Chapter 5

INCORPORATION OF NON-CANONICAL AMINO ACIDS INTO FILAMENTOUS M13 BACTERIOPHAGE

The work in this chapter was performed in collaboration with A. James Link.

Abstract:

Viral particles offer well-defined structural scaffolds for the development of bio-inspired nanotechnologies. The filamentous M13 bacteriophage has been successfully used to associate proteins with their genetic information in order to screen peptide sequences for binding to target molecules. We show here that M13 viral particles containing the non-canonical amino acid azidohomoalanine in multiple coat proteins can be produced, and the introduced azide moieties used as sites for chemoselective ligation. These virions can be modified using both copper-catalyzed and strain-catalyzed azide-alkyne [3+2] cycloadditions. Biotin can be tethered through either a terminal alkyne or a cyclooctyne to the azide-bearing phage particles and detected through western analysis. Aha-labeled bacteriophage retain their infectivity after modification with strain-catalyzed [3+2] cycloaddition.

5.1 Introduction

M13 bacteriophage are filamentous viruses that prey on male bacteria. These non-lytic phage infect the bacterium through the TolA receptor on the pilus membrane, see Figure 5-1. At the onset of infection, the host bacterium's cellular processes are subsumed, and the production of viral proteins begins rapidly and efficiently. During infection, the viral coat proteins accumulate in the membrane of the cell and are ultimately extruded through the membrane to form an infectious virion containing the phage DNA. These virions are comprised of five different coat proteins. The major coat protein, pVIII, interacts with the packaged DNA while forming an elongated cylinder around it. There are approximately 3000 pVIII proteins in every M13 bacteriophage. On either end of the virion the other coat proteins are presented; in particular, pIII and pVI are required for infection of bacteria. These four coat proteins are considered the "minor" coat proteins and are present in 4-6 copies each.

Filamentous bacteria have been a tool for molecular biologists and chemists for many years. Beginning with early uses for development of the first plasmids and extending through their current utility in display of peptide and protein libraries for selection, they continue to be studied and used in novel applications.[1] Not only are bacteriophage used for biochemical studies, but they have also found biomedical use for delivery of anti-β amyloid antibodies to detect amyloid plaques *in vivo*.[2, 3] Through phage display, peptides that can initiate the nucleation of inorganic crystals and nanowires have been identified.[4-6] The stability and simplicity of M13 bacteriophage have made them useful tools for both biology and materials science. Their functionality

as well as their utility can be expanded through the incorporation of non-canonical amino acids into M13 coat proteins.

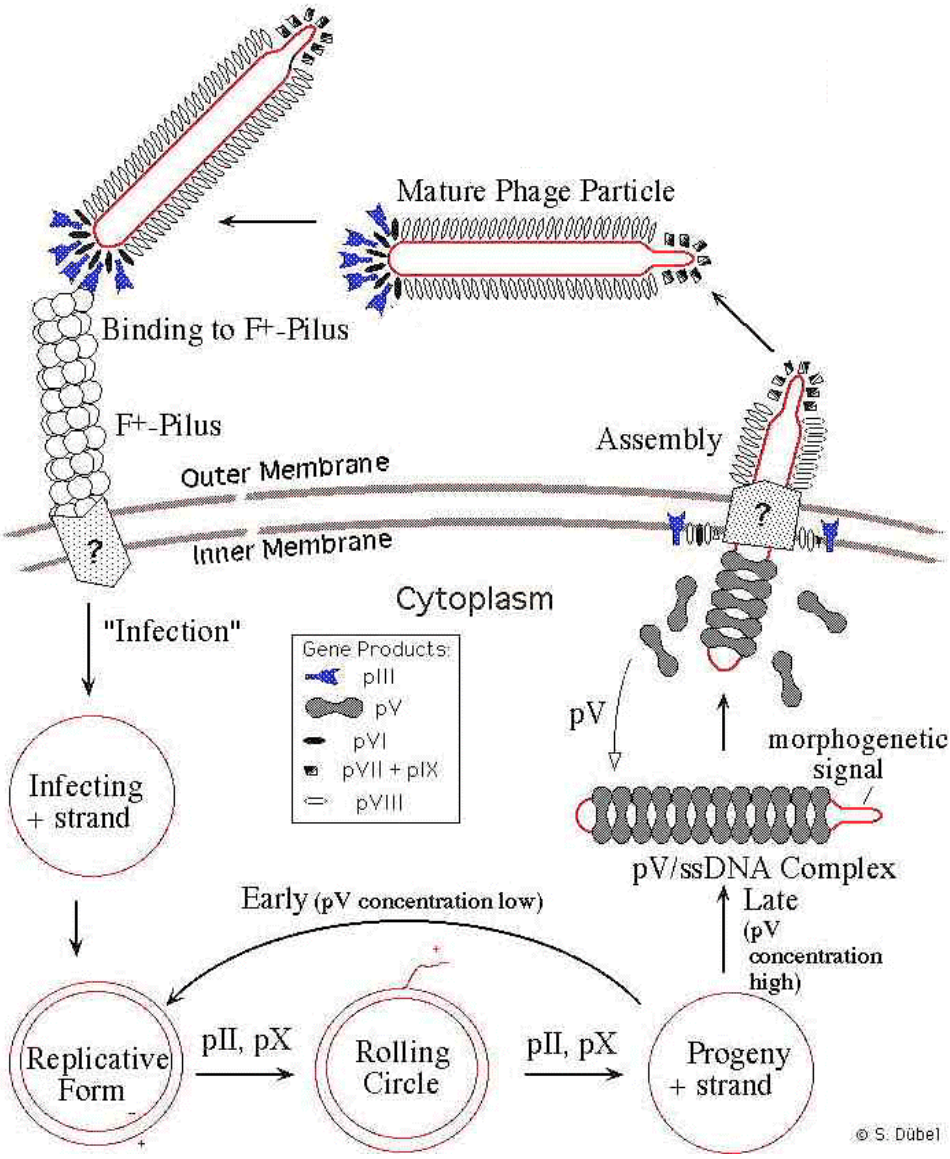

Figure 5-1. The life cycle of the M13 bacteriophage.

(from http://www.biochem.arizona.edu/classes/bioc471/pages/Lecture5/M13LiveCycle.gif)

5.1.1 Codon reassignment using Aha

The azide-bearing non-canonical amino acid Aha can act as a surrogate for methionine in recombinant proteins produced in *E. coli*.[7] In order to force the protein biosynthesis machinery to use Aha, the cells must be depleted of methionine through its removal from the growth medium and the use of a strain that is metabolically incapable of producing methionine. The incorporation of Aha into the outer membrane protein, OmpC in *E. coli* allows the presentation of azides on the cell surface.[8] The cells can subsequently be labeled with biotin via copper mediated [3+2] cycloaddition and detected with a streptavidin-horseradish peroxidase (streptavidin-HRP) conjugate.[9] This display technology was applied to the selection of mutant methionyl-tRNA synthetases that allow the incorporation of azide-bearing methionine analogues not well-activated by the endogenous synthetase.[10] The efficacy of the chemistry and the high affinity of avidin for biotin provide detection of small amounts of incorporated azidohomoalanine. This chemistry was used to label cowpea mosaic virus that was chemically derivatized with azides or alkynes.[11] The addition of azides to phage through the translational incorporation of azidohomoalanine allows the natural residues of the phage coat to remain unmodified.

5.1.2 The model pIII protein is pIII-APP

An initial goal was the localization of incorporation of Aha into one phage coat protein, ideally the low valency coat protein pIII. The pIII protein is known to tolerate the insertion of whole proteins into its outer loops for selection through binding assays. Inducible control over the expression of the pIII protein was desired to allow induction

upon the introduction of Aha to the growth medium. The phagemid vector, pJC20, encodes a recombinant pIII fusion protein containing the avian pancreatic peptide (APP) that has been inserted into phage for functional selection.[12] This model protein was chosen for its previous success with introduction into phage particles and relatively minor alteration of the phage protein.[13] This phagemid does not contain all the proteins necessary to produce phage particles, therefore it is necessary to introduce the other proteins through helper phage infection. The production of bacteriophage particles was initiated by infection with M13KO helper phage after expression of recombinant pIII.

5.1.3 Required E. coli strains

As mentioned above, metabolic incorporation of azidohomoalanine requires a methionine auxotrophic strain for expression. The M13 phage also require the F^+ pilus to infect the cells. An F^+ methionine auxotroph was developed from CAG 18491 using transposon stabilization to remove tetracycline resistance[14] and then conjugation with an F^+ strain to procure a copy of the F episome. Production of phage incorporating Aha was monitored by Western blot after copper-catalyzed azide-alkyne [3+2] cycloaddition, and infectivity was determined by transduction of kanamycin resistance. Aha-modified phage were determined to retain their infectivity; however, after copper-catalyzed chemistry, the infectivity was completely suppressed. A copper-free variant of the Huisgen cycloaddition, strain-catalyzed [3+2] cycloaddition,[15] was effective in producing infective biotinylated phage.

5.2 Materials and Methods

5.2.1 Materials

The phagemid vector pJC20 was obtained from Prof. Alanna Shepartz at Yale University. The helper phage, M13K07, were purchased from Amersham Biosciences. Azidohomoalanine was synthesized as previously reported.[9] *E. coli* strain ER2738 was obtained from New England Biolabs. Streptavidin-HRP, anti-E-tag-HRP and the Western development reagents were purchased from Amersham Biosciences.

5.2.2 Strains

The *E. coli* methionine auxotrophic strain, CAG18491, was co-transformed with pJC20 and pLysS-IQ by electroporation. The auxotrophy of the resulting CAG[pJC20/pLysS-IQ] cells was then stabilized, and the tetracycline transposon removed through growth in nonselective media and subsequent selection on chlortetracycline and fusaric acid media.[14] Following the loss of tetracycline resistance, the CAG[pJC20/pLysS-IQ] cells were mated with ER2738 for three hours at 37°C in order to transfer the F episome from ER2738 to CAG[pJC20/ pLysS-IQ]. Aliquots of the mating mixture were then plated on LB agar plates containing tetracycline to select the cells that had received a copy of the F-episome and ampicillin and chloramphenicol to separate CAG[pJC20/pLysS-IQ] from the ER2738. The resulting colonies were then cultured and tested for both methionine auxotrophy and resistance to tetracycline. A suitable strain was identified, stocked, and named CAGF+[pJC20/pLysSIQ].

5.2.3 Phage production

Single colonies of CAGF+[pJC20/pLysSIQ] were cultured overnight in M9+20 amino acids media and then diluted into 60 mL of 1x M9 media with 20 amino acids (40 mg/L) (M9+20). The culture was grown to an OD_{600}=1 and the cells centrifuged at 6000x g for 7 minutes. The cells were resuspended in one volume of M9 media + 19 amino acids and incubated with agitation for 10 minutes at 37°C. The cells were again pelleted and finally resuspended in 1 volume of M9 media with 19 amino acids, ampicillin (200 mg/L), chloramphenicol (35 mg/L), tetracycline (20 mg/L) and 1 mM IPTG. The resuspended cells were then separated into 3 flasks containing 40 mg/L Met, 40 mg/L Aha or no 20^{th} amino acid. Protein expression continued for 3 hours, and then super-infection with helper phage was begun by adding 2.4 x 10^{11} tu to the 20 mL of each culture. After one hour of infection, the medium was removed again and the cells resuspended in the final medium, M9+20, and kanamycin (35 mg/L) was added to the roster of antibiotics added. This culture was grown overnight, and the supernatant was retained after the cells were pelleted at 6000 rpm for 7 minutes at 4°C.

5.2.4 Phage precipitation

One-sixth volume of PEG-NaCl (20% PEG, 2.5 M NaCl) was added to the supernatant from phage preparation before incubation for at least 24 hours at 4°C. The precipitate was then collected at 9000 x g for 10 minutes at 4°C. The precipitated phage were resuspended in 1 mL phosphate-buffered saline (PBS), pH=7.4 and centrifuged at

4°C and 10,000 rpm for 5 minutes. The supernatant was retained and 1/6th volume PEG-NaCl added again. This mixture was incubated on ice for one hour, then centrifuged in a Beckman refrigerated microcentrifuge for 15 minutes at 10,000 x g. The supernatant was discarded and the phage pellet resuspended in 200 μl PBS, pH=7.4. A short final spin removed any remaining insoluble material.

5.2.5 Azide-alkyne copper-catalyzed [3+2] cycloaddition on precipitated phage particles

An aliquot (40 μL) from the resuspended phage solution was diluted to 200 μl in phosphate-buffered saline (1x PBS), pH=7.4. For copper bromide catalysis, the triazole ligand, tris-(benzyltriazolylmethyl)amine (TBTA, 200 μM final concentration), biotin PEO-propargylamide (50 μM) and approximately 10 μL of a fresh aqueous suspension of 40 mM CuBr were added to the phage solution. Similarly, 200 μM CuSO4, 200 μM TBTA and 400 μM TCEP were used for copper-sulfate catalysis. The total approximate yield of phage in one infection experiment was 10^8 transducing units. One-fifth of the total yield of phage was used for each copper-catalyzed reaction, thus approximately 2 x 10^7 transducing units were present. The reaction was allowed to proceed for 16 hours at 4°C with agitation. After the reaction, the precipitate from the reaction was pelleted for 1 minute in a microfuge and the supernatant retained.

5.2.6 Tricine gel electrophoresis and Western analysis

Phage particles labeled with PEO-biotin were diluted 1:1 in tricine loading buffer and boiled for 15 minutes before electrophoresis on a 12% tricine gel at 165 V. The transfer and detection were carried out as previously reported.[8] The phage proteins

were transferred to a nitrocellulose membrane, and the membrane was then blocked with 5% milk solution in PBS/Tween for one hour. The membrane was washed with PBS/Tween and probed with an avidin-HRP conjugate for one hour at room temperature. The membrane was again washed and the protein bands visualized with Western blot detection reagents.

5.2.7 Titering of bacteriophage by transduction of kanamycin resistance

ER2738 cells were grown to an OD_{600} of 0.8 and 70 μl of cell suspension was mixed with 2 μl of phage solution. The mixture of cells and phage was incubated for one hour and 45 minutes before serially diluting to 10^{-6}. The dilutions were plated on LB + tetracycline and kanamycin plates and grown overnight at 37°C. The count of resulting colonies corresponds to the number of diluted infectious phage.

5.2.8 Strain-catalyzed azide-alkyne [3+2] cycloaddition on Aha-bearing bacteriophage

Aha-bearing phage in 1x PBS, pH 7.4 were diluted 5 fold into 100 μL of 1x PBS, pH 7.4. To this solution, 5 μL of 5 mM cyclooctyne-biotin in DMSO was added. The reaction was incubated overnight at 37°C, while rotating. The bacteriophage were again precipitated with PEG-NaCl, resuspended in 1xPBS and used immediately for Western analysis and titering.

5.3 Results and Discussion

5.3.1 Creation of methionine auxotrophic strain bearing an F episome

M13 bacteriophage will only prey upon bacteria bearing a pilus, an appendage used for conjugation and transfer of genetic material between bacteria. The production of bacteriophage from an *E. coli* strain requires that it carry the F episome. Similarly, the maximal replacement of a natural amino acid with a non-canonical one in recombinant proteins requires the use of an *E. coli* strain deficient in the synthesis of the natural amino acid.[7, 16-18] Consequently, a strain with both methionine auxotrophy and an F episome was needed to effectively produce bacteriophage particles modified with the methionine analog, azidohomoalanine. The methionine auxotroph CAG14891 contains a tetracycline transposon in the MetE gene, which encodes a transmethylase that converts homocysteine into methionine. This strain was cured of the tetracycline transposon through growth in non-selective liquid media and subsequent plating on fusaric acid and chlortetracycline plates.[14] Cells presenting tetracycline resistance are sensitive to the fusaric acid and chlortetracycline, so any colonies on those plates are bacteria that have lost the tetracycline resistance. This procedure was necessary to allow selection for the transfer of the *tetR*-containing F-episome from *E. coli* strain ER2738 to CAG14891. A culture of the now tetracycline-sensitive strain CAG14891 was transformed with plasmids pJC20 and pLysSIQ to allow separation of the pilus-bearing CAG14891 cells from ER2738 cells, and selected transformants mixed with a culture of ER2738. Aliquots of the mixture were spread on plates containing tetracycline, chloramphenicol and ampicillin to select only those CAG14891 cells that had received an F episome. A

few of the resulting colonies were selected and checked for retention of methionine auxotrophy.

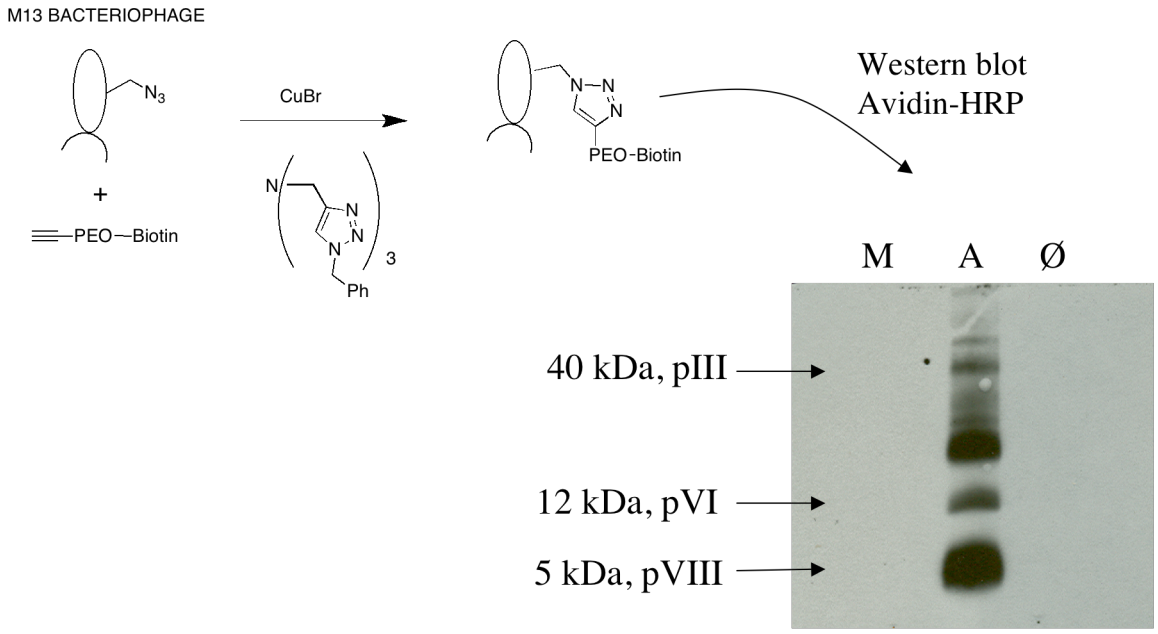

Figure 5-2. Western analysis of bacteriophage produced with methionine (M), azidohomoalanine (A) or no amino acid (Ø) during induction of pIII expression. Modification of bacteriophage virions with propargyl-PEO-biotin through copper-catalyzed azide-alkyne [3+2] cycloaddition is dependent on the presence of Aha during overexpression of pIII from phagemid pJC20 before infection with helper phage.

5.3.2 Production of Aha-bearing phage

The strain described above, CAGF+[pJC20/pLysSIQ] was grown in minimal media containing all 20 amino acids until reaching mid-log phase growth. At that point, the cultures were shifted into minimal medium containing the non-canonical amino acid Aha, and production of the recombinant avian pancreatic peptide- pIII fusion protein was induced with IPTG. This induction phase was carried out for 3 hours to allow the

production of recombinant protein, after which the culture was again medium shifted into M9+20 and infected with helper phage. This second medium shift was intended to promote the localization of Aha to the recombinant phage protein alone. After an overnight period of phage production, the cells were pelleted and the supernatant retained for isolation of the virions. The virions were precipitated and concentrated using PEG-NaCl. Confirmation of the presence of Aha in those coat proteins requires the ligation of affinity tags through chemistry specific to the non-canonical amino acid.

5.3.3 Attachment of affinity tag to Aha-bearing bacteriophage

Bioorthogonal reactions utilizing non-biological functionalities allow the specific introduction of exogenous molecules within the chemically complex milieu of proteins, nucleic acids and other cellular debris.[19-21] In particular, the azide-alkyne dipolar cycloaddition has proven both specific and effective for the creation of bioconjugates under mild conditions and in high yield.[22-26] In order to ascertain the incorporation of Aha into the coat proteins of the phage as produced above, a propargyl-PEO-biotin tag was incubated with the phage in the presence of copper (I) bromide as catalyst and TBTA ligand. The phage were incubated overnight at 37°C with agitation in the reaction mixture. After incubation, the insoluble particles were removed by centrifugation and the solution used directly for Western analysis. Figure 5-2 shows a representative blot of ligated phage proteins detected with streptavidin-HRP. Signal is observed only after treatment of the host bacteria with Aha. No signal appears in the methionine or 19 amino acid control lanes.

5.3.3 Comparison of copper sulfate and copper bromide as catalysts

The azide-alkyne [3+2] cycloaddition is catalyzed by copper (I), which can be delivered as either a copper (I) salt or by reduction of a copper (II) salt. Prior experiments have shown that the labeling of outer membrane proteins on the surface of *E. coli* cells was enhanced through the use of copper [I] bromide in place of copper [II] sulfate and TCEP.[9] The efficacy of labeling Aha-bearing phage particles with biotin through azide-alkyne [3+2] dipolar cycloaddition using either copper bromide or copper sulfate as a catalyst was tested. Figure 5-3a illustrates that the use of copper bromide rather than copper sulfate results in enhanced modification of the phage particles. This enhanced modification was not due to decreased specificity of the reaction, as shown by the lack of signal in either of the negative control lanes of the Western blot shown in Figure 5-3a.

5.3.4 Infectious phage titer after incubation with Aha

Phage particles produced after induction of recombinant pIII in the presence of Met, Aha and nineteen amino acids were titered to determine infectivity in transducing units with strain ER2738 after PEG-NaCl precipitation. As shown in Figure 5-4, the titer for phage produced with Aha present during pIII expression was comparable to those produced with Met, which indicates that the introduction of Aha into recombinant pIII does not adversely affect phage particle production. The titer of phage produced with only nineteen amino acids during pIII expression was barely lower than that produced during Met or Aha incubation. Presumably, infectious particles were produced due to the presence of all twenty natural amino acids during the overnight phage production.

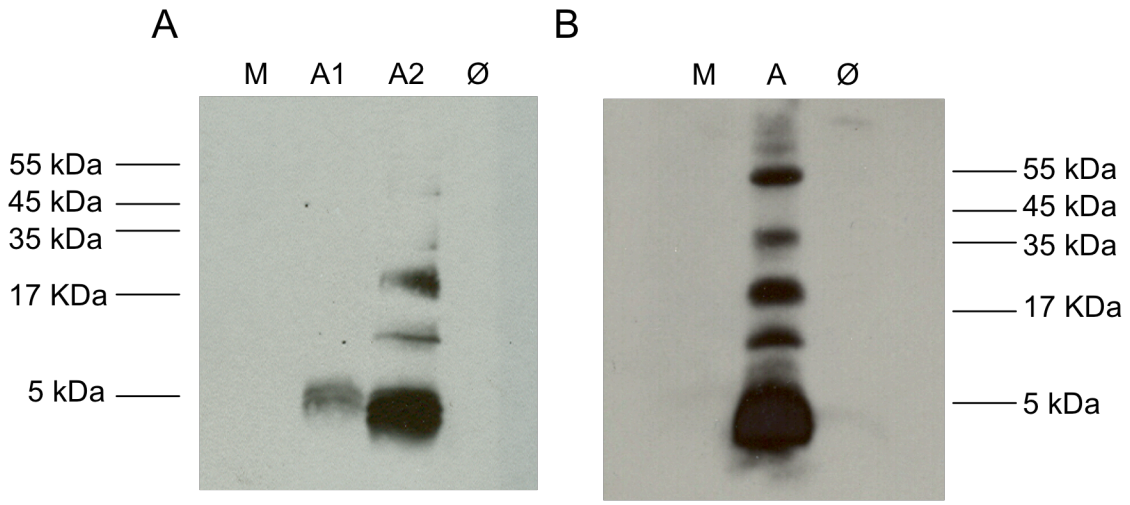

Figure 5-3. Comparison of catalysts for the azide-alkyne ligation of propargyl-PEO-biotin to Aha-enhanced bacteriophage proteins. A) Western analysis of bacteriophage produced with methionine (M), azidohomoalanine (A) or no amino acid (Ø) during induction of pIII expression. The methionine, A2 and no amino acid phage were treated with copper bromide conditions for the ligation of propargyl-PEO-biotin while copper (II) sulfate was used for catalysis of sample A1 with the alkyne biotin. B) Western analysis of bacteriophage produced as above. All samples were treated with cyclooctyne-biotin for the strain-catalyzed ligation of alkyne-biotin. The specificity of the reaction is borne out by signal only appearing in the Aha- treated lane. Streptavidin-HRP was used for the detection of both blots.

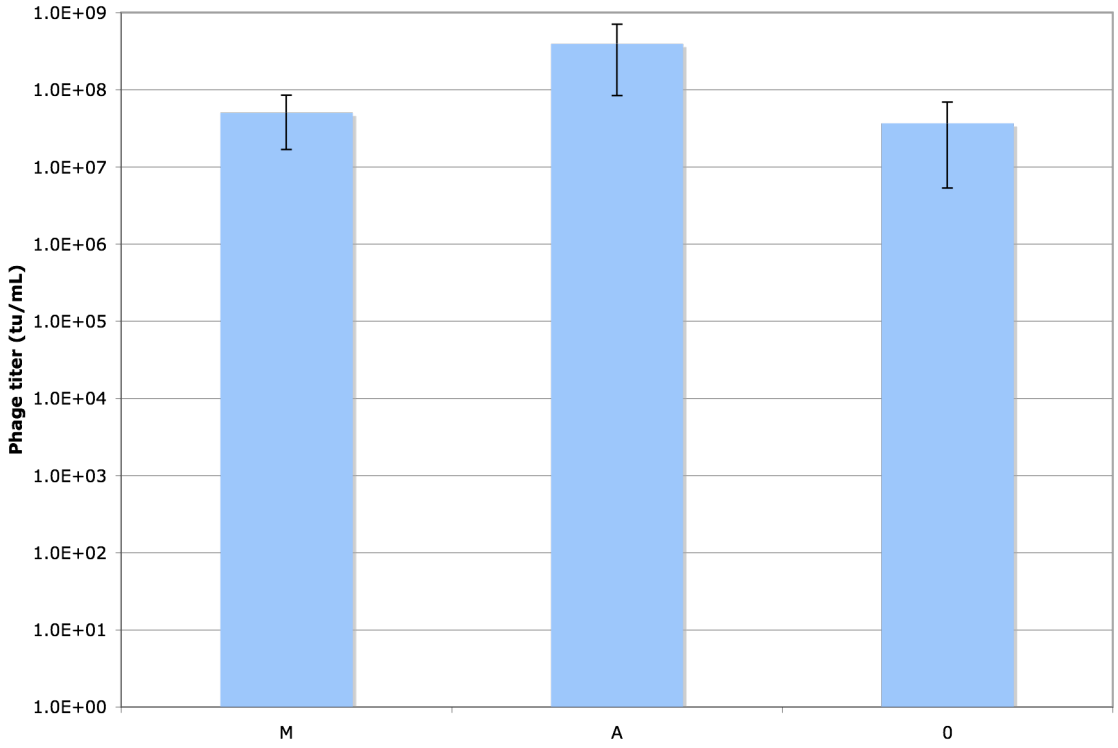

Figure 5-4. Infectious titer of bacteriophage produced with methionine (M), azidohomoalanine (A) or no amino acid (Ø) during induction of pIII expression. Infectious phage are produced with both Aha and Met during expression of pIII.

5.3.5 The effect of copper chemistry on phage titer

Treatment of the phage with either copper bromide or copper sulfate resulted in obliteration of the infectivity of the Aha-bearing bacteriophage. Removal of the copper by centrifugation of the reaction mixture and separation of the supernatant for PEG-NaCl precipitation was not sufficient to restore infectivity to the phage particles. Bertozzi and coworkers have devised a clever method to circumvent copper toxicity: strain-based catalysis of the Huisgen dipolar cycloaddition.[15, 27] Using cyclooctyne compounds, they found that molecular strain can be used to form a triazole between a cycloalkyne and an azide. Aha-bearing phage were successfully modified with the cyclooctyne-biotin

affinity tag synthesized by the Bertozzi laboratory (Figure 5-3b), and these phage retained their infectivity, as shown in Figure 5-5.

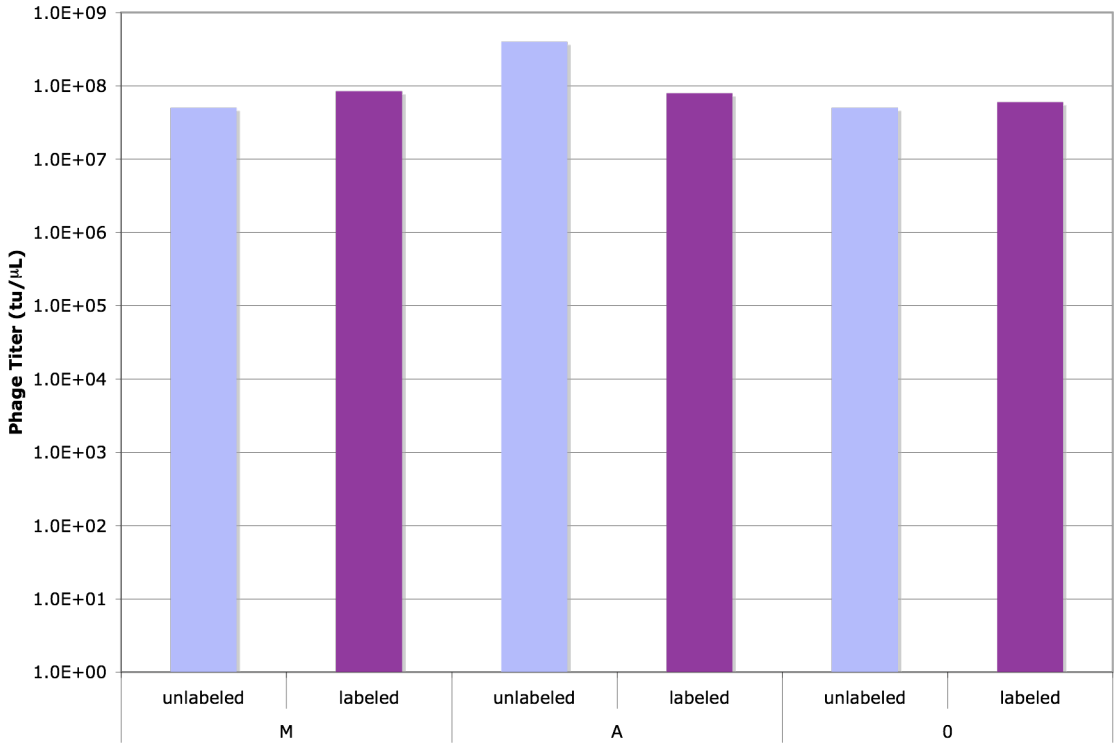

Figure 5-5. Bacteriophage modified with cyclooctyne-biotin remain infectious. The amino acid present during pIII expression is noted under the column labels: methionine (M), azidohomoalanine (A) or no amino acid (Ø). The blue bars represent the unlabeled phage titer for each of the three expression cultures. The purple bars represent the phage titer after labeling with cyclooctyne-biotin overnight.

5.4 Conclusions

A methionine auxotrophic strain carrying the F episome was created and called CAG-F+. It was transformed with pJC20 and pLysSIQ to produce recombinant pIII. In this strain, after induction of APP-pIII in the presence of Aha and infection with helper phage, infectious and modifiable phage particles could be isolated from the culture medium. The Aha-bearing virions were modified using both copper bromide- and copper sulfate-catalyzed Huisgen cycloaddition with an alkyne-biotin tag, as detected by Western analysis. Copper bromide gave more complete labeling than copper sulfate, but both catalysts completely obliterate phage infectivity. Copper-free addition of biotin through a cyclooctyne-biotin label allowed the retention of infectivity by the modified phage.

The localization of incorporation of the non-canonical amino acid to only one of the phage coat proteins would be a welcome extension of this work. Nonetheless, this work has shown that non-canonical amino acids can be globally incorporated into M13 bacteriophage without harming the infectivity of the resulting particles. These modified virions offer a new scaffold for the creation of novel bioconjugates.

5.5 References

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