DETAILED METHODOLOGY

1. Protein purification

Vibrio parahaemolyticus pirA and *pirB* genes were synthesized by BioBasic (Markham, Ontario) and subcloned into pET26b between the *Nde*I and *Xho*I sites. The proteins were recombinantly expressed with C-terminal 6X-histidine tags in *Escherichia coli* strain BL21, similarly to previously described methods (Lee et al. 2015). To purify the proteins, the *E. coli* cells were pelleted by centrifugation, resuspended in buffer A (20 mM Tris-HCl pH 8.0, 100 mM or 300 mM NaCl for PirA and PirB, respectively, and 20 mM Imidazole) plus 12.5 µg ml^{-1} DnaseI and 1 mM PMSF. Resuspended cells were lysed using sonication, the clarified soluble fraction was passed over a HisTrap column (Cytiva) equilibrated with buffer A, and the bound protein was eluted with an imidazole gradient up to 500 mM. For PirA, eluted protein was dialyzed against 10 mM Tris-HCl, pH 8.8, purified with anion exchange chromatography (HiTrapQ HP, Cytiva), dialysed into phosphate buffered saline (PBS), concentrated to ~10 mg ml^{-1}, aliquoted, flash frozen, and stored at –80°C. For PirB, eluted protein was purified by size exclusion chromatography using a HiPrep 16/60 Sephacryl S-100 HR (Cytiva) column equilibrated with PBS buffer, concentrated to ~10 mg ml^{-1}, aliquoted, flash frozen, and stored at –80°C.

Nanobodies were expressed with N-terminal TEV-cleavable 6X-histidine and Thioredoxin (TRX) tags in *E. coli* strain Rosetta-gami 2 grown overnight in autoinducing media (Formedium) at 30°C as previously described (Loutet et al. 2024). For each nanobody, the *E. coli* cells were pelleted by centrifugation, resuspended in 10 mM HEPES, pH 7.5, 250 mM NaCl, 20 mM imidazole, 50 mM $CaCl_2$, 12.5 µg ml^{-1} DnaseI, 1 mM PMSF, and lysed using sonication. The clarified soluble fraction was passed over a HisTrap column (Cytiva) equilibrated with 10 mM HEPES, pH 7.5, 250 mM NaCl, 20 mM imidazole, 50 mM $CaCl_2$, and the bound protein was eluted with an imidazole gradient (20 to 300 mM). TEV protease and EDTA were added to the eluted nanobody at concentrations of 40 µg ml^{-1} and 5 mM, respectively, and the nanobody was dialyzed overnight at 4°C into 10 mM HEPES, pH 7.5, 250 mM NaCl. After TEV cleavage, the nanobody was passed over a second HisTrap column equilibrated with 10 mM HEPES, pH 7.5, 250 mM NaCl and the nanobody protein was collected in the flowthrough. Depending on the pI of the nanobody, they were subsequently dialyzed against either 10 mM Tris-HCl, pH 8.8 and purified with anion exchange chromatography (HiTrapQ HP, Cytiva) or 10 mM HEPES, pH 7.0 and purified with cation exchange chromatography (HiTrap SP HP, Cytiva). The nanobodies were then eluted from the ion exchange columns using NaCl gradients of 5 mM to 1 M. Nanobodies were concentrated to ~10 mg ml^{-1} in a final buffer of 20 mM HEPES, pH 7.4, 150 mM NaCl, aliquoted, flash frozen, and stored at –80°C.

2. Nanobody discovery

The protocols used for the discovery of nanobodies are well-described (Baral et al. 2013) and were applied here to PirA and PirB. Briefly, llamas (Cedarlane Laboratories) were immunized with 100 µg of each antigen on days 1, 21, 42, and 63 and lymphocytes were collected from sera on days 28, 49, and 70. Lymphocyte RNA was converted to cDNA, which was subsequently used as a template for the PCR amplification of nanobody genes. The nanobody genes were used to create M13 phage libraries wherein each phage particle expresses a nanobody fused to the phage gIII tip protein and carries the corresponding nanobody DNA sequence internally. Phage were sorted into antigen-specific groups through three rounds of biopanning. Monoclonal phages were assessed by

ELISA for binding to wells coated with either 10 µg ml^{-1} of antigen or PBS and the nanobody genes from those phage clones with a four-fold greater binding to antigen-coated wells compared to PBS-coated wells were sequenced. Nanobodies with unique complementarity determining regions (CDRs) were selected for screening in *in vitro* experiments.

3. Gastrointestinal tract extract stability assay

Gastrointestinal tract stability assays were conducted as previously described (Loutet et al. 2024) using either shrimp GI tract or chicken jejunal extracts. Reactions containing 2.4 µL shrimp GI tract extract or chicken jejunal extract, 5 µg nanobody in 0.8 µL PBS, and 4.8 µL of 150 mM NaCl were set up on ice. Control reactions without jejunal extract were also set up containing 5 µg nanobody in 3.2 µL PBS and 4.8 µL of 150 mM NaCl. The tubes were incubated on ice for 5 min, followed by 42°C incubation for up to 24 h. After incubation, 8 µL of preheated 2X SDS sample buffer was added to stop the reaction and nanobody stability was assessed using SDS-PAGE.

LITERATURE CITED

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