

“Fishing” for Genes Encoding Noncytoplasmic Proteins in a *Vibrio* Fosmid Library

Les M. Hoffman and Dilara Begum, EPICENTRE

Vibrio species (*sp.*) are common in marine environments,¹ and the genus includes the causative agents of some human diseases. For example, *V. vulnificus* causes a significant number of infections following the consumption of contaminated oysters. *V. parahaemolyticus* causes gastroenteritis associated with the consumption of raw, improperly cooked, or cooked, recontaminated fish and shellfish. There are also *Vibrio sp.* without known pathogenicity, such as *V. rumoiensis*, isolated in a Japanese fish processing plant.

In 1994, we isolated a *Vibrio sp.* from a sample of kelp found in Carmel, California. Sequence analysis of ribosomal DNA regions revealed that its closest match was *Vibrio sp.* GWS-TZ-H188, found in the Wadden Sea of Germany. Here we describe how EPICENTRE’s products minimized the time and effort required to both construct a fosmid library for this novel marine bacterium and initiate a screen for noncytoplasmic protein genes. Figure 1 gives an overview of the process.

Fosmid library construction without sizing DNA

A genomic library, consisting of 30 to 40-kb inserts, was constructed for the *Vibrio sp.* using the CopyControl™ Fosmid Library Production Kit. For this kit, insert DNA is generated by random shearing of the genomic DNA rather than partial restriction enzyme digestion. Random shear libraries have unbiased sequence representation because fragmentation is not dependent on the random distribution of restriction sites and is not affected by methylation. Moreover, obtaining 30 to 40-kb inserts by random shearing is quite simple, since many protocols used to isolate genomic DNA produce a significant amount of DNA in this size range.

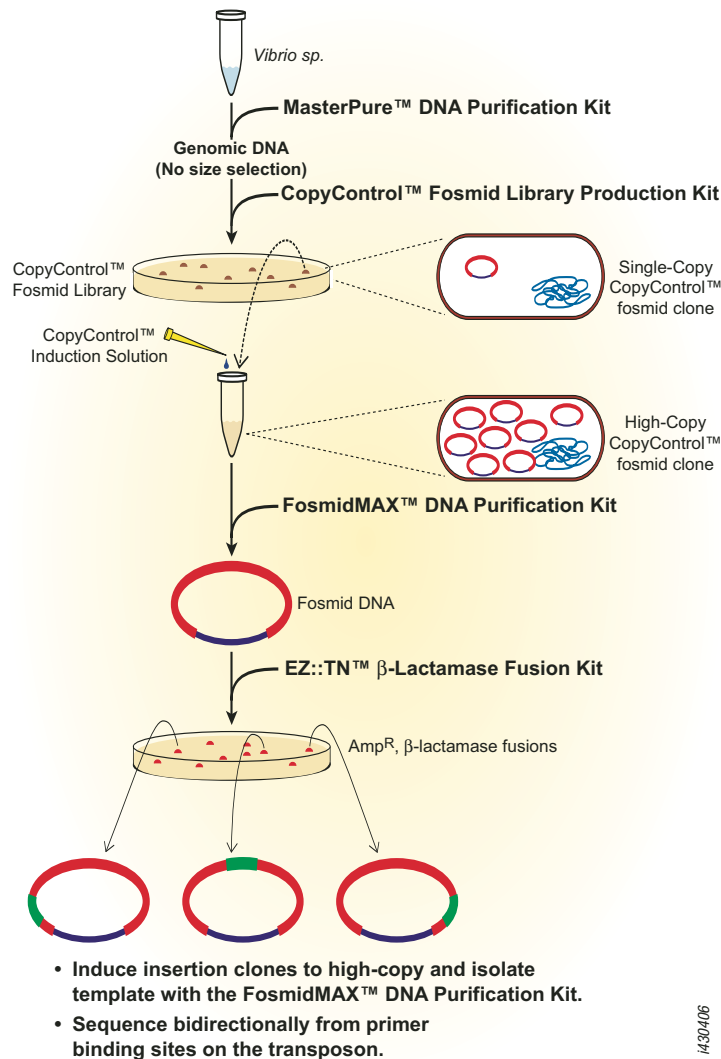


Figure 1. Overview of production of a *Vibrio sp.* CopyControl™ Fosmid Library and an EZ::TN™ Transposon-based screen for noncytoplasmic proteins.

The MasterPure™ DNA Purification Kit, for example, yielded *Vibrio* genomic DNA that, following end-repair and phosphorylation, was used directly in a ligation and subsequent packaging reaction. Even after being archived for over eight years, only about 300 ng of blunt-ended DNA was needed to generate a library of approximately 2,300 fosmid clones. Assuming an average insert size of 40 Kb and a 4-Mb genome, there are more than enough clones to ensure a 99% probability that any given DNA sequence is contained within the library.

High-yields of fosmid DNA

Low yields of DNA from single-copy fosmid clones is often a rate-limiting step in their analysis. CopyControl clones, however, can be induced from single copy to 10 to 50 copies per cell in a TransforMax™ EPI300™ host. Following a 3-hour induction, an average of 2 µg of fosmid DNA was isolated from a 2-ml culture using the new FosmidMAX™ DNA Purification Kit. EPICENTRE’s kit eliminates the need for columns, resins, or organic extractions and effectively removes contaminants that degrade DNA and interfere with downstream applications like *in vitro* transposition (below), restriction enzyme digests (Figure 2) and sequencing.

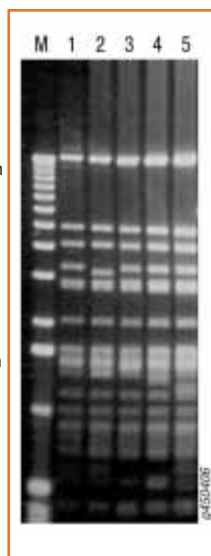
A transposon-based screen for noncytoplasmic protein genes

Transposon-based screens are one of the most efficient ways to “fish-out” genes of interest, especially from large insert clones like fosmids or BACs. The EZ::TN™ β-Lactamase Fusion Kit was developed for the direct selection of genes encoding cell envelope and secreted proteins. The kit features the EZ::TN™ <blaM/R6Kγori> Transposon which contains a β-lactamase gene (*blaM*) that lacks both promoter and secretory signal sequences. Two fosmid clones

were screened with a one-step *in vitro* reaction that randomly inserts a single EZ::TN <blaM/R6Kγori> Transposon into the fosmid DNA. TransforMax EPI300 cells were transformed with an aliquot of the reaction and cells were selected at single copy with standard concentrations of ampicillin (100 µg/ml) and chloramphenicol (25 µg/ml, vector marker). Only *blaM* fusions that generate hybrid proteins transported through the inner membrane will confer resistance to ampicillin.

1430406

Figure 2. Fosmid DNA isolated with the FosmidMAX™ DNA Purification Kit was used directly for downstream applications like fingerprinting. DNA isolated from induced CopyControl™ fosmid clones was digested with *Hind* III and analyzed by agarose gel electrophoresis. Lane 1, parental fosmid clone; Lanes 2-5, EZ::TN™ <blaM/R6Kγori> Transposon insertion clones derived from the parental clone in Lane 1. Differences in restriction patterns verify that the EZ::TN Transposon insertions occurred in different regions of the fosmid. M, 1-kb ladder.



Sequence analysis of β-lactamase fusions

To determine the exact site of an EZ::TN <blaM/R6Kγori> Transposon insertion, we sequenced bidirectionally from primer binding sites near the ends of the transposon. A BLAST search for homologies to known bacterial genes revealed that most of the *blaM* fusions were to “conserved hypothetical proteins” (Table 1). Nothing is known about the expression or function of these genes in related *Vibrio* species. Here we demonstrate that the genes are expressed, at least in another Gram-negative host cell, and, the argu-

ment could be strongly made, that they encode noncytoplasmic proteins.

Sequences from two insertion clones, pCC1/Vib1-2 and pCC1/Vib1-3, have homology to genes known to encode non-cytoplasmic proteins (Table 1). Membrane-bound Na⁺/H⁺ antiporters (pCC1/Vib1-2) have several valuable roles in bacteria: (1) maintenance of an electrochemical potential across the cytoplasmic membrane; (2) extrusion of Na⁺ and Li⁺, which are toxic at high levels within the cell; (3) regulation of intracellular pH under alkaline conditions; and (4) cell volume regulation.² TolC (pCCi/Vib1-3) is a large, trimeric envelope protein that acts as a cell exit duct for proteins and drugs in Gram-negative bacteria.³ Multidrug efflux proteins like TolC may be important for the design of antibacterial agents to treat multidrug-resistant infections. Interestingly, pCC1/Vib2-7 contains a transposon in a gene resembling *tral*. *Tral* encodes a conjugative relaxase, which is a cytoplasmic protein known to associate with the TraD membrane protein in *V. cholerae*.⁴ Perhaps, by virtue of its association with TraD, the *tral*-*blaM* fusion is ferried across the membrane.

Summary

We streamlined genomic library construction and clone characterization for a novel *Vibrio* sp. primarily by eliminating steps that are often thought to be required. The CopyControl Fosmid Library Production Kit, for example, enabled us to make a complete and unbiased library without doing partial restriction enzyme digests, sizing the DNA in a gel, or growing large volumes of culture to isolate fosmid DNA for downstream applications. Using the MasterPure DNA Purification Kit and the FosmidMAX DNA Purification Kit eliminated the need for columns, resins or organic extractions and gave good yields of high-quality DNA. Finally, the successful “fishing” expedition for noncytoplasmic protein genes using the EZ::TN β-Lactamase Fusion Kit resulted from a relatively effortless direct screen that did not require hybridization or enzyme assays.

References

1. Urakawa, H. *et al.* (1999) *Microbiology* **145**, 3305.
2. Vimont, S. and Berche, P. (2000) *J. Bacteriol.* **182**, 2937.
3. Koronakis, V. (2003) *FEBS Lett.* **555**, 66.
4. Dreiseikelmann, B. (1994) *Microbiol. Rev.* **58**, 293.

Fosmid Insertion Clone*	Results of BLAST Search
pCC1/Vib1-2	Putative Na ⁺ /K ⁺ antiporter
pCC1/Vib1-3	Outer membrane protein (TolC)
pCC1/Vib1-4	Conserved hypothetical protein
pCC1/Vib1-5	Conserved hypothetical protein
pCC1/Vib2-1	Hypothetical protein
pCC1/Vib2-2	Conserved hypothetical protein
pCC1/Vib2-3	Conserved hypothetical protein
pCC1/Vib2-7	Conjugative relaxase (Tral)

*Insertion clones are derived from two different fosmids, pCC1/Vib1 and pCC1/Vib2.

Table 1. Random EZ::TN™ <blaM/R6Kγori> Transposon insertions into CopyControl™ fosmid clones result in β-lactamase fusions to open reading frames with homology to hypothetical or noncytoplasmic proteins.

www.epicentre.com/ccfosmid.asp

CopyControl™ Fosmid Library Production Kit

CCFOS110 1 Kit

Kit contains sufficient reagents to produce up to 10 CopyControl Fosmid libraries.

Phage T1-Resistant EPI300™-T1^R *E. coli* cells, required for inducing CopyControl Fosmid clones to high-copy number, are supplied with the kit.

www.epicentre.com/masterpure_complete.asp

MasterPure™ DNA Purification Kit

MCD85201 200 Purifications

Contents:

- Red Cell Lysis Solution
- Tissue and Cell Lysis Solution
- MPC Protein Precipitation Reagent
- 2X T&C Lysis Solution
- TE Buffer
- RNase A
- Proteinase K

www.epicentre.com/fosmidmax.asp

FosmidMAX™ DNA Purification Kit

FMAX046 1 Kit

Reagents sufficient for 150 X 1.5-ml; 10 X 40-ml; or 5 X 100-ml purifications.

Contents:

- FosmidMAX™ Solutions 1 to 4
- RiboShredder™ RNase Blend
- TE Buffer

www.epicentre.com/fusion.asp

EZ::TN™ β-Lactamase Fusion Kit

EZ131BL 10 Reactions

Contents:

- EZ::TN™ Transposase
- EZ::TN™ <blaM/R6Kγori> Transposon
- EZ::TN™ 10X Reaction Buffer
- EZ::TN™ 10X Stop Solution
- Forward and Reverse Primers
- Control Target DNA
- Sterile Water