Engineering Synthetic Biotics to Secrete Therapeutic Proteins

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Summary

- Synthetic Biotics are live, non-pathogenic *E. coli* Nissle (EcN) bacteria designed with drug-like properties.
- Synlogic's synthetic biology platform allows the engineering of probiotic strains to express and secrete single or multiple proteins with immunomodulatory functions e.g., cytokines (IL2), peptide fusions (GLP2-IgA) and nanobodies.
- Secretion cargo is optimized using signal peptide libraries, protein fusions, or mutagenesis to improve stability and solubility.
- A variety of mechanisms enable enhanced secretion including:
  - Outer membrane architecture modifications
  - Expression of heterologous secretion systems
  - Process development e.g., fermentative growth optimization
- We demonstrate that secreted proteins maintain their bioactivity *in vitro* and in the colon effluent of mice dosed with an engineered EcN strain.

Engineered EcN secretes a high level of bioactive hIL2

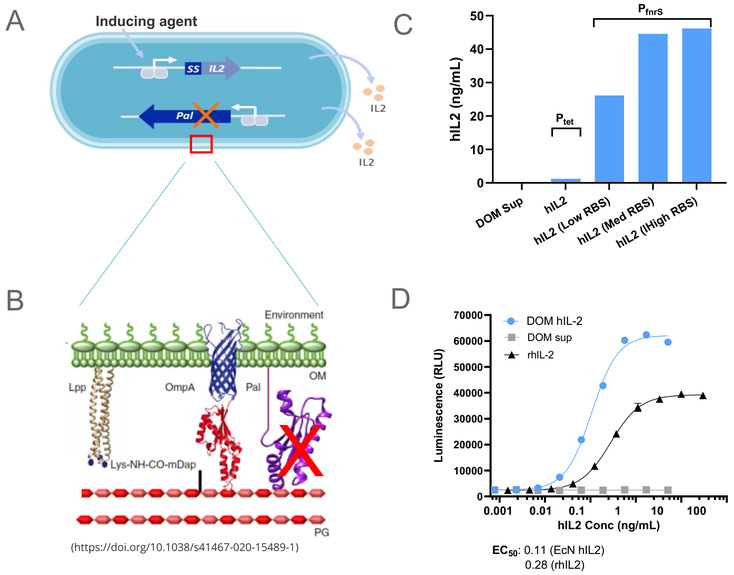

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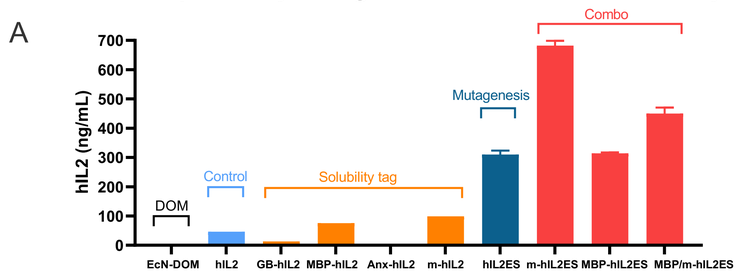
Figure 1. A. Schematic of EcN-IL2. **B.** EcN DOM (Diffusible Outer Membrane) mutants engineered by deleting *pal* gene make the membrane "leaky" and enhances protein secretion. **C.** *In vitro* secretion of hIL2 quantified by ELISA. **D**. Bioactivity analysis of EcN secreted hIL2 with Promega's IL2 bioassay cells and analyzed with GraphPad Prism.

Conclusions

- EcN is a versatile chassis organism for secretion of therapeutic proteins.
- These data demonstrate EcN is amenable to a variety of engineering strategies to optimize secretion, which can be tailored to the protein cargo, the selected secretion system, or both.
- These innovations are key to the development of a new modality of therapeutics for pathologies in the gut, such as Inflammatory Bowel Disease

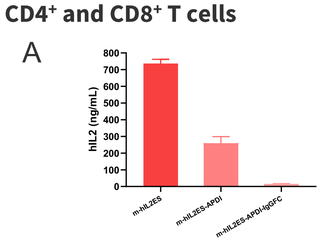
hIL2 secretion improved by mutagenesis and fusion with solubility tag



Strain	EC_{50} (ng/mL)
hIL2	0.09
hIL2ES	0.14
m-hIL2ES	0.11
MBP-hIL2ES	0.14
MBP-m-hIL2ES	0.27

Figure 2. A. hIL2 muteins (=mutant proteins) secreted from EcN DOM and quantified by ELISA. GB=protein G B1 domain, MBP=Maltose binding protein, Anx= annexin, m=mouse, ES=$K^{35}E$, $C^{125}S$ amino acid substitutions in IL2 protein. **B.** Alignment of N-terminal sequences of hIL2 and mouse IL2. m-hIL2 was created by replacing the N terminal sequence with mIL2's. **C & D.** Bioactivities of selected muteins were measured using an IL2 reporter assay and analyzed with GraphPad Prism.

EcN secreted bioactive hIL2 muteins, promoted expansion of primary CD4⁺ and CD8⁺ T cells



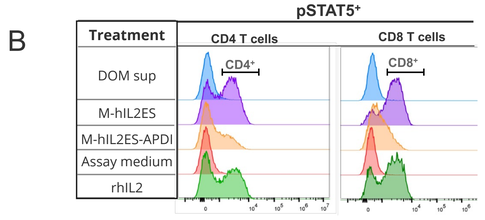
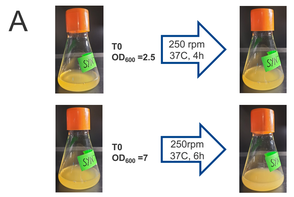


Figure 3. A. hIL2 muteins from EcN DOM quantified by ELISA. Mutations in APDI=$V^{69}A$, $Q^{74}P$, $N^{88}D$, $L^{118}I$ substitutions. **B.** Human peripheral blood pan-T cells incubated with medium containing 100 ng/mL of toxin-removed hIL2 mutein or rhIL2. Cells were immunostained, counted by flow cytometry and analyzed with FlowJo.

EcN secretion improved by bioprocess optimization



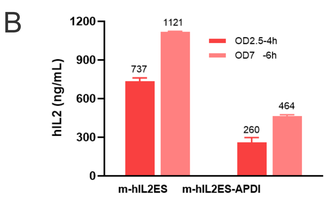
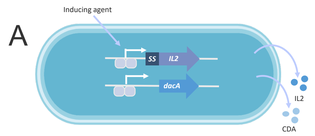


Figure 4. A. Schematic of flask manufacture of hIL2. **B.** Production of hIL2 muteins in broth quantified by ELISA.

EcN secretes multiple therapeutic agents for combination therapy



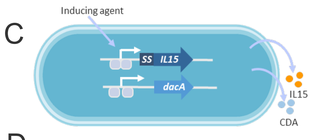
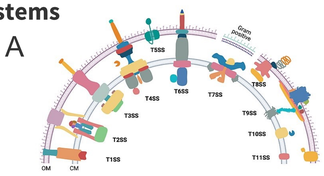
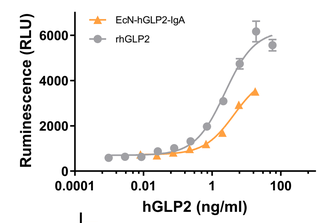


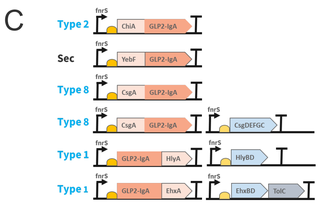
Figure 5. A, Schematic of EcN expressing hIL2-dacA strain**. B.** Human IL2 and CDA (Cyclic di-AMP, a STING agonist,) secreted simultaneously. **C ,** Schematic of EcN-hIL15-dacA strain. **D.** Human hIL15 and CDA secreted simultaneously. DacA (diadenylate cyclase) from *Listeria monocytogenes*.

EcN secretes bioactive hGLP2 via multiple heterologous secretion systems



Source: https://en.wikipedia.org/wiki/Bacterial_secretion_system





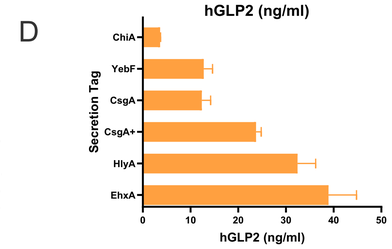
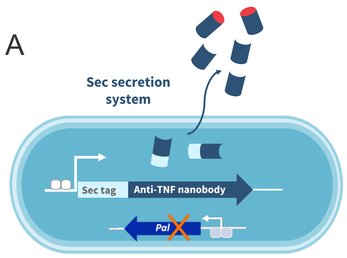
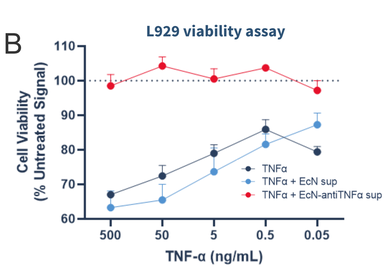


Figure 6. A. Illustration of protein secretion systems found in Gram negative bacteria. **B.** Bioactivities of selected muteins assayed with DiscoverX's PathHunter eXpress β-Arrestin GPCR cells and analyzed with GraphPad Prism. **C.** Schematic of over expressing operon(s) for tagged hGLP2-IgA and components of associated secretion system. **D.** *In vitro* secretion of hGLP2-IgA quantified by ELISA.

EcN secretion of a bioactive antiTNFα nanobody





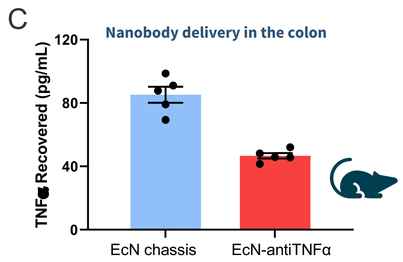


Figure 7. A. Schematic of EcN-antiTNFα. A TNFalpha specific nanobody was fused to a sec secretion signal and expressed in the DOM secretion chassis. **B.** Mouse L929 fibroblast cells, which are sensitive to killing by TNFalpha, were incubated overnight with a range of TNFalpha protein concentrations plus supernatant from EcN-WT or EcN-antiTNFα. **C.** Amount of spiked-in TNFalpha recovered from colon effluents of with mice dosed with 1e10 CFU for 4 hrs, demonstrating the recovery of active, secreted antiTNFα nanobody. Quantification by ELISA (Invitrogen).

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