

# Targeting anti-HIV-1 Peptides to Mucosal Surfaces with Commensal Probiotic Microbes

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## Abstract

Forty million people currently live with HIV-1 infection or AIDS with 5 million being new infections. These new infections occurred in countries that are poverty stricken, do not support sexual education and condom use, and are often adulterous and homophobic societies. Although, researchers strive to create an effective vaccine, there is no cure for HIV-1 infection. HIV-1 infected individuals rely heavily on antiretroviral drugs to prevent HIV-1 infection from developing into AIDS. These drugs are extremely expensive; therefore many impoverished communities do not have access to these therapies. Due to the expense of antiretroviral drugs, inadequate condom usage, intolerance of sexuality, and new scientific insights, new innovative therapies are needed for prevention of HIV-1.

There is an urgent need for agents that can block HIV infection at the primary sites of transmission; gastrointestinal and urogenital mucosa. On the mucosal surface, there are native microbes (flora) that assist in several metabolic functions, inhibit growth and metabolism of foreign organisms, and act as the first line of defense for invasion through the mucosal surfaces. Unfortunately, HIV-1 can successfully elude the flora. If the flora is improved or genetically enhanced, it may provide an adequate line of defense against HIV-1 infection. Therefore, we propose to use genetically modified, commensal microbes that secrete anti-HIV-1 peptides for this purpose.

Recently we have genetically altered the probiotic bacterial *E. coli* strain Nissle 1917 and the fungal *S. boulardii* strain Sb49 to enable both microbes to secrete HIV-1 fusion-inhibiting peptides. *E. coli* Nissle 1917 can express and secrete large quantities of HIV-1 fusion-inhibiting peptides fused to the carboxyterminal signal sequence of hemolysin A. *S. boulardii* also express HIV fusion-inhibiting peptides and secrete by route of the alpha-factor secretory pathway. The secreted peptides from both organisms successfully prevent HIV-1 infection *in vitro*. Moreover, *E. coli* Nissle 1917 colonize the mucosal surfaces of the rectum, colon, cecum, duodenum, ileum, jejunum, and vagina in mice. Bacteria isolated from the feces of the mice several months after initial colonization contain Nissle 1917 that still secrete HIV-1 fusion-inhibiting peptides. Genetically engineered probiotic microbes can successfully colonize the gastrointestinal

## Development of Anti-HIV Microbes

### AIM:

Develop commensal microbes that secrete anti-HIV peptides and can colonize the gut, vagina and mouth.

### Uses

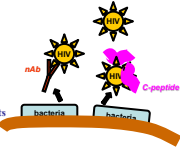
- Block new infections
- Prevent rebound
- Vaccination
- Peptide production

### Hosts

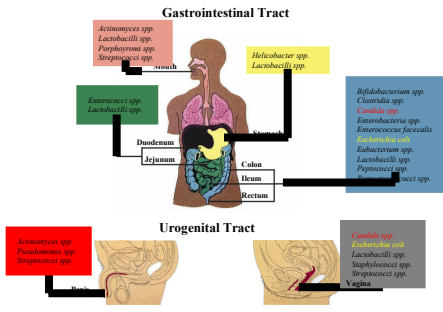
- Commensal nonpathogenic species that normally colonize cervico-vaginal and gastrointestinal tracts
- Bacteria
- Yeasts

### Secretion system

- Cell surface or complete secretion
- Type I, II, III or IV

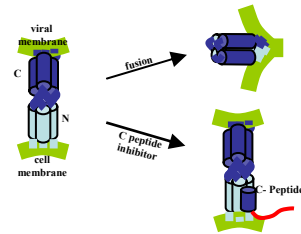


## A Potential Method of Targeting anti-HIV1 peptide to the mucosal surface: Commensal Microbes



Early HIV1 infection and viral replication happens primarily in mucosal surfaces of the gastrointestinal tract, vagina, and oral cavities. There are many commensal microorganisms, including *E. coli* and yeast, that live in these areas of the body.

## C Peptides are Potent Inhibitors of HIV Fusion



In the pre-hairpin intermediate, the C-terminal region of gp41 is anchored in the viral membrane and the N-terminal region is inserted into the host cell membrane. In the absence of inhibitor, this transient state collapses into a trimer-of-hairpins that brings the N- and C-terminal regions into proximity, promoting fusion of the viral and cellular membranes. In the presence of a C-peptide, fusion is inhibited by binding of the peptide to the exposed N-terminal region of gp41, thereby preventing formation of the trimer-of-hairpins.

## Design of Secretable HlyA Peptides Containing HIV Fusion Inhibitor Sequences

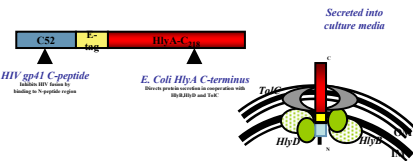
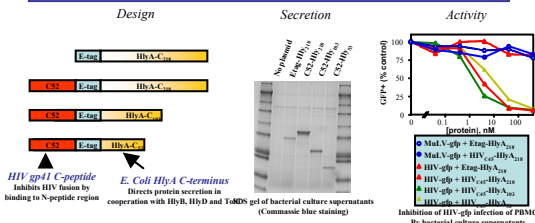


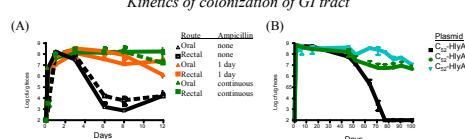
Diagram of the C52-HlyA218 peptide. Initiator and linker sequences are indicated in black, HIV gp41 C-terminal heptad repeat sequences in blue, E-tag epitope sequences in green, and the beginning of the HlyA218 C-terminal secretion signal in red.

## Secretion and Biological Activity of HlyA Fusion Peptides Containing HIV Fusion Inhibitor Sequences



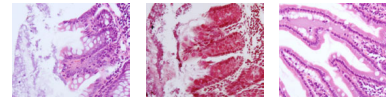
(A) Secretion of anti-HIV peptides by bacteria. Design of C52 cassettes: Etag-HlyA<sub>105</sub>, C<sub>52</sub>-HlyA<sub>116</sub>, C<sub>52</sub>-HlyA<sub>218</sub>, C<sub>52</sub>-HlyA<sub>103</sub>, and C<sub>52</sub>-HlyA<sub>218</sub>. (B) Commassie Blue stained SDS gel of supernatants of log-phase IPTG-induced cultures of *E. coli* Nissle 1917 transformed with the HlyB, HlyD plasmid pVDL9.3 plus (1) no additional plasmid; (2) Etag-HlyA<sub>116</sub>; (3) C<sub>52</sub>-HlyA<sub>218</sub>; (4) C<sub>52</sub>-HlyA<sub>103</sub>; (5) C<sub>52</sub>-HlyA<sub>105</sub>. (C) Inhibition of HIV infection by secreted peptides. PBMC were incubated with various dilutions of bacterial culture supernatants, infected with an HIV or MuLV-pseudotyped HIV-GFP reporter virus, and assayed 72 hours later for GFP reporter expression by FACS. Results are normalized to the percent of GFP<sup>+</sup> cells in the absence of inhibitor.

## Mouse Colonization by Nissle 1917 Expressing Anti-HIV Peptide



Mouse colonization. (A) Effects of antibiotic treatment and administration route on short-term colonization. Mice were orally or rectally inoculated with Nissle 1917 carrying the CnR HlyB, HlyD plasmid pVDL9.3 and the Amp<sup>R</sup> peptide expression plasmid pC52-HlyA218. The mice were given no antibiotic, one day pre-treatment with ampicillin, or continuous ampicillin treatment as indicated. At intervals, feces samples were assayed for Amp<sup>R</sup> CmR colony forming units (cfu). (B) Long-term colonization. Mice were orally and rectally inoculated with a total of Nissle 1917 carrying pVDL93 and the peptide expression plasmid pC52-HlyA218, pC52-HlyA103, or pC52-HlyA53 as indicated. Mice were treated for with ampicillin, then taken off antibiotic for the remainder of the experiment. Feces samples were periodically assayed for Amp<sup>R</sup> CmR cfu.

## Growth and mucus induction in colon



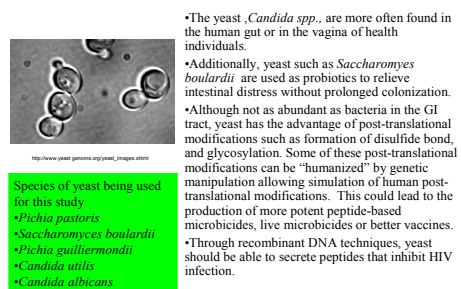
Histopathology and immunohistochemistry. Section of gastrointestinal tract from mice colonized with Nissle 1917 carrying the CnR HlyB, HlyD plasmid pVDL9.3 and the Amp<sup>R</sup> peptide expression plasmid pC52-HlyA218 were stained with Hematoxylin and Eosin (H & E) (for tissue staining) and gram (for bacterial staining). Displayed above are examples from the colon and duodenum.

## Anatomy of Colonization



Anatomical distribution of bacteria. Mice were orally or rectally inoculated with Nissle 1917 carrying pDL9.3 and pC52-HlyA218. After three days of oral ampicillin treatment, the animals were euthanized and dissected, and tissue segments were homogenized and assayed for Amp<sup>R</sup> CmR cfu. For vaginal inoculation, the vagina was rinsed with ampicillin then inoculated with bacteria. The vagina and colon were dissected 3 days later and assayed for Amp<sup>R</sup> CmR cfu. (A) Diagram of gastrointestinal tract. (B) Distribution of cfu in tissues.

## Using Genetically Altered Commensal or Probiotic Yeast for Anti-HIV Microbicide



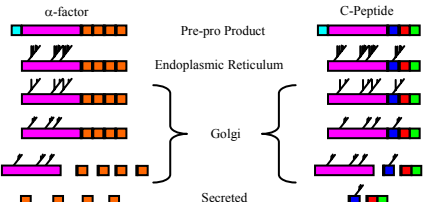
- Pichia pastoris*
- Saccharomyces boulardii*
- Pichia guilliermondii*
- Candida utilis*
- Candida albicans*

## Design of a General C-Peptide Yeast Secretion Cassette



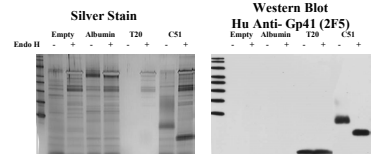
The mating pheromone  $\alpha$ -factor from *Saccharomyces cerevisiae* can be produced and secreted from the cell by various species of yeast such as *Candida spp.* and *Pichia spp.* Therefore we used integrating plasmids (Invitrogen) that contain the  $\alpha$ -factor secretory signal for secretion; spacers which are sites for proteolytic cleavage and enhanced secretions; MYC and hexahistidine tags. The cassette was put under a constitutive and inducible promoter for *Pichia pastoris*. *Pichia pastoris* is commonly used expression of heterologous proteins.

## Pathway for Secretion of C-Peptide



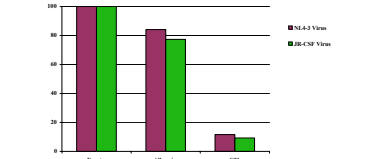
$\alpha$ -factor travels through the yeast secretory pathway going through organelles such as endoplasmic reticulum and golgi where post-translational modifications such glycosylation and proteolytic cleavage occurs to produce a mature mating pheromone. Similarly, the C-peptide secretion yeast cassette goes thru the same pathway producing and secreting C51 in the culture media.

## Secretion of a Glycosylated Form of C-Peptide (C51)



*Pichia pastoris* strains were integrated with plasmids containing albumin secretion (Albumin) cassette; C-peptide secretion (C51) cassette; or no plasmid at all (empty). Yeast cells were grown for 2 days and media was collected. Media was spun down and cells were removed from the media. 15 $\mu$ L of media was treated with (+) or without (-) Endo H to remove glycosylation. A commercial C-peptide T20 was also treated with (+) or without (-) Endo H. Samples were run on SDS-PAGE tricine gels. Gels were silver stain or transferred to nitrocellulose blot. The western blot was probed with 2F5, a human C-peptide antibody.

## The Glycosylated C-Peptide (C51) Maintains Biological Activity



*Pichia pastoris* strains expressing no foreign peptide, albumin or C51, were grown for 2 days and media was collected. Media was spun down and cells were removed from the media. 20 $\mu$ L of media from each sample was added to Peripheral Blood Mononuclear Cells (PBMC). PBMC samples were infected with X4 (NL4-3) R5 (JR-CSF) HIV-1-GFP viruses or no virus and incubated for 3 days. Cells were harvested and analyzed by FACS. The relative percentage of cells infected was calculated based upon without cell incubate with media from *Pichia pastoris* expressing no foreign proteins.

## CONCLUSIONS

1. Bacteria and yeast can be genetically engineered to secrete anti-HIV peptides.
2. A commensal strain of anti-HIV *E. coli* can colonize mice for months and secrete peptide in the GI tract.
3. The live microbial microbicide approach has several advantages for preventing mucosal transmission of HIV:
  - LONG-LASTING
    - Can colonize for weeks to months
    - No need to apply immediately before sex
  - INEXPENSIVE
  - SIMPLE TO STORE AND TRANSPORT
  - EASILY ASSAYED
  - CAN PROTECT AGAINST MULTIPLE AGENTS