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Enabling Glycosyltransferase Evolution: A Facile Substrate-Attachment Strategy for Phage-Display Enzyme Evolution

Kerry Routenberg Love,^[a] Jonathan G. Swoboda,^[a] Christopher J. Noren,^[b] and Suzanne Walker^{*[a]}

Directed enzyme evolution has the potential to generate novel catalysts able to compete with chemical methodologies in the synthesis of complex biomolecules. Directed evolution is often carried out in cells,^[1] but for many classes of enzymes, such as glycosyltransferases, it is difficult to couple enzymatic activity to a cell-based selection. M13 phage display^[2] is an in vitro methodology that potentially enables the selection of enzymes that do not provide cell-based phenotypes.^[3] In order to select phage-bound enzymes based on catalytic activity, the desired substrate needs to be immobilized near the displayed enzyme to allow for affinity capture of the desired product. Schultz and co-workers developed the first catalysis-based display method, in which the substrate molecule was attached next to the displayed enzyme by a coiled-coil interaction.^[4] Although this approach is promising, only a handful of enzymes have been displayed on phage in an active form, and there are still no facile, general methods for substrate immobilization on the phage (vide infra).^[5] Here we introduce a chemically straightforward method for substrate attachment using selenocysteine (Sec) residues. Implementation of this method required construction of a phagemid/helper phage system that allows the display of pIII fusions bearing Sec residues adjacent to a displayed enzyme. We also report the first display of an active glycosyltransferase on phage. This work lays the foundation for evolving glycosyltransferases to make novel glycoconjugates and should also enable the directed evolution of other enzyme classes for which ex vivo selection strategies are desirable.

Phage display is a convenient strategy to link phenotype and genotype; it enables amplification and identification of peptides and proteins following an in vitro selection.^[2] In phage-display enzyme evolution, proximal substrate attachment can be accomplished by using an M13 phagemid/helper phage system in which two different types of pIII fusions are presented on the phage surface (see Scheme 1). The enzyme is

displayed on a pIII fusion expressed from the phagemid; the adjacent pIII peptides expressed from the helper phage genome provide potential sites for substrate attachment. Widespread implementation of phage-display enzyme evolution requires a method of modifying a uniquely reactive handle with a functionalized substrate that can be accessed with minimal synthesis.

One of us (C.J.N.) has shown recently that selenocysteine (Sec), which is encoded by the TGA codon, can be expressed on phage by fusing a cassette consisting of a TGA codon followed by a selenocysteine insertion sequence (SECIS) to the 5' end of gIII.^[6] Because Sec is considerably more nucleophilic than cysteine and reacts at lower pH (pK_a of 5.2 versus 8.1 for Cys),^[7] Sec-bearing phage can be derivatized selectively in the presence of other potential side-chain nucleophiles.^[6] Encouraged by these results, we wanted to evaluate selenocysteine as a handle for substrate immobilization on phage displaying a pIII-enzyme fusion. We prepared helper phage encoding pIII bearing an N-terminal Sec residue by performing a vector swap between a M13KE vector containing the SECIS insert^[6] and M13KO7 using the *PacI* and *BsrGI* sites.

We also prepared a phagemid (pMurG-pIII) encoding the *E. coli* glycosyltransferase (Gtf) MurG, an enzyme essential in bacterial cell-wall biosynthesis.^[8] The *murG* gene was subcloned from a pET-21a expression vector (Novagen) into the pFAB5cHis.TT.HUI phagemid vector as a fusion to the 3'-end of a *pelB* leader sequence and the 5'-end of a truncated gIII. MurG was chosen as starting point to explore enzyme evolution by phage display because it is a member of the GT-B superfamily of Gtfs that have related three dimensional structures but different substrate selectivity.^[9] Many members of the GT-B superfamily are involved in antibiotic biosynthesis,^[10] and the ability to alter the substrate selectivity of these Gtfs could enable the production of new biologically active glycoconjugates that are synthetically difficult to access.^[11]

Infection of cultures containing the pMurG-pIII phagemid with the Sec-expressing helper phage resulted in the production of phage bearing both the enzyme and the Sec handle on the same end of the phage particle (Scheme 1). As a negative control for Sec modification, we also prepared phage expressing wild-type pIII by infecting bacteria containing the pMurG-pIII phagemid with the commercially available M13KO7 helper phage. All phage were purified by using CsCl gradient centrifugation.^[12]

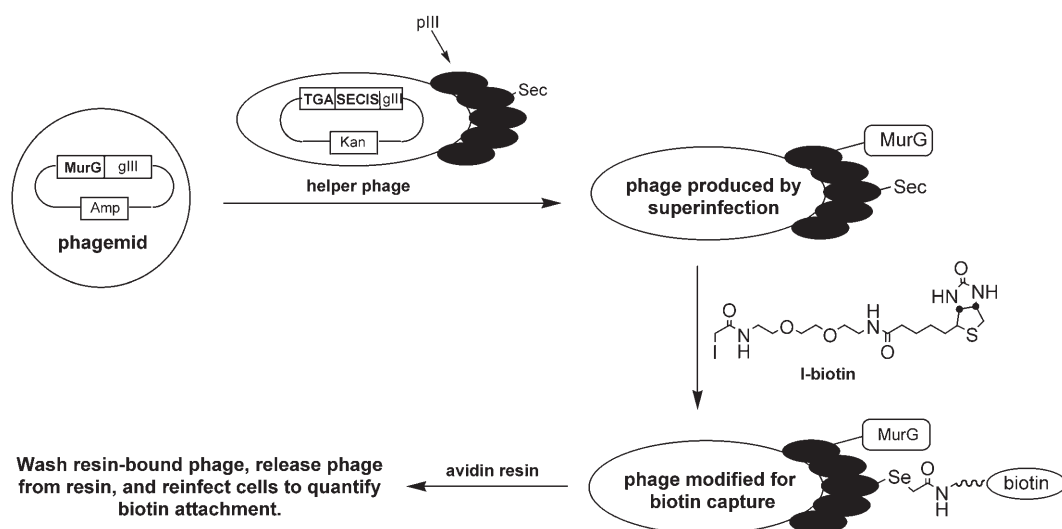
The activity of phage-bound MurG was established by incubating phage with UDP-[¹⁴C]-GlcNAc and a biotin-labeled lipid I analogue.^[8] Product formation was monitored by spotting the reaction mixtures onto streptavidin membranes at various time points (Scheme 2). The membranes were washed and counted to evaluate the product formation. Radioactive counts above background were detected on the membranes within a few minutes and continued to increase for approximately 1 h before leveling off (Figure 1). Using the specific activity of the radiolabeled UDP-GlcNAc (30 mCi mmol⁻¹), we estimate that 0.1 μ M product was formed within an hour; this means that each phage-bound MurG performed between 10³ and 10⁴ turnovers. Only a few phage-bound enzymes reported have been

[a] Dr. K. R. Love,⁺ J. G. Swoboda,⁺ Prof. S. Walker
Department of Microbiology and Molecular Genetics
Harvard Medical School
Boston, MA 02115 (USA)
Fax: (+1) 617-738-7664
E-mail: suzanne_walker@hms.harvard.edu

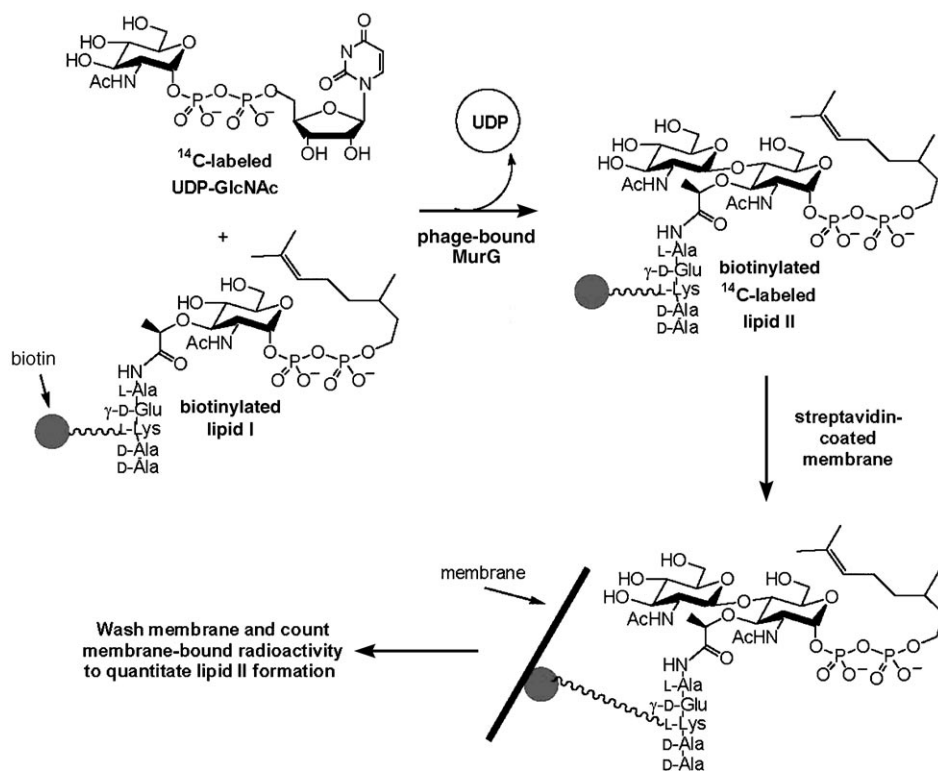
[b] Dr. C. J. Noren
New England Biolabs
Ipswich, MA 01938 (USA)

[*] These authors contributed equally to this work.

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Scheme 1. Method for concomitant enzyme and substrate display on phage (SECIS = selenocysteine insertion sequence, Kan = kanamycin, Amp = ampicillin).



Scheme 2. Biotin-capture assay for MurG activity. Phage particles displaying MurG were incubated with 33 μM UDP-[¹⁴C]-GlcNAc (specific activity = 30 mCi mmol⁻¹), 54 μM biotinylated lipid I, and 20 units alkaline phosphatase in a buffer (50 mM HEPES, 5 mM MgCl₂, pH 7.9; 20 μL total reaction volume). Reaction mixtures were spotted onto streptavidin-coated membranes (SAM²® Biotin Capture Membrane, Promega; binding capacity > 4 nmol biotin per cm²). Membranes were washed with TBS/Tween 20 (0.01%; 4 × 100 mL), TBS (4 × 100 mL), water (2 × 100 mL), and 20% ethanol (100 mL) prior to counting bound radioactivity.

characterized in solution^[13] because doing so requires both a sensitive assay and a robust enzyme capable of performing many turnovers. The display of active MurG on phage bodies well for phage-display evolution of related glycosyltransferases.

To demonstrate the presence of Sec in the pIII fusions, phage particles were treated with an electrophilic, selenol-specific reagent, iodoacetyl-PEO₂-biotin (I-biotin, Pierce), at pH 5.2 for 1 h at 25 °C.^[6] The reaction mixtures were immunoblotted, and a band corresponding to the expected biotinylated pIII fusion was observed in the Sec-derived phage that had been treated with I-biotin, but not in the M13K07-derived control phage (Figure 2).

The utility of the Sec-modification method depends on whether phage can be captured following derivatization of the Sec-pIII fusion. Capture of modified phage enables enrichment over background during the course of a selection. Substrate attachment was conducted by using I-biotin, as described above, and excess I-biotin was quenched by the addition of β -mercaptoethanol. As a control, reaction conditions were also applied to M13K07-derived phage, which does not contain a reactive handle. The treated phage were poly(ethylene glycol) precipitated for 1 h at 4 °C, resuspended in TBS (25 mM Tris-HCl, pH 7.4, 140 mM NaCl, 2.5 mM KCl) and incubated for 15 min at 37 °C with Soft-link soft-release avidin resin (Promega, biotin-binding capacity: 30 nmol mL⁻¹) blocked with bovine serum albumin and

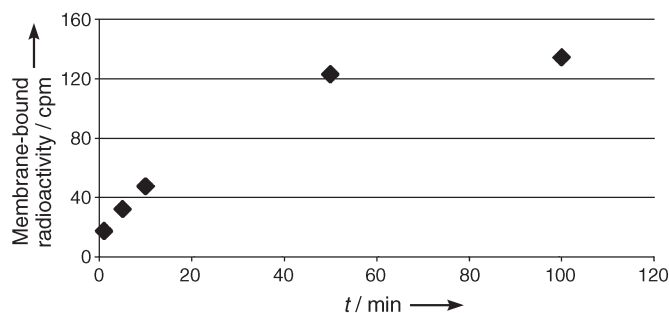


Figure 1. Time course for the formation of lipid II by MurG displayed on a Sec-derived phage, as described in Scheme 2. Phage was used at a concentration of 1.85×10^9 cfu μL^{-1} . Background (15 cpm) was subtracted from each data point.

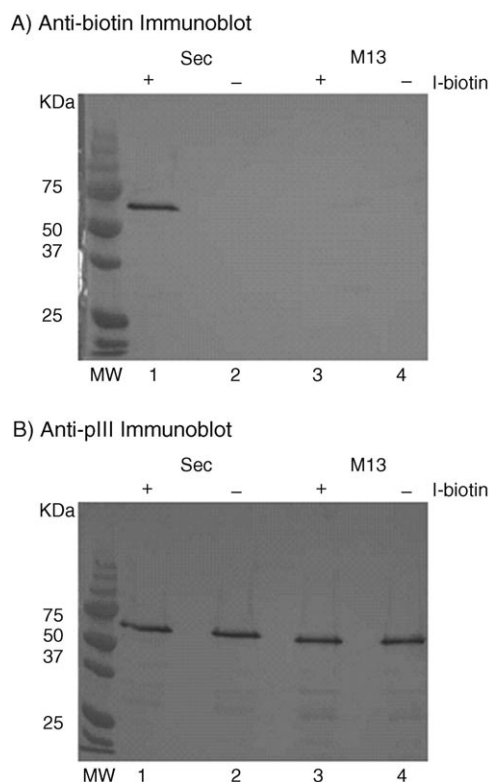


Figure 2. Immunoblots of biotinylated phage from reaction mixtures containing Sec- and M13KO7-derived control phage with and without I-biotin. Samples were run on a 10% SDS PAGE gel. Biotinylation was probed with HRP-conjugated anti-biotin antibody (Cell Signaling Technologies). Gels were visualized by chemiluminescence. MW = Precision Plus Protein Standard, BioRad; lane 1: Sec + I-biotin, lane 2 = Sec, lane 3: M13 + I-biotin, lane 4: M13.

M13KO7 helper phage, which lacks ampicillin resistance. The resin then was washed with TBS containing 0.01 % Tween 20 ($13 \times 200 \mu\text{L}$), followed by TBS ($2 \times 200 \mu\text{L}$).

The number of phage immobilized on the beads was determined under two sets of conditions. First, we titered phage after resuspending the beads in TBS and infecting diluted overnight cultures of *E. coli* NovaBlue (Novagen) directly with the bead suspension. Infected cultures were grown on ampicillin plates to determine the colony-forming units (cfu) per mL of

liquid culture. We also titered after releasing the phage from the beads using a biotin solution. The cfu values were compared to the number of input phage, that is, phage present after attachment and precipitation steps, but before bead capture. This comparison enabled us to determine the percentage of recovered phage in the presence and absence of I-biotin (Figure 3). The percentage of recovered phage was normalized within each set of data by comparing percentages as a ratio with the background recovery.^[14]

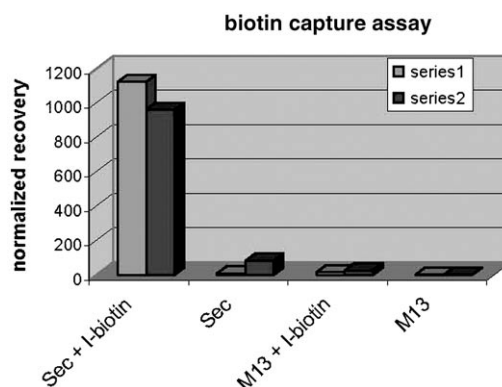


Figure 3. Normalized recovery of phage following chemical modification and biotin capture on avidin resin. Recovery was normalized by using input titer, that is, the number of phage present after attachment and precipitation steps, for each type of phage and set of reaction conditions.^[14] The four column pairs show relative rates of recovery for Sec- and M13KO7-derived phage following incubation with I-biotin. Two data sets from phage prepared independently are shown.

As shown in Figure 3, the recovery of biotin-treated Sec phage was significantly greater (100-fold or more) than the recovery of untreated Sec phage or biotin-treated M13KO7 control phage. Recovery of Sec-derived phage is tenfold higher when phage are released from the beads (data not shown); this indicates that the infectivity of bead-bound phage is compromised, as previously reported.^[4] These results demonstrate the efficiency of Sec modification by solution-phase substrates. Our method has important advantages over earlier methods for selective pIII modification. Other methods use large targeting elements for substrate immobilization; therefore they are limited in the molar quantities of substrate available for an attachment reaction. Large amounts of Sec-reactive substrates can be procured and used since their synthesis requires only one step—condensation of a substrate-bound amine with iodoacetic acid. In Gtf evolution, it is especially important to have a chemically straightforward method that is compatible with a wide variety of functional groups, as glycosyl donors and acceptors are densely functionalized biomolecules. We anticipate that the Sec-derived helper-phage system described here will prove generally useful for phage-display evolution of enzymes.

Directed evolution of glycosyltransferases has become an important goal since Gtfs with altered substrate selectivity could be used for the chemoenzymatic synthesis of complex carbohydrate-containing molecules. The GT-B superfamily of

glycosyltransferases is the most promising prototype for Gtf evolution due to the diversity of substrates utilized by this class of enzymes. In this paper, we have demonstrated that MurG, a GT-B family member, can be displayed in its active form on phage. We have also developed a phagemid/helper-phage system that should enable efficient substrate immobilization. This method can be easily adapted for any display technology, including M13 and T7 phage display, ribosome display, and cell-surface display. With active phage-bound enzyme and a strategy for substrate immobilization in hand, we can begin to evaluate whether a donor-bound or acceptor-bound approach is better for a successful glycosyltransferase evolution.

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[14] This treatment of data is best represented by the following equation: ((normalized immobilized phage/input phage) × 100)/background observed.

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