Use of *in vivo* Expression Technology to Identify Virulence Factors and Protective Antigens of *Vibrio cholerae* O1

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Background and Significance

- Cholera is endemic in over 100 countries and causes 100,000 to 150,000 deaths annually
- Current injectable vaccine is only about 50% effective and protection lasts about 3 months, so is not recommended and no longer used
- A single dose of live oral vaccine CVD 103-HgR provided 91% protection against moderate or severe diarrhea but little protection in field trial in Indonesia

Two pieces of information are needed to direct development of new live oral cholera vaccines

- What gene products are responsible for the virulence of *V. cholerae*?
- What gene products are responsible for inducing the protective immune response?

Hypothesis

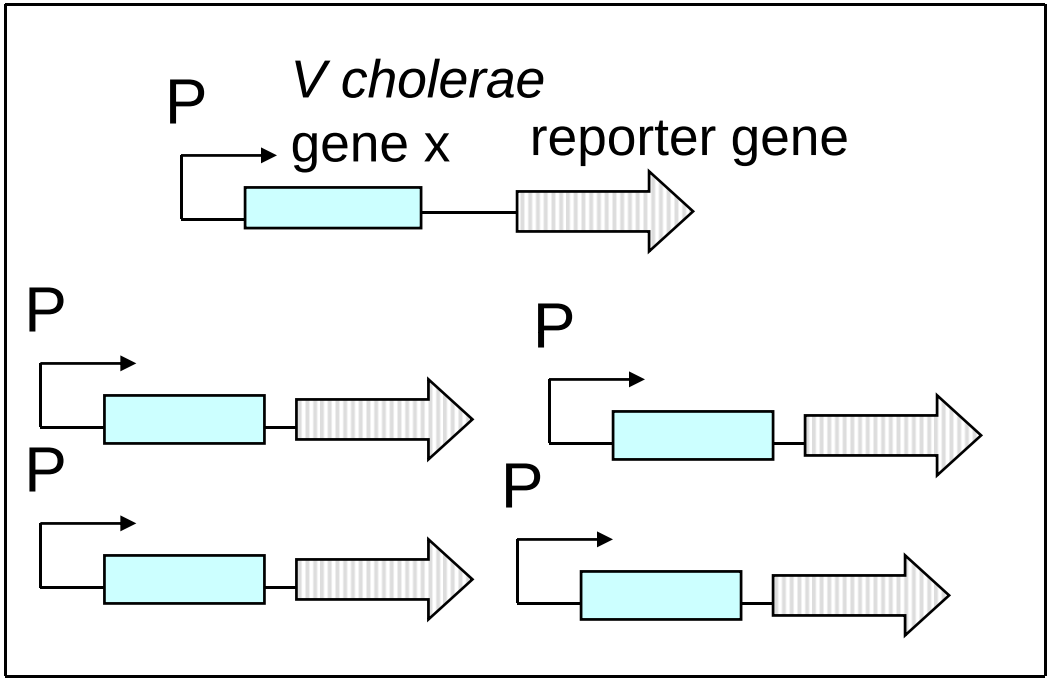
A defined subset of genes of *Vibrio cholerae* O1 are expressed *in vivo*, but not *in vitro*, and these genes play important roles both in pathogenesis and in stimulating the protective immune response.

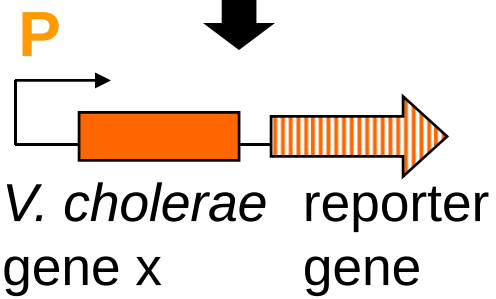
Specific Aim

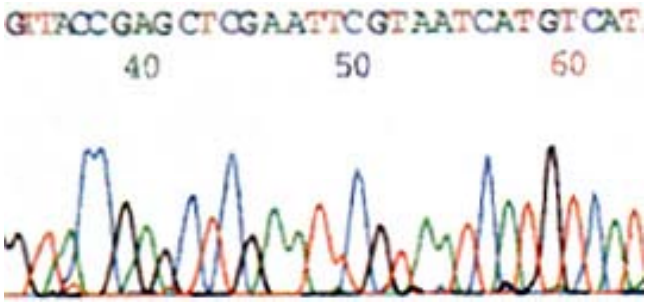
- To identify *V. cholerae* genes that are selectively induced *in vivo* during human infection, using *in vivo* expression technology (IVET) in a volunteer study.

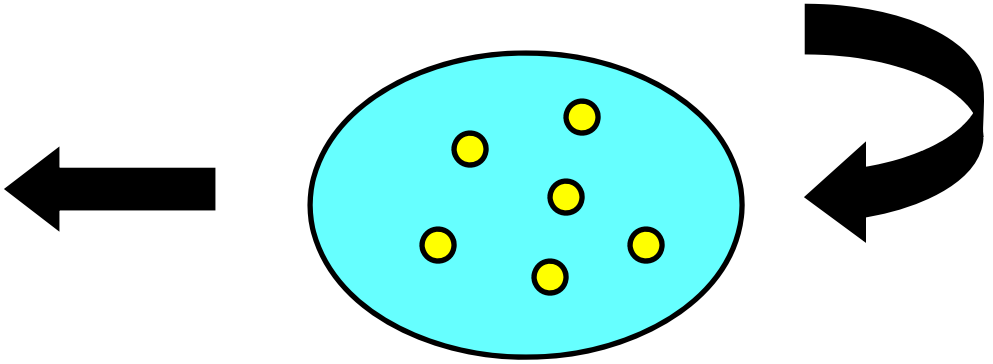
In vivo Expression Technology (IVET)

Library of *V. cholerae* genes









Resolvase IVET (RIVET)

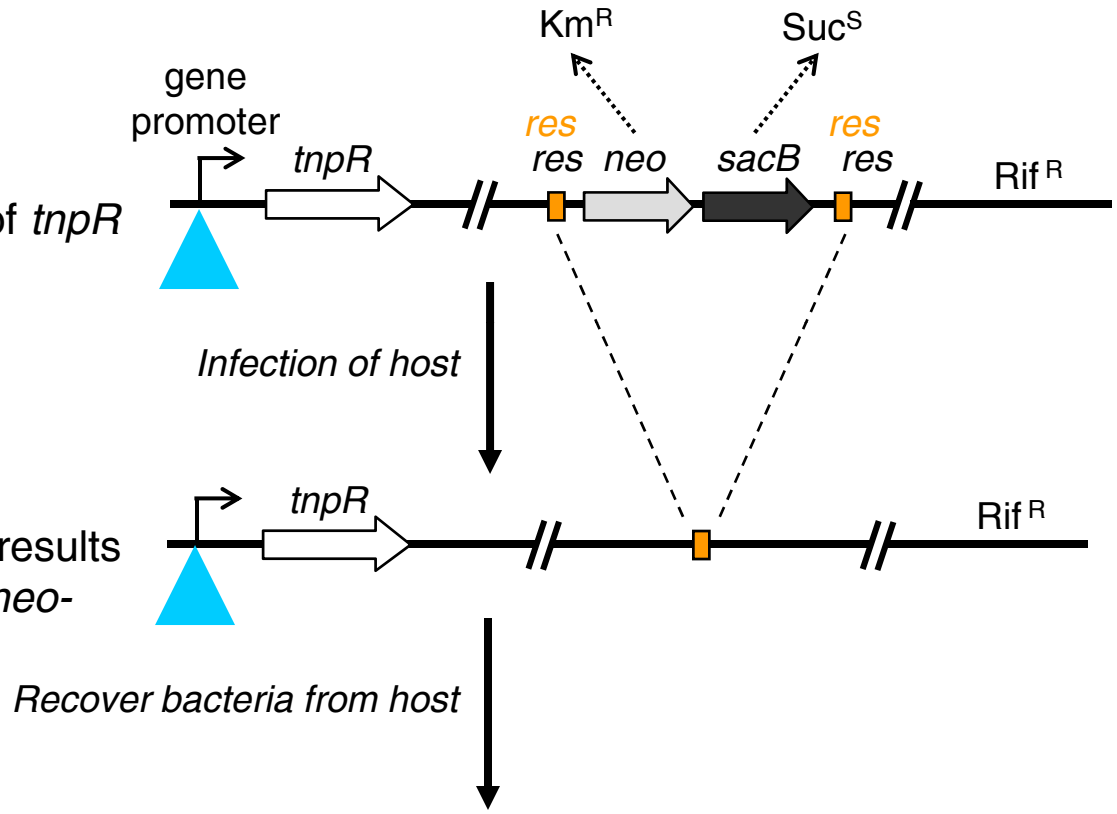
- Allows detection of transiently expressed genes by fusing promoters to promoterless *tnpR* gene encoding resolvase (TnpR)

RIVET method for identifying *Vibrio cholerae* gene promoters activated during infection

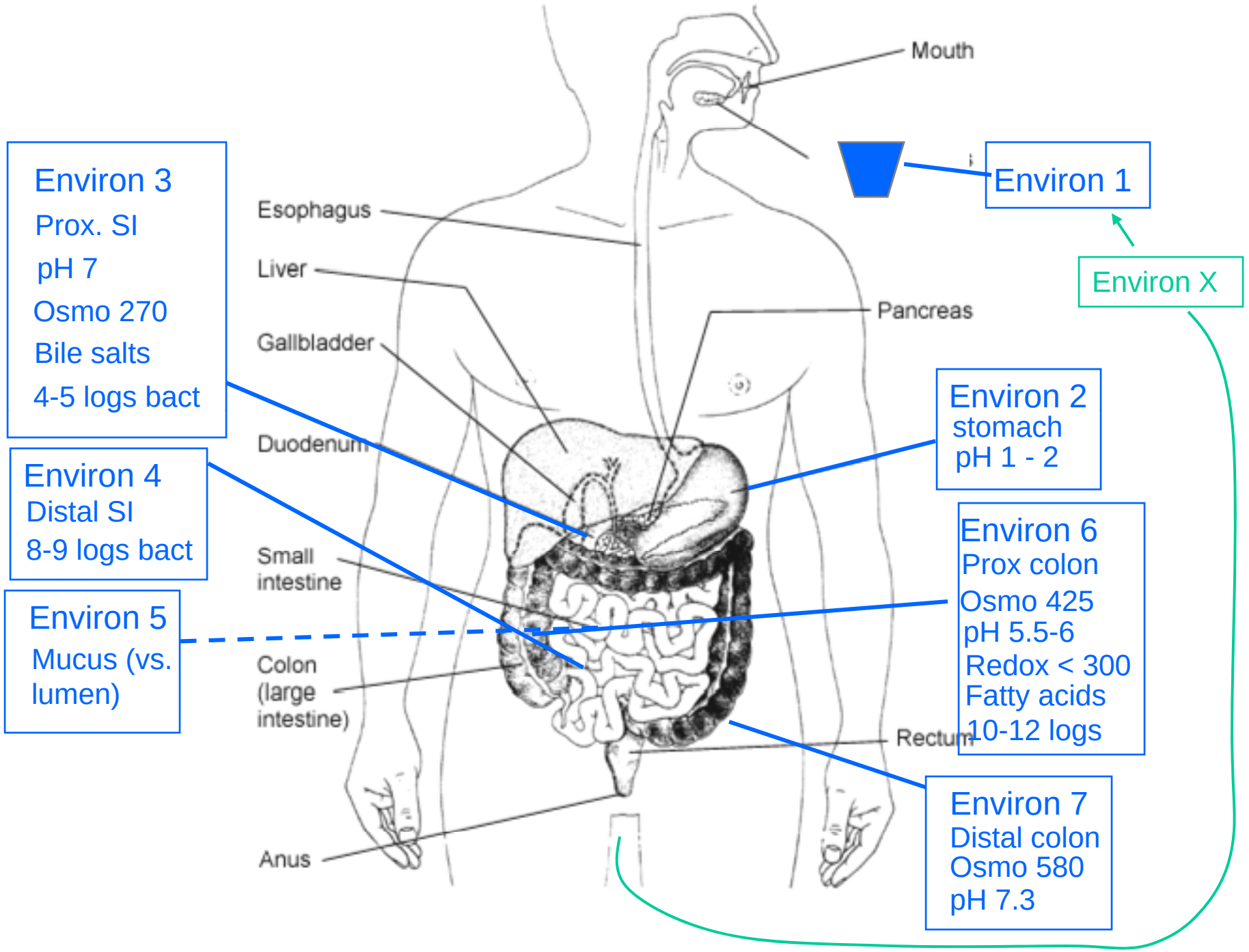
Reporter gene: *tnpR* encodes resolvase that mediates excision of DNA between *res* sites

<u>Step 1</u>. Construct library of *tnpR* fusion strains.

<u>Step 2</u>. Activation of gene promoter during infection results in permanent excision of *neo-sacB* genes by TnpR.



<u>Step 3</u>. Recombined strains are selected on plates with rifampicin and sucrose. DNA upstream of *tnpR* gene is sequenced.


Mouth
Esophagus
Liver
Gallbladder
Duodenum
Small intestine
Colon (large intestine)
Anus
Pancreas
Rectum
Environ 1
Environ X
Environ 2
stomach
pH 1 - 2
Environ 3
Prox. SI
pH 7
Osmo 270
Bile salts
4-5 logs bact
Environ 4
Distal SI
8-9 logs bact
Environ 5
Mucus (vs. lumen)
Environ 6
Prox colon
Osmo 425
pH 5.5-6
Redox < 300
Fatty acids
10-12 logs
Environ 7
Distal colon
Osmo 580
pH 7.3

Specific Aim

- To identify *V. cholerae* genes that are selectively induced *in vivo* during human infection, using in vivo expression technology (IVET) in a volunteer study.

Secondary Aims

- To determine the role of the newly identified gene products in pathogenesis of cholera in humans
- To determine the role of the newly identified gene products in the protective immune response to *V. cholerae*
- To compare *in vivo*-expressed proteins identified in humans with those identified by IVET in an animal model of cholera to validate the animal model

Study Design

- Healthy adult volunteers (n=3-5)
- Screened, counseled, informed
- Volunteers ingest a pool of $1x10^8$ *V. cholerae* strain CVD 110 (*ctxA*-deleted).
  - Each of the 10,000 strains in the pool will differ by the single gene fused to *tnpR*.

Study Design

- Observe as inpatients for 9 days; collect all stools to isolate *Vibrio*.
- Collect intestinal fluid twice by string capsule for *Vibrio* culture and collect blood for serology.
- Treat with tetracycline for 5 days as inpatients.
- Discharge if criteria met.
  - No diarrhea, completed course of tetracycline, stool culture negative
- Outpatient follow-up visits for serology.

Study Population: Healthy adult community volunteers

- Inclusion Criteria
  - **Age 18-40 years**
  - **Normal medical history, psychological and physical examination**
  - **Normal laboratory tests**
- Exclusion Criteria
  - **Significant medical history**
  - **Allergy to tetracycline**
  - **Recent antibiotic use**
  - **Poor peripheral vein access**
  - **Pregnancy**
  - **Positive HIV or hepatitis serology**
  - **Positive stool exam for pathogen**
  - **Failure to pass written exam**

Outcome Measures

- Isolate and identify *V. cholerae* stool and duodenal fluid isolates that have lost the *neo-sacB* cassette
- Collect blood for immune responses measurements before and after challenge

Laboratory Follow-up of Clinical Specimens

- Sequence *V. cholerae* gene insert, compare to genome sequence, and identify and express corresponding protein
- Determine immune response to implicated protein and relate to serum vibriocidal antibody titer

Statistical Considerations

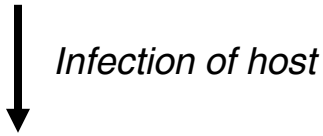
- Sample size: small
  - Limit risk of diarrhea to as few volunteers as possible to meet aims of the study
  - Large numbers of vibrio will be recovered
- Data analysis
  - From each daily stool specimen, at least 20 different vibrio colonies that have lost the *neo-sacB* cassette will be picked and the clone identified
  - No statistical comparisons

Summary

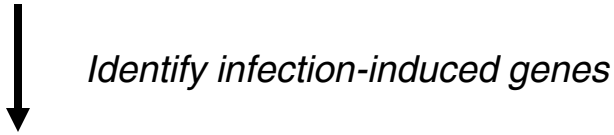
- A small number of healthy adult volunteers will be infected with a pool of single-gene variants of a cholera toxin-deleted strain of *V. cholerae* O1.
- The pool will include 10,000 variants, each containing a different gene that has been tagged so that its expression *in vivo* can be detected.
- Strains recovered from human stool and duodenal fluid which contain a gene expressed *in vivo*, but not *in vitro*, will be examined in the laboratory to detect new virulence factors of *V. cholerae* and new antigens that contribute to the protective immune response.

Summary of preliminary testing of IVET library in a mouse model of cholera

Step 1. The inoculum lot (library) was used to infect a group of sixteen 5-day-old outbred mice.



Step 2. After 24 hours, mice were euthanized, the small intestines were removed and homogenized, and the homogenates were plated on petri plates supplemented with rifampicin and sucrose to select recombined *V. cholerae* strains.



Step 3. 96 strains (6 individual colonies from each mouse intestine) were collected. The DNA upstream of *tnpR* in each strain was amplified by PCR and the products were sequenced. The resulting sequences were used to identify the corresponding genes present in the *V. cholerae* sequenced genome.

Results of initial testing of IVET library in mice

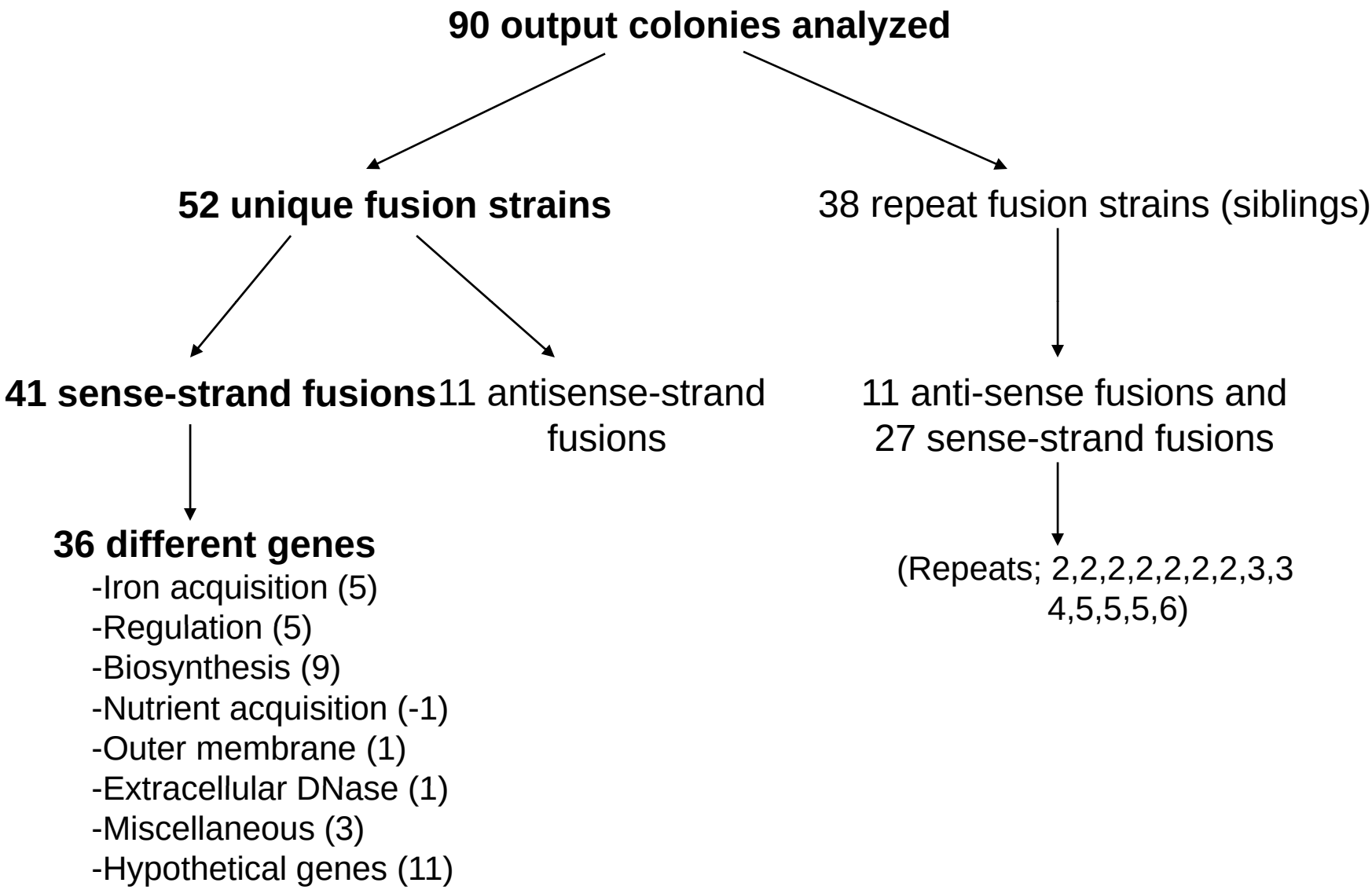


FIGURE 1. Genetic manipulations used to construct IVET library.

Step 1: Isolate spontaneous rifampicin-resistant derivative of CVD 110. This new strain is called AC-V1107. This step is not shown in figure below.

Step 2: Integrate *res-neo-sacB-res* and *res1-neo-sacB-res1* into the *V. cholerae* AC-V1107 *lacZ* gene to generate strains AC-V1245 and AC-V1246, respectively.

Step 3: Integrate pIVET plasmids into AC-V1245 and AC-V1246 genomes to generate IVET library.