THE *ESCHERICHIA COLI* INNER MEMBRANE PROTEIN YHIM IS NECESSARY FOR EFFICIENT ATTACHMENT OF BACTERIOPHAGE T4

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- *E. coli*
- Inner membrane protein
- T4

ABSTRACT

Bacteriophages are obligate intracellular parasites, but many of the cellular proteins involved in replication have not been identified. We have tested the role of the inner membrane protein YhiM in bacteriophage replication. YhiM is a conserved (21) membrane protein in *Escherichia coli (E. coli)* thought to be localized to the cytoplasmic membrane that is necessary for cell survival under conditions of cell stress, including acid shock, low osmolarity and high temperature. We show here that YhiM is necessary for replication of the bacteriophage T4. It also plays a modest role in the replication of T1, T3, and T5 but it does not play a role in the replication of ΦX174. Our data indicated that no replication of T4 occurs in cells missing YhiM. This block in infection is due to a block in attachment of the virus to the cell surface.

INTRODUCTION

Bacteriophages are viruses that infect bacteria. They have numerous strategies to infect their host cell and require many host proteins for their replication. All viruses require a receptor on the cell surface to identify a susceptible cell and begin infection (3). The bacteriophage then depends on numerous host proteins to reproduce. For example, the bacteriophage T4 requires the *E. coli* genes *tabB, groE* and *mop* for proper assembly of the virus (6, 9, 28). T4 replication is also dependent on RpoS (4), a sigma factor involved in regulating responses to cell stress and changes in the growth of *E. coli* (14). Finally, growth of the bacteriophage

T4 is dependent on environmental conditions, including temperature (12, 27). Given that the environment affects bacteriophage replication and that pathways involved in response to environmental stresses can affect viral replication, we were interested in determining whether other genes involved in responses to environmental stresses might also be involved in T4 replication.

YhiM is a conserved inner membrane protein in *E. coli*. It has 10 predicted transmembrane domains and homology to the DUF 2776 family of proteins, an uncharacterized family of proteins (24). YhiM expression is upregulated under conditions of cell stress, such as acid stress, high temperature and changes in osmolarity (22, 30-32). YhiM expression is regulated by the sigma factor RpoS (1, 31). YhiM is also necessary for survival during acid shock and conditions of high temperature and low osmolarity (2, 21). Finally, YhiM has also been shown to play a role in GABA transport (29).

Because YhiM seems to play a role in protecting the cell during conditions of cell stress (2, 21) and bacteriophage replication can be affected by RpoS, we wanted to know if YhiM might also play a role in infection by bacteriophages that infect *E. coli*. There are several well-characterized bacteriophages that infect *E. coli*, including T1, T3, T4, T5 and ΦX174. These viruses belong to different virus families, have different morphologies and require different proteins from the cell for infection. T1 belongs to the Siphoviridae family, has a ~49kb DNA genome and uses the FhuA protein for attachment (10). T3 has a short tail, a DNA genome of ~40kb, and uses lipopolysaccharides (LPS) for attachment to the cell surface (32). T4 is a member of the "T-even" phage group, has a large DNA genome (~170kb) and uses OmpC and LPS for attachment to cells (20). T5 has a long non-contractile tail, a DNA genome of ~121 Kb, and uses the FhuA receptor for attachments (15) (19). ΦX174 is a member of the *Microviridae* family, does not contain a tail, has a genome of ~5 Kb and uses LPS for attachment. T3 and T4 use the same glucose residue for binding, whereas ΦX174 uses a different site on LPS for attachment (23).

We used two *E. coli* strains, the wildtype UCB strain and a previously published strain with a Tn10 insertion into the *yhiM* gene (21) to test whether bacteriophage replication is dependent on YhiM. We tested a wide range of bacteriophages including T1, T2, T3, T4, T5, and ΦX174. These viruses were chosen because they are all well characterized and belong to different families of viruses. Our data indicated that replication of the bacteriophage T4 is dependent on YhiM. Attachment to the cell surface is blocked by a lack of YhiM, but this effect can be partially rescued by using a high virus number per cell or by prolonged incubation with the virus. This suggests that YhiM mediates efficient T4 attachment at the cell surface.

MATERIALS AND METHODS

STRAINS USED

The *E. coli* strains UCB and NVU31 have been previously described (21). UCB is the parental strain of NVU31 and NVU31 has a Tn10 insertion at position 167 of the *yhiM* gene (21). Strain B was obtained from Bruce Voyles, Grinnell College, Grinnell, IA. T1, T3, T4 and ΦX174 were obtained from Presque Isle Cultures, Erie, PA. T4 was a gift from Peter Gauss, Western State College, Gunnison, CO.

EFFICIENCY OF PLATING

These experiments were performed as previously described (18). Briefly, UCB or NVU31 was mixed with top agar (per liter: 10g tryptone, 1g yeast extract, 5g NaCl, 0.2g glucose, 7g agar) and then layered on top of bottom agar (per liter: 10g tryptone, 1g yeast extract, 5g NaCl, 0.2g glucose, 10g agar). All media components were obtained from Fisher Scientific. Ten µl of various dilutions of virus in T broth (per liter: 10g tryptone, 5 g NaCl) were spotted on top of the top agar overlay. Efficiencies are described relative to titer on UCB.

ATTACHMENT ASSAYS

Mid-log cultures of UCB or NVU31 were incubated at an MOI=0.01, 0.1, 1 or 10 and lysed with chloroform prior to titer using strain B. For experiments performed at 4°C, cells were incubated on ice with shaking during attachment. Cells were then shifted to 37°C prior to single step growth curve analysis (17).

COMPLEMENTATION ASSAYS

yhiM was amplified from genomic DNA using the primers RST201 (5'-GTGAACATATATATCG-GGTGG-3') and RST202 (5'- TTATTTTTTAG-CAGGAACCGCTTC-3) and was cloned into pTrc-HisTOPO (Invitrogen). pTrc-HisTO-PO-*yhiM* or pTrc-HisTOPO-LacZ (Invitrogen) was transformed into either UCB or NVU31. Protein expression was verified by SDS Page. Cells were induced for 2 hours with 1mM IPTG prior to attachment assays.

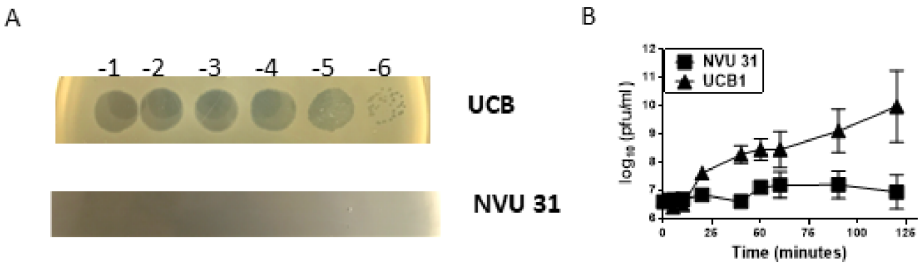
RESULTS

To determine whether *yhiM* plays a role in viral infections, we infected wildtype (UCB) and ΔYhiM (NVU31) with various viruses to see if a lack of *yhiM* affected virus growth (Table 1). A lack of *yhiM* had no effect on infection with T5. In contrast, a lack of *yhiM* had a modest effect on infection of T1, T3 and ΦX174, with a 10-fold reduction in efficiency of plating (EOP). T4 was the most dependent on *yhiM*, with a $>10^6$-fold decrease in efficiency of plating (Table 1 and Fig. 1A). No plaques were seen after infection of NVU31 cells with T4 (Fig. 1A).

Table 1: Efficiency of plating in cells missing *yhiM*. Efficiency of plating is relative to number of plaques during infection of UCB. Experiment was repeated at least 3 times.

	T1	T3	T4	T5	ΦX174
NVU31	10^{-1}	10^{-1}	$< 10^{-6}$	1	10^{-1}

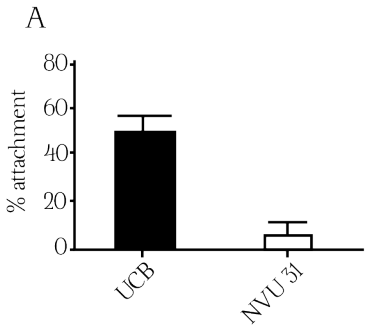
Figure 1: A) Efficiency of plating of T4 on NVU 31. B) Single step growth curve of T4 infection of both wildtype (UCB) and ΔYhiM (NVU 31). All samples were lysed in chloroform prior to plaque assay analysis. Each experiment was conducted at least 3 times. Error bars represent the standard error of the mean (SEM).



We were interested in determining the stage of the viral lifecycle that was blocked in NVU31 cells. One possible explanation for the lack of plaques on NVU31 is that T4 can enter and produce virus particles but cannot lyse NVU31. We tested this possibility by doing a single step growth curve on both NVU31 and UCB. Samples were lysed in chloroform before assaying the amount of virus to release any intracellular virus. Our data indicated that no virus was produced in NVU31 cells (Fig. 1B), suggesting that the virus may not be able to enter and/or replicate in NVU31.

We next wanted to determine if the lack of replication was due to a lack of attachment. We performed attachment assays using both UCB and NVU31 cells. We found that after 10 minutes of incubation at 37°C, 50% of T4 attached to UCB while <5% of T4 attached to NVU31 cells (p=0.0013) (Fig. 2A). These data suggested that the replication defect in *yhiM* deficient cells may be due to an inhibited attachment. To show that the block in attachment was due to *yhiM*, we constructed a plasmid that expresses *yhiM* (pTrc-HisTOPO-*yhiM*) and transformed both UCB and NVU31 cells. We also transformed both cell lines with pTrc-HisTOPO-LacZ as a control for protein overexpression. We then tested attachment on both cells (Fig. 2B). Consistent with previous data, the virus attached to UCB expressing lacZ but did not attach to NVU31 expressing lacZ (p=0.042). In contrast, overexpression of YhiM restored the ability of T4 to attach to NVU31 (p=0.4119), suggesting that the lack of attachment to NVU31 is due to the lack of YhiM (Fig. 2B). The previous data suggested that T4 could not attach to cells deficient in *yhiM*. We next determined whether this was a complete block in attachment or whether T4 attached inefficiently to the cell surface. We took two approaches to this question. First, we added increasing amounts of virus per cell to see if the addition of extra virus would complement the phenotype. Addition of additional virus resulted in an average percentage attachment of 2% at 0.01 and 0.1 pfu/cell (Fig. 3A and B) and increased to 7.5% at 1 pfu/cell (Fig. 3C) and 9% at 10 pfu/cell (Fig. 3D). Our data indicated that while adding additional virus slightly increases attachment, T4 does not attach to mutant cells to the same degree as it attaches to wildtype cells even when we add 10 pfu/cell (*p*=0.0026) (Fig, 3A-D). Another possibility to explain the lack of attachment is that the virus attaches slowly to the surface of cells. Because replication occurs when cells are incubated at 37°C, we decided to let the virus incubate at 4°C for 30 minutes and then shift the cells to 37°C for the rest of infection to occur. This allowed the virus to attach without replication and lysis of the host cell. We found

Figure 2: A) Attachment of T4 to UCB and NVU 31 cells after 10 minutes. B) Attachment of T4 to UCB and NVU31 cells expressing either LacZ or YhiM after 10 minutes. Each experiment was conducted at least3 times. Error bars represent SEM.



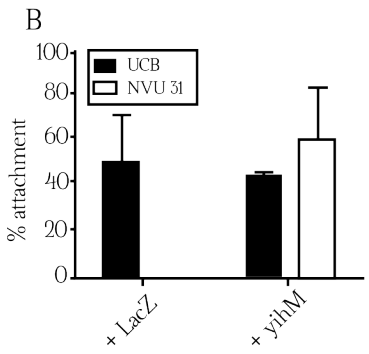
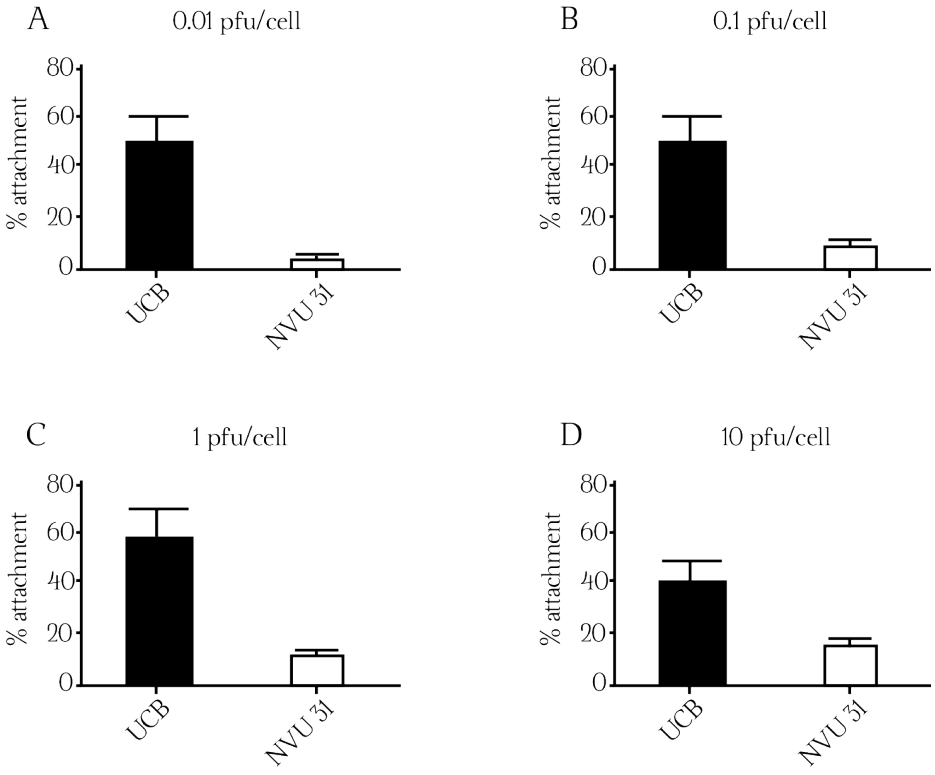


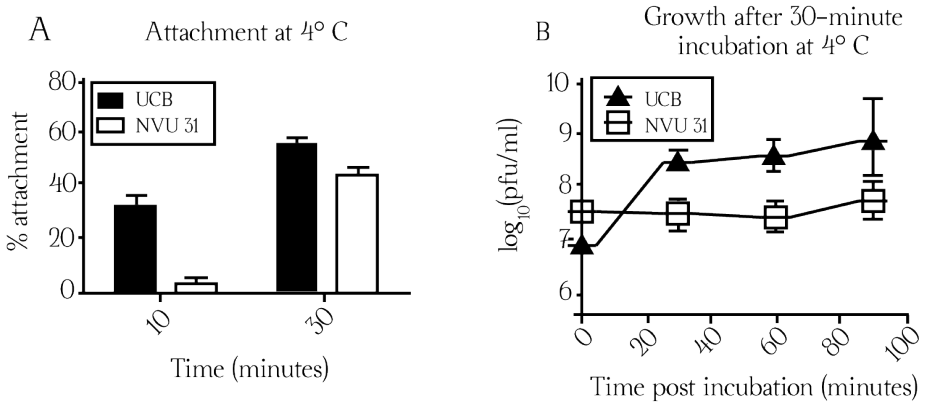
Figure 3: Attachment of T4 to UCB and NVU 31 cells using A) 0.01 pfu/cell, B) 0.1 pfu/cell, C) 1 pfu/cell or D) 10 pfu/cell. Attachment was measured at 10 minutes post addition of virus. Each experiment was conducted at least 3 times. Error bars represent SEM.



that while some of the virus attached to NVU31 after 30 minutes, it did not attach at the same level as UCB (p=0.0630) (Fig. 4A). We next tested whether the virus that had attached to the surface of NVU31 could enter and replicate within these cells. We incubated UCB and NVU31 cells with T4 for 30 minutes at 4ºC to let the virus attach and then shifted the cells to 37ºC to allow DNA entry and replication to occur. After attachment to UCB at 4ºC, T4 could enter and produce progeny when shifted to 37ºC (Fig 4B). In contrast, no progeny was produced after attachment to NVU31 cells. This suggests that while the virus can attach inefficiently to ΔYhiM cells, YhiM is also necessary for a post attachment step in viral replication.

Figure 4: Attachment of T4 at 4°C. A) % attachment after 30 minutes of attachment. B) T4 replication at 37°C after 30-minute incubation at 4°C. Each experiment was conducted at least 3 times. Error bars represent SEM.

Figure 4



DISCUSSION

These experiments describe the identification of the inner membrane protein *yhiM* as a cellular component necessary for T4 but not T1, T2, T3, T5 or ΦX174 replication. T4 resistant *E. coli* were previously described (6,9,13,16,20,25,26,28). Some of these mutations result in a block in head assembly (6, 9, 28). These mutations map to the *tabB, groE* and *mop* genes. Mutations have also been characterized that result in the delay of the onset of viral DNA replication and failure to produce late gene products (20, 25, 26). These mutations map to RNA polymerase. Some of the mutations have been linked to mutations in the LPS (13). Other mutations result in a block in T4 replication at increased temperature (16).

Bacteriophage adsorption involves three steps: initial contact, reversible binding and irreversible binding (7). If any of these three steps fail to occur, attachment will not occur and the virus will fail to replicate. Our data indicated that *yhiM* plays a role in one of these three steps. Given that multiple viruses infect our *yhiM* mutant (T1, T3, T5, ΦX174), it seems unlikely that major increases in capsule production are responsible for our lack of attachment. It is also possible that our mutants express abnormal levels of OmpC or LPS, the two receptors involved in T4 attachment on the cell surface. However, OmpC is not required for T4 attachment, so it is unlikely that changes in OmpC expression would lead to a decrease in T4 binding (11). T3, T4 and ΦX174 all use LPS as a binding site for attachment but only T4 is unable to bind to cells missing *yhiM* (8, 15). Furthermore, T3 and T4 bind to the same glucose residue on LPS (23), suggesting that the LPS synthetic pathway is not

significantly affected by a lack of *yhiM*.
Since YhiM belongs to a group of proteins regulated by RpoS, we suggest that a lack of YhiM may affect other pathways regulated by RpoS and that these pathways are necessary for normal attachment and replication of T4. YhiM is involved in regulating survival in many growth conditions, including response to increased temperature (2). T4 growth is also affected by changes in E. coli physiology and temperature (12). T4 replication is not regulated properly in the absence of RpoS (4). Since YhiM is one of many RpoS regulated proteins, one possibility is that the inhibition of replication in RpoS deficient cells is dependent on a lack of YhiM. This could be because the virus interacts directly with YhiM to promote attachment, which seems unlikely since YhiM is not on the outer membrane where attachment occurs. Alternatively, YhiM could be involved in other cell processes that are also necessary for T4 attachment. For instance, T4 replication is dependent on temperature (27), and previous T4 resistant mutants fail to replicate T4 at high temperatures (16). YhiM's role in mediating cell survival at high temperature (2) may indicate that T4 attachment depends on functional YhiM to maintain cell viability at high temperatures, therefore allowing T4 to successfully attach and replicate within *E. coli*.

We have shown that the inner membrane protein YhiM is important for efficient replication of T4 but not T1, T2, T3, T5 or ΦX174 bacteriophages. This block in replication is due to a defect in T4 attachment. Further studies will determine the mechanism by which YhiM mediates this function.

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