 2011). Chlamydial Inc proteins are most likely not the only proteins inserted into the inclusion membrane. Considering *Chlamydia*'s reduced metabolic activities (discussed in more detail in chapter 5) a tight exchange of metabolites at the host/pathogen interface is indispensable. Only one single host transporter, the mammalian sodium multivitamin transporter (SMVT) has been identified in the inclusion membrane so far, probably allowing

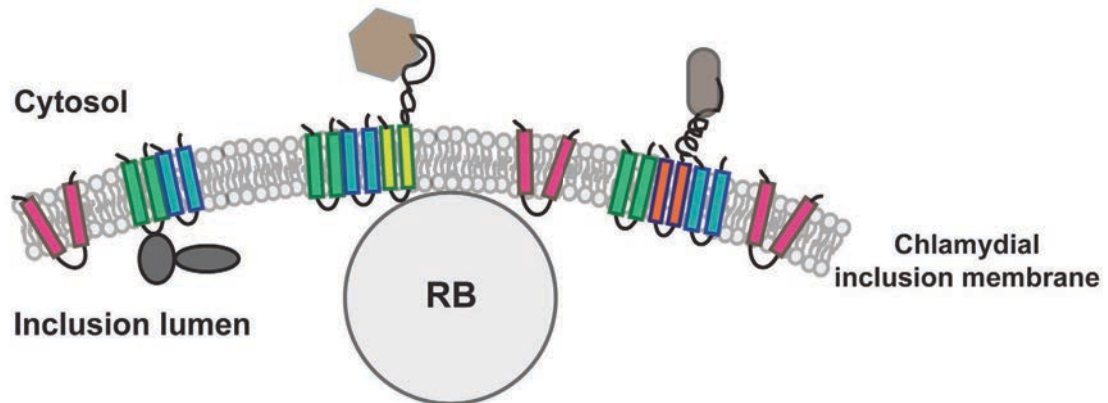


Figure 12: Inc proteins might serve as scaffolds to organize the membrane into microdomains supporting host-chlamydial interactions (Moore and Ouellette, 2014).

for a two-step import of biotin, lipoic acid and pantothenic acid into the inclusion lumen, where subsequently bacterial transporters (BioY and other unknown ones) facilitate the import into the bacteria (Fisher et al., 2012). Heinzen and Hackstadt could demonstrate that fluorescent markers ranging from 520 to 500 000 Da were not able to passively diffuse through the inclusion membrane, indicating that the inclusion membrane is impermeable to even low-molecular-weight compounds (Heinzen and Hackstadt, 1997). It is thus highly probable that the inclusion membrane contains many more transporters, either of bacterial or host origin, permitting nutrient exchange.

4.4 The host defence and chlamydial immune evasion

C. trachomatis usually infects the single-cell columnar layer of the epithelium in the endocervix of women and the urethra of men, resulting in an intense inflammation at the site of mucosal infection (Brunham and Rey-Ladino, 2005). In order to elucidate the immune reaction of humans against *C. trachomatis*, the mouse model using the closely related *C. muridarum* has been useful. After infection of the epithelial cells pro-inflammatory mediators are secreted, on one hand leading to a polarized immune response towards a protective T_H1 -type response, and on the other hand to the secretion of cytokines being involved in the pathology associated with *Chlamydia* infection (Darville et al., 2003; Johnson, 2004). Immune cells are recruited to the site of inflammation in response to the secreted cytokines leading to the accumulation of lymphocytes and the selection and expansion of naive B and T cells (Figure 13) (Brunham and Rey-Ladino, 2005). In the mouse model activity of T cells (mainly $CD4^+T_H1$ cells) is indispensable for the resolution of a *Chlamydia* infection

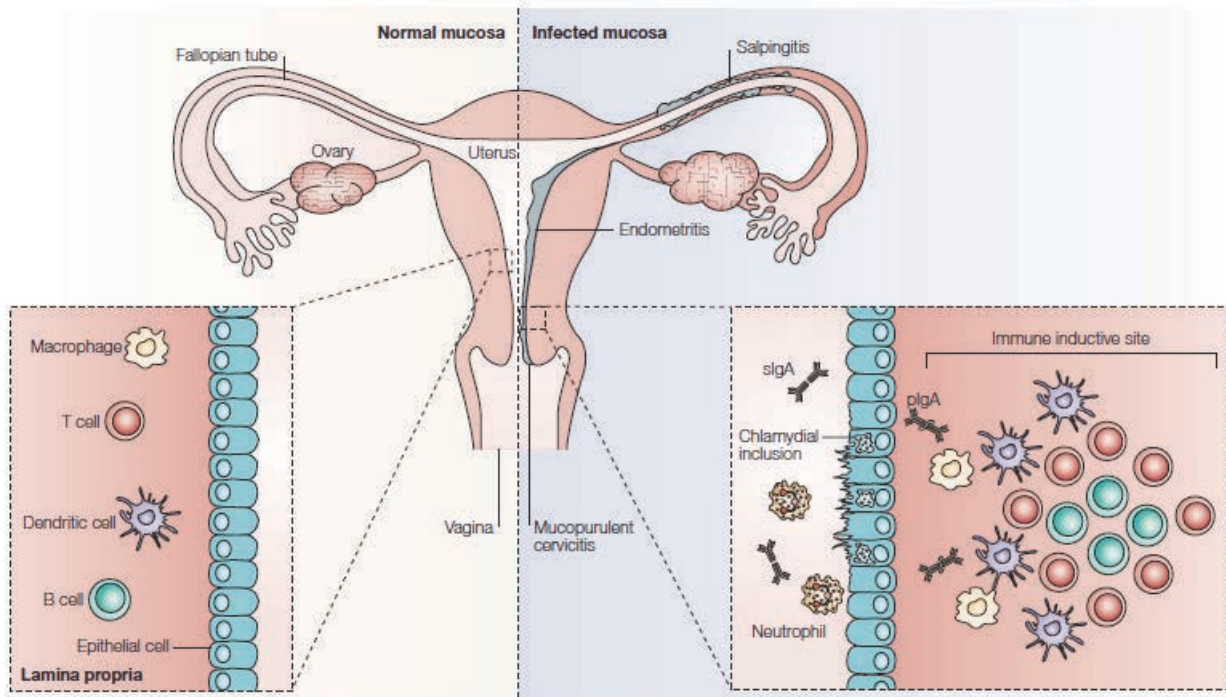


Figure 13: Female genital tract infection with *C. trachomatis*. EBs infect the epithelial cells of the cervix, with mostly little to no clinical symptoms. *Chlamydiae* ascend to the fallopian tubes causing pelvic inflammatory disease, salpingitis and scarring, with the possible outcome of infertility and ectopic pregnancy. The inflammation is characterized by a recruitment of neutrophils and macrophages and the formation of immune inductive sites in the submucosa, containing B cells, T cells, dendritic cells and macrophages, leading to the deployment of a secretory IgA (sigA) response. pIgA, polymeric IgA. Image is taken from (Brunham and Rey-Ladino, 2005).

(Ramsey and Rank, 1991; Rank et al., 1985; Su and Caldwell, 1995). While mice lacking B cells do not show a significantly different outcome of a primary infection, it appears that B cells are important for the resolution of a secondary infection, as mice lacking both CD4⁺ and CD8⁺ cells, while having a normal amount of B cells, are able to do so (Brunham and Rey-Ladino, 2005). It has been suggested that B cells and antibodies enhance a protective T cell response (Moore et al., 2003). Interestingly, the antibody titre found in cervical secretion of infected women correlates directly with the severity of the disease (Punnonen et al., 1979).

It is important to keep in mind that, while spontaneous clearance of the bacteria in mice occurs typically after around 4 weeks, infection in humans can last a couple of months before being resolved (Parks et al., 1997). Also, the human adapted strain *C. trachomatis* is rapidly cleared by mice. While studies in mice are useful to investigate several aspects of the immune response, and to identify potential markers of the clinical outcome of infection, studies on clinical samples from human patients are absolutely required.

C. trachomatis developed some strategies to evade the host immune response, some of which will be mentioned here. The major outer-membrane protein (MOMP) and the polymorphic

membrane proteins (Pmps) are both highly diverse surface proteins, facilitating the avoidance of detection by antibodies (Brunham et al., 1993). Also, chlamydial LPS appears to be 100 times less potent in eliciting an immune response than LPS from other bacteria (Ingalls et al., 1995). The bacteria equally interfere with the pathway of host cell apoptosis through different mechanisms, ensuring a viable surrounding for the duration of infection (Fan et al., 1998; Fischer et al., 2004; Kun et al., 2013). Enhanced survival inside the host is also guaranteed by formation of a niche with limited exposure to host cell cytoplasmic defence machineries, the inclusion.

5. Chlamydial metabolism

The first comprehensive insight into chlamydial metabolism was provided by the genome sequencing of *C. trachomatis* serovar D in 1998 and brought several surprises (Stephens et al., 1998). It was later followed by the description of the genome of one environmental *Chlamydiae* (Horn et al., 2004). Interestingly, many pathways are functional in the *Parachlamydiaceae* that are not functional in the *Chlamydiaceae*, reflecting the different environmental pressures (Omsland et al., 2014). In both cases, it turned out that the ability of *Chlamydia* spp. to metabolise certain substrates had previously been missed, mostly due to methodological constraints. It remains however that many metabolic pathways are incomplete or missing, reflecting the exploitation of the cellular environment to fulfil essential metabolic functions.

5.1 Energy parasite hypothesis

In 1962 Moulder coined the hypothesis of *Chlamydia* as an "energy parasite", as early studies failed to reveal their capacities to oxidise intermediates of central metabolic pathways, such as the tricarboxylic acid (TCA) cycle or glycolysis. The apparent lack of full pathways to produce ATP led him and colleagues to hypothesize that *Chlamydiae* are essential scavengers of their host's ATP pool (Moulder, 1962). Weiss et al demonstrated the utilisation of radioactively labelled D-glucose while oxygen consumption did not increase (Ormsbee and Weiss, 1963; Weiss et al., 1964). He concluded that *Chlamydiae* were unable to perform the TCA cycle as well as aerobic respiration, and that they most likely use the pentose phosphate pathway. Enzymatic activities related to the pentose phosphate pathway were detected soon after (Moulder et al., 1965), and it was confirmed that the TCA cycle existed only in an

incomplete way in *Chlamydia*, equally by determination of enzymatic activity (Weiss, 1967). Interestingly, Weiss noticed that purified *Chlamydiae* produced pyruvate from D-glucose in a way that could not be explained by the exclusive use of the pentose phosphate pathway, hinting towards the production of ATP through substrate-level phosphorylation during glycolysis. Paradoxically, they were only able to do so when ATP was added externally. It became obvious that throughout the purification step host-derived hexokinase had been co-purified, indicating that *Chlamydiae* do not possess a functional hexokinase catalysing the phosphorylation of glucose to glucose-6-phosphate (Glc6P). This was the first proof that *Chlamydia*'s host-independent glucose metabolism starts with the phosphorylated compound (Vender and Moulder, 1967; Weiss and Wilson, 1969). While these studies gave evidence that *Chlamydiae* were indeed able to produce their own ATP, host-free protein synthesis of purified RBs strictly depended on the external addition of nucleotides. Hatch et al suggested the requirement of import of host-derived nucleotides due to the inability of *de novo* synthesis and proved that the import of ATP occurred through an ATP-ADP exchange mechanism (Hatch, 1975; Hatch et al., 1982). He also proposed the necessity of ATP hydrolysis in fuelling the generation of a membrane potential required for the import of amino acids (Hatch et al., 1982). These early studies validated the "energy parasite" hypothesis only partially, suggesting that while *Chlamydia* does possess the capacity to produce ATP through glycolysis and NADPH (a reducing cofactor used in anabolic reactions such as lipid and dNTP generation) through the pentose phosphate pathway, they require at the same time the import of host-derived ATP.

5.2 Metabolic pathways in the post-genomic era

Genomic sequencing of *Chlamydia* revealed the existence of metabolic pathways previously thought to be non-functional. While efforts were undertaken to conduct more targeted functional studies via heterologous expression in *E. coli*, many pathways remain elusive. Figure 14 depicts most of the metabolic pathways known to occur in *C. trachomatis* to date.

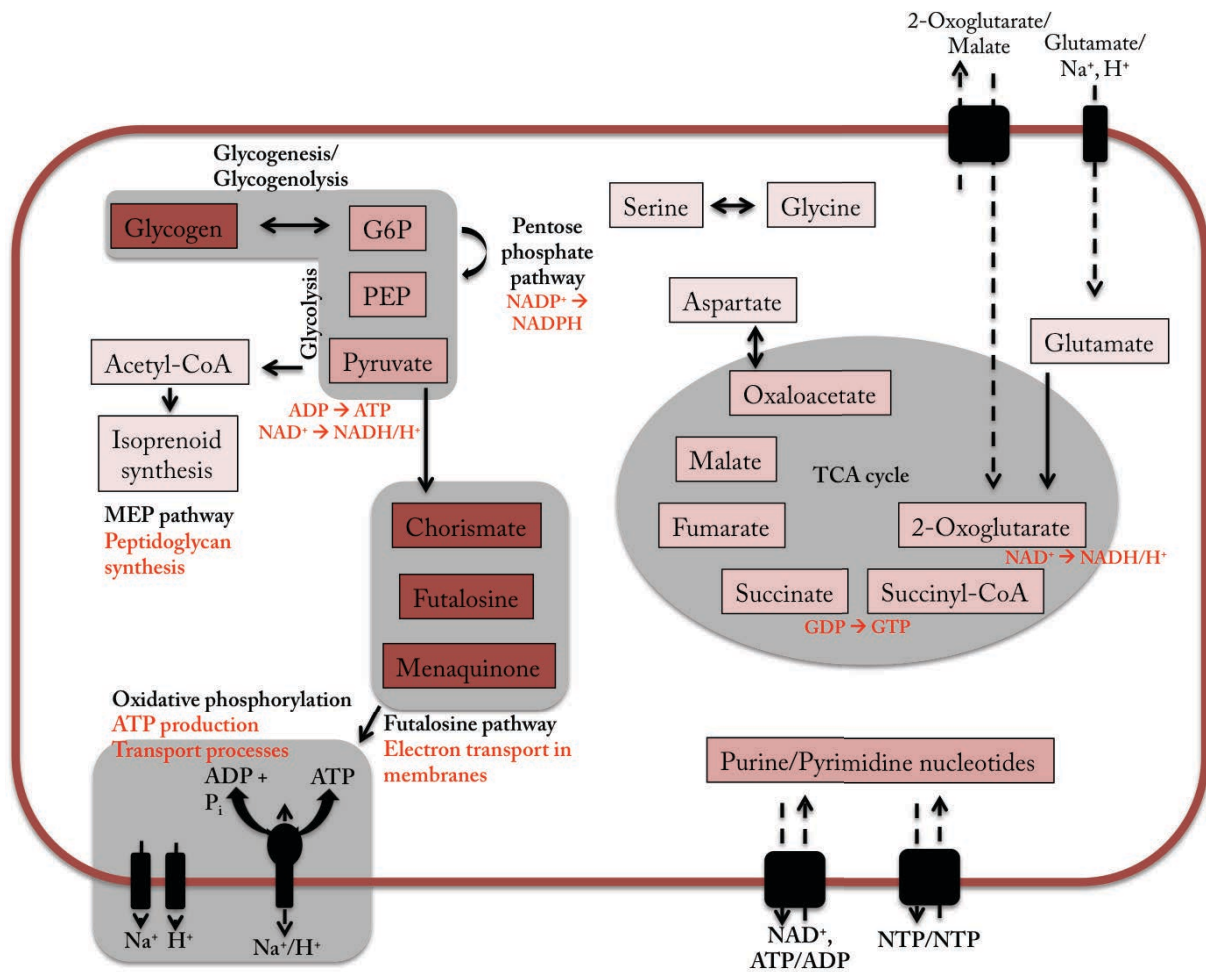


Figure 14: Schematic representation of the main metabolic pathways of *C. trachomatis*.

5.2.1 Substrate level phosphorylation

Genetic sequencing and homology predictions provided the final proof that *Chlamydia* encodes for all the enzymes necessary for glycolysis (Figure 15), but lacks the gene for a hexokinase. Heterologous expression in *E. coli* confirmed the functionality of all these enzymes (Iliffe-Lee and McClarty, 1999), demonstrating that the bacteria are able to metabolise Glc6P to pyruvate, leading to the production of ATP through substrate level phosphorylation. Import of Glc6P is most likely to occur through the hexosephosphate transporter UhpC (Schwoppe et al., 2002). While other bacteria such as *E. coli* also import glucose via a phosphotransferase system (PTS) having the ability to directly phosphorylate glucose upon import into the bacteria, *Chlamydiae* only possess certain subunits of the PTS, rendering it dysfunctional for glucose import (Barabote and Saier, 2005). The first step of glycolysis involving the hexokinase includes phosphorylation of glucose through use of ATP,

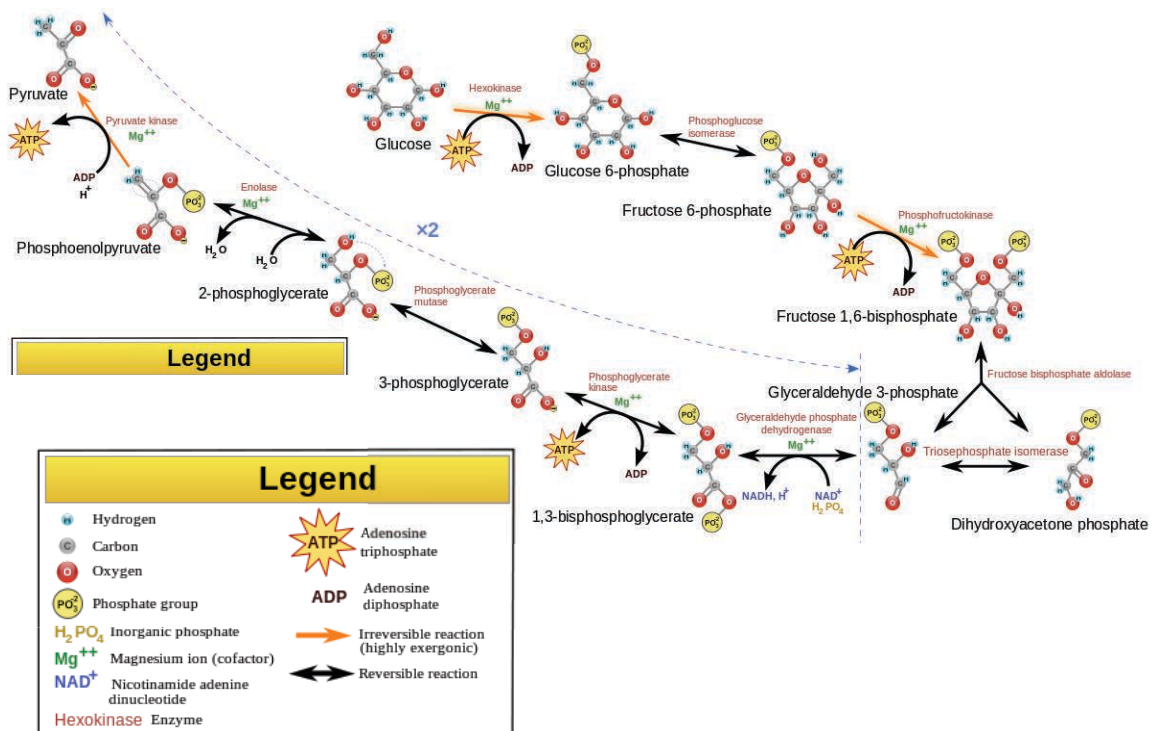


Figure 15: Glycolysis overview. Wikipedia.

which is more costly than importing Glc6P directly. Thus, while the absence of hexokinase is unusual in bacteria (environmental *Chlamydiae* retained this gene), as long as *Chlamydia* is able to obtain Glc6P from its host, it is only of benefit to import Glc6P rather than Glc, and to lose the superfluous gene for hexokinase.

While *Chlamydiaceae* are able to metabolise the product of glycolysis, pyruvate, into acetyl-CoA through a pyruvate dehydrogenase via oxidation of NAD^+ to NADH/H^+ , they do apparently lack the enzymes to enter the TCA (for the depiction of a complete TCA cycle see Figure 16). Three enzymes of the TCA are missing in all *Chlamydiaceae*: citrate synthase, aconitase and isocitrate dehydrogenase (Stephens et al., 1998), confirming early experimental evidence mentioned above. To complete the TCA cycle *Chlamydia* is therefore highly dependent on a constant exchange of metabolites with the host cell. McClarty proposed the exchange to occur through a dicarboxylate translocator (SodTi) importing 2-oxoglutarate while exporting malate (Iliffe-Lee and McClarty, 2000). Import of glutamate and conversion to 2-oxoglutarate might be an alternative pathway for *Chlamydia* to enter their reduced TCA cycle, a mechanism that had been experimentally demonstrated to function in *C. psittaci* (Weiss, 1967). All of the *C. trachomatis* genomes sequenced so far have revealed that

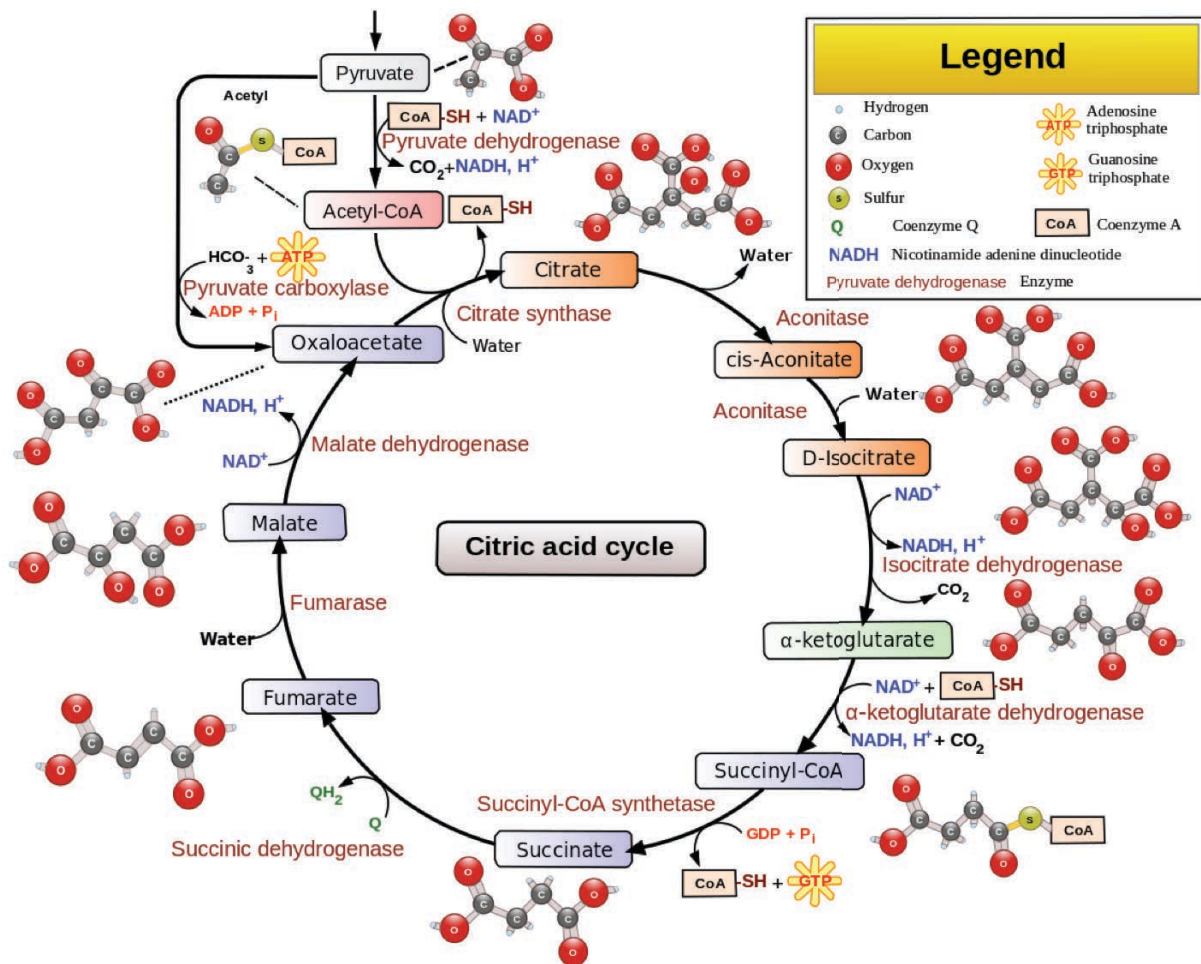


Figure 16: Citric acid or TCA cycle overview. Wikipedia.

reduction in the TCA cycle is not at its end. They carry a frameshift mutation in the gene encoding a subunit of the succinate dehydrogenase, rendering the enzyme dysfunctional (Cecchini, 2003; Thomson et al., 2008). Two frameshift mutations in the gene encoding fumarase for strains *C. trachomatis* L2/434/Bu and L2/UCH-1/proctitis might have the same consequence (Thomson et al., 2008). Not only does this reduce the TCA to a mere synthesis of succinate and succinyl-CoA from 2-oxoglutarate (still leading to the production of NADH/H^+ and GTP), but it also has consequences on the pathway of oxidative phosphorylation, as will be discussed in section 5.2.2.

5.2.2 Oxidative phosphorylation

To gain energy, an alternative way to the substrate level phosphorylation is oxidative phosphorylation, a process in which electrons are transferred from electron donors to electron acceptors. The released energy creates a membrane potential through the use of membrane spanning enzymes, water-soluble and lipid-soluble proteins. The set of proteins responsible for the energy transfer is referred to as electron transport chain or respiratory chain. This

membrane potential is generally used to generate ATP via different types of ATPases. Two different electron donors are used, NADH and succinate, both of which will be oxidised to NAD^+ and fumarate, respectively. Genome analysis revealed that *Chlamydiaceae* possess the capacity to produce ATP through oxidative phosphorylation. However, their respiratory chain is minimal. They encode for a NADH dehydrogenase (complex I, Nqr), succinate dehydrogenase (complex II, SdhA-C), cytochrome bd oxidase (complex IV, CydAB) and a V-type ATPase (complex V) (Omsland et al., 2014; Stephens et al., 1998). The chlamydial NADH dehydrogenase seems closely related to Na^+ -translocating NADH dehydrogenases, indicating that they rather use a sodium-driven force than a proton-driven force to build up a membrane potential. As mentioned above all *C. trachomatis* possess a reduction in their TCA cycle through truncation of the succinate dehydrogenase, rendering it dysfunctional. This enzyme is uniquely positioned acting as a regulator not only of the TCA cycle, but also of the oxidative phosphorylation and transfers electrons from succinate (oxidizing it to fumarate) to a ubiquinone, an electron carrier. The chlamydial cytochrome bd oxidase exhibits high oxygen affinity, indicating that *Chlamydia* may be confronted with low oxygen levels during infection. Interestingly, V-type ATPases are known to predominantly use sodium as ion, which yields less ATP than a proton-driven force. It has thus been suggested by Hatch that the chlamydial ATPase might be used for the opposite purpose: the generation of a membrane potential by ATP-hydrolysis fuelling transport processes (Hatch et al., 1982).

5.2.3 Other metabolic pathways

Additionally to the above-mentioned metabolic pathways, *Chlamydia* also encodes for the genes necessary for gluconeogenesis, the mechanism required for the regeneration of Glc6P out of substrates such as pyruvate or phosphoenolpyruvate, many of the reactions being reversible steps of glycolysis. Concordant with previous observations *Chlamydiaceae* possess the genes for a complete pentose phosphate pathway (Figure 17). The pentose phosphate pathway is required for the regeneration of NADPH and pentose phosphates. It is not involved *per se* in the acquisition of energy, but its products can enter glycolysis.

Isoprenoids are synthesized via the nonmevalonate pathway (methylerythritol phosphate (MEP) pathway) using pyruvate or acetyl-CoA as substrate (Grieshaber et al., 2004). This pathway allows for the production of precursors for peptidoglycan synthesis and for metabolites required for prenylation of proteins. In addition, a metabolite of the MEP pathway, IspE, plays a critical role in EB to RB conversion, as mentioned in 3.2.2. The

classical pathway of menaquinone synthesis (the chorismate pathway) is present in environmental *Chlamydiae*, but not in *Chlamydiaceae*, which led to the assumption that the latter have to import this lipid-soluble substance from the host cell, as it is indispensable for the electron transport in the oxidative phosphorylation process (Ilfie-Lee and McClarty, 1999). However, there is a recently described alternative pathway for menaquinone synthesis in bacteria, the futasoline pathway, (Hiratsuka et al., 2008), which appears to be present in all *Chlamydiaceae*. Metabolic pathways for pyrimidine and purine synthesis are lacking, but nucleotide transporters have been identified (Npt1Ct and Npt2Ct), importing ATP, GTP, UTP, CTP and NAD⁺ (Fisher et al., 2013; Tjaden et al., 1999). CTP can also be obtained by conversion of UTP (Tipples and McClarty, 1993). *Chlamydiae* are able to produce dNTPs out of NTPs by a chlamydial specific ribonucleotide reductase (McClarty and Tipples, 1991).

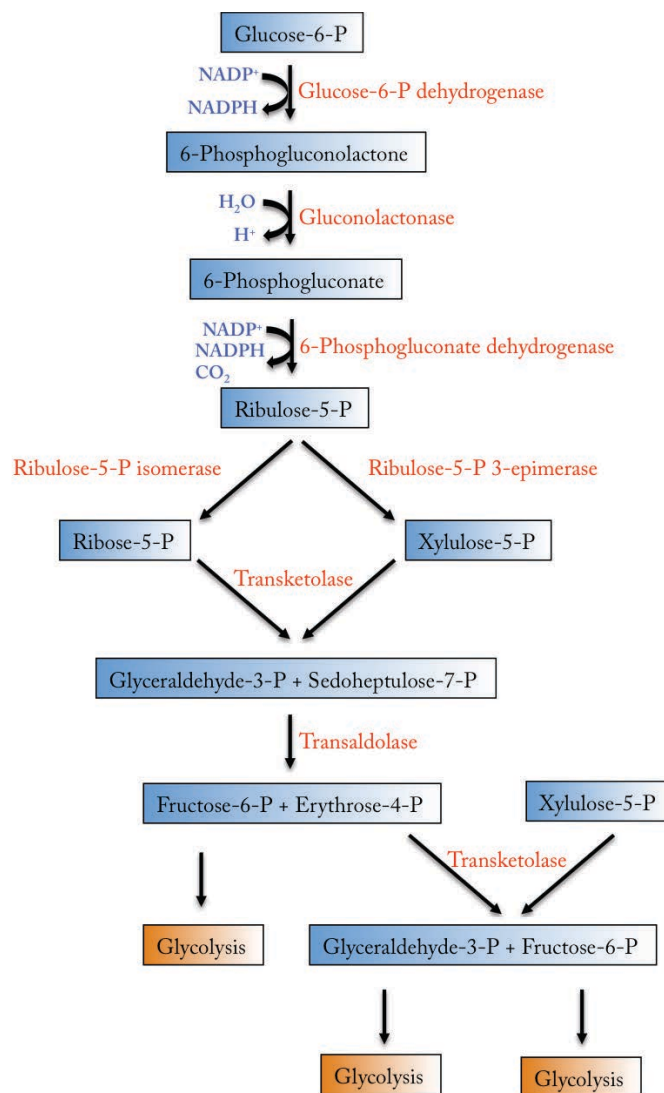


Figure 17: Pentose-phosphate pathway overview. P = phosphate

5.3 Glycogen

Glycogen is a storage polysaccharide that can easily be broken down into glucose molecules and is present in animals, fungi and bacteria, indicating its long evolutionary history. Its structure is similar to that of plant starch but is essentially a more branched version of the polymer. Glucose molecules are linked through an α -1,4 glycosidic bond with α -1,6 links at the branching points (Figure 18). Branching points of glycogen isolated from biological sources are not in precisely defined locations, but the average chain of mammalian glycogen counts 13 glucose residues, rather than 8-12 in bacteria (Roach et al., 2012) (Wilson et al., 2010). In mammals glycogen is an osmotically neutral means of storing glucose, mainly in the liver (discovered by Claude Bernard in 1857) and in muscle cells. The storage is under tight hormonal regulation, and glucose is released from glycogen when needed (Roach et al., 2012). Whereas the liver cells deliver glucose through the bloodstream to the rest of the body, the muscle cells retain their glycogen-derived glucose for moments of activity. Glycogen is less reduced and thus less energy rich than fatty acids, but bears some important advantages: glucose from glycogen is readily mobilized and depicts thus an energy source for sudden, strenuous activity; except in situations of starvation, glucose is the main energy source in the brain; in contrast to fatty acids released glucose can provide energy in anaerobic conditions, in the absence of oxygen (Berg JM, 2002). Glycogen is present in the cytoplasm under the form of individual β -particles with a diameter ranging from 10 to 40 nm (Figure 12) (Coimbra and Leblond, 1966). Several β -particles appear to be able to form bigger aggregates, called α -particles, even though chemical and physical characteristics of these α - particles are not well

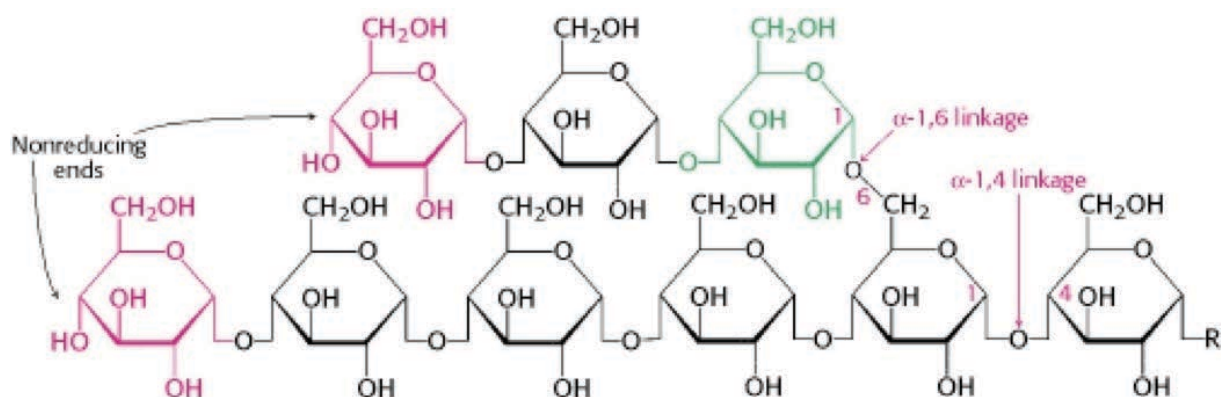


Figure 18: Structure of glycogen molecule and ramifications. Image taken from (Berg JM, 2002).

understood (Roach et al., 2012). Muscle glycogen can be broken down through two different ways: in the cytoplasm via the degradation by glycogen degrading enzymes (which will be explained in more detail in chapter 5.3.2) or through a lysosomal pathway involving a lysosomal α -glucosidase (Roach et al., 2012). The latter, autophagy-linked, mechanism has also been identified to occur in liver and heart of a foetus, probably to provide a vast amount of rapidly required energy. But whether this is ubiquitous or remains restricted to muscle cells and new-borns remains to be elucidated (Roach et al., 2012). Glucose-phosphates derived from glycogen breakdown in mammals have three different fates: glycolysis (ATP production); pentose phosphate pathway (yielding NADPH and ribose derivatives); conversion into free glucose and release into bloodstream. The role of bacterial glycogen on the other hand is less clear. While it has been demonstrated that glycogen can serve as carbon source in times of starvation (Strange, 1968) e.g. for *Vibrio cholerae* (Bourassa and Camilli, 2009) some bacteria might use it for different purposes, such as sporulation or a driving force for differentiation in the case of *Bacillus subtilis* or *Streptomyces coelicolor* (Kiel et al., 1994; Martin et al., 1997; Plaskitt and Chater, 1995). It was suggested that the latter also use glycogen to maintain turgor pressure through glycogen accumulation (Bruton et al., 1995). In *Salmonella enteritidis* a link could be established between glycogen synthesis, biofilm formation and virulence (Ballicora et al., 2003; Bonafonte et al., 2000).

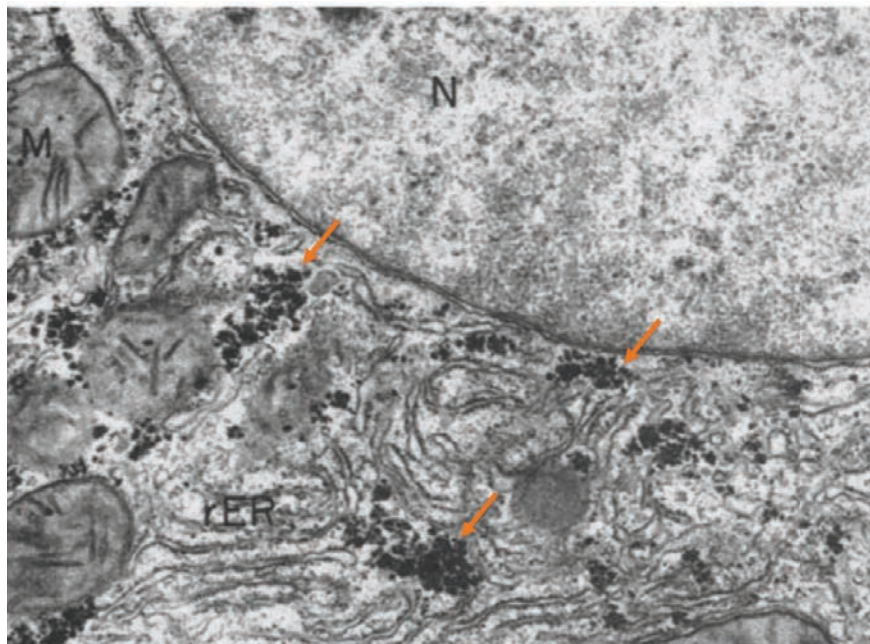


Figure 19: Electron micrograph of rat liver cells (Coimbra and Leblond, 1966). Black granules are glycogen (marked with a red arrow). N = nucleus; M = mitochondria; rER = rough endoplasmic reticulum.

5.3.1 Chlamydial glycogen metabolism

It has been known since the sixties that inclusions of *C. trachomatis* infected cells stain positive with iodine, a strong indication for glycogen accumulation (Gordon and Quan, 1965). In a more in-depth study by electron microscopy Chiappino and colleagues stated the presence of glycogen not only in the inclusion lumen, but also in EBs and, to a lesser extent, in RBs. They concluded that the intraluminal glycogen is released by bacteria through lysis, a hypothesis that has been widely accepted throughout the chlamydial research community (Chiappino et al., 1995). Among other *Chlamydiaceae* only the closely related *C. muridarum* equally accumulates glycogen within the inclusion lumen.

The enzymes for glycogen synthesis and degradation are well conserved in most bacterial species (Preiss, 2014; Wilson et al., 2010). Figure 20 gives an overview over the glycogen genes encoded by *Chlamydiaceae*. Interestingly, although *Chlamydia* has a comparably small genome, with evidence of on-going reduction, *Chlamydia* retains all the enzymes necessary for functional glycogen synthesis and degradation, with some differences to the well characterized *E. coli*. This bacterium imports glucose and phosphorylates it directly to Glc6P through the phosphotransferase system (PTS), which, as mentioned before, is incomplete in *Chlamydia*. Again, *Chlamydia* also misses hexokinase, implicating that the bacteria need to import phosphorylated glucose, glucose-1-phosphate (Glc1P) or Glc6P, presumably through the single annotated chlamydial hexose phosphate transporter, UhpC. Glc6P and Glc1P can be interconverted by a phosphoglucomutase (PGM). Glc1P is converted to ADP-Glucose (ADP-Glc) by an ADP-glucose pyrophosphorylase GlgC, in the presence of Mg^{2+} and ATP, releasing inorganic pyrophosphate. ADP-Glc is subsequently used as a sugar donor by the bacterial glycogen synthase, GlgA, producing a linear chain of glucose molecules through α -1,4 glycosidic bonds. Only one exception is known so far, *Prevotella bryantii*, a ruminal bacterium lacking GlgC. Its GlgA adapted to the exclusive use of UDP-glucose (UDP-Glc) from the ruminal host as a sugar donor (Lou et al., 1997; Wilson et al., 2010). The chlamydial GlgA was detected in the inclusion lumen 12 hpi and then in the host cell cytoplasm in an immunofluorescence assay using an antibody against the endogenous protein (Lu et al., 2013).

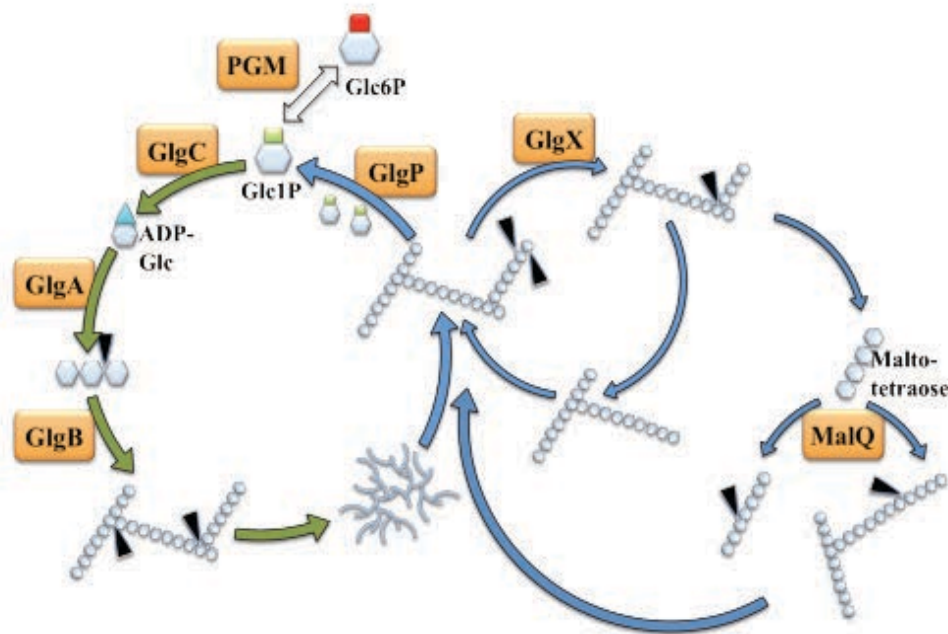


Figure 20: Chlamydial glycogen metabolism. In green: glycogen synthesis. In blue: glycogen degradation. Phosphoglucomutase (PGM) interconverts between Glc6P and Glc1P, the latter one serving as substrate for ADP-Glc synthesis through an ADP-glucose pyrophosphorylase (GlgC). The glycogen synthase (GlgA) subsequently produces linear glycogen chains via α -1,4 glycosidic bonds, and the branching enzyme (GlgB) introduces ramifications through α -1,6 linkages. The non-reducing end of the glycogen molecule is degraded through glycogen phosphorylase (GlgP) releasing Glc1P until it is 4 glucose units away from a branching point. In this scheme the debranching enzyme GlgX hydrolyses branching points and MalQ transfers the maltotetraoses to another chain. Black arrows point to the site of enzymatic activity.

GlgB, the branching enzyme, introduces the ramifications of the glycogen molecule in two steps: first, it removes a 6-9 unit long glucose chain from the linear glycogen molecule. It then transfers the oligosaccharide to a glucose residue somewhere in the molecule and links both with an α -1,6 glycosidic bond. A chlamydial *glgB* mutant with a dysfunctional GlgB enzyme was generated through chemical mutagenesis. It displayed a strong phenotype: the authors described the morphology of the inclusions as being large and granular, and upon glycogen staining they could confirm the identity of these granules as being precipitates of glycogen, presumably non-branched (Figure 21) (Nguyen and Valdivia, 2012). Intriguingly, they observed the same phenotype for *Chlamydia* bearing a mutation in a very different gene, *gspE*, a mutant with impaired type 2 secretion. Upon degradation of glycogen a glycogen phosphorylase (GlgP) releases single Glc1P molecules to a minimum chain length of 4 units. Interestingly, proteomics of *Chlamydia* infected cells revealed an enrichment of GlgP in the inclusion membrane-containing fraction (Saka et al., 2011), but this has never been

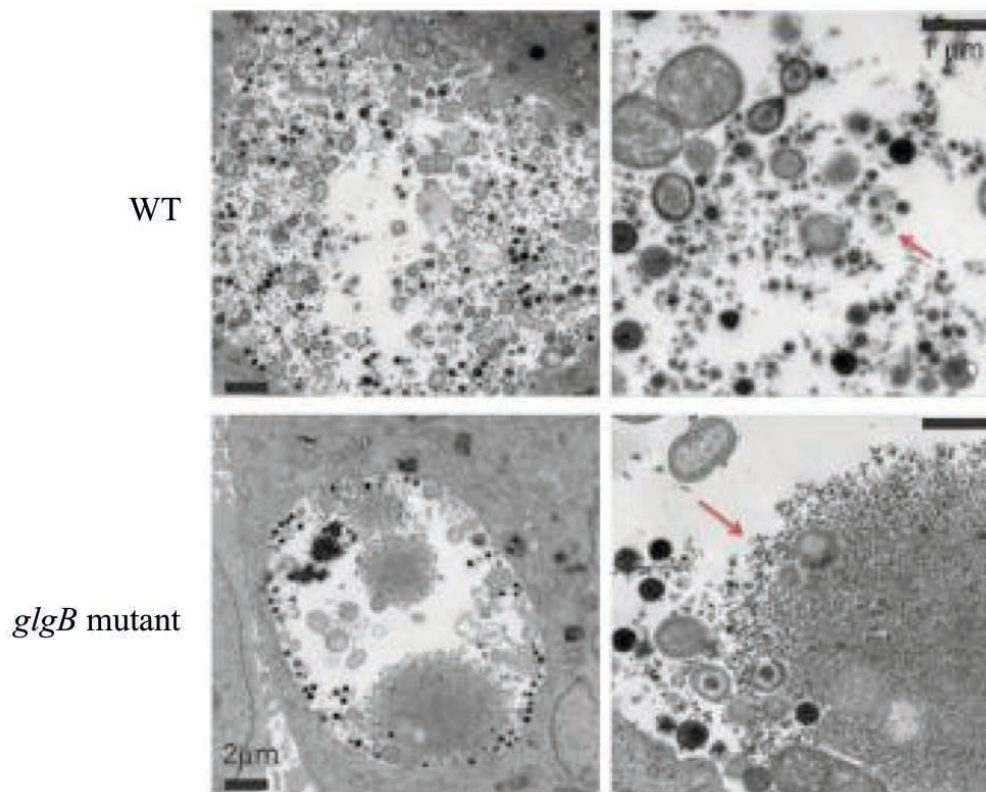


Figure 21: Inclusions of wild-type infected or *glgB* mutant infected cells. Note the precipitates of glycogen in the mutant (red arrow) compared to the wild-type. (Nguyen and Valdivia, 2012).

confirmed directly using antibodies. GlgX, a debranching enzyme, probably cleaves off the remaining maltotetraose and MalQ presumably transfers it to an already existing glycogen chain of various length, rendering it again accessible for further degradation through GlgP (pathway depicted in Figure 20). Alternatively, MalQ could cleave off a chain of three glucose units and transfer them to another chain in the glycogen molecule, while GlgX releases the remaining fourth glucose residue (Dauvillee et al., 2005; Hwang et al., 2013). The exact mechanisms of GlgX and MalQ are not very well conserved in the bacterial kingdom. Whether they are both required for degradation cannot be elucidated through sequence analysis. While a number of bacteria possess a single operon clustering genes related to glycogen metabolism together, chlamydial glycogen genes are highly dispersed throughout the genome (Stephens et al., 1998). It is not clear if the chlamydial glycogen enzymes or their synthesis are under some kind of regulation, as reports do not agree on it. RT-PCR analysis could not reveal a change of the expression pattern of *glgA* and *glgP* when infection occurred in absence or presence of glucose or another carbon source (Ilfie-Lee and McClarty, 2000). When O'Connell and colleagues incubated infected cells with the glucose analogue 2-

deoxyglucose (2DG) irreversibly binding to hexokinase and thus blocking the host synthesis of Glc6P, they observed a decrease of glycogen in the inclusion lumen, together with a decrease of transcription of *glgA* but not of *uhpC*, compared to non-treated cells, indicating a substrate-dependent transcriptional regulation of glycogen synthesis (O'Connell et al., 2011). In *E. coli* GlgP underlies an indirect regulation connected to the PTS. One of the PTS subunits, the cytoplasmic HPr, transfers a phosphoryl group to glucose. Interestingly, GlgP is able to bind to HPr, and displays reduced activity when HPr is phosphorylated, and maximum activity when HPr is unphosphorylated. This allows for glycogen accumulation when the substrate glucose is imported, and inhibits collateral degradation (Deutscher et al., 2006; Wilson et al., 2010). *Chlamydiae* do not encode for the permease subunit of the PTS and are thus not able to import glucose. However, they encode for different HPr enzymes, whose function as a part of an incomplete complex has not been determined yet.

A cryptic plasmid of 7.5 kb is found in almost all natural isolates of *C. trachomatis*, containing 8 open reading frames (ORFs). While it has been demonstrated that some of these ORFs encode for virulence factors, not all of the functions are understood yet. Matsumoto could show that a plasmid-less strain of *C. trachomatis* was not able to accumulate glycogen in the inclusion lumen, suggesting for the first time a link between the plasmid and chlamydial glycogen synthesis (Matsumoto et al., 1998). Ten years later it was shown that transcription of *glgA*, but not of the other glycogen related genes, was highly decreased in a natural isolate lacking the plasmid, compared to a wild-type strain (Carlson et al., 2008). Plasmid encoded *pgp4* appears to be a transcriptional regulator of chromosomal gene expression including *glgA* (Gong et al., 2013). When used to infect mice the plasmid-less strain displays a lower pathogenicity and virulence than the wild-type strain, and the very rare occurrence of a natural plasmid-less isolates highly speaks for its importance during infection (Carlson et al., 2008).

5.3.2 Eukaryotic glycogen metabolism

The eukaryotic glycogen metabolism resembles the bacterial one, but exhibits some major differences, which are going to be stressed here. To date there are 12 glucose transporters known in humans, the most important and best characterized being: GLUT1, ubiquitous and providing basal glucose levels; GLUT2, a bidirectional transporter mainly expressed in liver cells and pancreatic β -cells; GLUT3 being mainly expressed in the placenta and in neurons and GLUT4, mainly found in skeletal muscle and adipose tissues and being

insulin sensitive. It was shown that *C. caviae* infection up-regulates the host GLUT1 receptor upon infection and leads to an increase in glucose consumption (Ojcius et al., 1998). The imported glucose is subsequently phosphorylated to Glc6P by a hexokinase. Blocking of this pathway by addition of 2DG strongly impairs glycogen accumulation in the chlamydial inclusion lumen (O'Connell et al., 2011). A phosphoglucomutase is responsible for the conversion to Glc1P, which serves as substrate for UDP-glucose pyrophosphorylase, producing UDP-Glc on the expense of UTP. In contrast to the bacterial GlgA, the eukaryotic glycogen synthase is not able to initiate glycogen *de novo* synthesis. Initiation requires another enzyme, glycogenin, a self-glucosylating protein, that transfers glucose from UDP-Glc to a tyrosine residue within itself, and subsequently forms a chain of 10-20 glucose units through α -1,4 glycosidic bonds. Elongation of the short glycogen chain occurs through the glycogen synthase, which uses UDP-Glc as a substrate. Note that prokaryotes use ADP-Glc as a sugar donor. The glycogen synthase is highly regulated by the cellular concentrations of UDP-Glc, Glc6P and glycogen, and by its own phosphorylation status (Roach et al., 2012). Ramifications are introduced by the branching enzyme in a fashion similar to what happens in prokaryotes. The degradation of glycogen in eukaryotes occurs first through the glycogen phosphorylase cleaving off Glc6P units until reaching four residues before a glucose that is branched with an α -1,4 linkage, corresponding to the prokaryotic mechanism. However, the glycogen debranching enzyme AGL performs a dual role, in which it first removes three out of the four remaining glucose residues and transfers them elsewhere in the molecule, and then cleaves the remaining glucose unit through an α -1,6 glucosidase activity to render the linear glycogen molecule accessible to the glycogen phosphorylase for further degradation (Hwang et al., 2013; Markan et al., 2010).

A very interesting feature of eukaryotic glycogen is that the particles form higher-order assemblages with associated proteins, providing the particles with some kind of 'organelle features' (Roach et al., 2012; Stapleton et al., 2010). Some of these enzymes that stay tightly associated with the glycogen particle are glycogenin, glycogen synthase, glycogen phosphorylase, AGL and several regulatory proteins. Interestingly, albeit the branching enzyme interacts with glycogen, it does not undergo a stable association (Caudwell and Cohen, 1980).

Glycogen accumulation within the inclusion lumen was first detected around 50 years ago, but the question of its origin was rarely raised. Lysis of bacteria and thus release of intrabacterial glycogen into the lumen has been suggested to give rise to this phenotype, and has broadly been accepted by the chlamydial community. However, the sole indication that this would indeed be the case are electron microscopy images depicting the lysis of some of the bacteria and the subsequent release of glycogen into the inclusion lumen (Chiappino et al., 1995).

It has also been known for more than a decade now that glucose consumption is essential for *C. trachomatis*, as infection in the absence of glucose fails to produce any infectious forms (Harper et al., 2000; Iliffe-Lee and McClarty, 2000). This demonstrates the importance of the nutrient, and probably its polysaccharide glycogen, to the pathogen. We were intrigued by the lack of strong evidence supporting the hypothesis that bacterial lysis is the source of intraluminal glycogen. This work aimed to reconstitute the glucose flow in *C. trachomatis* infected cells and to understand the mechanisms for the intraluminal and bacterial glycogen accumulation.

MATERIAL AND METHODS

Cells and bacteria

HeLa cells (ATCC), wild-type mouse embryonic fibroblasts (MEF) and MEF *Atg5*^{-/-} (generous gift from N. Mizushima, Tokyo Medical and Dental University) were cultured in Dulbecco's modified Eagle's medium with Glutamax (DMEM, Invitrogen), supplemented with 10 % (v/v) fetal bovine serum (FBS). For experiments with medium containing different glucose concentrations DMEM without glucose (DMEM, Invitrogen) was used and complemented with 5 mM sodium pyruvate (Sigma-Aldrich), 10 % fetal bovine serum (FBS) and the indicated glucose concentration (Merck). *C. trachomatis* LGV serovar L2 strain 434 (ATCC), the plasmid-less strain LGV L2 25667R (Bowie, 1990) or L2^{IncD}GFP (Vromman et al., 2014) were purified on density gradients as previously described (Scidmore, 2005). The *ipaB* and *mxiD* strains are derivatives of M90T, the virulent wild-type strain of *Shigella flexneri*, in which the respective genes (*ipaB* and *mxiD*) have been inactivated (Allaoui et al., 1993). The *Escherichia coli* strain *DH5α* was used for cloning purposes and plasmid amplification. Both *S. flexneri* and *E. coli* strains were grown in Luria-Bertani medium with ampicillin at 0.1 mg/ml.

Electron microscopy and periodic acid-thiocarbohydrazide-silver proteinate reaction (PATAg)

HeLa cells were grown in wells, infected with *C. trachomatis* LGV serovar L2 strain 434 at an MOI of 0.1 and carefully trypsinized at the indicated time points. The cells were then washed with PBS once and fixed with 0.1 M cacodylate and 2.5 % glutaraldehyde at room temperature for at least 30 min. PATAg staining was performed as described elsewhere (Thiéry, 1967). Briefly, thin sections were incubated in 1 % periodic acid for 25 min and then washed several times in water, followed by an incubation step in 0.2 % thiocarbohydrazide in 20 % acetic acid for 45 min. Several washing steps in a graded acetic acid series to water were carried out and the thin section were stained with 1 % silver proteinate for 30 min. Samples were observed on a transmission electron microscope within a week after preparation.

Reinfection assay and flow cytometry

HeLa cells were cultured in the indicated glucose medium three days prior to the experiment and then infected with L2^{IncD}GFP at a multiplicity of infection (MOI) < 0.5.

Forty-eight hrs later cells were washed in PBS, detached and lysed with beads (1 mm). Fresh HeLa cells in full DMEM 10 % FBS were inoculated with a serial dilution of the cell lysate and 24 hrs later flow cytometry was used to determine the IFU as described elsewhere (Vromman et al., 2014). Briefly, cells were washed with PBS and gently trypsinized. After a washing step in PBS cells were fixed in PFA 2 % in PBS and incubated for 16 hrs at 4 °C. Flow cytometry analysis was carried out with a FACS Gallios (Beckton Coulter).

Quantitative Reverse Transcription PCR and Reverse Transcription PCR

Total RNA was isolated from 5×10^5 HeLa cells infected with *C. trachomatis* LGV serovar L2 after 1 h, 3 h, 8 h, 16 h, 24 h or 40 h of infection (MOI of 10 for 1 h, 3 h, 8 h and MOI of 0.1 for 16 h, 24 h, 40 h) with the RNeasy Mini Kit with DNase treatment (Qiagen) according to the manufacturer's protocol. RNA concentrations were determined by NanoDrop and normalized to equal contents. Reverse transcription (RT) was performed with SuperScript III Reverse Transcriptase (Life Technologies) and quantitative PCR (qPCR) undertaken with LightCycler 480 system using LightCycler 480 SYBR Green Master I (Roche). In parallel genomic DNA (gDNA) of each time point was purified with the DNeasy Blood and Tissue Kit (Qiagen), and the amount of bacteria in the samples determined by qPCR using chlamydial primers. This was done to normalize the cDNA of the different samples. Primers are listed in Table 1, and their specificity was ensured through the analysis of melting curves. For the evaluation of siRNA SLC35D2 efficiency the steps until reverse transcription were the same, but the PCR was run with PrimeStar (Clontech). Equal volumes were loaded on agarose gels and bands were revealed using UV-light visualizing ethidium bromide intercalated into DNA.

Table 1: List of primers used in qRT-PCR and qPCR

Name	Gene	Primers
glgA	CT798	AATGATTGGAATGCGTTACGG CGGTAGGTTGTCACTGCTTCC
glgB	CT866	GTGCATCATTGTTGGGGTAGGA CTCGCGATTTCAGGTGTAAGG
glgC	CT489	GCCTTTGCCTCAGAATTTTCC CCAAACCTGACTTCCATCTCG
glgX	CT042	CTCCCTTTAACCCCATTTTG CACGGTAGCATCCATTCCACT

malQ	CT087	CGTCGGTCTCTTTTCGATTTTG GGGATCGTCCCAGATCTTCT
pgm	CT295	AGCCCGAGTCTTAAAGCGAAG CCAAAGGCTCTGGAATCTGAA
uhpC	CT544	CGGCTTTACAGGATGGTTCGC CGGATGCATTCCATGTTGGCA
omcB	CT443	CTGCAACAGTATGCGCTTGTC GCTGTTGCTGTTCCCTTGGTTC
hctA	CT743	TAAAGCCGCAGCACAAAGAGT CGAACAGGCTTCTTAGCAGCAG
euo	CT446	TATGCTACACGCATTGGTGCT GCCTCAAAACCTTCTCTCATGG
slc35d2	slc35D2	CTGCCTCTCTCTACGTTGG CCCGAGAATAATGGCAAAGA

Transfection

siRNA transfections were performed with Lipofectamine RNAiMAX (Life technologies) according to manufacturer's protocol. A mixture of 2 to 6 siRNA sequences (Table 2), with a final concentration of 10 nM each, was used and transfection was done twice, 48 h and 4 h prior to infection. siRNA efficiency was determined by immunoblot or qPCR (see respective sections for more detail).

Cells were transfected with plasmid DNA 24 h after seeding using jetPRIME transfection kit (Polyplus transfection) according to the manufacturer's protocol. The constructs used were Flag-GlgA and SLC35D2-HA (pMKIT-neo-hRel8-cHA, kindly provided by Nobuhiro Ishida, Chiba Institute of Science, Japan) (Ishida et al., 2005).

Table 2: List of used siRNAs

siRNA	5' → 3'	Company
Gys1	GGG CGA GGA GCG UAA CUA A	Dharmacon
	CAA CGA CGC UGU CCU CUU U	Dharmacon
	GAA GCU UUA UGA AUC CUU A	Dharmacon
	GAA UCG GCC UCU UCA AUA G	Dharmacon
UGP2	GGU UCA AGA UUA UCU AAG A	Eurogentec
	GUG GAU CUG UAU AUU CUU A	Eurogentec
SLC35D2	CCA CAU AAG UGG AUU AUC A	Eurogentec
	GCC ACC AUA AUG AUA CUA U	Eurogentec

GCU AGG GAA AUA CGG AGU A	Dharmacon
AAU UAA GCC UAC CGA UGU U	Dharmacon
GAA AUG UAC UCG GGC GAC A	Dharmacon
AGC CAG AGG UUU CGG AUU C	Dharmacon

Construction of recombinant plasmids

Genomic DNA from *C. trachomatis* LGV serovar L2 strain 434 was prepared from bacteria using the RapidPrep Micro Genomic DNA isolation kit (Amersham Pharmacia Biotech). The first 20 to 30 codons of different chlamydial genes including about 20 nucleotides upstream from the translation start sites were amplified by PCR using primers listed in Table 3 and cloned into the pUC19cya vector as described (Subtil et al., 2001). attB-containing primers (table 3, Gateway®, Life technologies) were used to amplify and clone *C. trachomatis* L2 *glgA* gene into a destination vector derived from the mammalian expression vector pCiNeo, providing an amino-terminal 3xflag tag, and into pDEST15 (Gateway).

The pBOMB4-Tet-Flag vector was derived from pBOMB4-Tet-mCherry (Bauler and Hackstadt, 2014). The mCherry sequence was excised using NotI and SalI and replaced by a new multiple cloning site (MCS) containing SpeI, SacII and two SfiI sites, and a 3xflag tag downstream of the MCS. *glgX* and Δ *glgX* genes were cloned into pBOMB4-Tet-Flag using SpeI and SacII restriction sites. *glgP* and Δ *glgP* genes were inserted into pBOMB4-Tet-mCherry using NotI and KpnI restriction sites, eliminating the gene for mCherry. Constructs were C-terminally tagged with a 1xflag that was encoded for in the reverse primer. All constructs were verified by sequencing. Primers are listed in Table 3.

Table 3: Primers used for cloning purposes. Tet-Flag = pBOMB4-Tet-Flag; Tet-mCherry = pBOMB4-Tet-mCherry

Gene	Vector	Primers
glgC	pUC19cya	AGTCAAGCTTGTAATAGTTTTGTTTTATGGCTGGTAGACGGACG AGTCTCTAGATCCGCATAAGACAATAACTCCTAC
glgA	pUC19cya	AGTCAAGCTTGTAATAGTTTTGTTTTATGAAAATTATTCACACAGCTATCG AGTCTCTAGATGCTAGTCCGTATAGCGCG
glgB	pUC19cya	AGTCAAGCTTGTAATAGTTTTGTTTTATGGATCCTTTTTCTTAAATACTC AAC AGTCTCTAGAAGAAACAATTCCCAAAAGATCTTG
glgP	pUC19cya	AGTCAAGCTTGTAATAGTTTTGTTTTATGTATTTTCGATCGGACAAAG AGTCTCTAGATTGAGGAGTCTGGACTACCC

glgX	pUC19cya	AGTCAAGCTTGTAATAGTTTTGTTTTATGGAATCTTTGTCTGTTCGTT AGTCTCTAGAAGAAAATAGAGAAAAACGGTAGCG
malQ	pUC19cya	AGTCAAGCTTGTAATAGTTTTGTTTTATGCCGTCATTATCCCAAT AGTCTCTAGAGCCATGCTTAGGAGAAGTATCTATC
pgm	pUC19cya	AGTCAAGCTTTTAGG TTC TGA TCG GAA GGT TGT CTT AGTCTCTAGACAAAATGTTTTTGGCTGTTTGA
glgA	pCiNeo	AAAAAAGCAGGCTTCATGAAAATTATTCACACAGCTATCG AGAAAGCTGGGTCTCATTATTGTTTATAAATTTCTAAATATTTATTGG
	pDEST15	AAAAAAGCAGGCTTCATGAAAATTATTCACACAGCTATCG AGAAAGCTGGGTCTCATTATTGTTTATAAATTTCTAAATATTTATTGG
glgX	Tet-Flag	CGATACTAGTATGGAATCTTTGTCTGTTCGTTT CGATCCGCGGATCTAGATTCCAAGGAGGTTTTTCTT
ΔglgX	Tet-Flag	CGATACTAGTATGTCACAAGCCCAGCAGGTTAC CGATCCGCGGATCTAGATTCCAAGGAGGTTTTTCTT
glgP	Tet-mCherry	TTATCCGCGGCCGCATGATTTTCGATCGGACAAAGA CGATGGTACCCTATTACTTGTCTGCATCGTCTTTGTAGTCAGAAGTTGGTTCG GGAAACATT
ΔglgP	Tet-mCherry	TTATCCGCGGCCGCATGACTCCTCAATCGGCATCAAC CGATGGTACCCTATTACTTGTCTGCATCGTCTTTGTAGTCAGAAGTTGGTTCG GGAAACATT

Immunofluorescence and PAS staining

HeLa cells grown on coverslips were infected with *C. trachomatis* LGV serovar L2 strain 434 with an MOI < 1 (unless specified differently) at 37°C and fixed in 4 % paraformaldehyde (PFA) in PBS for 30 min at room temperature (except when staining with anti-CT813 was intended, which required fixation in 2% PFA). Cells were blocked and permeabilized in 0.05 % saponin and 0.1 % bovine serum albumin (BSA) in PBS for 10 min before being subjected to antibody staining. An additional 10 min incubation step in 0,3 % Triton X-100 (Sigma-Aldrich) in PBS was performed for experiments in which *Chlamydia* were transformed, to obtain good permeabilization of the inclusion membrane. The antibody against inclusion protein CT813 was kindly provided by Dr. G. Zhong (San Antonio, Texas). Polyclonal anti-Cap1 antibodies were obtained by immunization of New Zealand white rabbits with purified recombinant Cap1 deleted of its last 167 amino acids and fused to a N-terminal His tag (Agro-Bio). The polyclonal anti-GlgX antibody was equally purchased from Agro-Bio, and was directed against two peptides (KHNEENGEYNRDGTSA NC and

HEDFDWEGDTPLHLPKEC). To investigate its specificity anti-GlgX was preabsorbed with either the two GlgX peptides or control peptides. For this, 1 µg/ml antibody was incubated with 20 µg/ml of peptides for 15 min at room temperature prior to immunostaining of cells. The monoclonal rat anti-HA antibody was purchased from Roche Diagnostics. Goat secondary antibodies were conjugated to Alexa488 (Molecular Probes), or to Cy3 or Cy5 (Amersham Biosciences). For Periodic acid Schiff (PAS) stain cells grown on coverslips were fixed in 4 % PFA/PBS for 30 min at room temperature and staining was performed according to Schaart (Schaart et al., 2004). Briefly, cells were incubated in 1 % periodic acid (Sigma) for 5 min. Thereafter coverslips were put in tap water for 1 min, quickly rinsed in mQ-H₂O and then applied to Schiff's reagent (Sigma) for 15 min at room temperature. Afterwards the coverslips were rinsed again in mQ-H₂O, incubated in tap water for 10 min followed by an incubation step in PBS for 5 min. Periodic acid oxidizes the vicinal diols in sugars such as glycogen to aldehydes, which now in turn react with the Schiff reagent to give a purple/magenta colour. Images were acquired on an Axio observer Z1 microscope equipped with an ApoTome module (Zeiss, Germany) and a 63× Apochromat lens unless specified. Images were taken with a Coolsnap HQ camera (Photometrics, Tucson, AZ) using the software Axiovision.

Quantification of glycogen with CellProfiler

The cell image analysis software CellProfiler was used to quantify glycogen content in inclusions stained with PAS. Around 50 inclusions were manually encircled, and their size and total staining intensity was determined. PAS staining not linked to the presence of glycogen was estimated by doing the same procedure on inclusions in cells grown in the absence of glucose. The averaged value obtained was considered background value and was subtracted. Glycogen content of inclusions of cells treated with control siRNA was considered 100 %.

Western Blot and antibodies

Cell pellets were lysed with a lysis buffer (8 M urea, 30 mM Tris, 150 mM NaCl, 1 % v/v sodium dodecyl-sulfate) and proteins were subjected to sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane, which was blocked with 1× PBS containing 5 % milk and 0.01 % Tween-

20. The membranes were then immunoblotted with primary antibodies diluted in 1 x PBS and 0.01 % Tween-20. Primary antibodies used in the secretion assay were mouse anti-cya, rabbit anti-CRP and rabbit anti-IpaD and were generously given by Drs N. Guiso, A. Ullmann and C. Parsot, respectively (Institut Pasteur, Paris). Other antibodies used were rabbit anti-Gyl1 (Millipore #04-357), rabbit anti-UGP2 (GeneTex #GTX107967), mouse anti-Flag M2 (Sigma-Aldrich), goat anti-mouse IgG coupled to horseradish peroxidase (HRP) and goat anti-rabbit IgG-HRP (GE Healthcare). Blots were developed using the Western Lightning Chemiluminescence Reagent (GE Healthcare).

Zymogram

Glycogen synthase genes (*glgA*) were amplified from genomic DNA of *Escherichia coli* K12 and *Chlamydia trachomatis* D/UW-3/CX and cloned into the expression vector pGEX (GE Healthcare) and pDEST15, respectively.

Precultures of wild-type and transformed *E. coli* were grown overnight in LB medium at 37°C, then transferred to fresh LB medium and grown until the optical density (OD) 600 nm reached 0.6. Cultures were subsequently induced overnight with 0.5 mM of IPTG.

Cells were harvested by centrifugation and disrupted by a French press at 1250 psi. The lysate was centrifuged at 16000 g for 15 min at 4°C. Soluble proteins (such as GlgA) were found in the supernatant. Glycogen synthase activity was detected by zymogram analysis. The proteins in the supernatant were separated by non-denaturing polyacrylamide gel electrophoresis (PAGE) containing 0.6 % rabbit glycogen (Sigma-Aldrich). After electrophoresis, gels were incubated overnight at room temperature in glycogen synthase buffer (66 mM glycyl-glycine, 66 mM (NH₄)₂SO₄, 8 mM MgCl₂, 6 mM 2-mercaptoethanol, and 1.2 mM ADP-Glc or UDP-Glc). Glycogen synthase activity was then visualized as dark activity band after iodine staining.

Heterologous secretion assay in *Shigella flexneri*

Analysis of secreted proteins was performed as described elsewhere (Ball et al., 2013; Subtil et al., 2001). Briefly, 1 ml of a 30°C overnight culture of *Shigella flexneri ipaB* or *mxiD* transformed with different cya chimeras was inoculated in 30 ml of LB broth with 0.1 mg/ml ampicillin and incubated at 37°C for 4 h. Bacteria were then harvested by centrifugation and the supernatant was filtered through a Millipore filter (0.2 µm). To

precipitate the proteins 1/10 (v/v) of trichloroacetic acid was added to the supernatant and the precipitate as well as the bacterial pellet resuspended in sample buffer for analysis by SDS-PAGE and immunoblot.

Transformation of *C. trachomatis* L2

The transformation protocol for *Chlamydia* was adapted from Wang (Wang et al., 2011). Briefly, 6 µg plasmid DNA was added to a crude bacterial preparation (10^7 IFU) in 200 µl CaCl₂ buffer (10 mM Tris pH 7.4 and 50 mM CaCl₂) and incubated for 30 min at room temperature. 4×10^6 HeLa cells that were washed, detached and resuspended in 200 µl CaCl₂ buffer were mixed with the EB/DNA mixture, incubated for an additional 20 min and added to four single wells in a six-well plate where they were allowed to attach and incubate at 37°C in 5 % CO₂ for 48 hours. Infected cells were then lysed with beads and cell debris removed by centrifugation. A part of the supernatant was mixed with an equal volume of 4SP (16 mM NaH₂PO₄, 0.4 mM C₁₂H₂₂O₁₁) and stored at -80°C. Another part of the supernatant was used to infect a fresh monolayer of HeLa cells in a T150 flask in presence of 1 µg/ml cycloheximide and 1 U/ml penicillin G and the rest of the supernatant was mixed with an equal volume of 4SP and stored at -80°C. After 48 hours infected cells were again lysed and the supernatant used to infect cells in a T75 flask (with cycloheximide and penicillin). A third passage 48-72 hours later into a T25 flask often showed non-aberrant chlamydial inclusions, showing that the bacteria had taken up and maintained the plasmid. Induction of the Tet-promotor was achieved by addition of 2 - 20 ng/ml anhydrotetracycline (Sigma-Aldrich) 8 or 24 hpi.

Glucose uptake assay

Gradient purified Chlamydia EBs were incubated in an axenic medium (5 mM KH₂PO₄, 10 mM Na₂HPO₄, 109.6 mM K-gluconate, 8 mM KCl, 1 mM MgCl₂) (Omsland et al., 2012) supplemented with 0.2 mM α-D-[14C(U)]-Glucose 1-Phosphate, α-D-[14C(U)]-Glucose 6-Phosphate or D-[14C(U)]-Glucose (Perkin Elmer) (0.1 µCi per sample). In some samples 10 mM of the indicated cold monosaccharide was added in a competition assay. After two hours of incubation at 37°C the bacteria were pelleted (15000 g for 5 min) and washed twice in 50 mM K₂HPO₄/KH₂PO₄, 100 mM KCl and 150 mM NaCl. Radioactivity

associated to the bacterial pellet and to the supernatant was measured by a scintillation counter.

RESULTS

1. Glycogen detection in *Chlamydia trachomatis* inclusion

Glycogen has been frequently detected in *C. trachomatis* inclusions (Gordon and Quan, 1965). Infected cells contain about two-fold more glycogen than non-infected cells (Matsumoto et al., 1998; Ojcius et al., 1998). To see whether glycogen accumulation occurred in the host cytoplasm, the inclusion lumen, or in both compartments we made use of periodic acid Schiff (PAS) stain, a common method used to detect polysaccharides. Glycogen appears as a diffuse pink stain in the cytoplasm, as well as under the shape of one or two large particles (Fig. 22 A). While we could still detect glycogen in the cytoplasm of

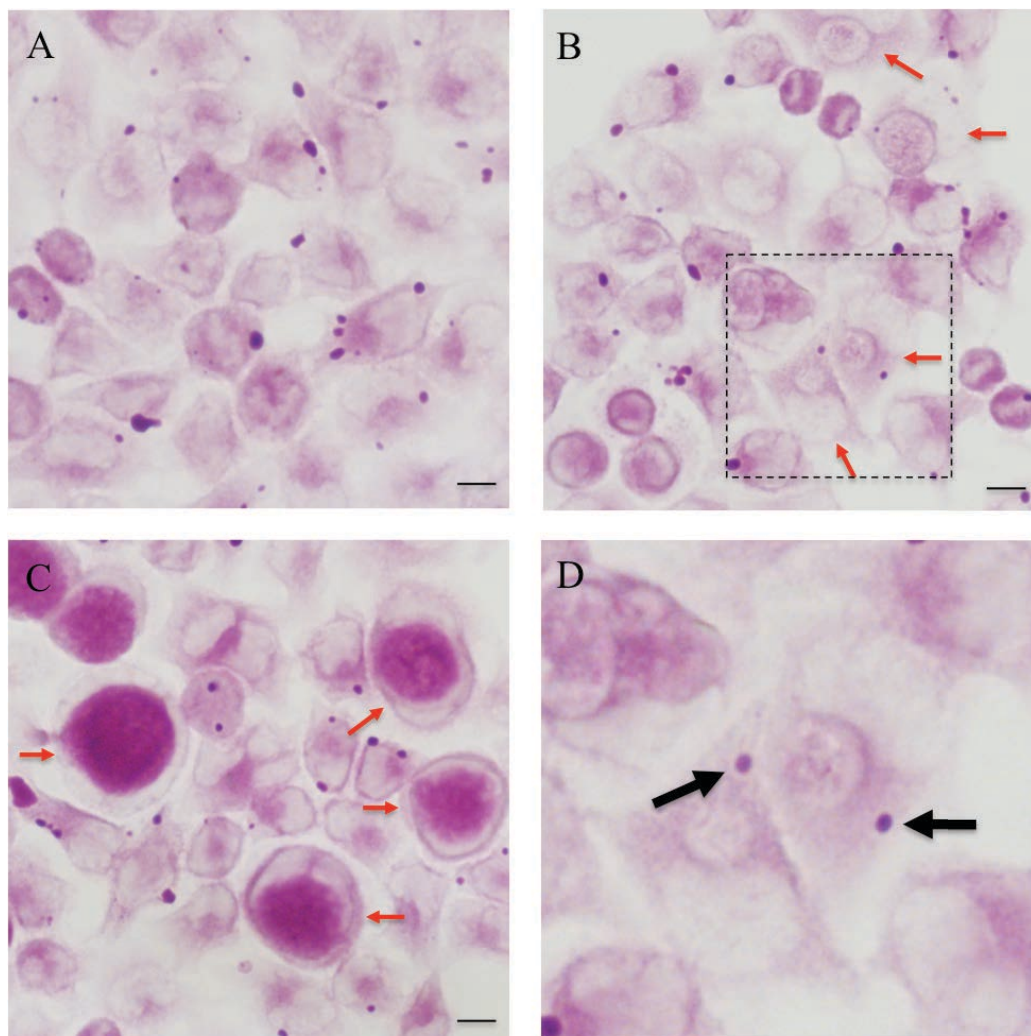


Figure 22: There is an overall decrease of cytoplasmic glycogen in infected cells, and a redistribution towards the inclusion. Cells were (A) non-infected, or infected with *C. trachomatis* for (B) 24 h or (C) 48 h, fixed in paraformaldehyde (PFA) and processed for periodic acid Schiff (PAS) stain. (D) is an enlargement of the boxed region in (B). Note the disappearance of glycogen particles (black arrows) still present at 24 hpi of infected cells (red arrows). Scale bar = 10 μ m.

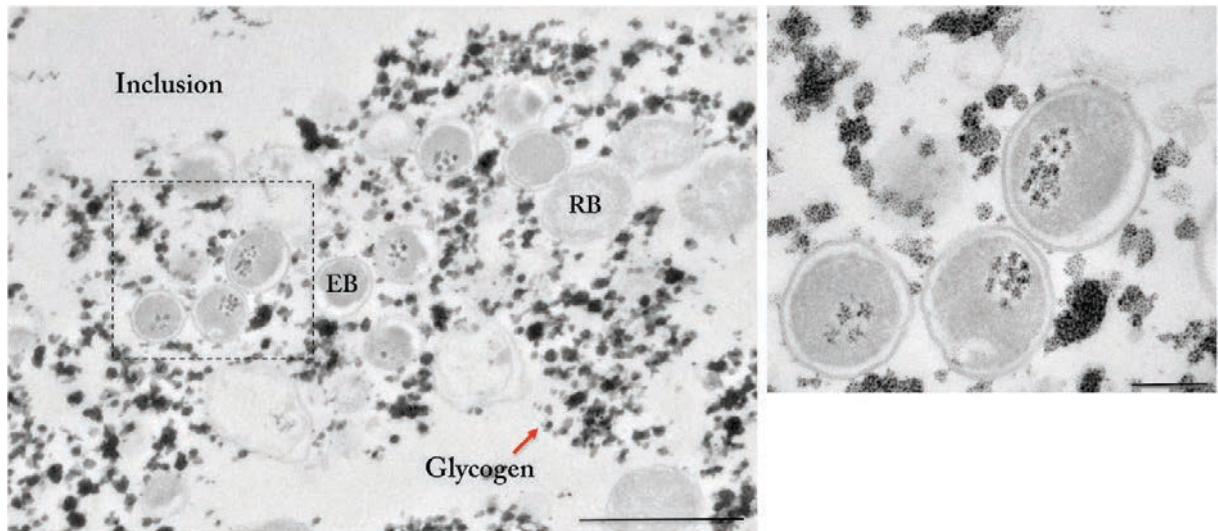


Figure 23: Glycogen accumulates in the inclusion lumen and in the EBs. HeLa cells were infected for 30 h with *C. trachomatis*. Glycogen (red arrow) is visualized by PATAg stain. TEM. Scale bar left = 1 μm. Scale bar right = 200 nm. The picture on the right shows an enlargement of the boxed region.

HeLa cells 24 hours post infection (hpi), large glycogen particles were no longer visible in cells infected for 48 hrs, and overall cytoplasmic glycogen levels had strongly decreased. Inclusions, on the other hand, showed a more prominent glycogen staining as the infection progressed (Figure 22 B-D), indicating that there is a redistribution of the glycogen pool towards the inclusion. In order to determine its subcellular localisation more precisely we used transmission electron microscopy (TEM). Glycogen was observed within the inclusion lumen as well as within EBs, but RBs were devoid of it (Figure 23). We did not observe an accumulation of glycogen around the inclusion membrane. It is also noteworthy that most of the intraluminal glycogen particles were bigger than those within the host cytoplasm (Figure 24). The very large majority of *C. trachomatis* isolates carry a plasmid, which has been described as necessary for glycogen accumulation in the inclusion (Carlson et al., 2008; Matsumoto et al., 1998). Indeed, we observed that the inclusions of the plasmid-less strain LGV 25667R contain only very few glycogen particles, similar in size to cytoplasmic glycogen particles. EBs of the plasmid-less strain contain detectable amounts of glycogen (Figure 25).

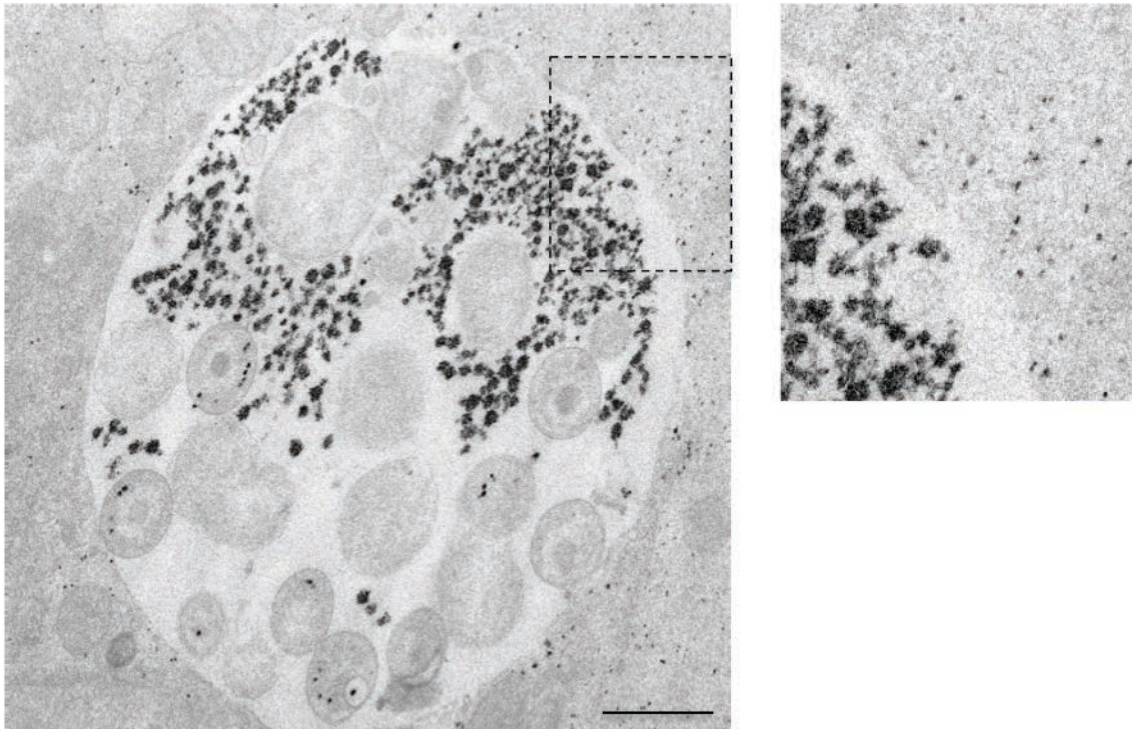


Figure 24: Glycogen particles in the inclusion lumen are in average of bigger size than in the host cell cytoplasm. HeLa cells were infected for 30 h with *C. trachomatis*. Glycogen is visualized by PATAg stain. TEM. Scale bar = 1 μ m. The picture on the right shows a 2-fold enlargement of the boxed region.

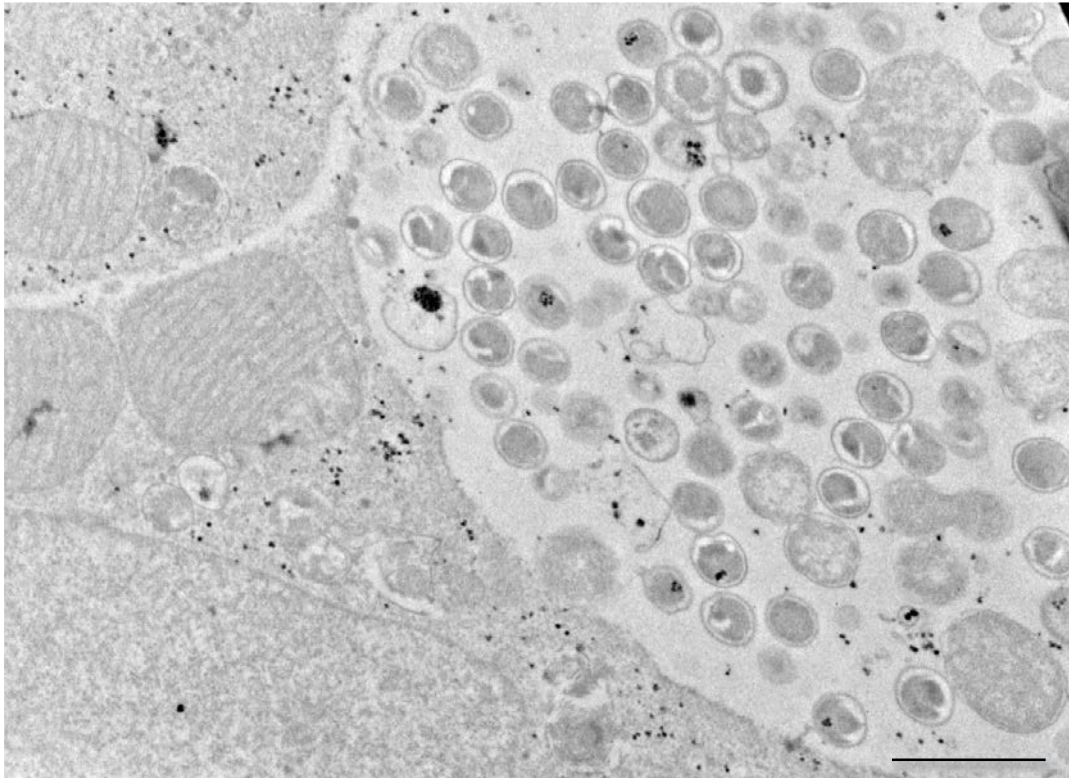


Figure 25: Glycogen accumulation is minimal in the inclusion of a plasmid-less strain. HeLa cells were infected for 30 h with *C. trachomatis* LGV 25667R. Glycogen is visualized by PATAg stain. Note that glycogen is also present in the EBs of the plasmid-less strain. TEM. Scale bar = 1 μ m.

2. Glucose is essential for *Chlamydia trachomatis* infection

According to previous reports there is no recovery of infectious EBs when cells are infected in the absence of glucose (Harper et al., 2000; Iliffe-Lee and McClarty, 2000). We wanted to determine the threshold of glucose that is required to obtain infectious progeny. To do so, we incubated cells three days prior to infection in medium containing glucose concentrations from 2 mg/ml to 0 mg/ml and kept them in the respective medium during the infection, which occurred in a low MOI of 0.5 to avoid multiple infections of a single cell. To minimize the effects that glucose starvation could have on the cells the culture media were complemented with sodium pyruvate, a substrate that can be used by host cells but not by *Chlamydiae* (Iliffe-Lee and McClarty, 2000; Weiss, 1967). Cells were lysed 48 hpi and the

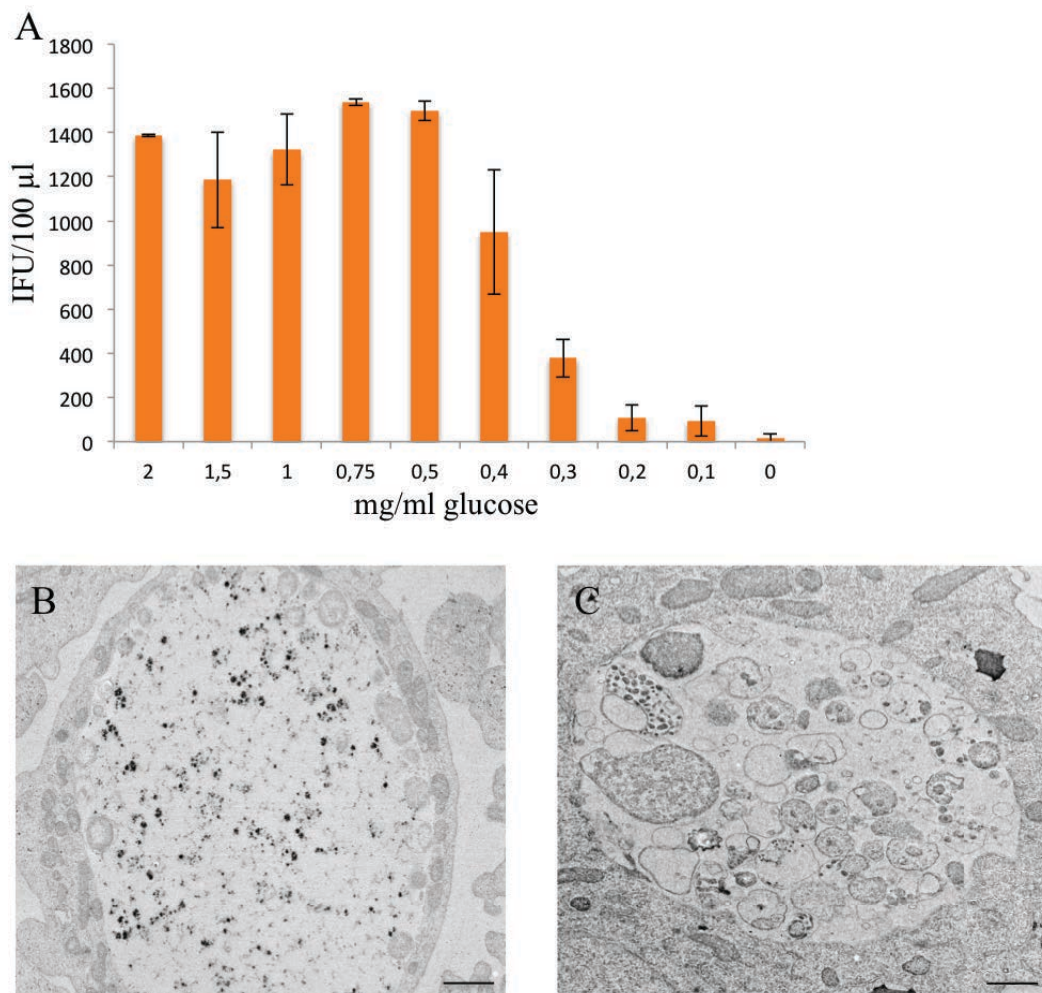


Figure 26: Effect of glucose concentration on infectivity of progeny. Lysats of cells in which infection occurred in media containing 0 mg/ml to 2 mg/ml glucose were used to reinfect fresh cells. (A) The histogram shows resulting IFUs/100 µl. Error bars depict the standard deviation (n=3). (B&C) Inclusions in cells incubated at (B) 0.4 mg/ml or (C) 0 mg/ml Glc were processed for PATAg staining. (B) Intraluminal glycogen accumulation still occurs. (C) When glucose is absent, bacteria develop in an aberrant manner. TEM. Scale bar = 1 µm.

supernatant was used to infect new cells and enumerate inclusion forming units (IFUs) one day later. Recovery of infectious particles was unchanged for cells grown in 2 mg/ml to 0.4 mg/ml of glucose. However, infectivity was reduced to around 30 % when incubated in 0.3 mg/ml glucose and quickly dropped to almost 0 % with lower glucose concentration (Figure 26 A). In order to determine if glycogen accumulation in the inclusion lumen still occurred at the threshold of 0.4 mg/ml glucose we used TEM, revealing that also at this concentration glycogen was still detected in the inclusion lumen (Figure 26 B). Markedly enlarged abnormal bacteria were observed in cells completely deprived of glucose (Figure 26 C).

3. Intraluminal glycogen is not derived from bacterial lysis

The provenance of the intraluminal glycogen of *C. trachomatis* has not been thoroughly examined yet, and only one report tackled that question. Chiappino and colleagues could observe rupture of some bacteria and release of their content, including glycogen, into the inclusion lumen, and concluded that intraluminal glycogen originates from the organisms themselves (Chiappino et al., 1995). Considering the abundance of intraluminal glycogen we thought this explanation very unlikely and we designed a simple experiment to test it. HeLa cells were deprived of glucose 48 h prior to infection and infected for 24 h still in the absence of glucose, before adding 10 mg/ml glucose. Cells were fixed at the time of glucose

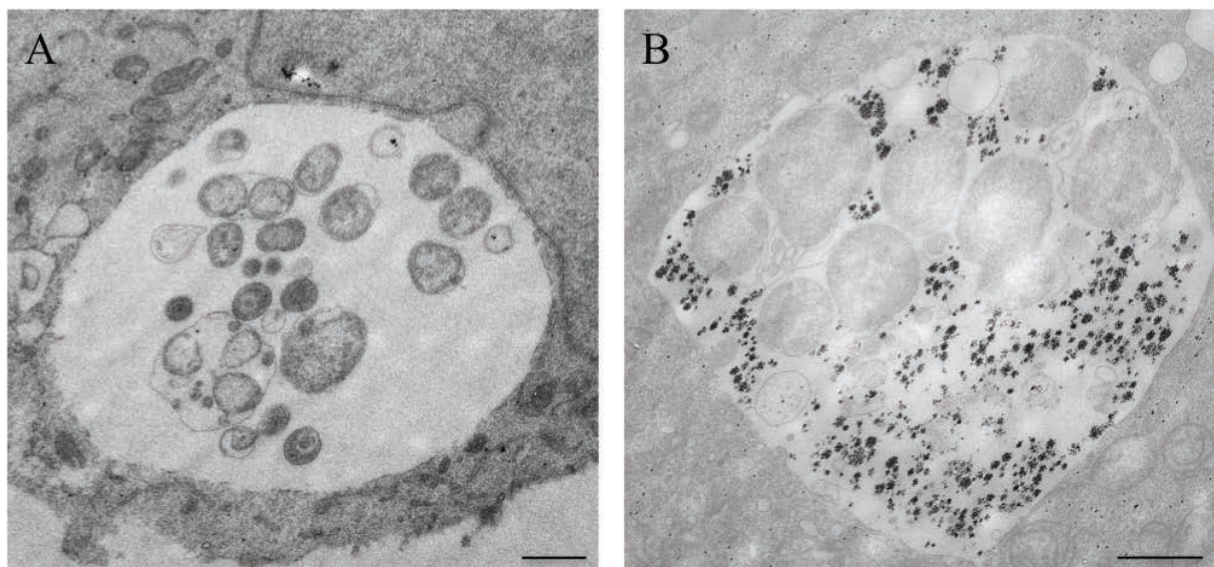
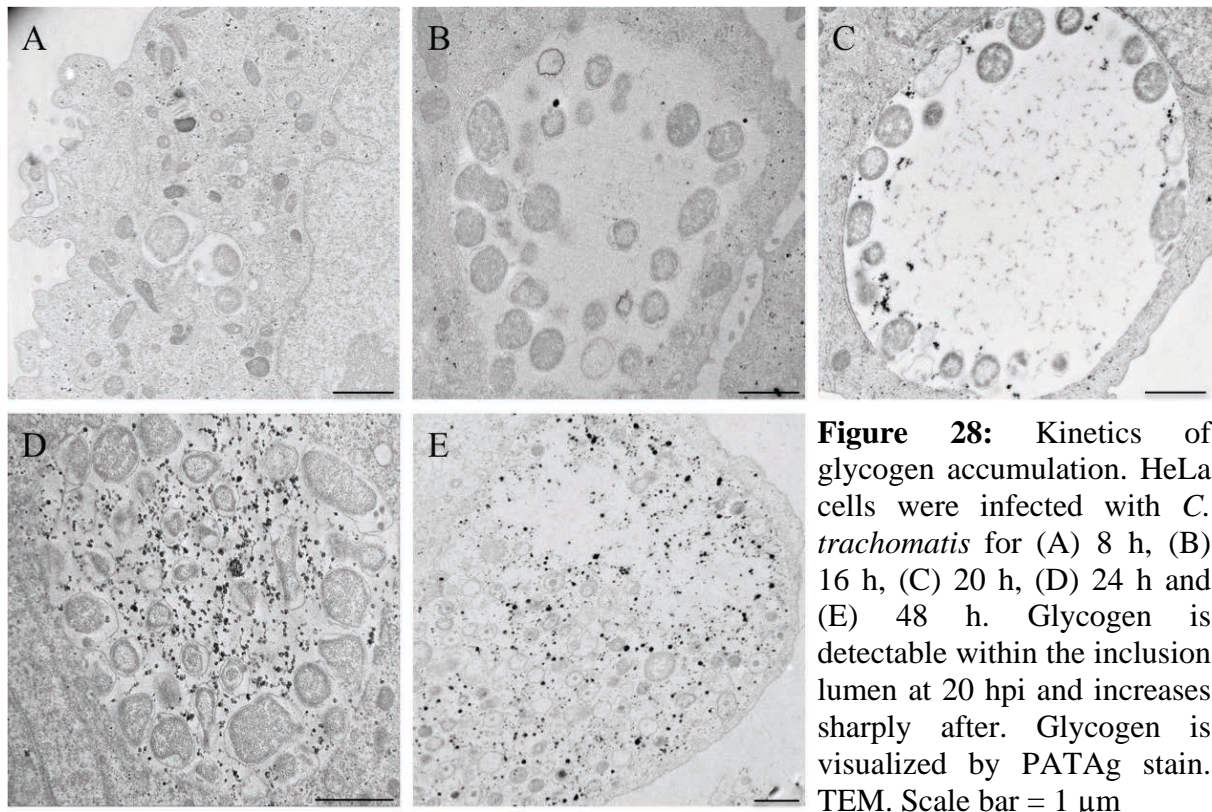


Figure 27: Intraluminal glycogen is not due to bacterial lysis. Cells were glucose-deprived 48 h prior to infection. 10 mg/ml glucose were added 24 hpi and cells were (A) fixed immediately or (B) 4 h after glucose administration. Note that no glycogen is detectable in the bacteria while it is highly abundant in the inclusion lumen. Glycogen is visualized by PATAg stain. TEM. Scale bar = 1 μ m.

administration and 4 h later. Before glucose addition the bacteria and the inclusion lumen were as expected devoid of glycogen, and many bacteria displayed an abnormal phenotype. Four h after glucose addition glycogen was detected in the inclusion lumen, but not in bacteria, disproving the hypothesis that intraluminal glycogen originates from bacterial stores (Figure 27).

4. Kinetics of glycogen accumulation

To investigate the time course of glycogen accumulation, we examined cells infected with *C. trachomatis* for increasing periods of time in TEM. No glycogen was detected in the nascent inclusion lumen nor in the bacteria up to 16 hpi. The onset of glycogen accumulation was between 16 and 20 hpi, in agreement with a previous report (Matsumoto et al., 1998), and glycogen continued to accumulate thereafter as the inclusions grew (Figure 28). This experiment, here in a glucose-rich medium, confirms our previous observation that glycogen accumulation occurs first in the inclusion lumen before being detectable in bacteria. Furthermore, before 20 h of infection inclusions contain mostly RBs, indicating that glycogen accumulation is triggered by the metabolically most active form of the bacteria.



In order to get more information on the expression pattern of chlamydial enzymes linked to glycogen metabolism, we undertook a transcriptional analysis by qRT-PCR. We reasoned that glycogen synthesizing enzymes might not be expressed at the same time as glycogen degrading enzymes, as these two activities would counteract each other. Belland and colleagues published a comprehensive study on the chlamydial transcriptome during infection revealing that almost all glycogen genes - synthesizing (*glgC*, *glgA*, *glgB*) and degrading (*glgP*, *glgX*, *malQ*), the phosphoglucomutase *pgm* and the transporter *uhpC* - have their transcriptional onset at 8 or 16 hpi and their maximum at 24 hpi and 40 hpi, without any transcriptional decline at the end of the infection when EBs predominate (Belland et al., 2003). Inconsistencies with another transcriptomics report (Nicholson et al., 2003) led us to perform the qRT-PCR ourselves on the genes of *glgC*, *glgA*, *glgB*, *glgP*, *glgX*, *malQ*, *pgm* and *uhpC*, including control genes *euo* ("early upstream ORF", early gene), *hctA* (histone-like protein A, late gene) and *omcB* ("outer membrane complex B", late gene) (Shaw et al., 2000). Linear standard curves for each gene were obtained with chromosomal DNA as template at concentrations ranging from 10 to 0.001 ng/ml. Melting curves showed only one peak for each primer pair indicating that only one amplicon was created, except for *glgP*, which was subsequently discarded from the analysis. Acquired values of the fluorescent intensity were first plotted against the standard curve, and this concentration then normalized to the amount of genomic DNA in the respective sample, to account for the raising number of bacteria throughout the developmental cycle. Expression of control genes followed the expected pattern and validating our experimental set-up: transcription of *euo* was detected 1 hpi, while expression of *hctA* and *omcB* started around mid-cycle and reached the peak at late time points (Figure 29). Most glycogen related genes showed a very similar pattern: *glgC*, *glgX*, *malQ*, *pgm* and *uhpC* were detected early on at 3 hpi, mostly peaked at 16 hpi and declined at 40 hpi, indicating that expression of these genes follows the pattern of highest overall activity in RBs. Only *glgA* and *glgB* seemed to be subject to a different regulation. *glgB* had its highest level at 1 and 3 hpi, and drastically declined thereafter. The onset of expression of *glgA* was at around 16 hpi, followed by a sharp increase. This pattern of expression correlates with the kinetics of glycogen accumulation (Figure 28), indicating that *GlgA* might be a master gene to control glycogen stores in the inclusion lumen. Somewhat surprisingly there

does not seem to be a tight regulation on the transcription of the other glycogen enzymes, with concomitant expression of synthesis and degradation enzymes at all stages of the developmental cycle.

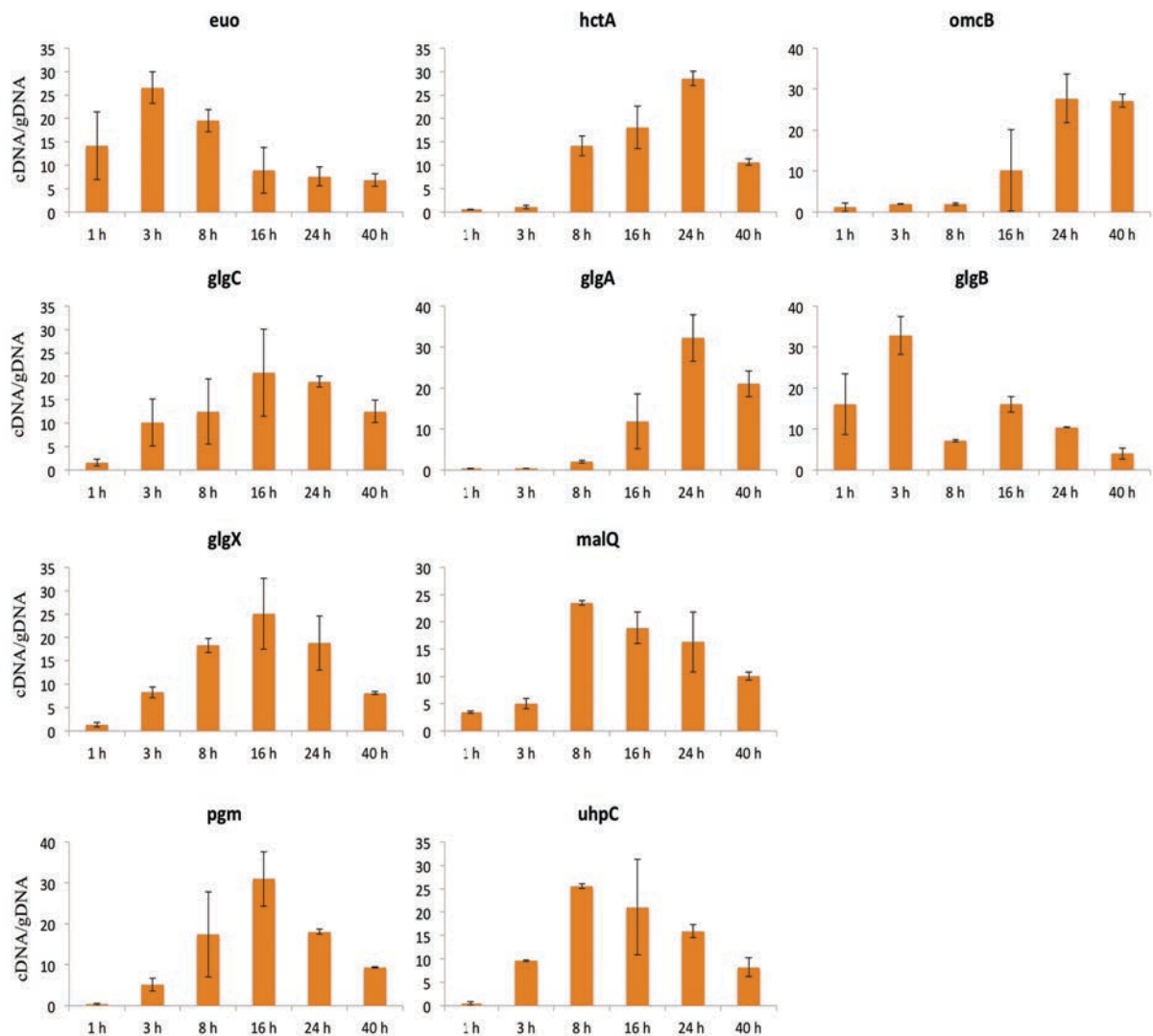


Figure 29: qRT-PCR of selected genes related to glucose metabolism. *euo*, *hctA* and *omcB* serve as reference genes (see text). Fluorescent values were plotted against standard curves and cDNA normalized with the overall chlamydial genomic gDNA present in parallel samples (cDNA/gDNA). Time points were 1 hpi, 3 hpi, 8 hpi, 16 hpi, 24 hpi and 40 hpi. Values were obtained from three independent experiments, each performed in triplicates. Error bars depict the standard deviation.

5. Mechanisms of glycogen accumulation in inclusion lumen

5.1 Hypotheses

Our observations raised an obvious question: how is glycogen accumulating within the inclusion lumen? We envisioned two different pathways depicted in Figure 30. In the first scenario, a glucose-derivative (such as a hexose phosphate or nucleotide sugar) is imported into the inclusion lumen through a transporter. Subsequently, *de novo* glycogen synthesis would take place through the activity of glycogen enzymes within the lumen. An alternative scenario consists in the import of host glycogen, in bulk through inward invagination of the inclusion membrane. We describe below experiments demonstrating that these two pathways indeed take place.

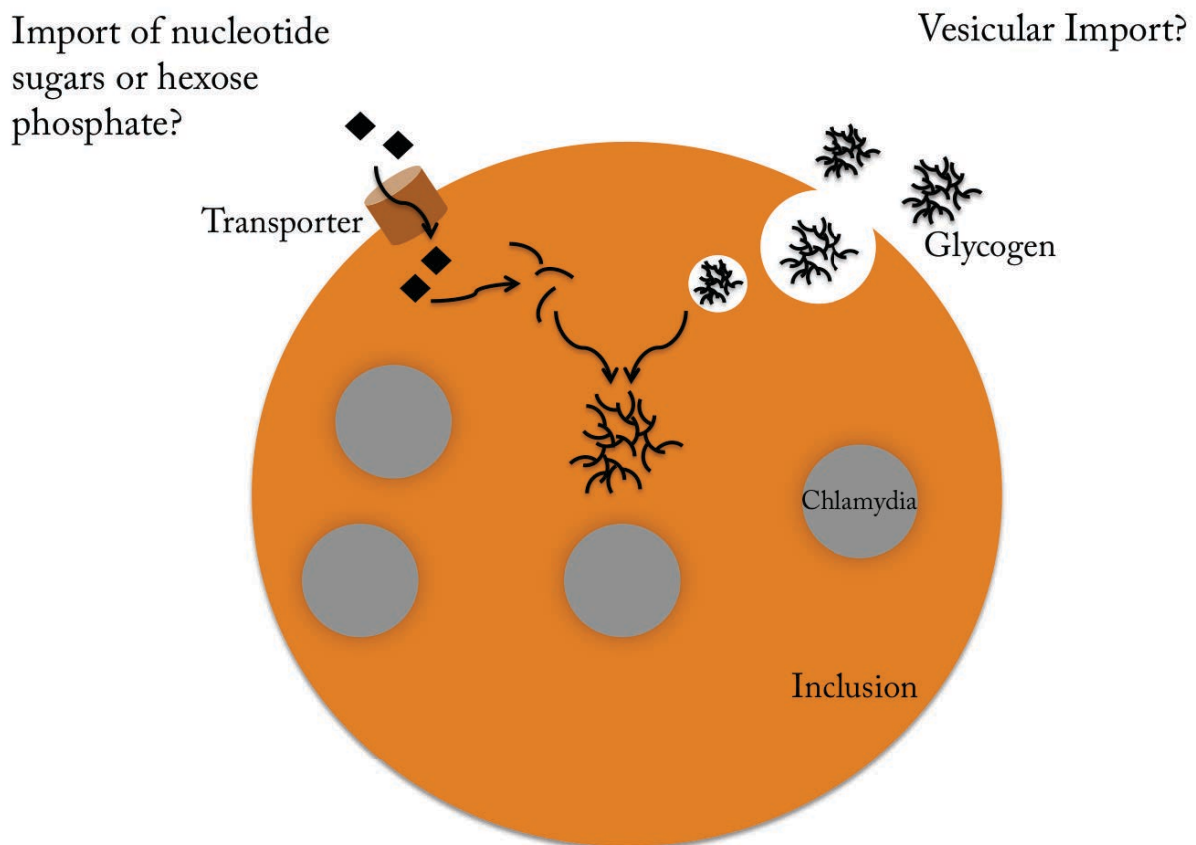


Figure 30: Two possible scenarios for intraluminal glycogen accumulation. Left: glucose-derivatives are imported through a transporter and *de novo* glycogen synthesis takes place in the inclusion lumen. Right: host glycogen is imported through invagination of the inclusion membrane.

5.2 Vesicular import of host glycogen

5.2.1 Part of luminal glycogen is translocated in bulk from the host cytoplasm

Intriguingly, we frequently observed glycogen filled vesicular structures within the inclusion (around 25 % of inclusions contained at least one of these structures). These vesicles have an average size of around 500 nm, ranging from 200 nm to 1 μm . Some of them appear to have only one membrane (Figure 31 D, E, H), while others had several, the outer one often loosened up (Figure 31 A, B, C, F, G, I). These observations strongly support the bulk import of glycogen, since it is difficult to see how membranes could appear around

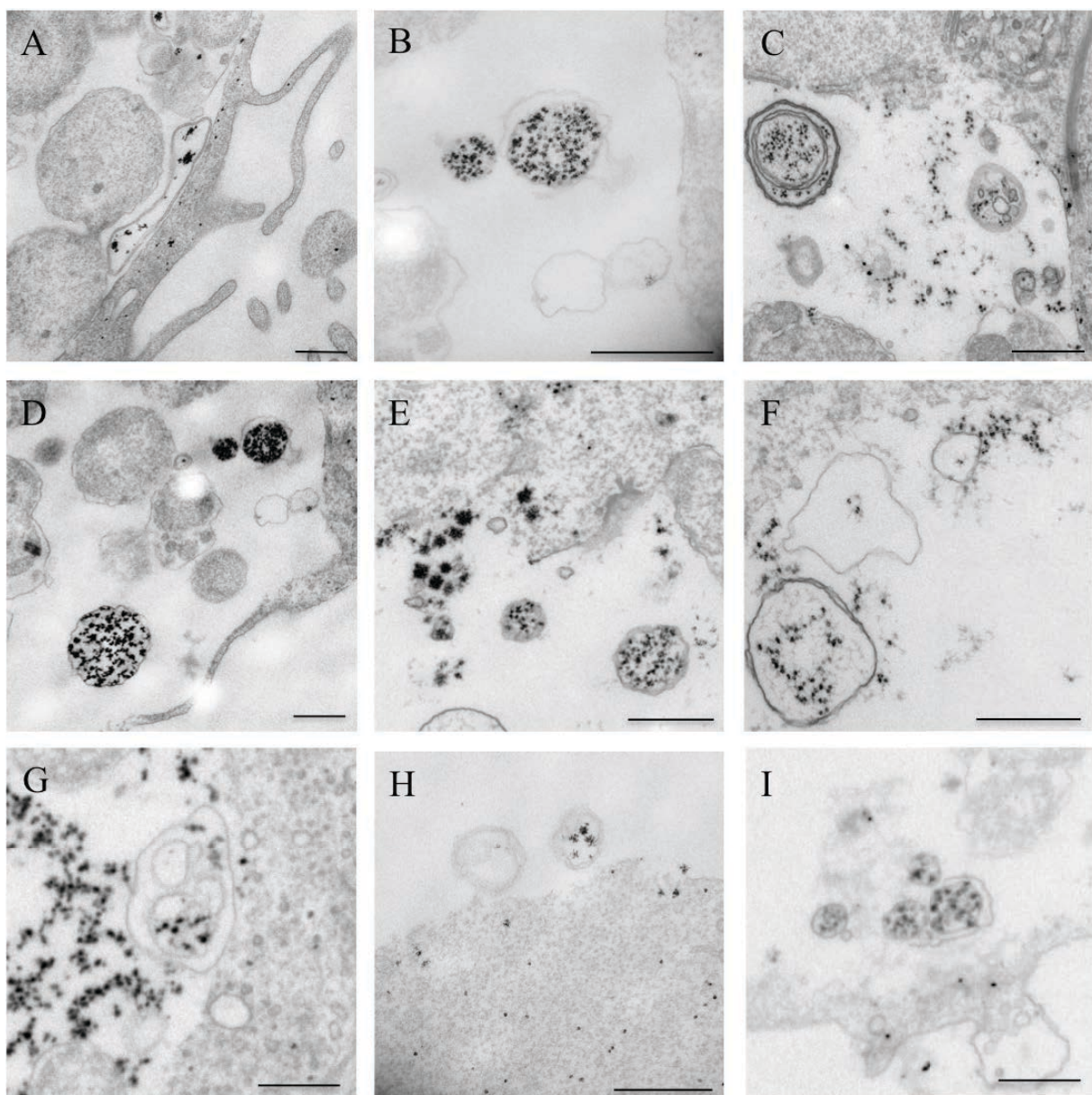


Figure 31: TEM images of glycogen-filled vesicles in the inclusion. HeLa cells 30 hpi with *C. trachomatis*. Glycogen is visualized through PATAg staining. Scale bar = 500 nm.

glycogen particles synthesized *de novo* in the inclusion lumen.

In another experiment we stained *C. trachomatis* infected cells for the endogenous Gys1 (host glycogen synthase) with a specific antibody. Gys1 was detected in the host cytoplasm, as expected, and was also detected in the majority of the inclusions observed (Figure 32). To ensure that the signals were indeed within the lumen, we used an apotome with increased axial resolution allowing the imaging of 250 nm thick Z-stacks. Depletion of host Gys1 by siRNA treatment led to the disappearance of the signal, confirming the specificity of the antibody. Interestingly, we obtained the same intraluminal staining for cells infected with the plasmid-less strain LGV 25667R (images not shown).

To confirm that glycogen synthase could transfer from the host cytoplasm into the inclusion lumen we transfected cells with a N-terminally tagged construct of the bacterial glycogen synthase, Flag-GlgA, before infecting them with *C. trachomatis*. Thirty h later, the cells were fixed and processed for immunofluorescence microscopy. The anti-Flag antibody detected a signal in the inclusion lumen of transfected cells, but not in non-transfected cells, ruling out a non-specific reaction with a bacterial antigen (Figure 33). A control transfection with a different construct bearing the same N-terminal tag (Flag-CT621) did not show any inclusion staining (not shown). We suggest a simple explanation for this phenotype: as it has been previously shown that at least Gys1 undergoes a stable association with glycogen molecules (Caudwell and Cohen, 1980), we think it is likely that import of host glycogen would equally lead to import of glycogen-bound enzymes, such as Gys1.

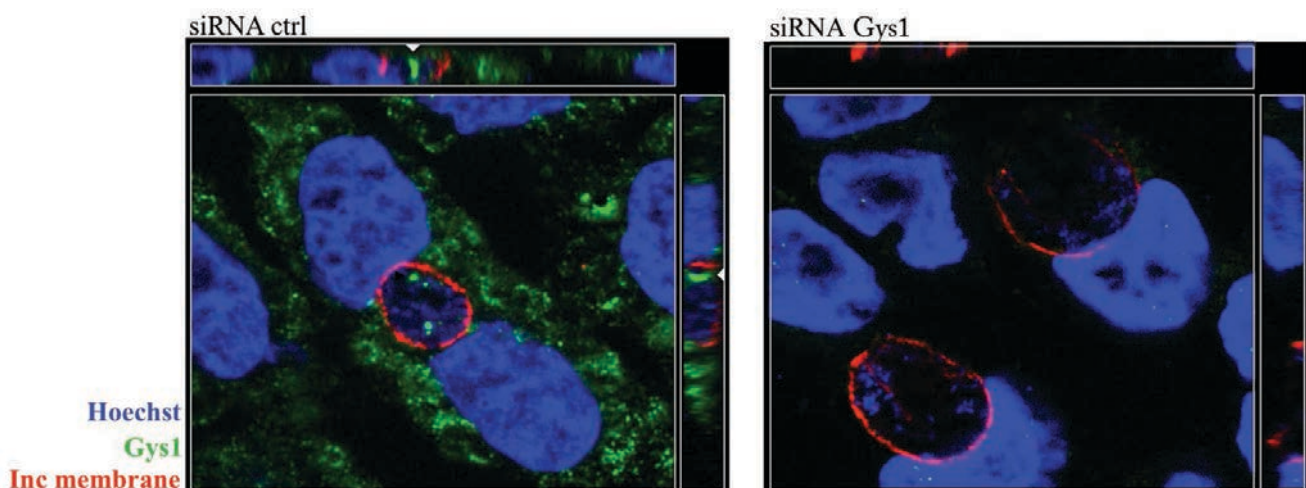


Figure 32: Gys1 is imported into the inclusion lumen. HeLa cells were treated with siRNA control or directed against the host glycogen synthase Gys1 prior to infection for 30 h with *C. trachomatis*. Hoechst in blue, staining DNA. Gys1 in green, an inclusion membrane protein (CT813) in red. Gys1 was detected not only in the cytosol but also in the inclusion lumen. Staining was specific since it disappeared upon Gys1 silencing by siRNA.

As the above mentioned observations point into the direction of vesicular import of host glycogen, we reasoned that knocking-down the host Gys1 by siRNA and thus depleting the host cell of glycogen should lead to a decrease of intraluminal glycogen. siRNA treatment was performed and repeated 48 h later. The cells were infected after the second treatment. Western Blot analysis and PAS staining of cells before infection showed that Gys1 had been completely depleted and glycogen strongly decreased in treated cells (Figure 34 A &B). However, inclusions of cells treated with Gys1 siRNA did not differ from those of cells treated with control siRNA (Figure 34 C). We measured the intensity/pixel in the inclusion via the cell image analysis software CellProfiler, and used inclusions of cells grown in absence of glucose to measure the background. The difference in intraluminal glycogen content between siRNA control and siRNA Gys1 treated cells was only marginal (Figure 34 D), strongly indicating that bulk import of host glycogen cannot account for all intraluminal glycogen accumulation.

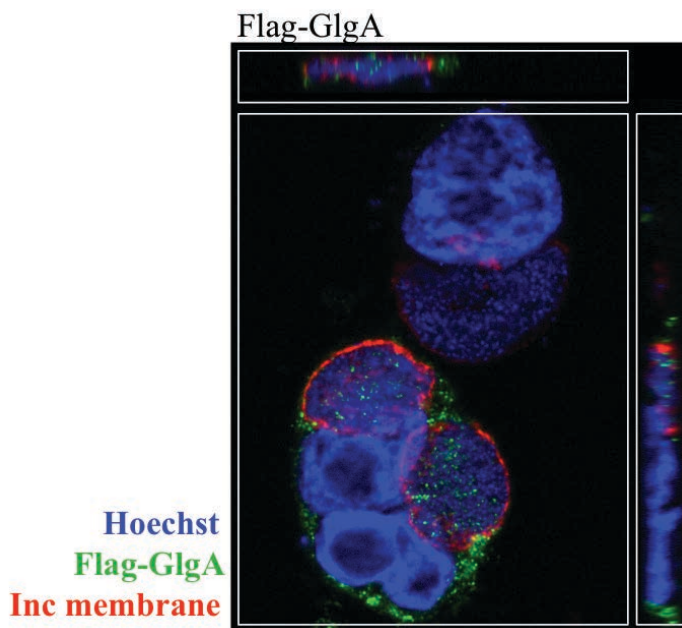


Figure 33: Flag-GlgA import into the inclusion lumen. HeLa cells were transfected with Flag-GlgA prior to infection with *C. trachomatis*. Hoechst in blue, staining DNA. Flag-GlgA in green, depicting cytoplasmic and inclusional staining. An antibody against an inclusion membrane protein (Cap1) was used to visualize the inclusion membrane (red).

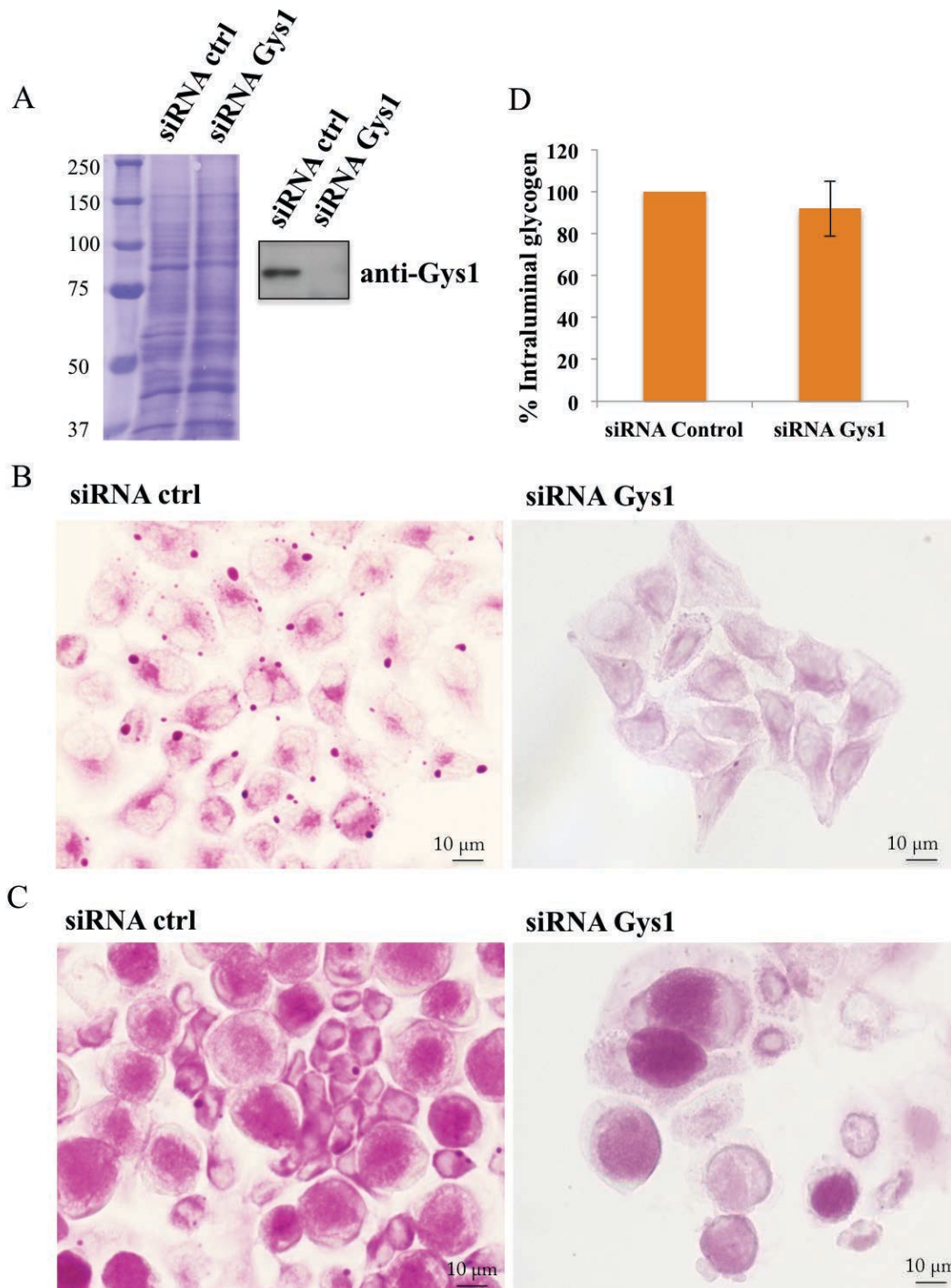


Figure 34: Host glycogen import is not the only source of intraluminal glycogen. HeLa cells were treated with either siRNA control or siRNA against Gys1 48 h prior to infection. (A) Coomassie staining of whole cell lysates (left) and immuno blot with an anti-Gys1 antibody of siRNA treated cells before infection. Gys1 was completely depleted after siRNA treatment. (B) PAS staining of the same samples as in (A). Glycogen strongly decreased upon siRNA treatment compared to control cells. (C) PAS staining on cells 48 hpi. Inclusions displayed the same coloration in cells treated with siRNA control and siRNA Gys1. (D) Quantification of intraluminal glycogen with CellProfiler. The average intensity/pixel of inclusions stained by PAS was determined and expressed as % of the value in control siRNA treated cells. Around 50 inclusions were counted for each experiment. Error bars correspond to the standard error of the mean (n=3).

5.2.2 Import of host glycogen and glycogen enzymes is autophagosome independent

We consequently aimed to elucidate the mechanism by which host cell glycogen is imported into the inclusion lumen. Two possibilities are conceivable: the direct uptake of host glycogen, or the uptake of host vesicles prefilled with glycogen, both through an inward invagination of the inclusion membrane (Figure 35). Many reports confirm the existence of autophagy of glycogen in neonatal mammals, providing quick energy for postnatal hypoglycemia. This pathway underlies a highly hormonal regulation, mediated by cAMP/protein kinase A (leading to induction) and phosphoinositides/mTOR (leading to inhibition). Autophagic glycogen-filled vacuoles have been described to contain a double or, more often, a single limiting membrane and fusion with a lysosome leads to degradation through the glycogen-hydrolysing acid glucosidase (Kotoulas et al., 2006; Schiaffino and Hanzlikova, 1972). Autophagy is a highly regulated process involving the concerted action of cytoplasmic proteins that in a first step create isolation membranes, which envelop the cargo (Zhao et al., 2008). The closing of the isolation membrane gives rise to a double membrane structure, the autophagosome. The autophagosome then eventually fuses with a lysosome to deliver its cargo for degradation and recycling. The cytosolic protein Atg5 is required for the elongation of the isolation membrane (Mizushima et al., 2002), and a Atg5 knockout mutant is autophagy-deficient (Kotoulas et al., 2006; Mizushima, 2007). We made use of a *Atg5* *-/-*

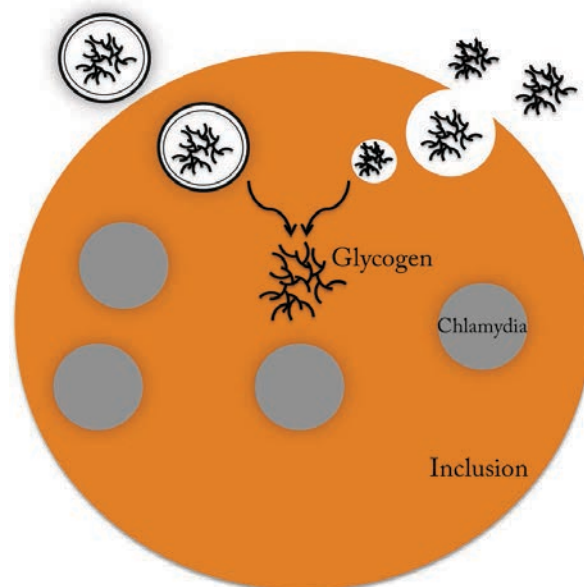


Figure 35: Two different potential sources of host glycogen uptake are conceivable: Cytoplasmic glycogen-filled vesicles possibly of autophagic origin, might be taken up (left). Alternatively, free cytoplasmic glycogen might be sampled at the inclusion membrane. Both mechanisms imply the inward invagination of the inclusion membrane.

mouse embryonic fibroblast (MEF) cell line and sought to determine if there was a difference in intraluminal glycogen accumulation, the import of host Gys1 or the presence of glycogen-filled vesicles. *Atg5*^{-/-} MEF showed similar luminal glycogen levels as WT MEFs (Figure 36 A), an expected result since we have shown that bulk import of glycogen is only marginal. Import of host Gys1 into the inclusion lumen still took place in *Atg5*^{-/-} MEF (Figure 36 B). In addition, glycogen-filled vesicles could still be detected in the inclusions of the autophagy-deficient cell line (Figure 36 C), demonstrating that autophagy is not required for bulk import of host glycogen.

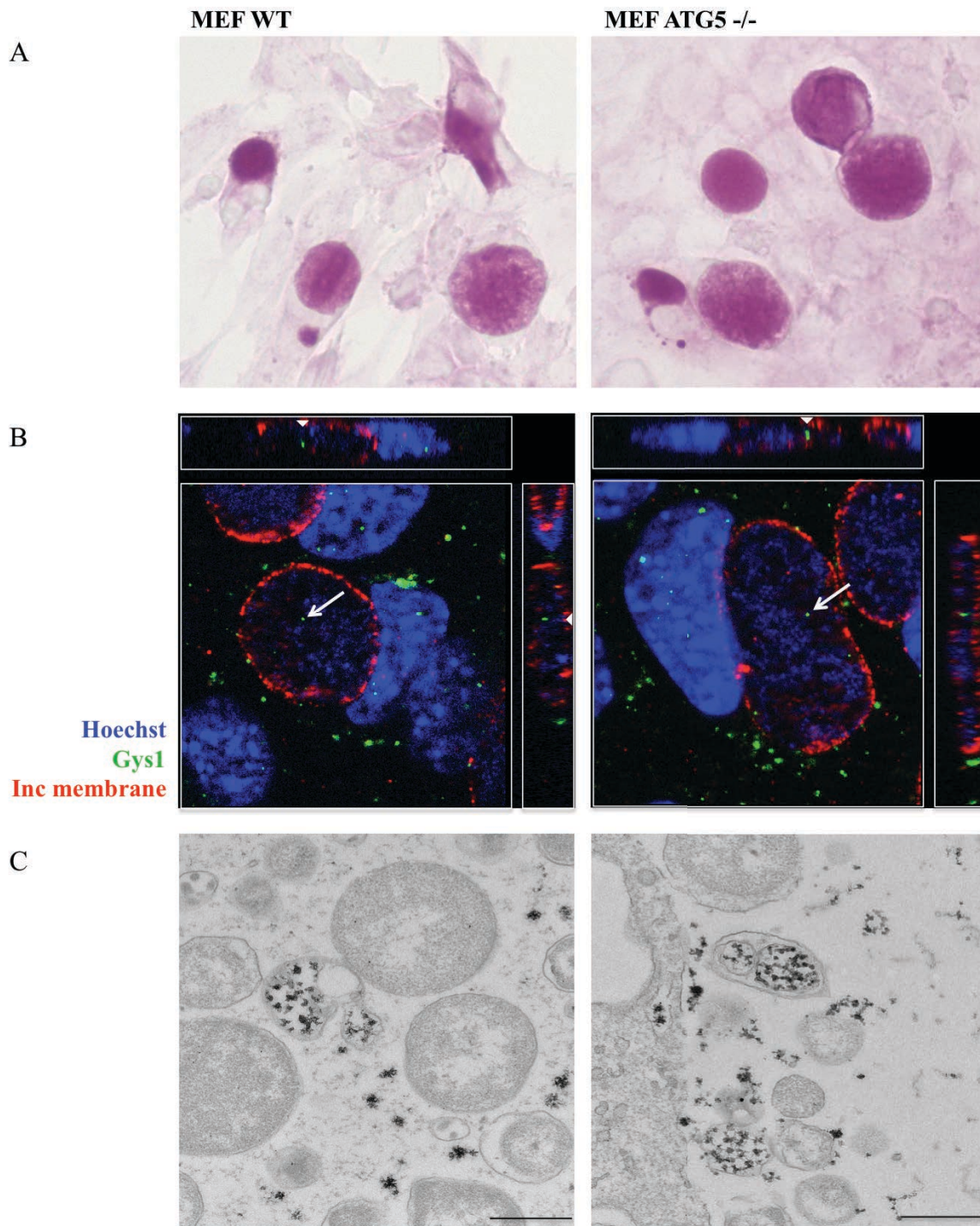


Figure 36: Gys1 import into the inclusion lumen is autophagy independent. Wild-type (WT) mouse embryonic fibroblasts (MEFs) or *Atg5*^{-/-} MEF were infected for 30 h with *C. trachomatis*. (A) PAS staining of WT MEF and *Atg5*^{-/-} MEF. (B) Hoechst in blue, staining DNA. Gys1 in green. An antibody against an inclusion membrane protein (CT813) was used to visualize the inclusion membrane (red). While less abundant than in HeLa cells, Gys1 (white arrows) was detected inside inclusions of both WT MEF and *Atg5*^{-/-} MEF. (C) TEM images of WT MEF and *Atg5*^{-/-} MEF. Glycogen-filled vesicles were discovered in inclusions of either cell line. Scale bar represents 500 nm.

5.3 Import of host glucose derivative

5.3.1 UDP-glucose is the host sugar transported into the inclusion lumen

We subsequently aimed at identifying the second mechanism for glycogen accumulation in the inclusion lumen. As proposed above (Figure 30), this alternative pathway could be the import of glucose derivatives and *de novo* glycogen synthesis within the inclusion lumen. To test this hypothesis, we looked at the effect of silencing the host enzyme UDP-glucose pyrophosphorylase (UGP2) on glycogen accumulation. UGP2 is responsible for the generation of UDP-Glc, the building block of eukaryotic glycogen (thus the direct substrate of Gys1). Similarly to the Gys1 siRNA treatment, siRNA transfection was performed for a first time, and then 48 h later for a second time, 4 h before infection. Western Blot analysis showed that UGP2 had been completely depleted before infection. Cytoplasmic glycogen stores decreased, as expected since UDP-Glc is the substrate of Gys1, but to a much lesser extent than when treated with siRNA against Gys1 (Figure 37 A&B). Very strikingly, glycogen content decreased strongly in inclusions of cells depleted of UGP2 (Figure 37 C) and had a reduction of almost 60 % in glycogen staining in the inclusions compared to the inclusions of cells treated with siRNA ctrl (Figure 37 D). This cannot be entirely accounted for by the reduction in the cytoplasmic pool of glycogen since Gys1 depletion had a much stronger impact on cytoplasmic glycogen stores, yet did not lead to a significant reduction in intraluminal glycogen stores. Thus this experiment strongly indicated that UDP-Glc is the substrate for *de novo* glycogen synthesis in the inclusion lumen.

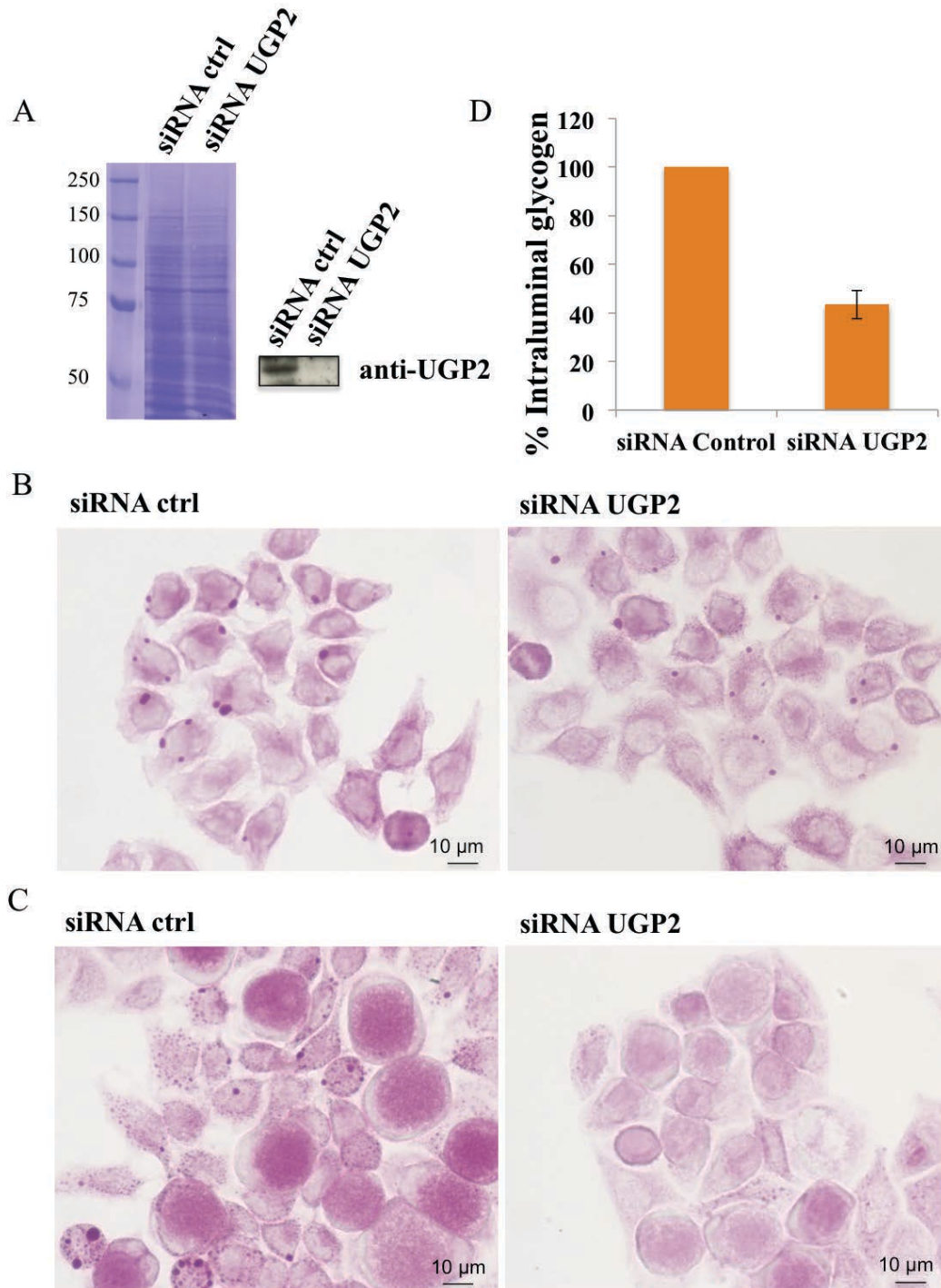


Figure 37: UDP-Glc is the substrate for intraluminal glycogen synthesis. HeLa cells were treated with either siRNA control or siRNA against UGP2 48 h prior to infection. (A) Coomassie staining of whole cell lysates (left) and immuno blot with an anti-UGP2 antibody of siRNA treated cells before infection. UGP2 was completely depleted after siRNA treatment. (B) PAS staining of the same samples as in (A). Glycogen decreased slightly upon siRNA treatment compared to control cells. (C) Intraluminal glycogen content was strongly reduced 48 hpi in cells treated with siRNA UGP2. (D) Quantification of intraluminal glycogen with CellProfiler. The average intensity/pixel of inclusions stained by PAS was determined and expressed as % of the value in control siRNA treated cells. Around 50 inclusions were counted for each experiment. Error bars correspond to the standard error of the mean (n=3).

5.3.2 UDP-glucose is a substrate for chlamydial GlgA

De novo glycogen synthesis in the inclusion lumen implies that glycogen synthesizing enzymes are present within this compartment. We could demonstrate that host Gys1 is indeed imported into the inclusion lumen, but also that intraluminal glycogen accumulation is independent of Gys1 (Figure 34), suggesting that intraluminal glycogen synthesis relies on chlamydial enzymes. As described before, bacterial glycogen synthase (GlgA) uses ADP-Glc as substrate, and not UDP-Glc. We thus sought to determine if the chlamydial GlgA is able to function on UDP-Glc, in contrast to other bacterial GlgAs. To do so, HeLa cells were transfected with the N-terminally Flag-tagged construct of chlamydial GlgA, fixed 24 h later and processed for PAS staining. Cytoplasmic glycogen content was massively increased in cells transfected with Flag-GlgA compared to mock-transfected cells (Figure 38 A), demonstrating that chlamydial GlgA is able to use host UDP-Glc for glycogen synthesis. A zymogram analysis was performed to further compare chlamydial GlgA activity towards either UDP-Glc or ADP-Glc. Briefly, lysates of *E. coli* lacking their endogenous *glgA* and transformed with either *E. coli glgA* or chlamydial *glgA* were separated on non-denaturing polyacrylamide gels that contained rabbit glycogen. The gels were subsequently incubated in buffer containing either UDP-Glc or ADP-Glc and glycogen production was visualized by iodine staining. While *E. coli* GlgA only showed activity upon incubation with ADP-Glc, the chlamydial GlgA showed activity with both substrates, UDP-Glc and ADP-Glc (Figure 38 B; zymogram experiment was performed by Mathieu Ducatez, University of Lille 1).

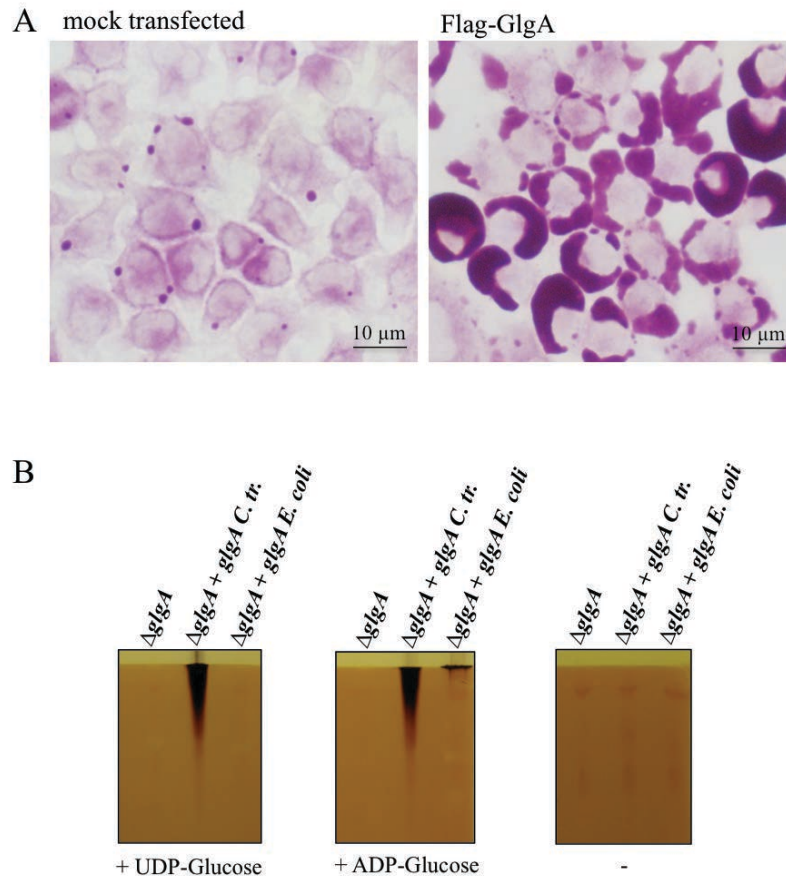


Figure 38: Chlamydial GlgA can use UDP-Glc as substrate for glycogen synthesis. (A) PAS staining was performed on HeLa cells transfected with chlamydial Flag-GlgA. Note the extensive glycogen production in Flag-GlgA transfected cells compared to mock transfected cells. (B) Zymogram analysis of chlamydial and *E. coli* GlgA activity. Lysates of *E. coli* lacking the *glgA* gene ($\Delta glgA$) alone, of $\Delta glgA$ transformed with chlamydial *glgA* (*glgA C. tr.*) and of $\Delta glgA$ transformed with *glgA* of *E. coli* (*glgA E. coli*) were separated by native polyacrylamide electrophoresis and incubated in either UDP-Glc, ADP-Glc or buffer only (-). Glycogen production was visualized by iodine staining.

5.3.3 Identification of UDP-Glc transporter at inclusion membrane

To further understand the mechanism of UDP-Glc import we aimed to identify its transporter at the inclusion membrane. As *Chlamydia* does not encode for any kind of nucleotide sugar transporter we focused on the host cell. Nucleotide sugar transporters are localized at either the ER or Golgi-apparatus to allow the import of nucleotide sugars serving as substrates for glycosylation of proteins, lipids and proteoglycans. They all belong to a family of highly conserved hydrophobic proteins with several transmembrane domains, the solute carrier family 35 (SLC35). Only a few of its members have been well characterized yet, and while some of them exert high specificity for one substrate, some of them utilize

several different nucleotide sugars containing mostly the same nucleotide (Handford et al., 2006; Song, 2013). The only UDP-Glc transporter that has been well characterized to date is the Golgi-localized SLC35D2, which transports UDP-Glc, UDP-N-acetylglucosamine and GDP-mannose in exchange for the corresponding nucleoside monophosphates (Ishida et al., 2005; Suda et al., 2004). It is known that UDP-Glc import also takes place in the ER, however the ER-localized transporter has yet to be identified. We transfected an SLC35D2 expressing plasmid with a C-terminal HA-tag, infected the cells for 30 hrs, fixed them and processed them for immunofluorescence microscopy. The HA-tagged transporter was in the ER and in the Golgi apparatus and was also enriched around the inclusion membrane (Figure 39 A). At this level of resolution we could not determine if the transporter was present in the inclusion membrane or whether it remained associated to ER- or Golgi-derived structures, which are known to accumulate around the inclusion. We depleted the host SLC35D2 by siRNA to assess the effect on intraluminal glycogen staining. Evaluation of the transcript level by RT-PCR showed that the efficiency of the knock down was high (Figure 39 B). PAS staining of infected cells and quantification via CellProfiler revealed a 40 % decrease of glycogen in the inclusions of siRNA SLC35D2 treated cells (Figure 39 C&D). However, there was no significant difference in the amount of glycogen in the cytoplasms of siRNA ctrl and siRNA SLC35D2 treated cells (not shown).

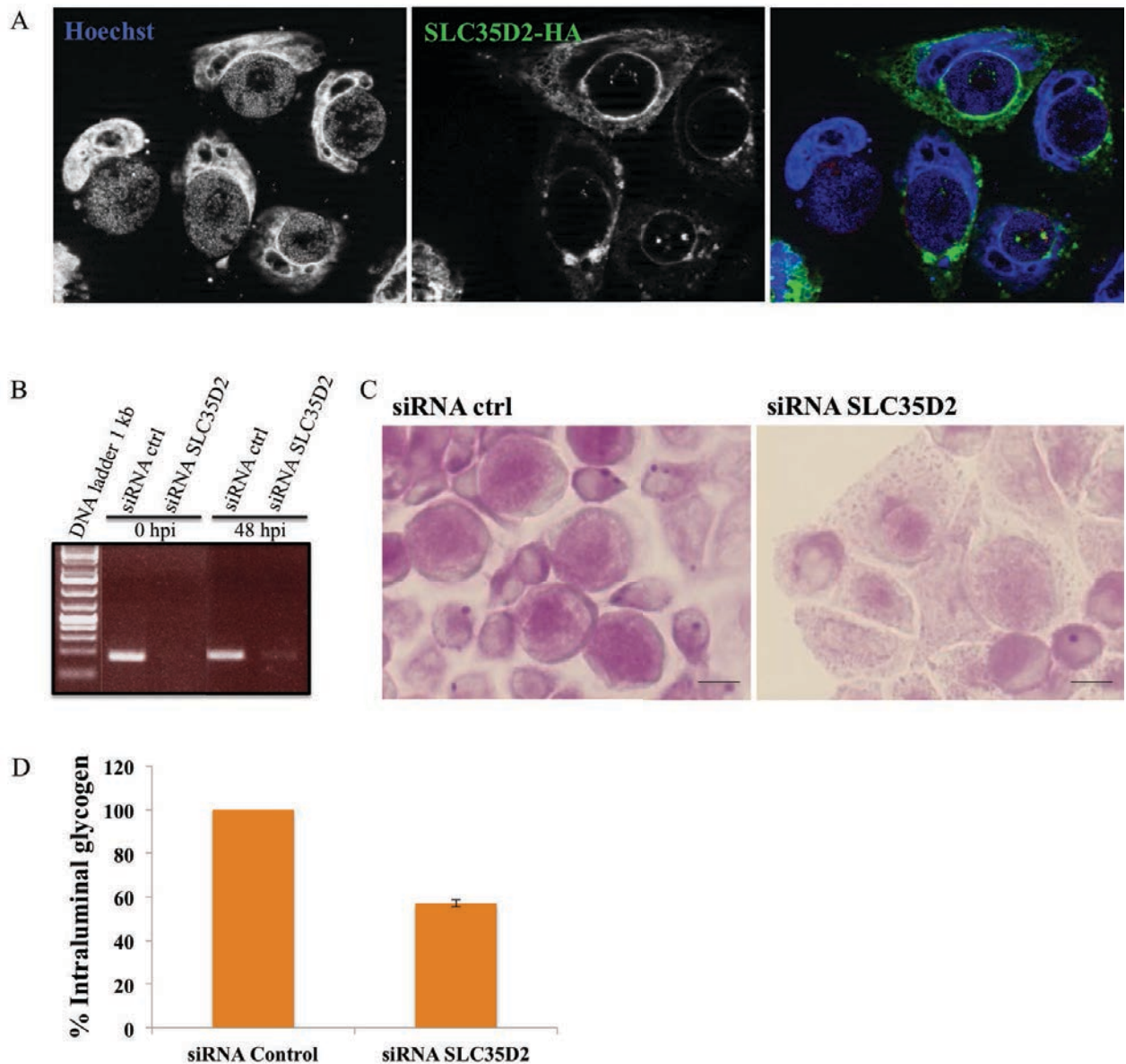


Figure 39: SLC35D2 imports UDP-Glc into the inclusion lumen. (A) HeLa cells were transfected with SLC35D2-HA prior to infection. Anti-HA partially labels the inclusion membrane or its close vicinity in transfected cells, but not in non-transfected cells. (B) Cells were treated with siRNA control (ctrl) or siRNA SLC35D2 48 h and 4 h prior to infection. Samples were taken at the moment of infection (0 hpi) and 48 hpi, and RT-PCR was performed with primers specific to SLC35D2. (C) PAS staining of siRNA ctrl or SLC35D2 treated cells 48 hpi. Error bar 10 μ m. (D) Quantification of intraluminal glycogen with CellProfiler. The average intensity/pixel of inclusions stained by PAS was determined and expressed as % of the value in control siRNA treated cells. Around 50 inclusions were counted for each experiment. Error bars correspond to the standard error of the mean (n=3).

6. Chlamydial glycogen enzymes are secreted for *de novo* glycogen synthesis

6.1 Heterologous test of secretion in *Shigella flexneri*

Taken together our data strongly indicate that UDP-Glc is imported into the inclusion lumen, where *de novo* glycogen synthesis takes place. This observation implies that glycogen synthesis enzymes are present in the inclusion lumen. In addition, intraluminal glycogen stores are only of benefit for the bacteria if the storage polysaccharide eventually becomes degraded, making glucose available for bacterial import. Thus, our data imply the presence of glycogen synthesizing and degrading enzymes in this compartment. We therefore checked first if the amino acid sequences of the chlamydial glycogen enzymes GlgC, GlgA, GlgB, GlgX, GlgP and MalQ possess a secretion signal peptide using the program SignalP 4.1 with neural network (NN) and a hidden Markov model (HMM) algorithm (<http://www.cbs.dtu.dk>). All of them were predicted to be negative, indicating that they were substrates to neither type II nor type V secretion, both being dependent of the Sec-pathway. Computational predictions of type III secretion signals (T3SS) are more difficult, as to date different hypotheses exist to whether the T3S is an mRNA-based signal, a chaperon mediated process, or an N-terminal signal peptide (Arnold et al., 2009). We made use of a machine-learning approach taking into account N-terminal sequence features (<http://www.effectors.org/>) and analysed the amino acid sequences of the glycogen enzymes. Only MalQ was predicted to possess a T3SS with this method. However, the most reliable way to detect a T3SS remains *in vitro* secretion assays based on the high conservation of the T3S apparatus and the recognition of the secretion signals throughout different species (Arnold et al., 2009; Subtil et al., 2005). Previous reports confirmed that chlamydial effectors can equally be translocated by the T3S apparatus of other pathogenic species such as *Yersinia* or *Shigella* (Fields and Hackstadt, 2000; Subtil et al., 2005; Subtil et al., 2001). We made use of a protocol established in our lab in which the N-terminal part including the first 20 amino acids of the chlamydial protein is fused to a reporter molecule, the calmodulin-dependent adenylate cyclase of *Bordetella pertussis* (Cya). The fusion with Cya allows for the detection of the chimera with a specific anti-Cya antibody. Constructs were transformed into two different *Shigella flexneri* mutant strains: *ipaB*, lacking regulation of T3S and thus being constitutive secretors, and *mxiD*, a mutant in which T3S is totally impaired (Allaoui et al., 1993; Menard et al., 1994). Liquid cultures of the transformed strains were fractionated into pellet and supernatant and the presence of the chimeras assessed in both fractions by Western Blot. To ensure that transformation did not hamper secretion a positive control was included: IpaD localizes to the tip of the T3S apparatus serving as a plug

and is amongst the first secreted proteins (Espina et al., 2006). To exclude that a signal in the supernatant fraction was due to lysis of the bacteria we also probed the membranes with an antibody against a cytosolic protein, the cAMP receptor protein (CRP). All chimeras were expressed in both strains, *ipaB* and *mxiD*, and could be detected in the bacteria (pellet fraction). GlgA/cya, GlgB/cya, GlgX/cya, GlgP/cya and MalQ/cya were also present in the supernatant fraction of the *ipaB* strain, but not of the *mxiD* strain, demonstrating that their secretion in the culture supernatant was T3S dependent. In contrast, GlgC/cya was restricted to the pellet fraction. IpaD could be found in the *ipaB* supernatant fractions for all constructs, demonstrating that transformation with the chimeras did not hamper secretion. Finally, CRP was present only in the pellets, excluding the possibility that detection of the chimera in the culture supernatant resulted from bacterial lysis (Figure 40). These results suggest that the full-length glycogen enzymes GlgA, GlgB, GlgX, GlgP and MalQ, but not GlgC, are substrates of T3S. We thus propose that those glycogen enzymes are secreted by *C. trachomatis* and that their presence in the inclusion lumen eventually leads to glycogen accumulation and subsequent degradation therein.

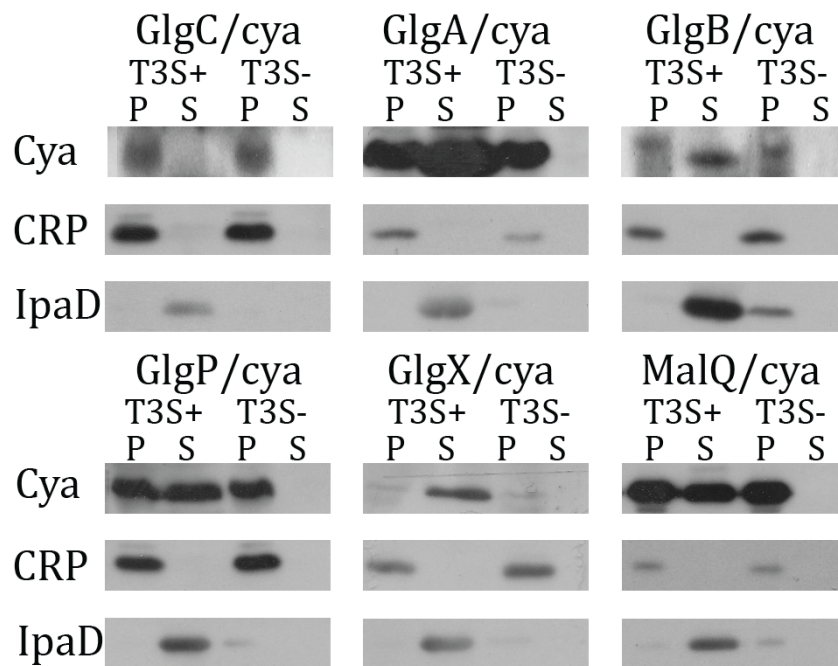


Figure 40: Heterologous test of secretion in *Shigella flexneri* reveals T3S signals in several of the glycogen enzymes. The N-terminal 20 amino acids of the indicated proteins were fused to the reporter Cya, and constructs were transformed into two different *S. flexneri* strains. The *ipaB* mutant constitutively secretes T3S substrates (T3S+), whereas the *mxiD* mutant is totally T3S impaired (T3S-). The transformants were grown in liquid culture, which was fractionated into pellet (P) and supernatant (S). Proteins were loaded on a SDS-PAGE gel and membranes blotted with anti-Cya antibodies to detect the chimeras in the different fractions. CRP, a *S. flexneri* cytosolic protein, served as lysis control and was only detected in the pellets. IpaD is subject to T3S and could be detected in the *ipaB* supernatant, demonstrating that secretion was not impaired by transformation with the various constructs. All chimeras were expressed in *ipaB* and *mxiD* and were found in the pellets. Chimeras of GlgA, GlgB, GlgX, GlgP, GlgX and MalQ but not GlgC were equally detected in the supernatant in the *ipaB* background, but not in the *mxiD* background, demonstrating that secretion occurred through a T3S mechanism.

6.2 GlgX is present in the inclusion lumen

We aimed to verify the results obtained for the N-termini in the heterologous test of secretion by using specific antibodies against the chlamydial glycogen enzymes. Anti-peptide antibodies against GlgX and GlgP were designed and purchased. Unfortunately, only the anti-GlgX antibody was of sufficient quality to be used in immunofluorescence or Western Blot. Staining of infected cells with the anti-GlgX antibody depicted a very interesting phenotype (Figure 41). At both 24 hpi and 48 hpi GlgX was found in the inclusion lumen, free of bacterial signal. Inclusion membrane staining was visible at 24 hpi, but not at 48 hpi. When we pre-incubated the anti-peptide antibody with the peptides against which it was directed, we could abolish the signal, demonstrating its specificity. Pre-incubation with random peptides did not decrease the signal. We could thus verify the secretion of GlgX by *Chlamydia* and the presence of the endogenous enzyme in the inclusion lumen.

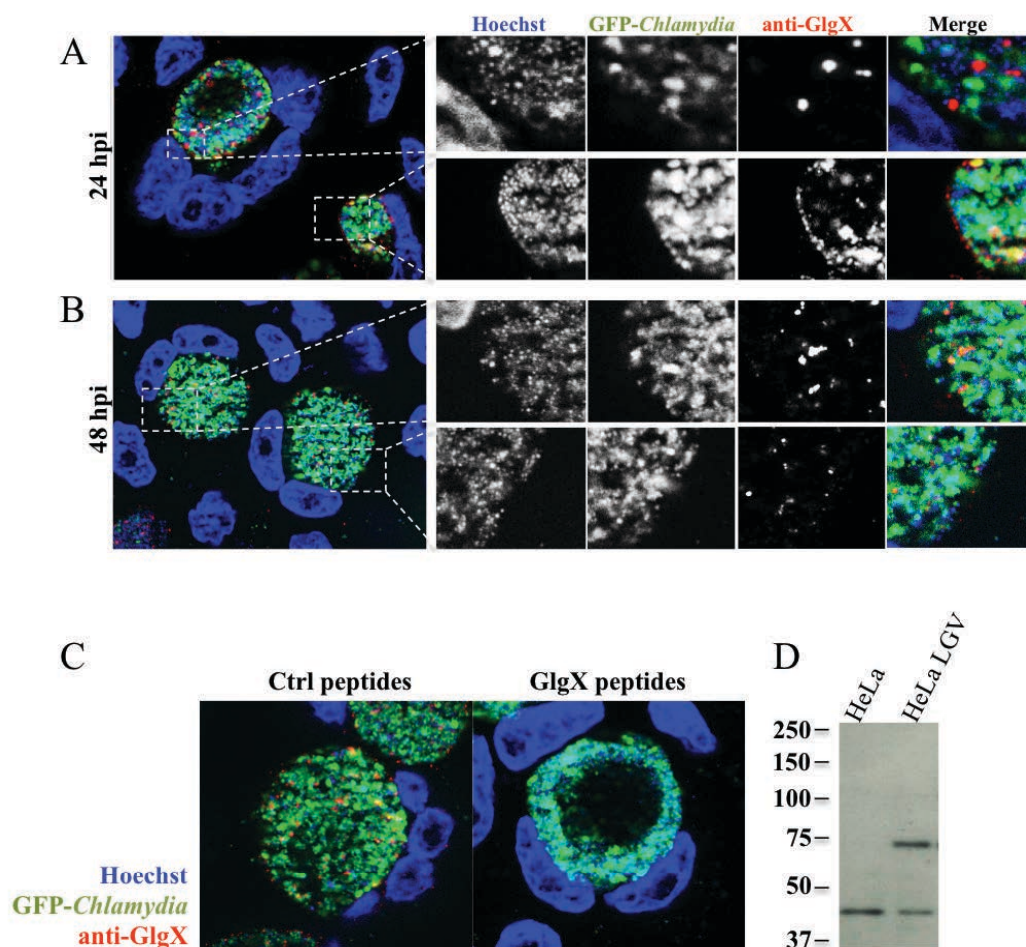


Figure 41: Staining of infected cells with an anti-GlgX antibody. HeLa cells infected for (A) 24 h or (B) 48 h with GFP-*Chlamydia* (green) and stained with an anti-GlgX antibody (red) and Hoechst (blue) to visualize the host and bacterial DNA. (C) Anti-GlgX was pre-incubated with either unrelated control peptides, or with the peptides the antibody was raised against. (D) Western Blot of non-infected HeLa or LGV infected HeLa cells. The anti-GlgX antibody detected a band of the expected molecular weight (73 kDa).

6.3 Overexpression of glycogen enzymes in *C. trachomatis*

We subsequently tried to verify secretion of the remaining glycogen enzymes by transforming *C. trachomatis* with a plasmid containing the glycogen genes under a tetracycline (Tet) inducible system. A recent report has demonstrated the ability of *C. trachomatis* to secrete IncD fused to a C-terminal Flag when the gene was inserted into the plasmid pBOMB4-Tet-mCherry. This plasmid is a modified version of the endogenous *C. trachomatis* plasmid pL2, containing all 8 ORFs (*pgp1-pgp8*), a gene for GFP expression, a β -lactamase gene, a pUC19 origin of replication, a small multiple cloning site (MCS) containing a gene for mCherry expression with the Tet-inducible promoter at its 5' end, and the Tet repressor TetR (Figure 42 A) (Bauler and Hackstadt, 2014). TetR binds tightly to the tetracycline operators (TetO) within the promoter and thus represses transcription. Addition of tetracycline leads to the release of the repressor and enables transcription. We adapted this plasmid to our own needs by removing the mCherry gene via the restriction sites NotI/SalI and substituting it with another small MCS (comprising two different SfiI sites and the unique restriction sites SpeI and SacII) more apt to our use. 3' of this MCS we introduced a *3xflag*, giving rise to the plasmid pBOMB4-Tet-Flag (Figure 42 B). In order to validate the system, we amplified the *glgX* gene from *C. trachomatis* and cloned it into the vector pBOMB4-Tet-Flag via the SpeI and SacII restriction sites. As negative control for secretion a truncated version of *glgX* lacking the first 90 base pairs, Δ *glgX*, was created. Tetracycline is toxic for *Chlamydia*, but its derivative anhydrotetracycline (ATc) has less antibacterial effects and displays a ~30 fold higher affinity towards the TetR, allowing for the use of lower doses (Wickstrum et al., 2013). A previous report analysed the effects of ATc on *C. trachomatis* and revealed a 50 % reduction of progeny when as little as 30 ng/ml were used. No or only small effects on inclusion size or progeny were observed when 10 or 20 ng/ml were used (Wickstrum et al., 2013). Transformation into *Chlamydia* yielded green bacteria with no apparent difference in inclusion size or bacterial growth, concordant with other reports (Bauler and Hackstadt, 2014). In absence of ATc no or only very little staining was detected when infected cells were probed with an anti-Flag antibody (Figure 43 A). Induction with 10 or 20 ng/ml ATc led to a higher level of expression of GlgX than of Δ GlgX. GlgX-Flag and Δ GlgX-Flag were detected within the inclusion lumen, both in the vicinity of or overlapping with GFP-*Chlamydiae*, but both constructs were absent from the inclusion membrane (Figure 43 B). The duration of exposure to ATc did not influence this phenotype, as there was no difference in the pattern of the Flag-distribution when ATc was added for 22 or only 6 h.

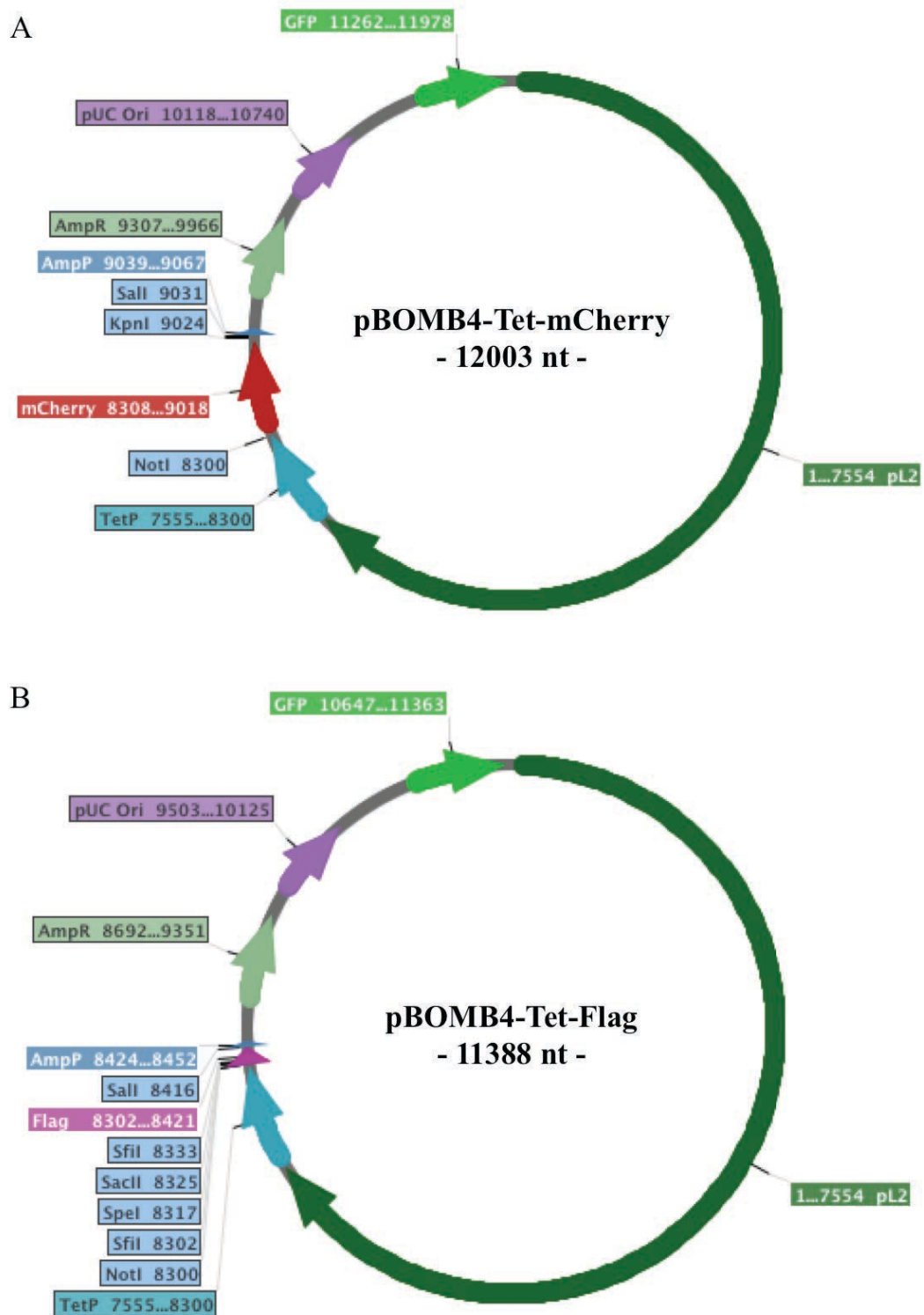


Figure 42: Plasmid maps of (A) pBOMB4-Tet-mCherry and (B) pBOMB4-Tet-Flag. pL2 = the 8 ORFs of the endogenous *C. trachomatis* L2 plasmid. TetP = Tetracycline-inducible promoter. Flag = 3xflag-tag. AmpP = β -Lactamase promoter. AmpR = β -Lactamase gene. pUC Ori = origin of replication of the pUC19 vector. mCherry = gene for mCherry expression. GFP = gene for GFP expression.

The extrabacterial Flag-staining was also present in the Δglx -transformant lacking its T3S signal. We could therefore not exclude that the extrabacterial signal we detected upon induction in the *glgX*-transformants was due to bacterial lysis. A similar approach was equally tested, in which we used the original pBOMB4-Tet-mCherry and introduced the genes *glgP* and $\Delta glgP$ (lacking the first 90 base pairs) via the NotI and KpnI restriction sites, excising *mCherry*. Both genes were cloned in a way that they were fused to a *1xflag* at their 3' end. While GlgP-Flag was expressed at 20 ng/ml ATc, Δ GlgP-Flag could not be detected at that concentration. The staining of GlgP-Flag was similar to GlgX-Flag and Δ GlgX-Flag and was found in the inclusion lumen, overlapping with bacteria or in bacteria-free regions. As the Δ GlgP-Flag control was not expressed we could not exclude that the extrabacterial signal we saw for GlgP-Flag was due to bacterial lysis. We concluded from this set of experiments that the multiple cloning site we had engineered to clone these genes was sub-optimal, and that more work on the plasmid will be needed to create a reliable tool to express tagged bacterial proteins in infection.

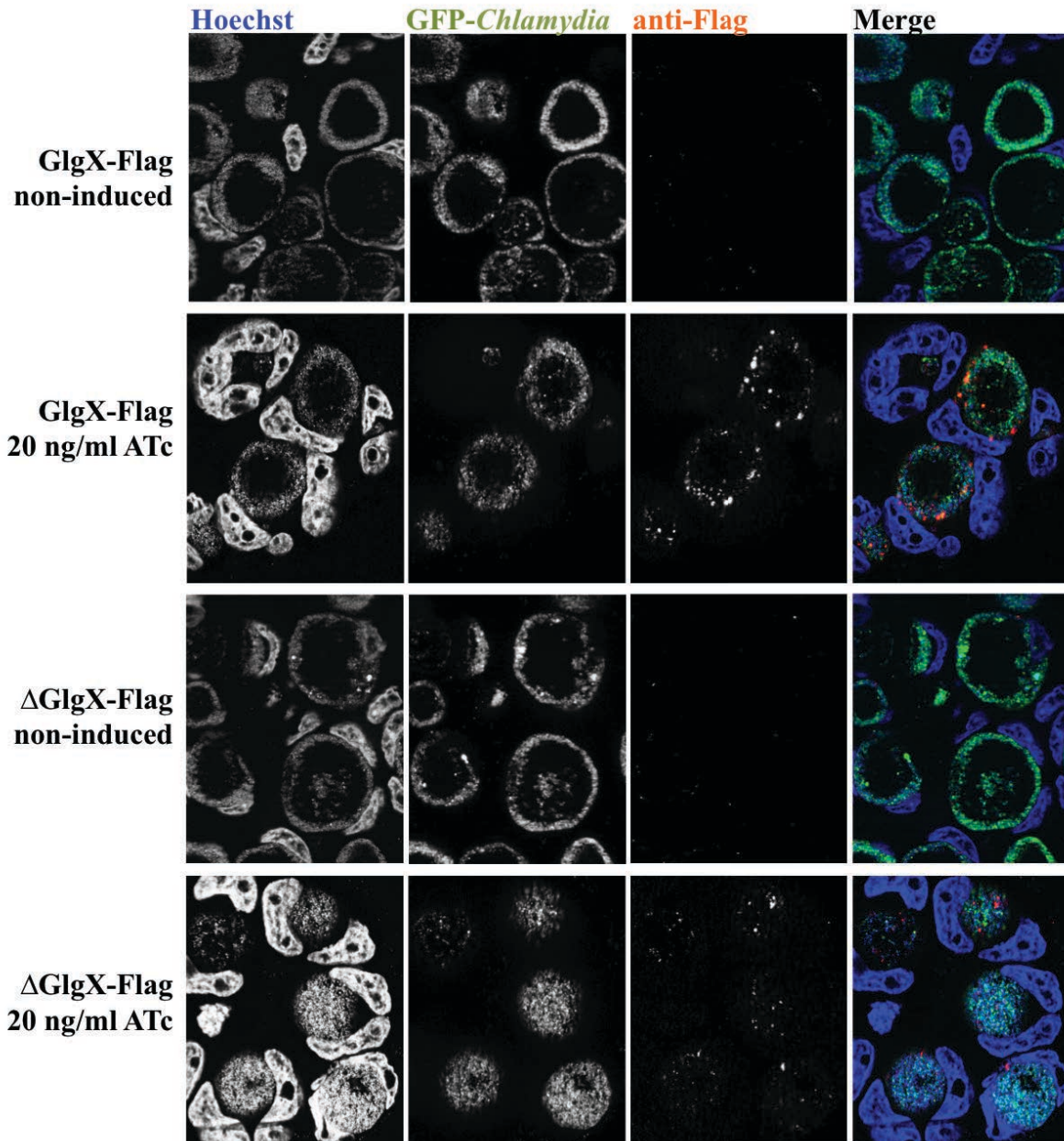


Figure 43: Overexpression of GlgX-Flag or Δ GlgX-Flag in *C. trachomatis*. HeLa cells were infected with *C. trachomatis* transformed with either GlgX-Flag or Δ GlgX-Flag in the shuttle vector pBOMB4-Tet-Flag expressing GFP. 8 hpi expression of the insert was induced with 20 ng/ml ATc. Constructs are stained with an anti-Flag antibody. Note that extrabacterial staining is found in both transformants, GlgX-Flag and Δ GlgX-Flag, lacking the first 30 amino acids.

6.4 Ectopically expressed GlgA compensates for the plasmid-less deficiency in glycogen accumulation

Plasmid-less strains have an intriguing phenotype since the inclusions they form have a very low glycogen content. This has been linked to a low level of expression of GlgA, in agreement with our finding that GlgA is the master gene controlling glycogen synthesis. GlgA secretion has been reported in the inclusion lumen and in the host cytoplasm (Lu et al., 2013). GlgA could thus control intraluminal glycogen stores at two levels: cytoplasmic GlgA could boost host glycogen production, thus fuelling bulk import. Alternatively, GlgA present in the inclusion lumen could enhance local glycogen synthesis. We think that bulk glycogen import into the inclusion only marginally contributes to intraluminal stores, and that the *de novo* synthesis pathway predominates. Indeed, cytoplasmic stores of glycogen are similar in cells infected with wild-type or plasmid-less strain, even in the vicinity of the inclusion where we could expect a local effect of GlgA secretion on host glycogen. Thus bulk import is presumably the same for the two strains, and if it made a significant contribution to intraluminal glycogen one would not expect such a difference in intraluminal glycogen accumulation between the two strains. In addition, the depletion of host glycogen by siRNA Gys1 did not lead to a decrease of intraluminal glycogen, indicating that cytoplasmic glycogen concentration does not directly affect intraluminal glycogen accumulation (chapter 5.2.1). Still, we wondered if GlgA expression in the host cytoplasm might compensate for the low endogenous expression of GlgA in the plasmid-less strain. To test this, we transfected cells with Flag-tagged GlgA before infecting them with either wild-type or plasmid-less *C. trachomatis*. Inclusions of cells infected with the wild-type strain did show a slight increase in glycogen content, as revealed by PAS staining (Figure 44). Strikingly, glycogen staining of inclusions from the plasmid-less strain was now indistinguishable from that of the wild-type strain. Increase in luminal glycogen likely results both from an increased bulk import of cytoplasmic glycogen (simply because cytoplasmic glycogen is much more abundant) and from the translocation of Flag-GlgA into the inclusion lumen (as stowaway of cytoplasmic glycogen bulk import, demonstrated in chapter 5.2.1), where it is no longer limiting for *de novo* glycogen synthesis. In either case, it is quite remarkable that a host-expressed gene can compensate for a bacterial deficiency.

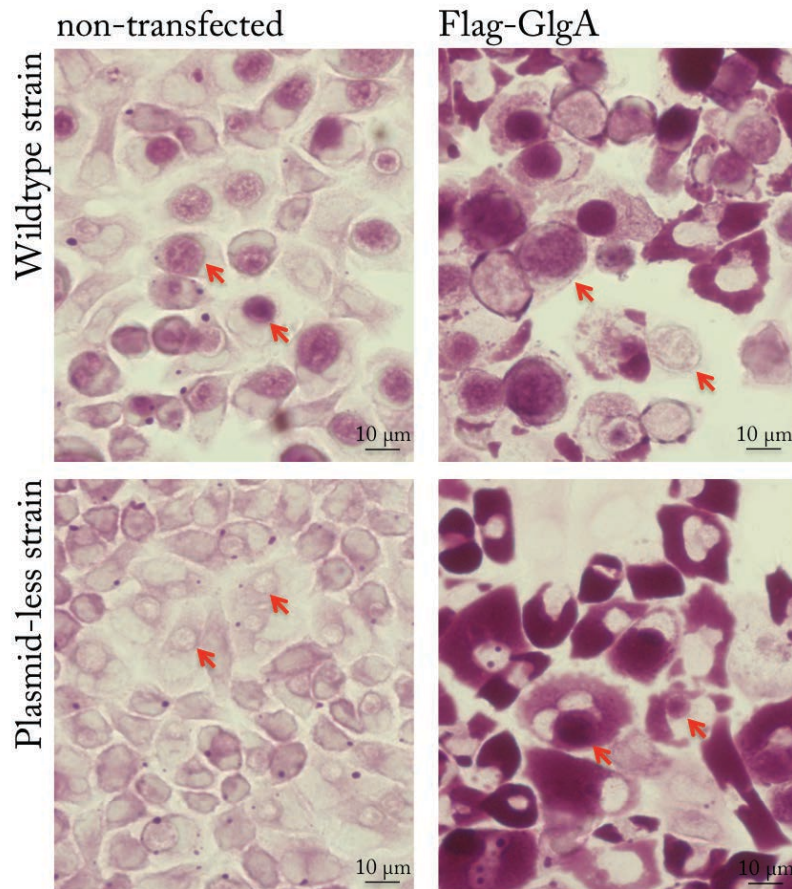


Figure 44: Flag-GlgA transfection leads to an increase in intraluminal glycogen accumulation. HeLa cells were transfected with Flag-GlgA before infection with either the wild-type LGV strain or the plasmid-less strain LGV 25667R. PAS staining of glycogen revealed an increase of intraluminal glycogen upon transfection, notably in the case of the plasmid-less strain. Red arrows = inclusions.

7. *Chlamydia* import Glc6P, but not Glc1P nor Glc

Accumulation of glycogen in the inclusion lumen precedes its appearance within EBs. Moreover, we have shown that bacterial glycogen degradation enzymes are likely T3S substrates. These data suggest that *Chlamydiae* trigger degradation of intraluminal glycogen into monomers amenable for subsequent uptake into the bacteria, where a new circle of glycogen synthesis starts. Degradation of glycogen through GlgP leads to the release of Glc1P. *Chlamydia* encodes for a hexose phosphate transporter, UhpC, annotated as a Glc6P transporter. Schwöppe and colleagues demonstrated that the UhpC of *C. pneumoniae* does indeed transport Glc6P, but not Glc1P (Schwoppe et al., 2002). How can the glycogen degradation product Glc1P then enter the bacteria? The amino acid identity between the C.

pneumoniae and *C. trachomatis* UhpC lies at 79 %, which could account for a difference in substrate specificity. We therefore aimed to determine which form of Glc was imported into *C. trachomatis*. Purified EBs were incubated with radioactively labelled C¹⁴-Glc, C¹⁴-Glc6P or C¹⁴-Glc1P in the absence or presence of a 50-fold excess of cold Glc, Glc6P or Glc1P. Radioactivity was measured in the bacteria after 2 h at 37 °C. Only C¹⁴-Glc6P did accumulate in the bacteria. A 50-fold excess of non-radioactive Glc6P out-competed C¹⁴-Glc6P accumulation, but not cold Glc1P nor Glc (Figure 45 A). This experiment demonstrates that *C. trachomatis* can exclusively take up Glc6P, while glycogen degradation yields Glc1P. To solve this paradox we hypothesized that Glc1P might be converted into Glc6P within the inclusion lumen prior to import into the bacteria. The *Chlamydia* encoded phosphoglucomutase (PGM) can fulfil this enzymatic activity, prompting us to test this enzyme for an N-terminal T3SS in the *Shigella flexneri* background. Intriguingly, a T3SS could be detected in PGM (Figure 45 B), supporting our hypothesis of conversion from the glycogen degradation product Glc1P to Glc6P within the inclusion lumen prior to uptake of Glc6P by the bacteria.

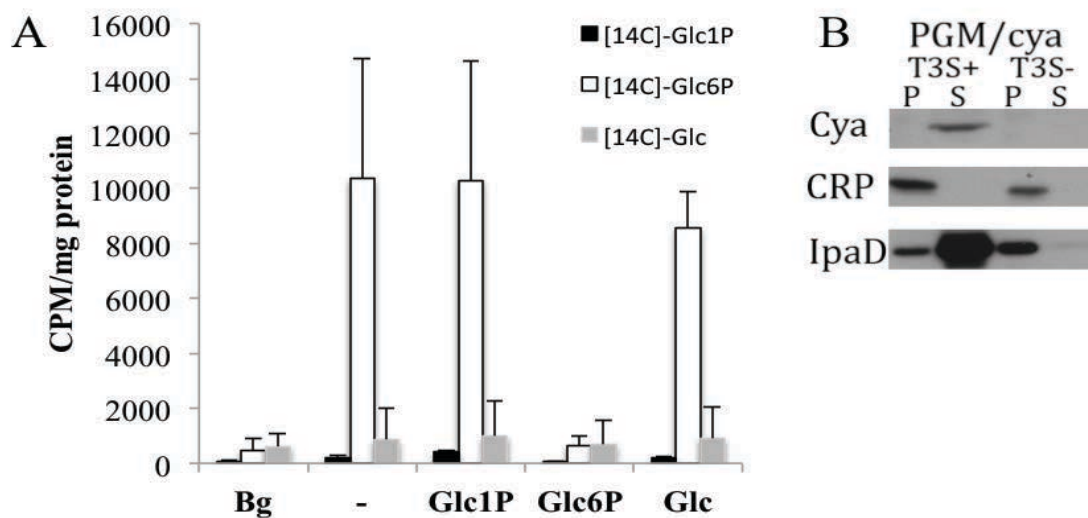


Figure 45: *C. trachomatis* secretes PGM and takes up Glc6P. (A) Purified EBs were incubated for 2 h with C^{14} -Glc, C^{14} -Glc6P or C^{14} -Glc1P in absence or presence of a 50-fold excess of non-radioactive Glc, Glc6P or Glc1P. Bacteria were subsequently washed and pelleted and radioactivity measured with a scintillograph. Background (Bg) = bacteria were incubated for 1 min instead of 2 h. - = absence of competitors. +Glc1P/+Glc6P/+Glc = presence of non-radioactive competitors. A radioactive signal could only be detected when bacteria were incubated with C^{14} -Glc6P, and signal was depleted upon addition of cold Glc6P. Counts per minute (CPM) were determined in the bacterial pellet and normalized to 1 mg of bacterial proteins. Error bars correspond to the standard deviation (n=3). (B) Heterologous test of secretion in *S. flexneri* was performed with a chimera containing the first 20 amino acids of PGM. The chimera was detected in the supernatant in the *ipaB* (T3S+) but not in the *mxiD* (T3S-) context, indicating that secretion was T3S dependent. See Figure 40 for details.

DISCUSSION

During all stages of infection, *Chlamydiae* must acquire nutrients from the surrounding microenvironment to live and multiply. Some of the nutrient uptake processes have been elucidated, but many remain unaccounted for. Glycogen accumulation in the inclusion lumen of *C. trachomatis* has first been described more than 80 years ago, but no in-depth study of this remarkable phenomenon had been undertaken. This work brings to light the glycogen's provenance and its fate. We will also discuss some hypotheses as to why this extraordinary way of "metabolic outsourcing" could be of benefit to this bacterium.

Glucose is essential for *C. trachomatis* and is stored as glycogen in the inclusion lumen and the EBs.

C. trachomatis has stringent nutrient requirements for growth. While incubation with various carbon sources such as glutamate, malate, α -ketoglutarate and oxaloacetate leads to the production of infectious progeny, the presence of glucose in the medium is highly favourable (Iliffe-Lee and McClarty, 2000). We could observe that there was no statistical difference in the production of infectious particles when infection occurred in media containing 2 to 0.4 mg/ml glucose (Figure 26 A). In absence of glucose no infectious particles were formed. The normal glucose level in the blood serum of a healthy human is around 0.6 to 1 mg/ml, and of the female vaginal fluid around 0.6 mg/ml, thus in the range of the saturation effect that we observed (Exley et al., 2007). It appears that even under low sugar concentrations (0.4 mg/ml) *C. trachomatis* still accumulates glycogen in the inclusion lumen (Figure 26 B). In high glucose conditions we detected glycogen accumulation in the EBs and in the lumen, but not in the RBs (Figure 23), in contrast to a previous report (Chiappino et al., 1995). Consistent with our finding, it was demonstrated that RBs require external addition of ATP in order to perform protein biosynthesis (Omsland et al., 2012). In the same study it was not only revealed that EBs do indeed possess metabolic activity, but also that they require the presence of Glc6P for that and that no activity could be detected in the sole presence of ATP. These intriguing data suggest that the two different developmental forms use different sources of energy, and go thus hand in hand with our observation that only EBs accumulate glycogen, the polysaccharide of glucose.

Dynamics of intraluminal glycogen accumulation reveal that it is not lysis derived.

Intraluminal glycogen accumulation had previously been attributed to bacterial lysis and to the release of chlamydial glycogen into the surrounding environment (Chiappino et al., 1995). In the present work we observed that glycogen accumulation occurred first in the inclusion lumen, before it started in the bacteria (Figure 27). Thus, the bacterial lysis that Chiappino and colleagues had observed might contribute to the overall glycogen content, but is not the main source. While it had already been shown that glycogen accumulation rapidly increased 18 hpi, light microscopy was used to do so and small concentrations could have remained undetected (Matsumoto et al., 1998). We could confirm in TEM that intraluminal glycogen accumulation started between 16 and 20 hpi and rapidly increased thereafter (Figure 28). At that stage, we only detected RBs and no EBs, which strongly suggests that the accumulation is triggered by RBs. Our transcriptomics analysis by qRT-PCR revealed that *glgA* most likely acts as a master gene to control the onset of glycogen synthesis, as its transcription start coincides with the onset of intraluminal glycogen accumulation (Figure 29). All the other glycogen synthesizing and degrading enzymes essentially depict a similar transcription profile, correlated to the increase in the number of the metabolically more active RB between 8 and 24 hpi. The only other exception is *glgB*, which is highly transcribed early on in the infectious cycle (1 to 3 hpi) and declines thereafter. The reason why GlgB is synthesized before GlgA (GlgA lying upstream of GlgB in the glycogen synthesis pathway) is not clear. We hypothesized that *glgB* was clustered in an operon with other genes whose transcription might be required early, but this is not the case (Albrecht et al., 2010).

A minor pathway imports host glycogen in bulk in an autophagy-independent way.

Our main interest was to identify the mechanism of intraluminal glycogen accumulation. We initially proposed two different and non-exclusive hypotheses. One implies the uptake of host glycogen in bulk, through either uptake of glycogen-filled vesicles or uptake of free host glycogen through invagination of the inclusion membrane. The second hypothesis is that substrates of glycogen synthesis are transported into the inclusion lumen and that synthesis takes place *de novo* within this compartment. Our observation of glycogen-filled vesicles within the inclusion strongly supports the hypothesis of bulk import of host glycogen (Figure 31). Additionally, glycogen enzymes such as the host glycogen synthase Gys1, or transfected Flag-GlgA, but not glycogen unrelated proteins we used as controls,

were detected in the inclusion lumen (Figure 32&33). We propose that these enzymes are an "accidental" by-product of import of host glycogen, as they are known to bind tightly to glycogen (Caudwell and Cohen, 1980). An attempt to see if imported Gys1 colocalized with imported Flag-GlgA failed, as transfection with Flag-GlgA appeared to lead to a massive downregulation of Gys1 and thus to an almost complete loss of its detection by immunofluorescence. Gys1 expression is likely controlled by the cellular concentrations of UDP-Glc, Glc6P and glycogen, which are strongly affected by Flag-GlgA expression.

We attempted to characterize these glycogen-filled vesicles further. As the PATAg staining used to visualize glycogen in TEM is incompatible with the immunogold procedure, we could not determine the exact provenance of the vesicular membrane(s) of those glycogen-filled vesicles with specific antibodies (for instance it would have been informative to stain with antibodies against Inc proteins, to see if the surrounding membrane originated from the inclusion membrane). Because several glycogen-filled vesicles showed multi-layered membranes, reminiscent of autophagosomes, we tested whether the presence of these vesicles is autophagy-dependent. In mouse embryonic fibroblasts deficient for autophagy (*Atg5*^{-/-} mutant cell line) we still observed intraluminal glycogen-filled vesicles, highlighting that those vesicles are not autophagosomes (Figure 36). In addition, Gys1 import still took place in this cell line. These results clearly demonstrate that the glycogen-filled vesicles seen in TEM in the inclusion lumen are not of autophagic origin. Additionally, we did not observe glycogen-filled vesicles outside the inclusion in the host cytoplasm. Therefore, we favour the hypothesis that the inclusion membrane invaginates through an unknown mechanism to take up free host glycogen. *Chlamydia* could use many different pathways for this mechanism of invagination. It could either be an unspecific "sampling" of the host cytoplasm, or a glycogen-specific uptake. Proteomics analysis showed an enrichment of GlgP in the inclusion membrane containing fraction (Saka et al., 2011), and we could demonstrate the presence of GlgX at the inclusion membrane by immunofluorescence (Figure 41). It is worth noting that neither GlgP nor GlgX contain a transmembrane domain. A pure speculation would be that both enzymes serve as some kind of "sensor" at the inclusion membrane, or anchor glycogen molecules to the membrane before invagination takes place.

The major pathway for intraluminal glycogen accumulation requires UDP-Glc import.

However, the vast majority of intraluminal glycogen is not host derived, as depletion of host glycogen via the Gys1 knockdown did not significantly change the amount of

glycogen found in the bacterial compartment (Figure 34). In contrast, when we knocked-down the human enzyme responsible for the generation of UDP-Glc (UGP2) we observed a strong decrease (~ 60 %) in intraluminal glycogen accumulation (Figure 37). Diminution of host glycogen levels could not account for this result, as the Gys1 knockdown was a lot more efficient in depleting glycogen in the host cytoplasm than the UGP2 knockdown. The host hexokinase is responsible for the phosphorylation of Glc into Glc6P, which can be converted to Glc1P, the substrate of UGP2. If Glc6P or Glc1P were the substrate of import into the inclusion, a UGP2 knockdown should not produce any difference in intraluminal glycogen accumulation because both Glc6P and Glc1P would remain available. Thus our results strongly support the hypothesis that UDP-Glc is imported in the inclusion lumen, to serve as a substrate for glycogen synthesis. Energetically speaking, it is beneficial to import UDP-Glc rather than Glc6P or Glc1P, as it relieves the bacteria from the costly reaction of transferring a nucleotide to the sugar molecule. These data were further strengthened by our finding that the chlamydial glycogen synthase GlgA was able to use UDP-Glc as substrate, in contrast to the vast majority of other bacterial glycogen synthases (Figure 38). This is highly intriguing, as it is yet another demonstration of how *Chlamydia* adapted evolutionary to its intracellular life style. We also showed that knocking down the Golgi-apparatus based UDP-Glc transporter SLC35D2 led to a 40 % reduction of intraluminal glycogen staining (Figure 39). In addition, we observed the accumulation of SLC35D2 at the periphery of the inclusion in cells transfected with a HA-tagged version of the transporter. Our data thus strongly indicate that SLC35D2 is at least partially responsible for the import of UDP-Glc. We have several different explanations as to why intraluminal glycogen accumulation was not completely abolished. As we used siRNA to deplete SLC35D2, the knockdown might not have been complete or the transporter displays a high stability, and the remaining SLC35D2 could be enough to account for the intraluminal glycogen. It is also conceivable that other Golgi- or even ER-based transporters from the SLC35-family of nucleotide sugar transporters use UDP-Glc as substrate, as not all of them are well characterized and no comprehensive study exists that has systematically tested the identified transporters for their substrates. Similarly, we cannot exclude that other glycogen building blocks are equally imported. Thus, SLC35D2 might not account for all sugar import into the inclusion. How the recruitment of the transporter is taking place remains to be elucidated.

As mentioned before GlgX and GlgP could be detected at the inclusion membrane. We propose that their function besides the hypothetical “anchoring” of host glycogen to the inclusion membrane could also lie in the degradation of host glycogen in the vicinity of the

inclusion, leading to the release of Glc1P. This monomer can now be converted to Glc6P and then to UDP-Glc by a host phosphoglucomutase and UGP2, respectively.

Glycogen metabolism is conferred to the inclusion lumen by secretion of chlamydial enzymes.

Intraluminal glycogen synthesis requires the presence of glycogen enzymes within this compartment. We detected T3SS in the amino-terminal sequence of GlgA, GlgB, GlgP, GlgX and MalQ, but not of GlgC, indicating that the former are secreted by *Chlamydia* (Figure 40). We transformed *C. trachomatis* with C-terminal Flag-tagged version of these enzymes to study their localization. Unfortunately, this approach using the pBOMB-Tet-mCherry vector developed by the Hackstadt's group was not successful, because all our constructs gave a similar pattern of intra-inclusion staining, including a negative control in which the first 20 amino-acids of GlgX were deleted (which should abrogate T3S) (Figure 43). We think that this pattern may result from bacterial lysis due to the overexpression conditions, as we had to use the inducer anhydrotetracyclin at concentrations close to the threshold of sensitivity of *C. trachomatis* to this antibiotic. In these conditions, we could not distinguish secretion of an enzyme from release of the enzyme through bacterial lysis. However, heterologous tests of T3S have been widely used in many studies, and the rate of false positives is below 5 % (Subtil et al., 2005). We propose that the glycogen enzymes are indeed secreted by a T3S system, and that they ultimately function within the inclusion lumen. The literature suggests that the T3S system is only triggered upon contact with a membrane, and intraluminal secretion of enzymes was not expected. However, GlgA was detected with a specific antibody within the inclusion lumen, not overlapping with bacteria (Lu et al., 2013). Strikingly, we could demonstrate the presence of GlgX in the inclusion lumen (in bacteria-free regions) and at the inclusion membrane (Figure 41). Loose membrane-like structures can frequently be seen in the inclusion lumen, and might trigger the luminal secretion of some effectors. Alternatively, secretion of glycogen enzymes into the vicinity of the inclusion membrane, and subsequent uptake through a vesicular mechanism, might take place, but is lacking evidence. If some of the enzymes, such as GlgA and GlgX, are secreted both in the inclusion lumen and in the host cytoplasm, the mechanism of regulation for such a dual pathway remains to be investigated.

Our data show that glycogen synthesis takes place within the inclusion lumen, even in the absence of host Gys1. Moreover, GlgA was detected in the inclusion lumen (Lu et al., 2013).

Remarkably GlgC, the chlamydial enzyme producing ADP-Glc from Glc1P, does not contain a T3SS. This observation fits perfectly with our finding that UDP-Glc is imported into the inclusion lumen. Secretion of GlgC into the inclusion lumen would be superfluous, with GlgA being able to produce unbranched glycogen out of UDP-Glc. That the glycogen branching enzyme GlgB is present in the inclusion lumen can also be derived from another interesting observation. Nguyen and colleagues obtained a *C. trachomatis* loss-of-function mutant lacking GlgB activity (Nguyen and Valdivia, 2012), with the particular phenotype that massive precipitates of unbranched glycogen were detected in the inclusion lumen. If GlgB was exclusively secreted into the host cytoplasm a GlgB loss-of-function mutation should be largely compensated for by the host branching enzyme. However, the precipitates found in inclusions of the *glgB*-mutant consist out of unbranched glycogen, and thus indicate strongly that GlgB failed to work within the inclusion lumen. Additionally, we could localize GlgX to the inclusion lumen and its membrane with specific antibodies. Thus, irrespective of their exact way of secretion, all data support the hypothesis that the glycogen synthesizing enzymes are active within the inclusion lumen.

The plasmid-less strain accumulates only minor amounts of the polysaccharide compared to the wild-type strain and has a strongly reduced expression level of GlgA. For neither of the strains did we observe glycogen accumulation around the inclusion membrane in TEM (chapter 1). When we ectopically expressed Flag-GlgA in cells infected with either the wild-type or the plasmid-less strain we observed increased intraluminal glycogen accumulation for both strains (Figure 44). These data strongly suggest that the difference between the two strains does not result from different levels of GlgA-triggered glycogen synthesis on the cytoplasmic side of the inclusion membrane. Instead, they indicate that GlgA is present in the wild-type inclusion and absent in the plasmid-less inclusion. When Flag-GlgA import into the inclusion took place, likely together with bulk glycogen uptake, the absence of GlgA in the plasmid-less inclusion was compensated for and high amounts of glycogen were subsequently detected in the inclusion lumen. These data confirm our finding that chlamydial glycogen enzymes are active in the chlamydial compartment.

***Chlamydia* take up Glc6P, and not Glc or Glc1P, and the latter is converted to Glc6P by a secreted PGM.**

We demonstrated that *de novo* glycogen synthesis takes place in the inclusion lumen, triggered by the presence of GlgA and GlgB. Similarly to those two glycogen synthesizing

enzymes, the degrading enzyme GlgX is present in the inclusion lumen and GlgP and MalQ are most likely T3S substrates, as we could show in the heterologous test of secretion. Glycogen storage in the inclusion lumen would only be of benefit for the bacteria if the glycogen were subsequently degraded into monomers amenable to bacterial uptake when needed. Also, old observations report that glycogen staining decreases at very late infection times, consistent with a late consumption of the stores (Gordon and Quan, 1965). Therefore we hypothesize that not only GlgX but also GlgP and MalQ as degrading enzymes are active in the inclusion lumen, which would eventually give rise to free Glc1P. We could clearly demonstrate that *Chlamydiae* are not able to take up Glc nor Glc1P, but exclusively Glc6P (Figure 45 A). This apparent contradiction can be explained by the fact that the chlamydial PGM (interconverting Glc1P and Glc6P) does possess a TS3 signal, and is thus most likely secreted (Figure 45 B). In our scenario, intraluminal glycogen is degraded into Glc1P and conversion to Glc6P occurs, which is now in turn imported into the bacteria.

Glycogen metabolism is partially outsourced into the inclusion lumen.

Figure 46 illustrates the mechanisms of intraluminal glycogen accumulation that we propose. Early during the infectious cycle SLC35D2, and possibly other transporters, are recruited to the inclusion membrane and UDP-Glc is translocated into the inclusion lumen. The activity of chlamydial glycogen enzymes, secreted by RBs into the inclusion lumen, leads to the onset of luminal glycogen synthesis between 16 and 20 hpi. In addition, host glycogen is imported in bulk into the inclusion lumen through invagination of the inclusion membrane. In culture cells, this second pathway only makes a minor contribution to the overall luminal glycogen content. At this stage of infection the inclusion contains mostly RBs. This developmental form does not accumulate glycogen and probably hardly uses Glc6P for growth (Omsland et al., 2012). Later on, EBs appear and accumulate intrabacterial glycogen. This happens via the degradation of intraluminal glycogen into Glc1P, which is subsequently converted to Glc6P, a substrate amenable to chlamydial uptake. It is not clear whether Glc6P is taken up by RBs, EBs, or both, but glycogen accumulation could only be detected in EBs. Proteomic analysis revealed that the chlamydial Glc6P transporter UhpC is mainly present in RBs, and to a lesser extent in EBs (Saka et al., 2011). We obtained similar results with our transcriptomic data, which showed that expression of *uhpC* is around five times higher at 8 hpi compared to 40 hpi (Figure 29). Maybe Glc6P is imported into the late RB, and its accumulation is one of several activators of RB-to-EB conversion. Once conversion is

terminated, glycogen could be synthesized in the EB. However, this is only a hypothesis, and the probably best way to test it would be to add radioactively labelled Glc to infected cells and to follow up on its transit to bacteria through electron microscopy. As EBs highly rely on Glc6P as energy source for metabolic reactions (Omsland et al., 2012), the intrabacterial glycogen store presumably allows for survival of EBs, in the inclusion lumen and after having exited the cell.

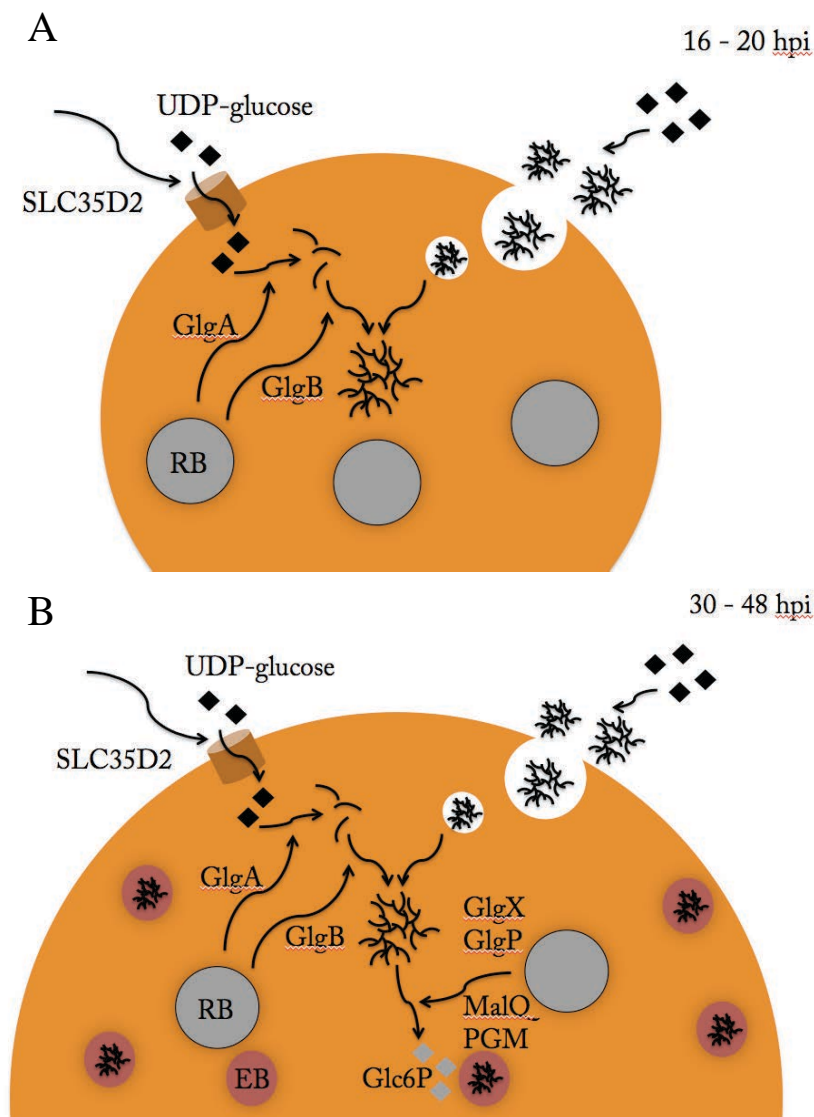


Figure 46: Scheme of mechanism proposed for intraluminal glycogen accumulation. (A) Early on in the infectious cycle host glycogen is imported via vesicular import (right), and UDP-Glc is imported via the host UDP-Glc transporter SLC35D2. Glycogen accumulation in the inclusion lumen is triggered by GlgA and GlgB. (B) Later on in the cycle RBs convert to EBs and intraluminal glycogen is degraded to Glc1P by GlgP, GlgX and MalQ. PGM now converts Glc1P to Glc6P, which is taken up by the bacteria, probably through UhpC. Glycogen accumulation in the EBs starts.

What is the benefit?

At a first glance it seems counter intuitive that *Chlamydia* would go through all these complicated steps of intraluminal glycogen accumulation prior to intrabacterial glycogen accumulation. Why not import Glc6P directly from the host cell cytoplasm into the inclusion lumen and then directly into the bacteria? We have strong indications that UDP-Glc import and intraluminal glycogen accumulation are triggered by RBs, as we could see the onset of accumulation in the absence of EBs and the transcriptional peak of most glycogen enzymes at around 16 hpi, a time when RBs predominate. However, Glc6P consumption seems to be of higher importance for EBs than for RBs. This delay between supply and need would lead to the accumulation of Glc6P, and with it an increase in osmotic pressure within the inclusion lumen, with possibly drastic consequences. Storing Glc in the form of glycogen in the inclusion lumen solves this problem. We thus propose that *Chlamydia* uses the inclusion lumen to store glycogen in a way that makes it inaccessible to the host cell, but keeps it in reach for future use. Why it is beneficial for *C. trachomatis*, amongst all the *Chlamydia* species, to accumulate these high amounts of glycogen within their parasitophorous vacuole, remains an open question. The explanation can possibly be found in the natural habitat, the actual site of *C. trachomatis* infection and its coexisting microbiome. The microflora of the female genital tract (the vagina as well as the cervix) is, in a healthy woman, heavily populated by lactobacilli (Corbishley, 1977). Their protective effect for the host towards viral and bacterial infections is well established, and lies partially in their production of H₂O₂ and lactic acid. The latter is responsible for the low pH of 4 in the female genital tract, which also exerts a protective effect against other organisms that cannot thrive in an acidic environment (Mirmonsef et al., 2012). When the microflora in the vagina or cervix is perturbed women can establish a bacterial vaginosis (BV), in which other bacteria increase. This BV is directly linked to a loss in the normal lactobacilli population and an increase in pH. The most frequent bacteria occurring during this imbalance are *Gardnerella*, *Prevotella*, *Atopobium* and *Leptotrichia* (Danielsson et al., 2011). It has been shown that the probability to become infected with *C. trachomatis* is 3.5 times higher in patients with BV (Wiesenfeld et al., 2003) and the elevated risk holds true for many other STIs, such as HIV (Mirmonsef et al., 2012). All the before mentioned bacteria emerging during a BV metabolize Glc as carbon source (Patel et al., 1999; Piot et al., 1982; Rainey et al., 1994; Takahashi and Yamada, 2000) and could thus indirectly have an impact on the Glc availability for *C. trachomatis*. In this scenario it would be of benefit for *C. trachomatis* to store as much Glc as possible in the form

of glycogen early on in the infection, to ensure a constant supply of this essential nutrient. The same applies to the microflora in the conjunctiva, which mainly consists out of *Pseudomonas*, *Propionibacteria*, *Bradyrhizobium* and *Corynebacteria* (Dong et al., 2011), all of which are equally able to metabolize Glc (Daddaoua et al., 2009; Koussemon et al., 2003; Lindner et al., 2011; Salminen and Streeter, 1987). However, if there is indeed a competition of Glc between *C. trachomatis* and the concurrent microflora remains to be proven.

One independent advantage of re-routing the host energy stores towards the inclusion lumen is that it might overall “weaken” the host cell in its fight against the intruder. Indeed, many autonomous host cell defence mechanisms rely on processes that require energy (including host protein synthesis or phosphorylation cascades) and that might be compromised in cells enduring sustained hijacking of its energy stores.

Finally, one question raised by our data, which is linked to the “why” question, is “how” did *Chlamydiae* acquire the ability to secrete enzymes, resulting in the ability to metabolize glycogen in the inclusion lumen, and to import UDP-Glc. Secretion of each protein, individually, does not appear to come to any advantage as long as the whole synthesis/degradation cycle is not completed. Ancestral *Chlamydiae* were more similar to the extant environmental *Chlamydiae*, and infected protozoans. Secretion of some or all of the chlamydial glycogen synthesizing enzymes into the host would fuel glycogen synthesis in the cytoplasm, building up an energy stock for both, the host and the bacterium. The glycogen-degrading functions could have been performed by the host cell, and only later on did *Chlamydiae* evolve its sophisticated system of relocating its glycogen metabolism into the inclusion lumen, requiring the activity of chlamydial degrading enzymes. A particular scenario, in which secretion of the chlamydial glycogen synthase GlgA would bring a definite advantage to an improbable biological system, was proposed by Steven Ball, and is known as the “Ménage à trois” hypothesis (Ball et al., 2013). Briefly, in this scenario a symbiotic relationship between a eukaryotic protozoan and a cyanobiont (later on giving rise to a first plant cell and its plastid) are only of benefit for the protozoan if it can make use of the cyanobiont's "dowry", i.e. the ability to make ADP-Glc (a strictly bacterial metabolite). It was proposed that an ancestral *Chlamydia* provided the missing link, in that it infected the cell and secreted GlgA, allowing for cytoplasmic synthesis of glycogen out of the cyanobiont's ADP-Glc, which was then available for the host cell. Later on *glgA* was integrated into the protozoans genome by horizontal gene transfer and slowly developed to become starch synthase in the course of evolution.

Conclusion

Several intracellular pathogens develop inside a vacuolar compartment. The recognized advantage of this “sheltered life” (Kumar and Valdivia, 2009) is to offer protection from cytosolic immune surveillance pathways. Our work reveals another advantageous feature of this lifestyle: the possibility to sequester molecules inside the vacuole, out of reach of the host. Here we describe sequestration of an energy storage molecule, and the transformation of the vacuole into a metabolically active compartment. For other intracellular bacteria or parasites, the vacuole could also serve as a storage space, not only for use by the parasite but also to deplete the host from selected components, thereby disrupting signalling pathways or other important host functions.

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ANNEXE

Use of a parasitophorous vacuole as a storage compartment

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ABSTRACT

Residence in a vacuole provides a shelter against cytosolic host defense to a number of intracellular parasites. Parasitophorous vacuoles of cells infected with the human pathogen *Chlamydia trachomatis* are rich in glycogen. Here we disprove the common view that luminal glycogen originates from bacterial lysis. Instead, we reveal two pathways for vacuolar glycogen storage: bulk uptake from cytoplasmic stores, generating membrane-bounded glycogen particles, and *de novo* synthesis. We provide evidence that bacterial glycogen synthesis and degradation enzymes are secreted into the inclusion lumen through type 3 secretion. Depletion of the host UDP-glucose pyrophosphorylase, or of the UDP-glucose transporter SLC35D2, leads to a strong decrease in intraluminal glycogen stores, designating this sugar as the substrate for *de novo* glycogen synthesis. Remarkably, the chlamydial glycogen synthase is able to use UDP-glucose, a nucleotide sugar only produced by eukaryotic cells. Thus, our data demonstrate for the first time metabolic activity within a vacuole lumen, through the action of bacterial enzymes on a host substrate. These two pathways allow the bacteria to build an energy store, out of reach for the host. Based on these findings we propose that parasitophorous vacuoles can serve to selectively deplete the host from essential resources or to disrupt signaling pathways.

INTRODUCTION

Several intracellular parasites reside in a membrane-bounded compartment called the parasitophorous vacuole. While this situation limits access to nutrients, the vacuole membrane is usually seen as advantageous since it offers protection against cytosolic host defense mechanisms (Kumar and Valdivia, 2009). *Chlamydiae* are Gram-negative obligate intracellular bacteria found as symbionts and pathogens in a wide range of eukaryotes, including protists, invertebrates and vertebrates. The human adapted strain *C. trachomatis* is the leading cause of infectious blindness (Taylor et al., 2014) as well as of sexually transmitted infections caused by bacteria. Infections of the urogenital mucosae often stay asymptomatic causing irreparable damage leading to ectopic pregnancies or tubal factor infertility (Brunham and Rey-Ladino, 2005).

The developmental cycle of *Chlamydiae* involves two morphologically distinct forms. Infectious particles, called elementary bodies (EBs), are small and adapted to extracellular survival. After invasion of the host cell, they establish a parasitophorous vacuole called an inclusion, and convert within the first hours into larger organisms with higher metabolic activity, called reticulate bodies (RBs). RBs replicate several times within the inclusion, until they differentiate back into EBs, in a non synchronous manner (AbdelRahman and Belland, 2005).

An obligate intracellular organism with a genome of only around one million base pairs *C. trachomatis* highly relies on the host with regard to several essential metabolic pathways, such as nucleotide or amino acid biosynthesis (Stephens et al., 1998). Lipid droplets and peroxisomes have been observed in the inclusion lumen, indicating that this compartment is able to engulf large particles to meet bacterial needs (Boncompain et al., 2014; Kumar et al., 2006). *C. trachomatis*, and the closely related mouse and hamster pathogen *C. muridarum*, are unique amongst *Chlamydiae* for their ability to accumulate glucose (Glc) under the form of glycogen in the inclusion lumen (Gordon and Quan, 1965). Glc deprivation leads to a complete loss of infectivity (Harper et al., 2000; Iliffe-Lee and McClarty, 2000). The *C. trachomatis* genome encodes for all the enzymes necessary for a functional glycogenesis and glycogenolysis (Figure 1A) (Stephens et al., 1998). Although they do not accumulate glycogen in the inclusion, other *Chlamydia* species also have a complete set of enzymes, a surprising observation given that this pathway is absent in most intracellular bacteria. Intriguingly, pathogenic *Chlamydiae* lack a hexokinase, the enzyme phosphorylating Glc, and therefore rely on the import of phosphorylated sugars for glycolysis or glycogenesis. During

bacterial glycogenesis, Glc-1-phosphate (Glc1P) serves as a substrate for the enzyme ADP-Glc pyrophosphorylase (GlgC) giving rise to ADP-Glc, which in turn is the building block for a linear chain of α 1,4-bonded Glc molecules. Ramifications through an α 1,6-glycosidic bond are introduced by the branching enzyme GlgB, giving rise to glycogen. The glycogen phosphorylase GlgP, the debranching enzyme GlgX and the amylomaltase MalQ participate in the degradation of the glycogen particle to Glc1P (Colleoni et al., 1999; Seibold and Eikmanns, 2007; Wilson et al., 2010).

The common view is that glycogen is produced inside the bacteria, and that bacterial lysis accounts for the free luminal glycogen (Chiappino et al., 1995). Here we show that luminal glycogen is derived from bulk import of host glycogen and *de novo* intraluminal synthesis, through the action of secreted bacterial enzymes. We reconstruct Glc metabolism in infected cells and demonstrate the ability for a microbe to convert its vacuole lumen into a compartment for regulated metabolic activity.

RESULTS

Glycogen accumulation in the inclusion lumen is not the result of bacterial lysis

Over the course of the developmental cycle, Glc consumption increases, and infected cells harbour higher amount of glycogen than non-infected cells (Ojcius et al., 1998). To determine whether glycogen accumulated in the host cytosol, in the inclusion, or both, we labeled polysaccharides using periodic-acid-Schiff (PAS) staining at different times of infection. Large glycogen particles were detected in most non-infected cells (Figure 1–figure supplement 1). Twenty-four hours post infection (hpi), glycogen was still detected in the cytoplasm of some of the infected cells, and the inclusions only showed weak PAS staining. However, 48 hpi, no glycogen particle was detected in the cytoplasm of infected cells, while inclusions heavily stained with PAS, indicating that the global increase in glycogen content is accompanied by a shift in its original cytosolic localization, to the benefit of the bacterial inclusion. We used transmission electron microscopy (TEM) to determine more precisely its subcellular localization and detected the polysaccharide in two locations: in the inclusion lumen, and within EBs (Figure 1B). Intraluminal glycogen appeared to be bigger in size than cytoplasmic glycogen (Figure 1–figure supplement 2). We did not observe glycogen in RBs, in contrast to an earlier report (Chiappino et al., 1995). In that publication, the presence of glycogen in the inclusion lumen was interpreted as the result of glycogen release from lysed

bacteria. Considering the abundance of glycogen in the inclusion lumen relative to its amount in bacteria we considered this hypothesis unlikely. We tested it by depriving the cells of Glc for 48 hrs before infecting them. Under these conditions, the inclusions contained no glycogen (Figure 1C). Restoring Glc availability for 4 h was sufficient to trigger the accumulation of glycogen in the inclusion lumen, but not in the bacteria (Figure 1D). This experiment demonstrates that glycogen in the inclusion lumen does not result from the release of bacterial stores through bacterial lysis. We next carefully examined the kinetics of glycogen appearance in the inclusion. Glycogen accumulation started between 16 and 20 hpi (Figure 1–figure supplement 3A), at a time when RBs largely predominate over EBs. RBs are devoid of glycogen and can therefore not be a source of glycogen accumulation through lysis. In addition, these observations suggest that, while glycogen accumulation in the inclusion is most obvious at late times of infection, the process is initiated by RBs, earlier than previously proposed (Moulder, 1991).

Part of luminal glycogen is translocated in bulk from the host cytoplasm

Our conclusion raised an obvious question: how could a large polymer reach the inclusion lumen? Two mechanisms are conceivable: bulk translocation of glycogen through inward invagination of the inclusion membrane, or transport of monomeric substrates (such as nucleotide sugars or hexose phosphates) across this membrane for *de novo* glycogen polymerization in the inclusion. We frequently observed glycogen-filled vesicular structures in the inclusion lumen in TEM (Figure 2A). Additionally, antibodies against the host glycogen synthase (Gys1) revealed that the enzyme is imported in the inclusion lumen of infected cells. Staining was specific since it disappeared when Gys1 expression was knocked down using siRNA prior to infection (Figure 2B). Altogether these data strongly support the hypothesis of bulk glycogen import. To determine if the glycogen-filled vesicles were of autophagic origin, we used an *Atg5*^{-/-} mutant mouse embryonic fibroblast (MEF) cell line, which is deficient in autophagy. Inclusions of *Atg5*^{-/-} MEFs still harboured glycogen, Gys1 and glycogen-filled vesicles, demonstrating that the pathway of bulk host glycogen uptake is autophagy-independent (Figure 2–figure supplement 1).

Chlamydial glycogen enzymes are secreted into inclusion lumen for *de novo* glycogen synthesis

If bulk glycogen import is the only mechanism at work, depleting host glycogen stores should dramatically reduce intraluminal glycogen content. Cells were transfected with siRNA

against Gys1 two days before infection. Even though only very little glycogen was left in the host cytoplasm at the time of infection, the decrease in glycogen content inside the inclusion 48 hpi was only marginal (Figure 2C,D). This experiment indicated that the alternative scenario, *de novo* glycogen synthesis in the inclusion lumen, does also take place.

Synthesis of glycogen, and possibly its degradation into Glc monomers amenable to bacterial uptake, implies that glycogen synthesis and degradation enzymes are present in the inclusion lumen. While Gys1 import in the inclusion lumen might contribute, it cannot account for the glycogen accumulation observed in Gys1 depleted cells. To test if *C. trachomatis* enzymes for glycogen metabolism are secreted in the inclusion lumen we looked for the presence of a type 3 secretion (T3S) signal in their amino terminal domain, as T3S is the prominent pathway for protein secretion in *Chlamydia* (Subtil et al., 2005). The N-termini of the proteins of interest were fused to a reporter (calmodulin-dependent adenylate cyclase of *Bordetella pertussis* Cya), and the secretion of the resulting chimera was evaluated in *Shigella flexneri*, in an assay validated previously (Subtil et al., 2005; Subtil et al., 2001). Five out of the six chimera tested, GlgA/Cya, GlgB/Cya, GlgX/Cya, GlgP/Cya and MalQ/Cya, were secreted in the supernatant of cultures when transformed in a *S. flexneri* mutant with constitutive T3S (*ipaB* strain), and not when transformed in a mutant deficient for T3S (*mxiD* strain). The endogenous T3S substrate of *S. flexneri*, IpaD, was also secreted only in the *ipaB* background (positive control), while cAMP receptor protein (CRP), a non-secreted protein, was found exclusively in the bacterial pellet, excluding the possibility of non-specific leakage into the supernatant. The sixth chimera tested, GlgC/Cya, was not detected in the culture supernatant, indicating that GlgC, which converts Glc1P into ADP-Glc, is not a substrate of T3S (Figure 3A). Secretion of the endogenous GlgA into the inclusion lumen, as well as into the host cytoplasm, has recently been confirmed using specific antibodies (Lu et al., 2013). To determine the localization of one of the degrading enzymes, we designed an anti-peptide antibody that recognized a single species in EB lysates tested by western blot, corresponding to the expected size for GlgX (Figure 3–figure supplement 1A). In cells infected for 24 hpi or 48 hpi GlgX was found in the inclusion lumen, with mostly no overlap with bacteria, demonstrating secretion within the inclusion lumen (Figure 3B). In addition, GlgX was detected on the inclusion membrane 24 hpi, but not 48 hpi. We controlled the specificity of the staining by preincubating the anti-peptide antibody with the peptides against which it was raised (Figure 3–figure supplement 1B). Altogether, these data confirm that GlgX is a substrate of T3S in *C. trachomatis*.

***Chlamydia* import Glc6P, not Glc1P nor Glc**

At late infection times luminal glycogen content decreases (Gordon and Quan, 1965). Chlamydial glycogen degradation enzymes, GlgX, GlgP and MalQ, possess T3S signals (Figure 3), and we demonstrated the presence of GlgX in the inclusion lumen. It is very likely that they are responsible for glycogen depolymerization, to feed EBs monomeric sugars at late infection times. There is only one annotated hexose phosphate transporter (UhpC) in *C. trachomatis*. We investigated if this transporter can transport the product of glycogen degradation, i.e. Glc1P. We incubated purified bacteria with radioactively labeled [C^{14}]-Glc, [C^{14}]-Glc1P or [C^{14}]-Glc6P, in absence or presence of a 50-fold excess of non-radioactive Glc, Glc1P or Glc6P. Only [C^{14}]-Glc6P was taken up by bacteria, and only Glc6P could compete it out (Figure 4A), demonstrating that the uptake was saturable, as expected for a transporter. Therefore we face a discrepancy between the glycogen degradation product, Glc1P, and the substrate of import into the bacteria, Glc6P. To solve this conundrum we hypothesized that Glc1P might be converted into Glc6P in the inclusion lumen. *C. trachomatis* encodes for a putative phosphoglucomutase (PGM), an enzyme allowing the interconversion between Glc1P and Glc6P. Using the heterologous test of secretion we found that *C. trachomatis* PGM contains a functional T3SS (Figure 4B). This strongly argues for the conversion, inside the inclusion lumen, of Glc1P into Glc6P followed by uptake through UhpC.

UDP-Glc is a substrate for chlamydial GlgA and is the host sugar transported into the inclusion lumen

GlgA expression is delayed compared to other glycogen enzymes, and coincides with luminal glycogen accumulation (Figure 1–figure supplement 3B), indicating that GlgA serves as master gene controlling the timing of glycogen accumulation in the inclusion. It has been shown that this enzyme is secreted into the host cell cytoplasm and into the inclusion lumen (Lu et al., 2013). Intriguingly, eukaryotic glycogen synthases function on UDP-Glc and the cytoplasm contains no ADP-Glc, the substrate of bacterial glycogen synthases. Transfection of cells with flag-tagged chlamydial GlgA led to massive glycogen accumulation, indicating that this bacterial glycogen synthase is very unusual in that it is functional on UDP-Glc (Figure 5A). Interestingly, when transfected cells were subsequently infected, we observed an increase in glycogen accumulation in the inclusion lumen, and Flag-GlgA was detected in the bacterial compartment (Figure 5–figure supplement 1). High glycogen content upon ectopic GlgA expression was also observed when infecting with a strain devoid of the natural plasmid

of *C. trachomatis*. Plasmid loss is associated with decreased GlgA expression and impaired glycogen accumulation (Carlson et al., 2008). Glycogen recovery upon GlgA transfection indicates that the low level of expression of GlgA in the plasmid-less strain accounts for the defect in glycogen storage. It is quite remarkable that a host-expressed gene can compensate for a bacterial deficiency.

A zymogram analysis was performed to further compare chlamydial GlgA activity towards either UDP-Glc or ADP-Glc. Briefly, lysates of *E. coli* lacking their endogenous *glgA* and transformed with either *E. coli glgA* or *C. trachomatis glgA* were separated on non-denaturing polyacrylamide gels that contained rabbit glycogen. The gels were subsequently incubated in buffer containing either UDP-Glc or ADP-Glc, and glycogen production was visualized by iodine staining. While *E. coli* GlgA only showed activity upon incubation with ADP-Glc, the chlamydial GlgA showed activity with both substrates, UDP-Glc and ADP-Glc (Figure 5B).

We next asked which Glc derivative might be translocated across the inclusion membrane for *de novo* glycogen synthesis. UDP-Glc stood as the best candidate: it is a substrate for GlgA, and its import would relieve the bacteria from the cost of nucleotide sugar synthesis. To test the requirement for UDP-Glc for luminal glycogen synthesis we silenced the expression of the host UDP-Glc pyrophosphorylase UGP2 by siRNA (UGP2 catalyzes the conversion of Glc1P into UDP-Glc) for two days before infection. We measured that glycogen accumulation within the inclusion lumen decreased by 57 % (s.e.m. = 5.8) compared to cells treated with control siRNA (Figure 5C,D). These data strongly argue for UDP-Glc being the substrate for sugar import into the inclusion. Note that while UGP2 silencing also impacted host glycogen stores, as expected, it did so to a lesser extent than Gys1 silencing (Figure 2D). Thus the greater impact of UGP2 silencing on luminal glycogen stores cannot be explained by its indirect effect on bulk glycogen import. Instead it very likely reflects the requirement for UDP-Glc for *de novo* luminal glycogen synthesis.

***C. trachomatis* co-opts host transporter SLC35D2 to import UDP-Glc into the inclusion lumen**

We have demonstrated that *de novo* glycogen synthesis takes place in the inclusion lumen, initiated by chlamydial glycogen synthase GlgA, using UDP-Glc as substrate. This implies that UDP-Glc is translocated from the host cytoplasm into the inclusion lumen. Among the large family of annotated sugar transporters, only SLC35D2 was experimentally shown to be able to transport UDP-Glc (Suda et al., 2004). In cells infected with *C.*

trachomatis, the transporter was enriched around the inclusion, indicating that it might play a role in UDP-Glc import (Figure 6A). To test this hypothesis we silenced SLC35D2 expression using siRNA prior to infection. In these conditions, glycogen accumulation in the inclusion decreased by 43 % (s.e.m. = 1.7) (Figure 6B,C), thus to a level similar to when UGP2 had been silenced (Figure 5C,D). These data strongly suggest that *C. trachomatis* co-opts the host nucleotide sugar transporter to favor UDP-Glc import into the inclusion lumen.

DISCUSSION

This work shows that two independent processes contribute to glycogen accumulation in the *C. trachomatis* inclusion lumen: bulk uptake from the host cytoplasm and *de novo* synthesis (Figure 7). It is a unique example of a bacterium utilizing the compartmentation of eukaryotic cells, to the extent that energy stores are radically shifted toward the bacterium and made inaccessible to the host.

Intraluminal glycogen accumulation had previously been attributed to bacterial lysis and to the release of chlamydial glycogen into the surrounding environment (Chiappino et al., 1995). In the present work we observed that glycogen appears in the inclusion lumen first, and at a stage when only RBs, that do not accumulate glycogen, are present. Thus, bacterial lysis is not the source of luminal glycogen.

We identified two mechanisms by which glycogen accumulates in the inclusion lumen. The presence of glycogen-filled vesicles in the inclusion lumen strongly argues for the uptake of host glycogen in bulk. In addition, host enzyme Gys1, which is known to bind to glycogen (Roach et al., 2012), is translocated into the vacuole. These findings confirm the ability for the inclusion to take up large particles from the host cytoplasm, already illustrated with the uptake of lipid droplets and peroxisomes (Boncompain et al., 2014; Kumar et al., 2006). These events likely occur through invagination of the inclusion membrane, but the underlying mechanisms remained to be investigated. While multi-layered glycogen filled vesicles were observed, they still occurred in Atg5 deficient cells, implicating that they are not of autophagic origin.

The observation that depletion of host glycogen (through silencing of the host glycogen synthase Gys1) does not impact luminal glycogen stores demonstrates that bulk uptake only marginally contributes to glycogen accumulation in the inclusion lumen in culture cells. We identified the second, and main, mechanism for luminal glycogen accumulation as *de novo* synthesis, through the action of chlamydial enzymes. Using a heterologous secretion assay,

we have identified T3S signals in all but one (GlgC) glycogen metabolism enzymes of *C. trachomatis*. We had previously demonstrated the robustness of this assay, with an estimated 5 % of false positives (Subtil et al., 2005). Secretion of GlgA in the inclusion lumen and in the host cytoplasm was reported recently (Lu et al., 2013). Indirect evidence for the secretion of the branching enzyme GlgB was brought by the observation that a GlgB mutant strain shows massive precipitation of glycogen in the inclusion (Nguyen and Valdivia, 2012). Precipitation occurs as a result of unbranched glycogen accumulation, implicating that GlgB normally functions in the inclusion lumen. Finally in this paper, we confirm the secretion of GlgX using specific antibodies. Interestingly, GlgX localizes not only to the inclusion lumen, but also to the inclusion membrane. Remarkably, GlgP location at the inclusion membrane was also reported (Saka et al., 2011), indicating that the two enzymes might work in concert, degrading host glycogen in the vicinity of the inclusion membrane. Thus, altogether these data demonstrate that *C. trachomatis* uses T3S to transform the lumen of the inclusion into a glycogen storage compartment.

So far, T3S is mostly described as a mechanism for protein translocation across a eukaryotic membrane, either the plasma membrane or the membrane of a parasitophorous vacuole (Galan and Wolf-Watz, 2006). The ability for chlamydial glycogen enzymes to reach the inclusion lumen slightly modifies this view. Loose membrane-like structures can frequently be seen in the inclusion lumen, and might trigger the secretion of some effectors into the inclusion lumen, especially from bacteria not in contact with the inclusion membrane. Also, it has been demonstrated, in *Yersinia pseudotuberculosis*, that T3S substrates can translocate first on the surface of the bacteria (Akopyan et al., 2011). Noticeably, at least GlgX and GlgA are secreted both inside the inclusion and across its membrane. Clearly, more work is needed to understand how T3S is regulated in *Chlamydia*, and what determines substrate translocation across the inclusion membrane.

Our transcription analysis revealed that *glgA* most likely acts as a master gene to control the onset of glycogen synthesis, as its transcription start coincides with the onset of intraluminal glycogen accumulation (between 16 and 20 hpi). All the other glycogen enzymes depict a similar expression profile, correlated with an increase in bacterial metabolic activity between 8 and 24 hpi. The only other exception is *glgB*, which is one of the few early genes. *glgB* is not in an operon (Albrecht, Sharma, 2010). It is not clear at this stage why the protein needs to be made long before GlgA is present, when GlgA lies upstream of GlgB in the glycogen synthesis pathway.

The very few *C. trachomatis* isolates that do not have the plasmid accumulate only minor amounts of glycogen compared to the wild-type strain, and GlgA expression is strongly reduced in these strains (Carlson et al., 2008). When we ectopically expressed Flag-GlgA in cells infected with either the wild-type or the plasmid-less strain, we observed increased intraluminal glycogen accumulation for both strains. These data strongly suggest that different levels of GlgA expression between the two strains fully account for their difference in terms of glycogen accumulation. When Flag-GlgA import into the inclusion took place, likely together with bulk glycogen uptake, the low level of endogenous GlgA in the plasmid-less inclusions was complemented, allowing for high glycogen accumulation.

In vitro polymerization assays using *E. coli* expressing *C. trachomatis* GlgA, as well as transfection experiments in a eukaryotic background where only UDP-Glc is available, proved that *C. trachomatis* GlgA can use UDP-Glc as substrate. This was highly unexpected, because bacterial glycogen synthases normally use ADP-Glc. Consistent with this finding, we observed a strong decrease in intraluminal glycogen accumulation when we knocked-down the human enzyme responsible for the generation of UDP-Glc (UGP2). Diminution of host glycogen level (and thus the reduction in the bulk import pathway) could not account for this result, as the Gys1 knockdown was a lot more efficient in depleting glycogen in the host cytoplasm than the UGP2 knockdown. This experiment points to UDP-Glc as being the substrate imported in the inclusion lumen. If, as we had initially hypothesized, Glc6P or Glc1P were the substrate of import into the inclusion, a UGP2 knockdown should not produce any difference in intraluminal glycogen accumulation because both Glc6P and Glc1P would remain available, as they lie upstream of UDP-Glc generation. Energetically speaking, it is beneficial to import UDP-Glc rather than Glc6P or Glc1P, as it relieves the bacteria from the costly reaction of transferring a nucleotide to the sugar molecule. It also fits perfectly with the absence of a T3S signal in GlgC, suggesting that this enzyme, which makes ADP-Glc out of Glc1P, remains restricted to the bacteria and only serves bacterial glycogen production. Secretion of GlgC into the inclusion lumen would be superfluous, with GlgA being able to produce unbranched glycogen out of UDP-Glc.

The identification of UDP-Glc as the sugar imported into the inclusion lumen was further comforted by our observation that knocking down the UDP-Glc transporter SLC35D2 led to a significant reduction of intraluminal glycogen staining. In addition, we observed that SLC35D2-HA accumulates at the periphery of the inclusion. Our data thus strongly indicate that SLC35D2 is at least partially responsible for the import of UDP-Glc. The fact that the

reduction was only partial, and slightly less pronounced than after UGP2 silencing, might be due to residual SLC35D2 expression. In addition, other Golgi- or ER-based transporters from the SLC35-family of nucleotide sugar transporters might transport UDP-Glc and be recruited to the inclusion membrane. How this recruitment is taking place remains to be elucidated.

We demonstrated that *de novo* glycogen synthesis takes place in the inclusion lumen, triggered by the presence of GlgA and GlgB. Glycogen storage in the inclusion lumen would only be of benefit for the bacteria if they were subsequently degraded into monomers amenable to bacterial uptake if needs be. Also, old observations report that glycogen decreases at very late infection times, are consistent with a late consumption of the stores (Gordon and Quan, 1965). Similarly to the glycogen synthesizing enzymes, the degrading enzymes GlgP, GlgX and MalQ possess T3S signals, and we demonstrated GlgX secretion using specific antibodies. We therefore hypothesize that these enzymes are active in the inclusion lumen, and generate free Glc1P. We could clearly demonstrate that *Chlamydiae* are not able to take up Glc nor Glc1P, but exclusively Glc6P. This is in agreement with data obtained on the homologous protein in *C. pneumoniae*, which transports Glc6P and not Glc1P (Schwoppe et al., 2002). This apparent contradiction can be explained by the fact that the chlamydial PGM (interconverting Glc1P and Glc6P) also possesses a TS3 signal, and is thus most likely secreted. Thus, we propose that intraluminal glycogen is degraded into Glc1P and conversion to Glc6P occurs, which is now in turn imported into the bacteria.

Altogether, our work revealed the origin of glycogen in the inclusion lumen and brought to light the complexity of the Glc flux in *C. trachomatis* infected cells (Figure 7, see legend for details). At a first glance it seems counter intuitive that *Chlamydia* would go through all these complicated steps of intraluminal glycogen accumulation prior to intrabacterial glycogen accumulation. Why not import Glc6P directly from the host cell cytoplasm into the inclusion lumen and then directly into the bacteria? UDP-Glc import is initiated by RBs, which, importantly, use ATP as an energy source, while Glc6P is preferred by EBs (Omsland et al., 2012). Thus, uptake of Glc6P into the inclusion lumen, before EBs appear, would lead to a high osmotic pressure within the inclusion lumen, which could be detrimental. Importing UDP-Glc, instead of Glc6P, and stocking it in the shape of the osmotically inert glycogen brings an elegant solution to the need to store Glc for later use. Why do *C. trachomatis*, amongst all the *Chlamydia* species, accumulate these high amounts of glycogen within their parasitophorous vacuole? Clearly, glycogen accumulation is not necessary for growth *in vitro*

(the plasmid-less strain grows very well). However, the ability for all *C. trachomatis* clinical isolates to accumulate glycogen speaks for a selective advantage of doing so. Many bacterial strains of the microflora of the female genital tract metabolize Glc as carbon source. Whether Glc can become limiting, especially during bacterial vaginosis, where the risk of becoming infected with *C. trachomatis* is elevated, is not known. It is an attractive hypothesis to explain why the ability for *C. trachomatis* to store Glc could come to an advantage. One independent advantage of re-routing the host energy stores towards the inclusion lumen is that it might overall “weaken” the host cell in its fight against the intruder. Indeed, many autonomous host cell defence mechanisms rely on processes that require energy (including host protein synthesis or phosphorylation cascades) and that might be compromised in cells enduring sustained hijacking of its energy stores.

MATERIALS AND METHODS

Cells and bacteria

HeLa cells (ATCC), wild-type and *Atg5*^{-/-} mouse embryonic fibroblasts (MEF) (generous gift from N. Mizushima, Tokyo Medical and Dental University) were cultured in Dulbecco's modified Eagle's medium with Glutamax (DMEM, Invitrogen), supplemented with 10 % (v/v) fetal bovine serum (FBS). For experiments with medium containing different Glc concentrations DMEM without Glc (DMEM, Invitrogen) was used and complemented with 5 mM sodium pyruvate (Sigma-Aldrich), 10 % fetal bovine serum (FBS) and the indicated Glc concentration (Merck). *C. trachomatis* LGV serovar L2 strain 434 (ATCC), the plasmid-less strain LGV L2 25667R (Bowie, 1990) or GFP-expressing L2 (L2^{IncD}GFP) (Vromman et al., 2014) were purified on density gradients as previously described (Scidmore, 2005). The *ipaB* and *mxiD* strains are derivatives of M90T, the virulent wild-type strain of *Shigella flexneri*, in which the respective genes (*ipaB* and *mxiD*) have been inactivated (Allaoui et al., 1993). The *Escherichia coli* strain *DH5 α* was used for cloning purposes and plasmid amplification. Both *S. flexneri* and *E. coli* strains were grown in Luria-Bertani medium with ampicillin at 0.1 mg/ml.

Electron microscopy and periodic acid-thiocarbohydrazide-silver proteinate reaction (PATAg)

HeLa cells were grown in wells, infected with *C. trachomatis* LGV serovar L2 strain 434 at an MOI of 0.1 and carefully trypsinized at the indicated time points. The cells were

then washed with PBS once and fixed with 0.1 M cacodylate and 2.5 % glutaraldehyde at room temperature for at least 30 min. PATAg staining was performed as described elsewhere (Thiéry, 1967). Briefly, thin sections were incubated in 1 % periodic acid for 25 min and then washed several times in water, followed by an incubation step in 0.2 % thiocarbohydrazide in 20 % acetic acid for 45 min. Several washing steps in a graded acetic acid series to water were carried out and the thin section were stained with 1 % silver proteinate for 30 min. Samples were observed on a transmission electron microscope within a week after preparation.

Immunofluorescence and PAS staining

HeLa cells grown on coverslips were infected with *C. trachomatis* LGV serovar L2 strain 434 with an MOI < 1 (unless specified differently) at 37°C and fixed in 4 % paraformaldehyde (PFA) in PBS for 30 min at room temperature (except when staining with anti-CT813 was intended, which required fixation in 2% PFA). Cells were blocked and permeabilized in 0.05 % saponin and 0.1 % bovine serum albumin (BSA) in PBS for 10 min before being subjected to antibody staining. The antibody against inclusion protein CT813 was kindly provided by Dr. G. Zhong (San Antonio, Texas). Polyclonal anti-Cap1 antibodies were obtained by immunization of New Zealand white rabbits with purified recombinant Cap1 deleted of its last 167 amino acids and fused to a N-terminal His tag (Agro-Bio). The polyclonal anti-GlgX antibody was equally purchased from Agro-Bio, and was directed against two peptides (KHNEENGEYNRDGTSANC and HEDFDWEGDTPLHLPKEC). To investigate its specificity anti-GlgX was preabsorbed with either the two GlgX peptides or control peptides. For this, 1 µg/ml antibody was incubated with 20 µg/ml of peptides for 15 min at room temperature prior to immunostaining of cells. The monoclonal rat anti-HA antibody was purchased from Roche Diagnostics. Goat secondary antibodies were conjugated to Alexa488 (Molecular Probes), or to Cy3 or Cy5 (Amersham Biosciences). For periodic-acid-Schiff (PAS) stain cells grown on coverslips were fixed in 4 % PFA/PBS for 30 min at room temperature and staining was performed as described (Schaart et al., 2004). Briefly, cells were incubated in 1 % periodic acid (Sigma) for 5 min. Thereafter coverslips were put in tap water for 1 min, quickly rinsed in mQ-H₂O and then applied to Schiff reagent (Sigma) for 15 min at room temperature. Afterwards the coverslips were rinsed again in mQ-H₂O, incubated in tap water for 10 min followed by an incubation step in PBS for 5 min. Periodic acid oxidizes the vicinal diols in sugars such as glycogen to aldehydes, which now in turn react with the Schiff reagent to give a purple/magenta colour. Images were acquired on an Axio observer Z1 microscope equipped with an ApoTome module (Zeiss, Germany) and a

63× Apochromat lens unless specified. Images were taken with a Coolsnap HQ camera (Photometrics, Tucson, AZ) using the software Axiovision.

Glucose uptake assay

Gradient purified *Chlamydia* EBs were incubated in an axenic medium (5 mM KH₂PO₄, 10 mM Na₂HPO₄, 109.6 mM K-gluconate, 8 mM KCl, 1 mM MgCl₂) (Omsland et al., 2012) supplemented with 0.2 mM α-D-[¹⁴C(U)]-Glc 1-Phosphate, α-D-[¹⁴C(U)]-Glc 6-Phosphate or D-[¹⁴C(U)]-Glc (Perkin Elmer) (0.1 μCi per sample). In some samples 10 mM of the indicated cold monosaccharide was added in a competition assay. After two hours of incubation at 37°C the bacteria were pelleted (15000 g for 5 min) and washed twice in 50 mM K₂HPO₄/KH₂PO₄, 100 mM KCl and 150 mM NaCl. Radioactivity associated to the bacterial pellet and to the supernatant was measured by a scintillation counter.

Construction of recombinant plasmids

Genomic DNA from *C. trachomatis* LGV serovar L2 strain 434 was prepared from bacteria using the RapidPrep Micro Genomic DNA isolation kit (Amersham Pharmacia Biotech). The first 20 to 30 codons of different chlamydial genes including about 20 nucleotides upstream from the translation start sites were amplified by PCR using primers listed in Suppl. table 1 and cloned into the pUC19cya vector as described (Subtil et al., 2001). attB-containing primers (Suppl. table 1, Gateway®, Life technologies) were used to amplify and clone the *C. trachomatis* L2 *glgA* gene into a destination vector derived from the mammalian expression vector pCiNeo, providing an amino-terminal 3xflag tag, and into pDEST15 (Gateway).

Transfection

siRNA transfections were performed with Lipofectamine RNAiMAX (Life technologies) according to the manufacturer's protocol. A mixture of 2 to 6 siRNA sequences (Suppl. table 2), with a final concentration of 10 nM each, was used and transfection was done twice, 48 h and 4 h prior to infection. siRNA efficiency was determined by immunoblot or RT-PCR (see respective sections for more detail).

Cells were transfected with plasmid DNA 24 h after seeding using jetPRIME transfection kit (Polyplus transfection) according to the manufacturer's protocol. The constructs used were Flag-GlgA and SLC35D2-HA (pMKIT-neo-hRel8-cHA, kindly provided by Nobuhiro Ishida, Chiba Institute of Science, Japan) (Ishida et al., 2005).

Quantification of glycogen with CellProfiler

The cell image analysis software CellProfiler was used to quantify glycogen content in inclusions stained with PAS. Around 50 inclusions were manually encircled, and their size and total staining intensity was determined. PAS staining not linked to the presence of glycogen was estimated by doing the same procedure on inclusions in cells grown in the absence of Glc. The averaged value obtained was considered background value and was subtracted. Glycogen content of inclusions of cells treated with control siRNA was considered 100 %.

Quantitative Reverse Transcription PCR and Reverse Transcription PCR

Total RNA was isolated from 5×10^5 HeLa cells infected with *C. trachomatis* LGV serovar L2 after 1 h, 3 h, 8 h, 16 h, 24 h or 40 h of infection (MOI of 10 for 1 h, 3 h, 8 h and MOI of 0.1 for 16 h, 24 h, 40 h) with the RNeasy Mini Kit with DNase treatment (Qiagen) according to the manufacturer's protocol. RNA concentrations were determined by NanoDrop and the samples normalized to equal RNA content. Reverse transcription (RT) was performed with SuperScript III Reverse Transcriptase (Life Technologies) and quantitative PCR (qPCR) undertaken with LightCycler 480 system using LightCycler 480 SYBR Green Master I (Roche). In parallel, genomic DNA (gDNA) of each time point was purified with the DNeasy Blood and Tissue Kit (Qiagen), and the amount of bacteria in the samples determined by qPCR using chlamydial primers. This was done to normalize the cDNA of the different samples. Primers are listed in Suppl. table 3, their specificity was ensured through the analysis of melting curves.

For the evaluation of siRNA SLC35D2 efficiency the steps until reverse transcription were the same, but the PCR was run with PrimeStar (Clontech). Equal volumes were loaded on agarose gels and bands were revealed using UV-light visualizing ethidium bromide.

Western Blot and antibodies

Cell pellets were lysed with a lysis buffer (8 M urea, 30 mM Tris, 150 mM NaCl, 1 % v/v sodium dodecyl-sulfate) and proteins were subjected to sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane, which was blocked with $1 \times$ PBS containing 5 % milk and 0.01 % Tween-20. The membranes were then immunoblotted with primary antibodies diluted in $1 \times$ PBS and 0.01 % Tween-20. Primary antibodies used in the secretion assay were mouse anti-cya, rabbit

anti-CRP and rabbit anti-IpaD and were generously given by Drs N. Guiso, A. Ullmann and C. Parsot, respectively (Institut Pasteur, Paris). Other antibodies used were rabbit anti-Gys1 (Millipore #04-357), rabbit anti-UGP2 (GeneTex #GTX107967), mouse anti-Flag M2 (Sigma-Aldrich), goat anti-mouse IgG coupled to horseradish peroxidase (HRP) and goat anti-rabbit IgG-HRP (GE Healthcare). Blots were developed using the Western Lightning Chemiluminescence Reagent (GE Healthcare).

Zymogram

Glycogen synthase genes (*glgA*) were amplified from genomic DNA of *Escherichia coli* K12 and *Chlamydia trachomatis* D/UW-3/CX and cloned into the expression vector pGEX (GE Healthcare) and pDEST15, respectively.

Precultures of wild-type and transformed *E. coli* were grown overnight in LB medium at 37°C, then transferred to fresh LB medium and grown until the optical density (OD) 600 nm reached 0.6. Cultures were subsequently induced overnight with 0.5 mM of IPTG.

Cells were harvested by centrifugation and disrupted by a French press at 1250 psi. The lysate was centrifuged at 16000 g for 15 min at 4°C. Soluble proteins (such as GlgA) were found in the supernatant. Glycogen synthase activity was detected by zymogram analysis. The proteins in the supernatant were separated by non-denaturing polyacrylamide gel electrophoresis (PAGE) containing 0.6 % rabbit glycogen (Sigma-Aldrich). After electrophoresis, gels were incubated overnight at room temperature in glycogen synthase buffer (66 mM glycyl-glycine, 66 mM (NH₄)₂SO₄, 8 mM MgCl₂, 6 mM 2-mercaptoethanol, and 1.2 mM ADP-Glc or UDP-Glc). Glycogen synthase activity was then visualized as dark activity band after iodine staining.

Heterologous secretion assay in *Shigella flexneri*

Analysis of secreted proteins was performed as described elsewhere (Ball et al., 2013; Subtil et al., 2001). Briefly, 1 ml of a 30°C overnight culture of *Shigella flexneri ipaB* or *mxiD* transformed with different Cya chimeras was inoculated in 30 ml of LB broth with 0.1 mg/ml ampicillin and incubated at 37°C for 4 h. Bacteria were then harvested by centrifugation and the supernatant was filtered through a Millipore filter (0.2 µm). To precipitate the proteins 1/10 (v/v) of trichloroacetic acid was added to the supernatant and the precipitate as well as the bacterial pellet resuspended in sample buffer for analysis by SDS-PAGE and immunoblot.

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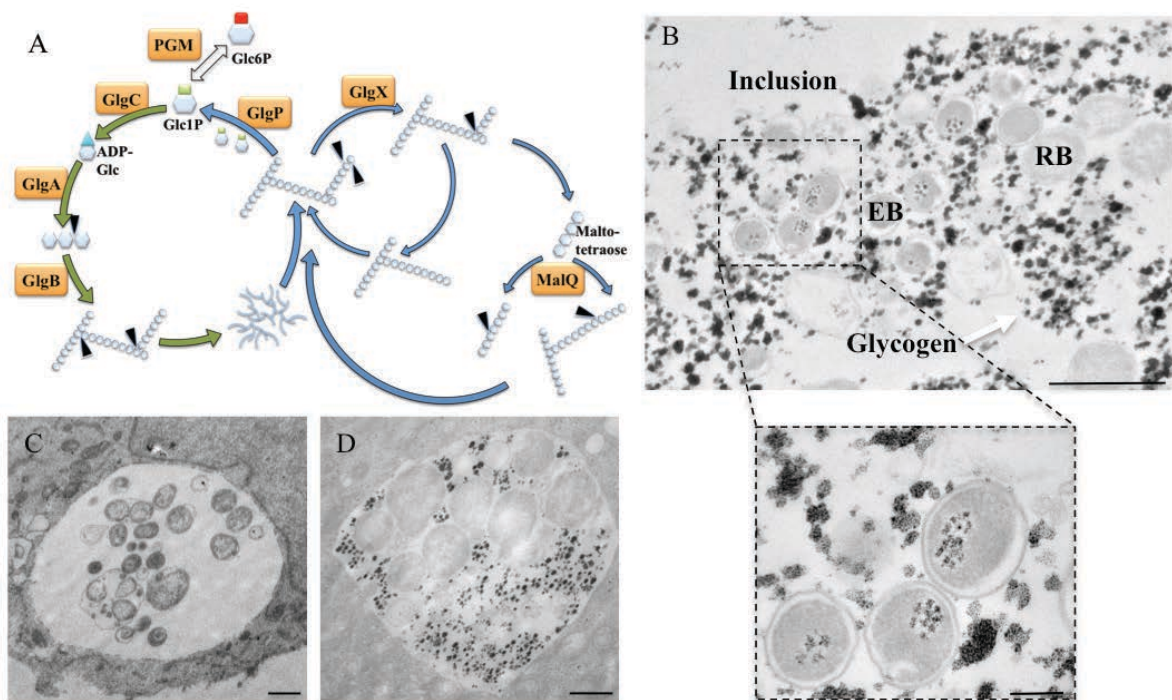


Figure 1: Glycogen accumulation in *C. trachomatis* inclusions. (A) Glycogen metabolism in bacteria. In green: glycogen synthesis. In blue: glycogen degradation. Glc1P is the substrate of GlgC for ADP-Glc synthesis. The glycogen synthase (GlgA) subsequently produces linear glycogen chains via α -1,4 glycosidic bonds, and the branching enzyme (GlgB) introduces ramifications through α -1,6 linkages. Glycogen depolymerization in Glc1P is the result of the activity of GlgP, GlgX and MalQ. Note that the phosphoglucomutase PGM allows for Glc1P conversion to Glc6P. Black arrows point to the site of enzymatic activity. Genes for all these enzymes are present in *C. trachomatis*. (B) HeLa cells were infected for 30 h with *C. trachomatis*. White arrow = glycogen. The picture on the bottom shows a 2-fold enlargement of the boxed region. Scale bar top = 1 μ m. Scale bar bottom = 200 nm. (C&D) Cells were glucose-deprived 48 h prior to infection. 10 mg/ml glucose were added 24 hpi and cells were (C) fixed immediately or (D) 4 h after glucose administration. Note that no glycogen is detectable in the bacteria while it is highly abundant in the inclusion lumen. Scale bar = 1 μ m. Glycogen is visualized by PATAg stain. TEM.

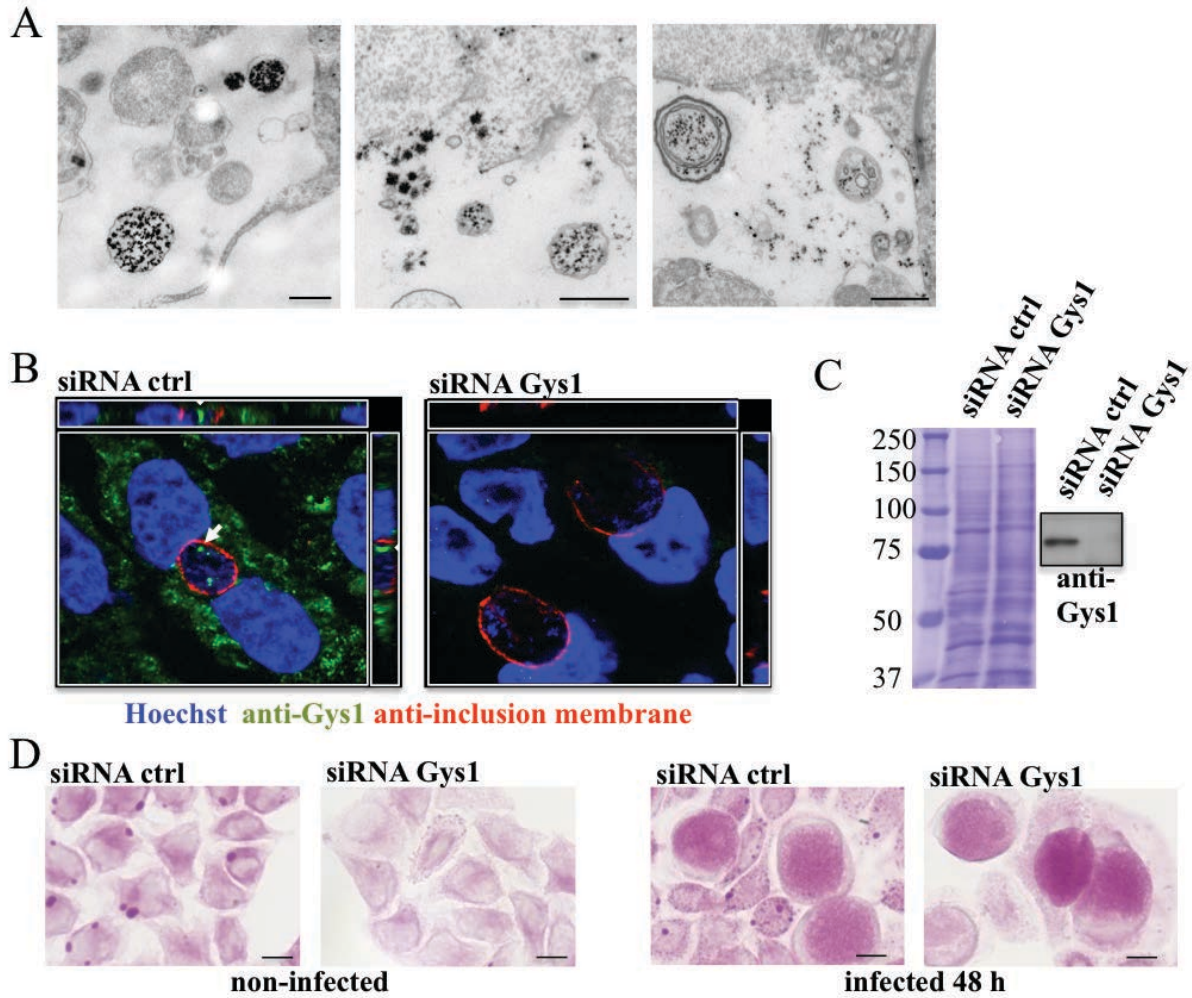


Figure 2: Bulk import of cytoplasmic glycogen contributes marginally to the accumulation of glycogen in inclusions. (A) TEM images of glycogen-filled vesicles in the inclusion. HeLa cells 30 hpi with *C. trachomatis*. Glycogen is visualized through PATAg staining. Scale bar = 500 nm. (B) Gys1 is imported into the inclusion lumen. HeLa cells were treated with siRNA control or against the Gys1 prior to infection for 30 h with *C. trachomatis*. DNA was stained in blue, Gys1 in green and the inclusion membrane in red. The white arrow points to intraluminal Gys1, see also xz (top) and yz (right) projections. (C,D) HeLa cells were treated with either siRNA control or siRNA against Gys1 48 h prior to infection. (C) Coomassie staining of whole cell lysates as loading control (left) and immunoblot with an anti-Gys1 antibody (right). (D) PAS on non-infected cells fixed at time of infection (left) and on cells infected for 48 h (right). Inclusions displayed the same coloration in cells treated with siRNA control and siRNA Gys1. Scale bar 10 μm.

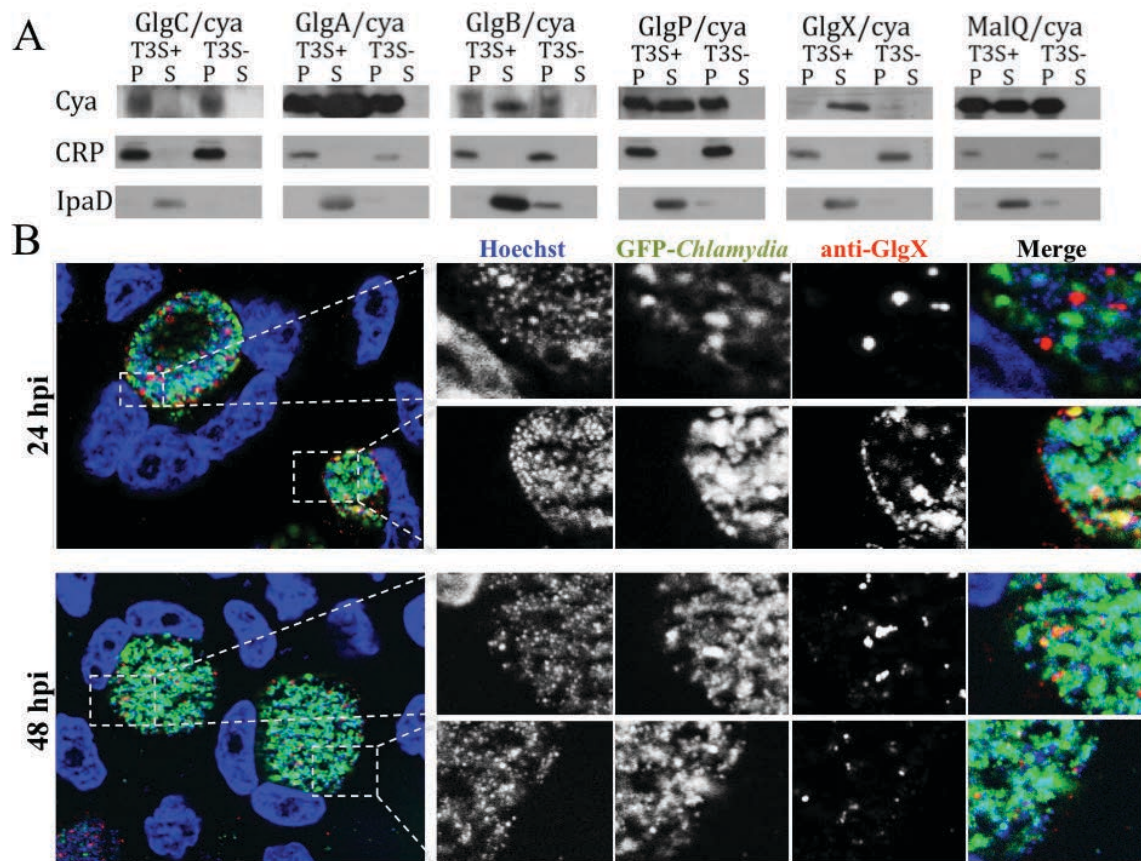


Figure 3: Chlamydial glycogen enzymes are putative T3S effectors. (A) Heterologous test of secretion in *Shigella flexneri* reveals T3S signals in some of the glycogen enzymes. The N-terminal 20 amino acids of the indicated proteins were fused to the reporter Cya, and constructs were transformed into the *S. flexneri* strains *ipaB* (T3S+) and *mxiD* (T3S-). Liquid cultures were fractionated into pellet (P) and supernatant (S) and analysed by western blot. All chimeras except GlgC/cya were detected in the supernatant in T3S competent bacteria and not in T3S defective bacteria. CRP and IpaD serve as negative and positive controls, respectively. (B) HeLa cells infected for (A) 24 h or (B) 48 h with GFP expressing L2 (green) and stained with an anti-GlgX antibody (red) and Hoechst (blue). Insets to the right show enlargements of the boxed areas.

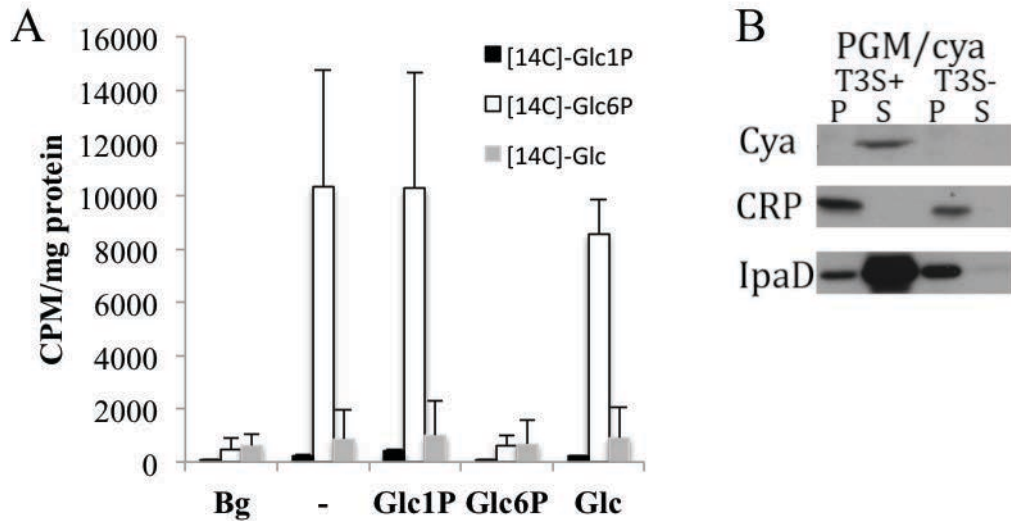


Figure 4: *C. trachomatis* takes up Glc6P and secretes PGM. (A) Purified EBs were incubated for 2 h with [¹⁴C]-Glc, [¹⁴C]-Glc6P or [¹⁴C]-Glc1P in absence or presence of a 50-fold excess of non-radioactive Glc, Glc6P or Glc1P. Bacteria were subsequently washed and pelleted and radioactivity measured with a scintillograph. Background (Bg) was measured after 1 min of incubation instead of 2 h. Error bar corresponds to the standard deviation (n=3) (B) Heterologous test of secretion in *S. flexneri* was performed on the first 20 amino acids of PGM as described in Figure 3A.

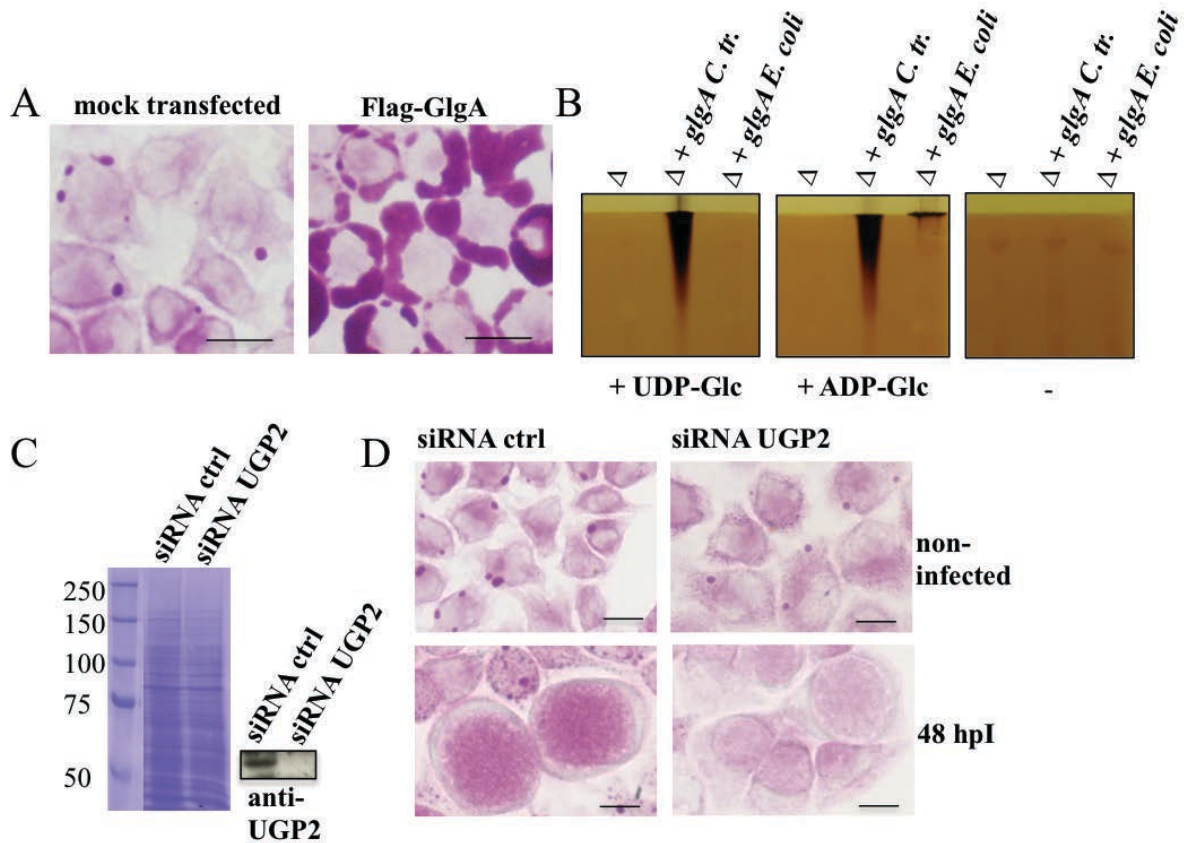


Figure 5: Chlamydial GlgA can use UDP-Glc as substrate for glycogen synthesis. (A) PAS staining was performed on HeLa cells 24 h after transfection with chlamydial Flag-GlgA. (B) Zymogram analysis. Lysates of *E. coli* lacking *glgA* (Δ) alone, or transformed with chlamydial *glgA* (*glgA C. tr.*) or *E. coli glgA* (*glgA E. coli*) were separated by native polyacrylamide electrophoresis and incubated in either UDP-Glc, ADP-Glc or buffer only (-). Glycogen production was visualized by iodine staining. (C&D) HeLa cells were treated with either siRNA control or siRNA against UGP2 48 h prior to infection. (C) Coomassie staining of whole cell lysates as loading control (left) and immunoblot with an anti-UGP2 antibody (right). (D) PAS on non-infected cells fixed at time of infection (top) and on cells infected for 48 h (bottom). Inclusions displayed the same coloration in cells treated with siRNA control and siRNA Gys1. Scale bar 10 μ m.

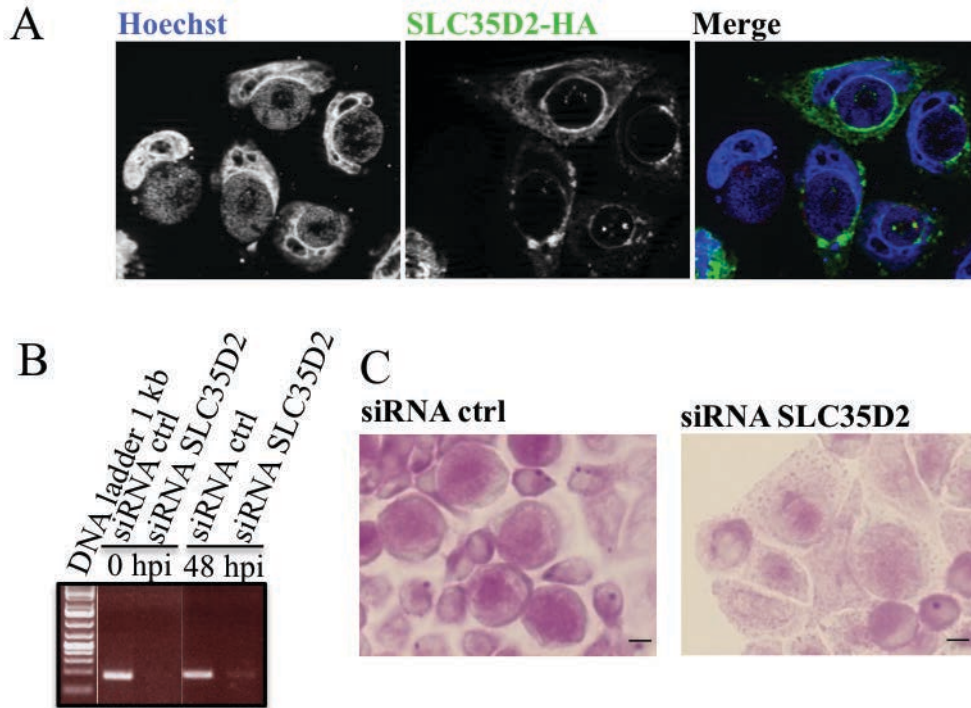


Figure 6: SLC35D2 imports UDP-Glc into the inclusion lumen. (A) HeLa cells were transfected with SLC35D2-HA prior to infection. SLC35D2-HA localizes to the inclusion membrane. (B) Cells were treated with siRNA control (ctrl) or siRNA SLC35D2 48 h and 4 h prior to infection. Samples were taken at the moment of infection (0 hpi) and 48 hpi, and RT-PCR was performed with primers specific to SLC35D2. (C) PAS staining of siRNA ctrl or SLC35D2 treated cells 48 hpi.

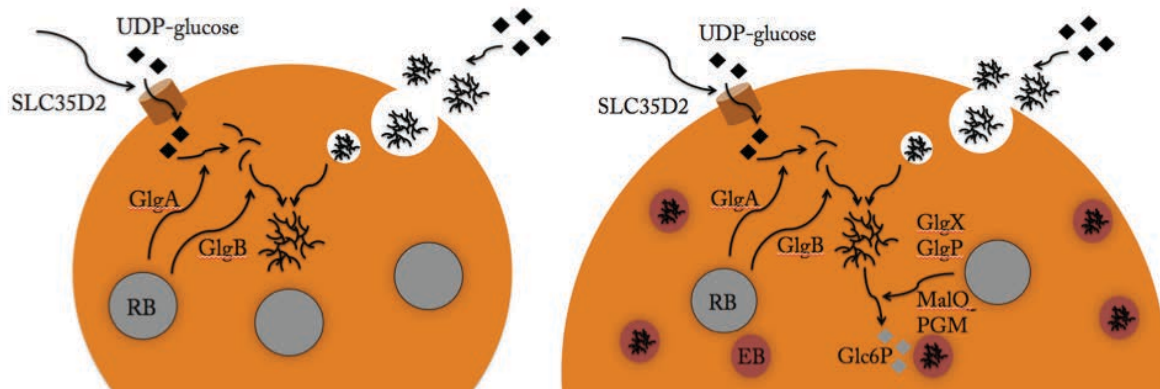


Figure 7: Scenario of Glc flux in *C. trachomatis*. Early during the infectious cycle (left) SLC35D2, and possibly other transporters, are recruited to the inclusion membrane and UDP-Glc is translocated into the inclusion lumen. The activity of chlamydial glycogen enzymes, secreted by RBs into the inclusion lumen, leads to the onset of luminal glycogen synthesis between 16 and 20 hpi. Probably at the same time, small amounts of host glycogen are imported into the inclusion lumen through invagination of the inclusion membrane. At this stage of infection the inclusion contains mostly RBs. This developmental form does not accumulate glycogen and probably hardly uses Glc6P for growth. Later on (right), EBs appear and accumulate intrabacterial glycogen. This happens via the degradation of intraluminal glycogen into Glc1P, which is subsequently converted to Glc6P, a substrate amenable to chlamydial uptake. As EBs highly rely on Glc6P as energy source for metabolic reactions, the intrabacterial glycogen store presumably allows for survival of EBs, in the inclusion lumen and after having exited the cell.

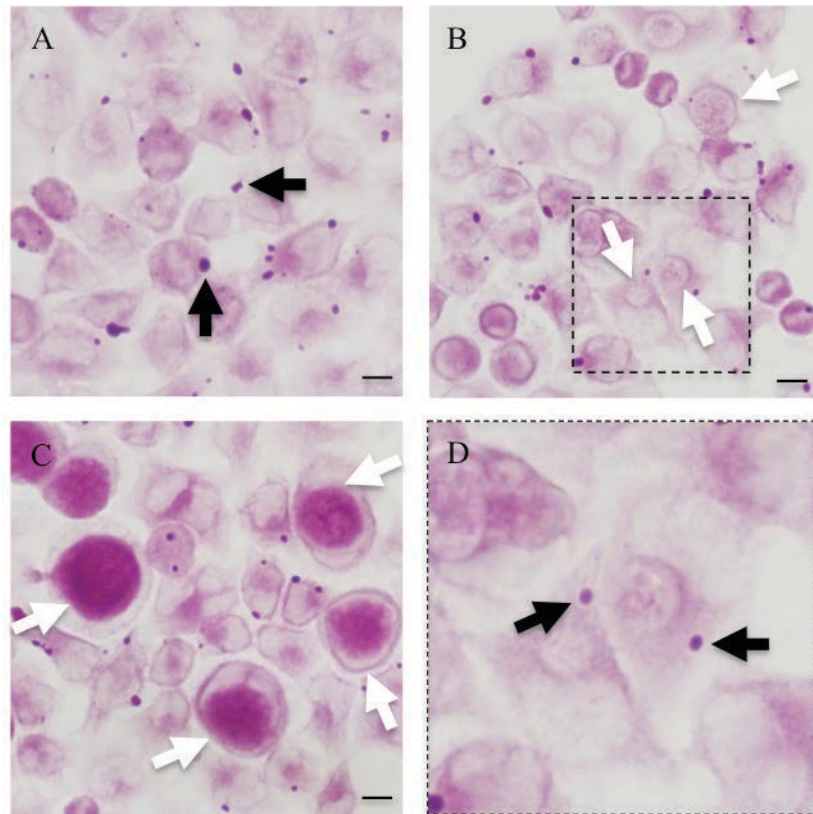


Figure 1 - figure supplement 1: There is an overall decrease of cytoplasmic glycogen in infected cells, and a redistribution towards the inclusion. Cells were (A) non-infected, or infected with *C. trachomatis* for (B) 24 h or (C) 48 h, fixed in PFA and processed for PAS stain. (D) is an enlargement of the boxed region in (B). Note that glycogen particles (black arrows) are still detected in cells infected for 24 h but not later, while inclusion glycogen content strongly increases with infection time. White arrows point to examples of inclusions. Scale bar = 10 μm .

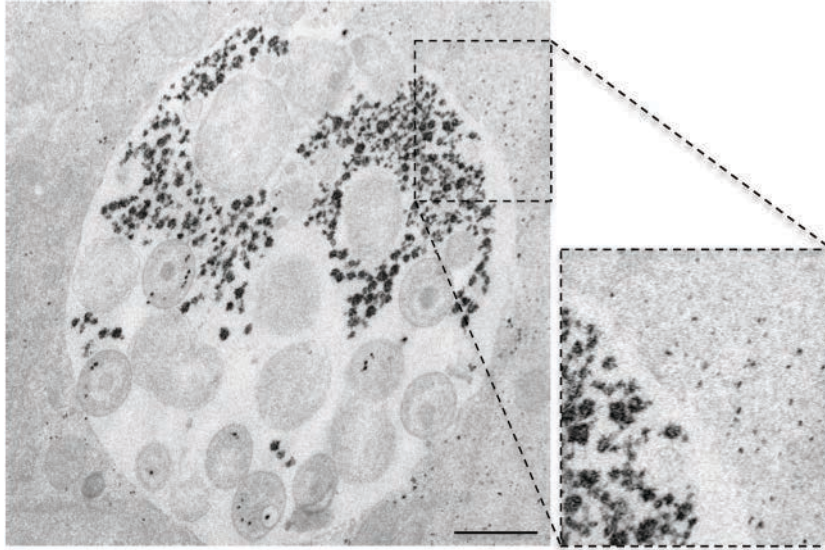


Figure 1 - figure supplement 2: Glycogen particles in inclusion lumen are in average of bigger size than in host cell cytoplasm. HeLa cells were infected for 30 h with *C. trachomatis*. The picture on the right shows a 2-fold enlargement of the boxed region. Glycogen is visualized by PATAg stain. TEM. Scale bar = 1 μ m.

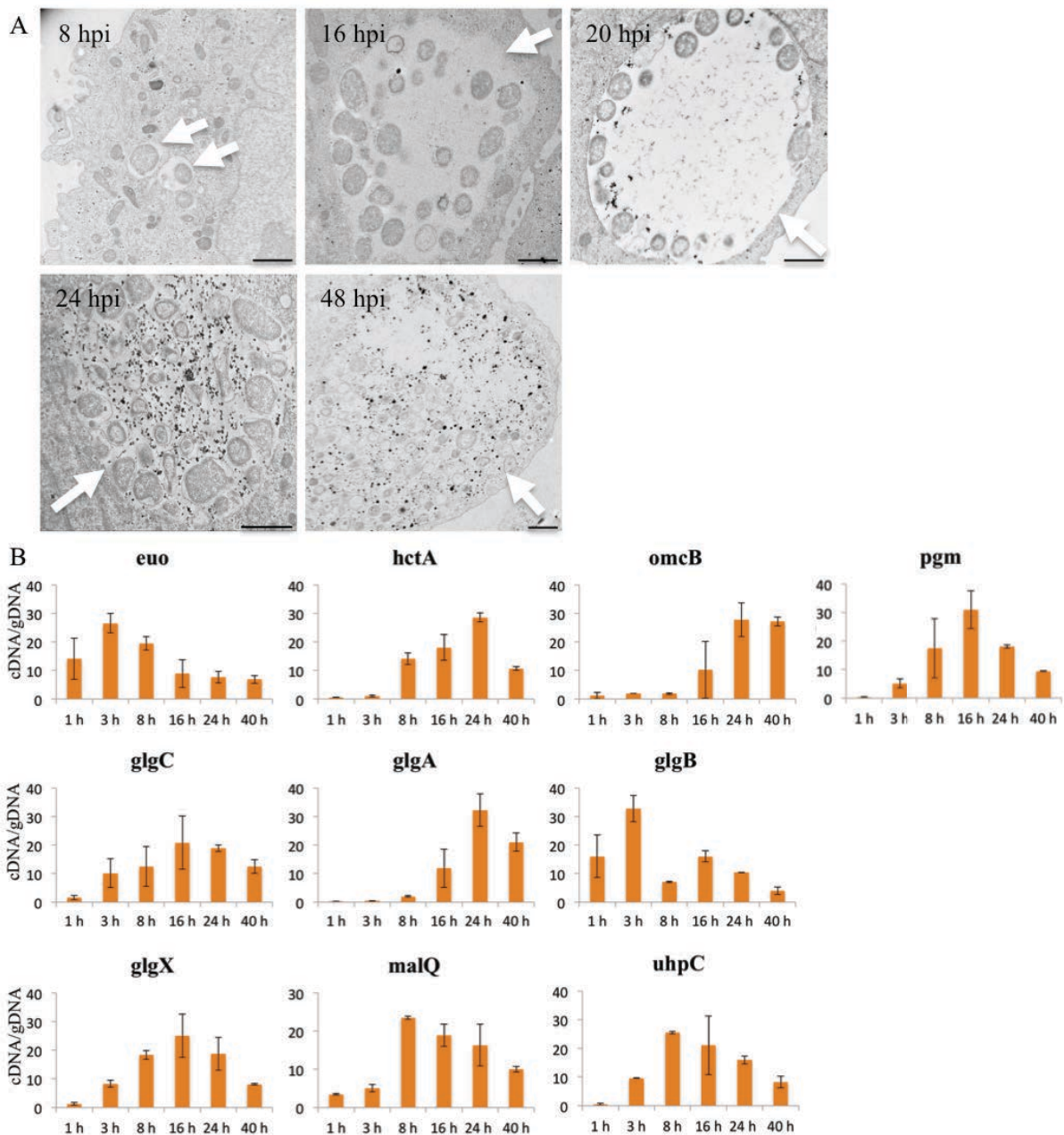


Figure 1 - figure supplement 3: Kinetics of glycogen accumulation and transcriptomics of genes related to glycogen metabolism. (A) HeLa cells were infected with *C. trachomatis* for 8, 16, 20, 24 or 48 h. Glycogen is visualized by PATAg stain. It is first detected the inclusion lumen (white arrows) at 20 hpi, and increases strongly after. TEM. Scale bar = 1 μ m. (B) qRT-PCR of selected genes related to glycogen metabolism. Fluorescent values were plotted against standard curves and cDNA normalized with the overall chlamydial genomic DNA (gDNA) present in parallel samples (cDNA/gDNA). An early chlamydial gene, *euo*, and two late genes, *hctA* and *omcB*, served as controls. Values were obtained from three independent experiments, each performed in triplicates. Error bars depict the standard deviation (n=3).

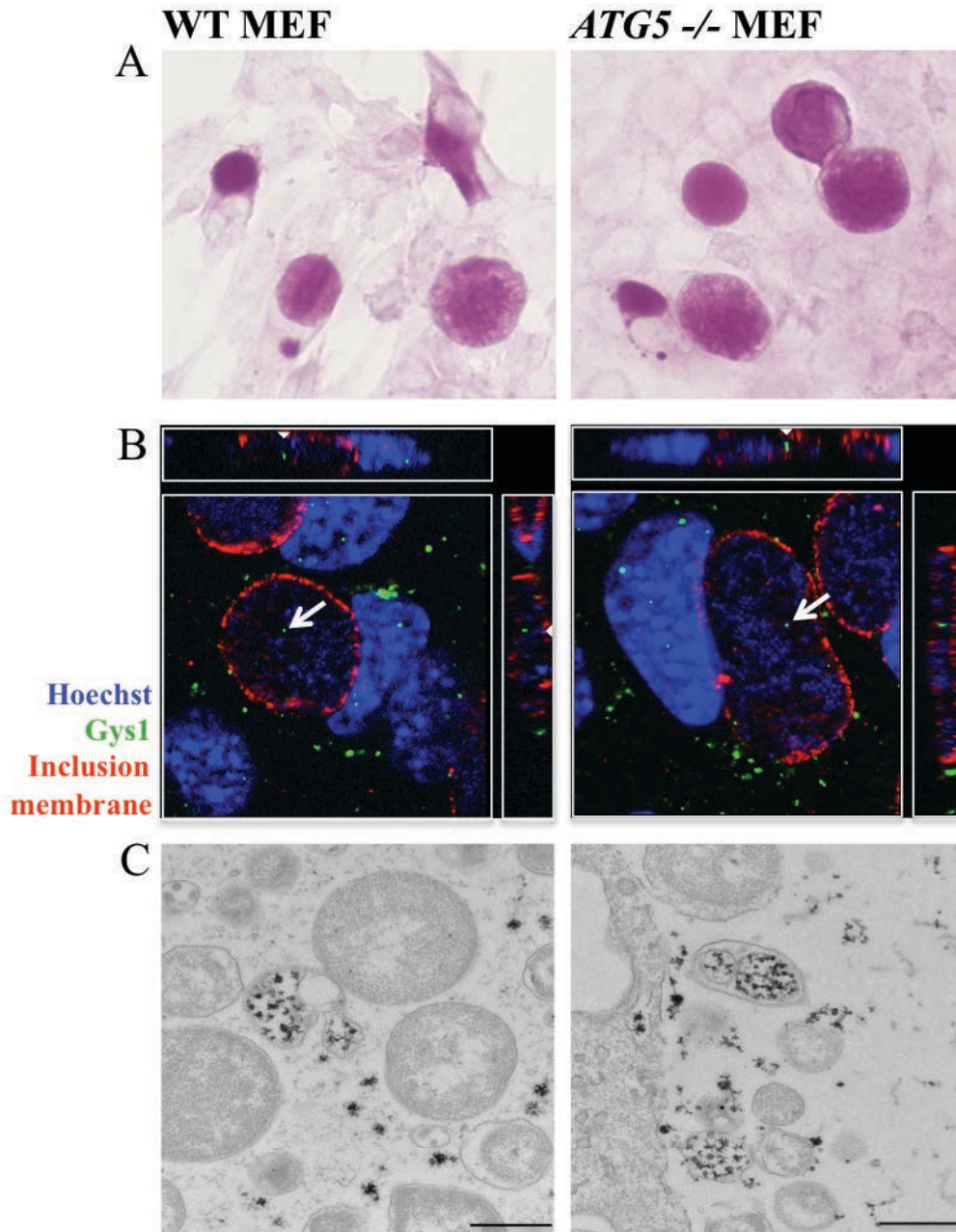


Figure 2 - figure supplement 1: Gys1 import into the inclusion lumen is autophagy independent. Wild-type (WT) or *Atg5*^{-/-} mouse embryonic fibroblasts (MEFs) were infected for 30 h with *C. trachomatis*. (A) PAS staining. (B) DNA is stained in blue, Gys1 in green and the inclusion membrane in red. While less abundant than in HeLa cells, Gys1 (white arrows) was detected inside inclusions of both cell lines. (C) TEM images. Glycogen-filled vesicles were observed in inclusions of both cell lines. Scale bar represents 500 nm.

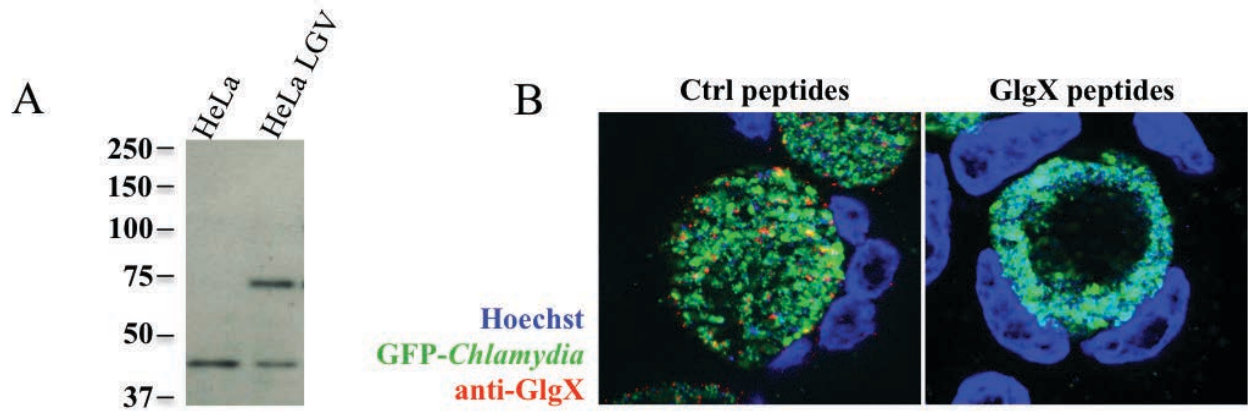


Figure 3 - figure supplement 1: The anti-GlgX antibody is specific. (A) Western Blot of non-infected HeLa or LGV infected HeLa cells. The anti-GlgX antibody detected a band of the expected molecular weight (73 kDa). (B) Anti-GlgX was preincubated with either unrelated control peptides, or with the peptides the antibody was raised against. DNA is in blue, bacteria in green and GlgX in red. The GlgX staining disappears upon preincubation with the GlgX peptides, demonstrating its specificity.

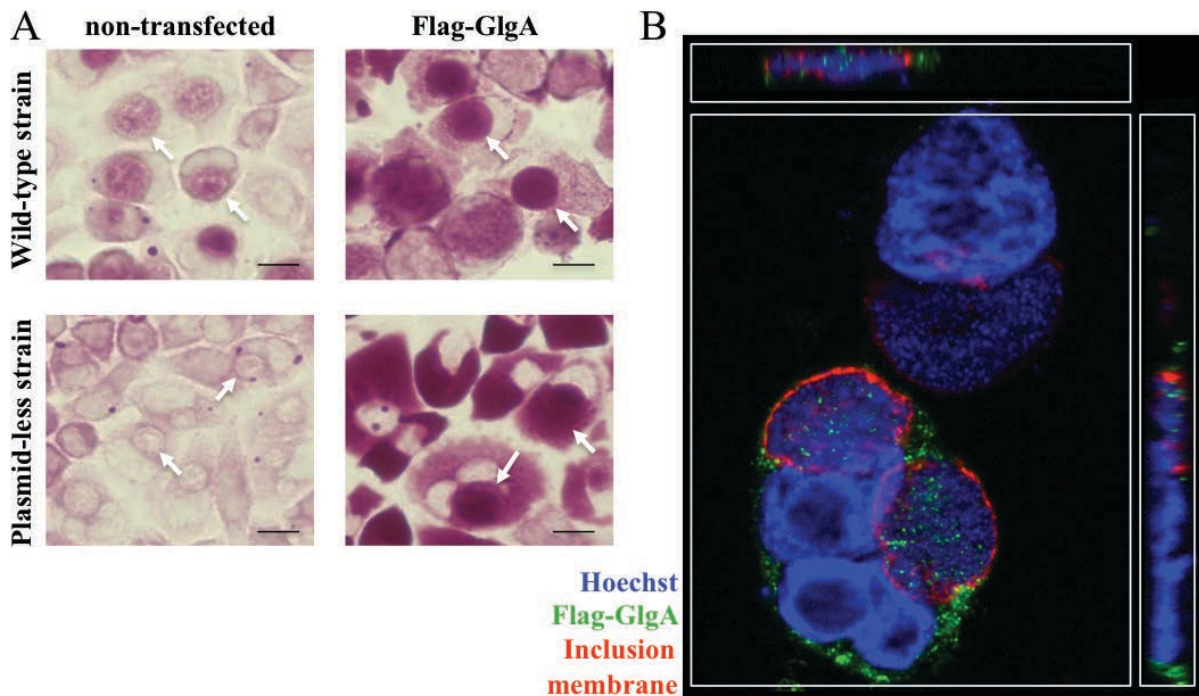


Figure 5 - figure supplement 1: Flag-GlgA is imported into the inclusion lumen. (A) Flag-GlgA transfection leads to an increase in intraluminal glycogen accumulation. HeLa cells were transfected with Flag-GlgA before infection with either the wild-type LGV strain or the plasmid-less strain LGV 25667R. PAS staining revealed an increase of intraluminal glycogen (white arrows) upon transfection. Remarkably the plasmid-less strain now showed similar glycogen staining to the wild-type strain. Scale bar 10 μm . (B) DNA is stained in blue, Flag-GlgA in green and the inclusion membrane in red. Flag-GlgA is abundant in the host cytoplasm and the inclusion lumen (see also xz (top) and yz (right) projections).

Suppl. Table 1: Primers used for cloning purposes.

Gene	Vector	Primers
glgC	pUC19cya	AGTCAAGCTTGTAATAGTTTTGTTTTTATGGCTGGTAGACGGACG AGTCTCTAGATCCGCATAAGACAATAACTCCTAC
glgA	pUC19cya	AGTCAAGCTTGTAATAGTTTTGTTTTTATGAAAATTATTCACACAGCTATCG AGTCTCTAGATGCTAGTCCGTATAGCGCG
glgB	pUC19cya	AGTCAAGCTTGTAATAGTTTTGTTTTTATGGATCCTTTTTTCTTAAATACTCAAC AGTCTCTAGAAGAAAACAATCCCAAAAGATCTTG
glgP	pUC19cya	AGTCAAGCTTGTAATAGTTTTGTTTTTATGTATTTTCGATCGGACAAAG AGTCTCTAGATTGAGGAGTCTGGACTACCC
glgX	pUC19cya	AGTCAAGCTTGTAATAGTTTTGTTTTTATGGAATCTTTGTCTGTTTCGTT AGTCTCTAGAAGAAAATAGAGAAAAACGGTAGCG
malQ	pUC19cya	AGTCAAGCTTGTAATAGTTTTGTTTTTATGCCGTCATTATCCCAAT AGTCTCTAGAGCCATGCTTAGGAGAAGTATCTATC
pgm	pUC19cya	AGTCAAGCTTTTAGGTTCTGATCGGAAGGTTGTCTT AGTCTCTAGACAAAATGTTTTTGGCTGTTTGA
glgA	pCiNeo	AAAAAAGCAGGCTTCATGAAAATTATTCACACAGCTATCG AGAAAGCTGGGTCTCATTATTGTTTATAAATTTCTAAATATTTATTGG
	pDEST15	AAAAAAGCAGGCTTCATGAAAATTATTCACACAGCTATCG AGAAAGCTGGGTCTCATTATTGTTTATAAATTTCTAAATATTTATTGG

Suppl. Table 2: List of siRNAs.

siRNA	5' → 3'	Company
Gys1	GGG CGA GGA GCG UAA CUA A	Dharmacon
	CAA CGA CGC UGU CCU CUU U	Dharmacon
	GAA GCU UUA UGA AUC CUU A	Dharmacon
	GAA UCG GCC UCU UCA AUA G	Dharmacon
UGP2	GGU UCA AGA UUA UCU AAG A	Eurogentec
	GUG GAU CUG UAU AUU CUU A	Eurogentec
SLC35D2	CCA CAU AAG UGG AUU AUC A	Eurogentec
	GCC ACC AUA AUG AUA CUA U	Eurogentec
	GCU AGG GAA AUA CGG AGU A	Dharmacon
	AAU UAA GCC UAC CGA UGU U	Dharmacon
	GAA AUG UAC UCG GGC GAC A	Dharmacon
	AGC CAG AGG UUU CGG AUU C	Dharmacon

Suppl. Table 3: List of primers used in qRT-PCR and qPCR.

Name	Gene	Primers
glgA	CT798	AATGATTGGAATGCGTTACGG CGGTAGGTTGTCAGTCTTCC
glgB	CT866	GTGCATCATTGTTGGGTAGGA CTCGCGATTCAGGTGTAAGG
glgC	CT489	GCCTTTGCCTCAGAATTTTCC CCAAACCTGACTTCCATCTCG
glgX	CT042	CTCCCTTTAACCCCATTTTG CACGGTAGCATCCATTCCACT
malQ	CT087	CGTCGGTCTCTTTCGATTTTG GGGATCGTTCAGATCTTCT
pgm	CT295	AGCCCGAGTCTTAAAGCGAAG CCAAAGGCTCTGGAATCTGAA
uhpC	CT544	CGGCTTTACAGGATGGTTCGC CGGATGCATTCCATGTTGGCA
omcB	CT443	CTGCAACAGTATGCGCTTGTC GCTGTTGCTGTTCTTGGTTC
hctA	CT743	TAAAGCCGCAGCACAAAGAGT CGAACAGGCTTCTTAGCAGCAG
euo	CT446	TATGCTACACGCATTGGTGCT GCCTCAAAACCTTCTCTCATGG
slc35d2	slc35D2	CTGCCTCTCTCTACGTTGG CCCGAGAATAATGGCAAAGA