The Development of an In-frame Deletion System in *Desulfovibrio vulgaris* Hildenborough

H-132

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ESPP2 Environmental Stress Pathway Project

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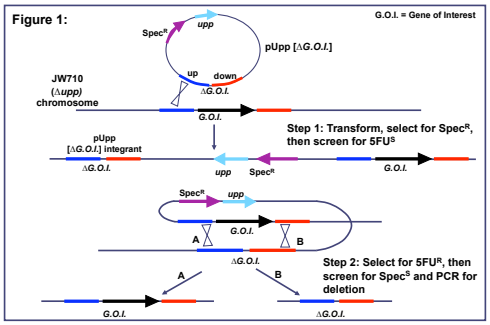
ABSTRACT

In recent years, genetic manipulation of the sulfate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough has seen enormous progress; however, the current method of deletion construction via marker exchange mutagenesis does not allow for easy selection of multiple sequential gene deletions because of the low number of selectable markers now available in *D. vulgaris*. To broaden the repertoire of genetic tools for manipulation of *D. vulgaris*, an in-frame markerless deletion system is being developed based on the *upp* -encoded uracil phosphoribosyltransferase as an element for a counterselection strategy. In wild-type *D. vulgaris*, growth is inhibited by the toxic pyrimidine analog 5-fluorouracil (5 -FU), whereas a mutant bearing a deletion of the *upp* gene is resistant to 5-FU. The introduction of a plasmid containing the wild-type *upp* gene expressed constitutively from the *aph*(5')-III promoter (the promoter for the kanamycin resistance gene in Tn5) into the *upp* deletion strain restored sensitivity to 5-FU. This observation is the basis for the establishment of a two-step integration and excision strategy for the deletion of genes of interest. Since this in-frame deletion does not contain an antibiotic cassette, multiple gene deletions can be generated in a single strain using this method. To construct such a markerless deletion for the R-subunit (DVU1703) of a type I restriction-modification system, Gateway Technology methods (Invitrogen) are being used. A destination vector containing the constitutively expressed wild-type *upp* gene has been constructed and is available for generating deletion vectors. Its use is reported here.

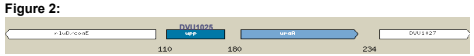
Markerless Deletion Strategy

Figure 1:



Construction of JW710 (Δ*upp*) Strain

Figure 2:



The predicted operon (http://www.microbesonline.org) containing the uracil phosphoribosyltransferase gene (*upp*: VIMSS206459, DVU1025) and the uracil permease gene (*uraA*: VIMSS206460, DVU1026). The genes in this operon are responsible for scavenging uracil, and WT *DvH* is extremely sensitive to even low concentration (0.1 ug/ml) of the toxic base analog 5-fluorouracil (5-FU)

Regions upstream and downstream from *upp* gene were amplified from WT *DvH* via two, separate PCR experiments. The primers (P2 and P3) were designed to contain two barcodes to be introduced on the end of each region, as well as containing overlapping regions.

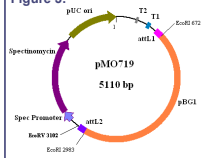
A third PCR experiment was used to join the products from PCR1 and PCR2 together to form the *upp* deletion construct. The deletion construct was ligated into the pBluescript plasmid, the sequences verified, and the plasmid named pMO711.

The plasmid pMO711 was then electroporated into WT *DvH*. Cells that had successfully integrated the deletion construction were selected by growing the cells on LS4 medium containing 40 ug 5-FU/ml.

Several isolated, single colonies were screened for the deletion using Southern Blot analysis. One of the isolates was chosen and named JW710.

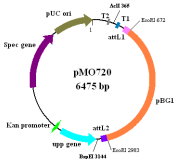
Construction of the kan promoted *upp* gene

Figure 3:

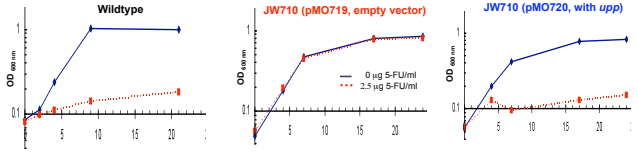


Plasmid pMO719 is a stable plasmid in *Desulfovibrio* strains because it contains the SRB replicon, pBG1, originally isolated as a cryptic plasmid from *Desulfovibrio desulfuricans* G100A.

The *upp* gene from *DvH* was placed under the control of the the *aph*(5')-III promoter (the promoter for the kanamycin resistance gene in Tn5) for constitutive expression. The construct was cloned into pMO719 to make pMO720.



Both pMO719 and pMO720 were electroporated into JW710 (Δ*upp*) and sensitivity to 5-FU determined.



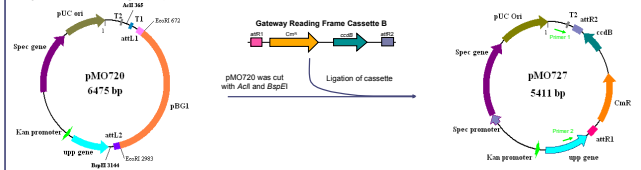
Graphs demonstrate that when the pMO720 plasmid (Stable, SpecR with *kan* promoter/*upp* gene construct) was transformed into strain JW710 (Δ*upp*) sensitivity to 5-FU was restored to wild-type levels. JW710 containing only the pMO719 plasmid remained resistant to 5-FU.

Gateway Technology

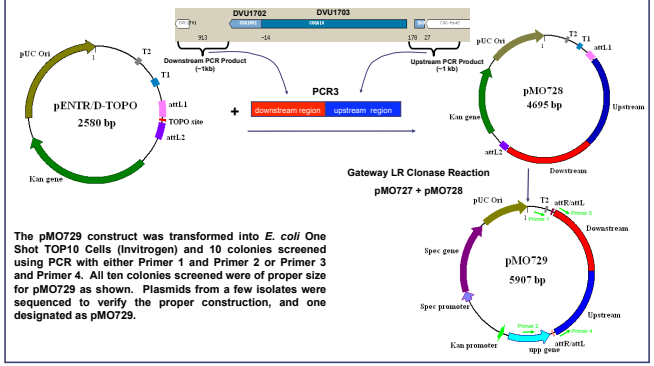
Figure 4:

A. Construction of Inframe Markerless Deletion Destination Vector

Using the Gateway Vector Conversion System (Invitrogen), we ligated the Gateway Reading Frame B (1713 bp) and then transformed it into *ccdB* Survival Cells (Invitrogen) by selecting for chloramphenicol and sequencing to verified proper construction of pMO727. The bacteriophage λ-derived *attR* sites in the cassette will allow recombinational cloning of markerless deletion constructs from an entry clone vector having appropriate *attL* sites.



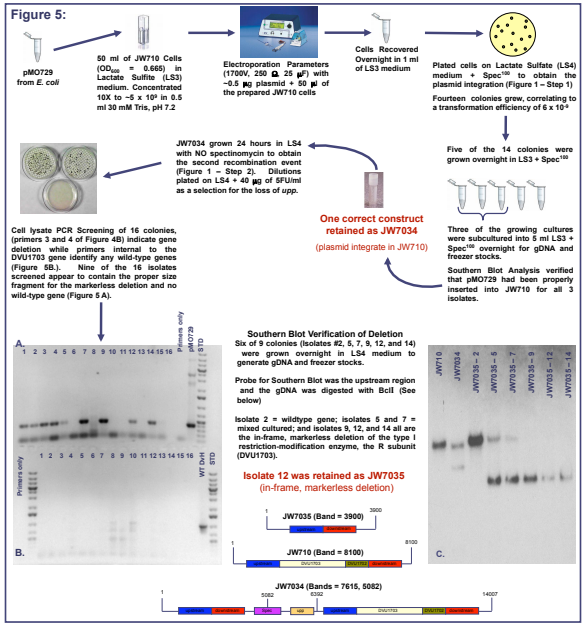
B. Entry clone construction (eg: ΔCOG610, type I restriction-modification enzyme, R subunit [DVU1703])



The pMO729 construct was transformed into *E. coli* One Shot TOP10 Cells (Invitrogen) and 10 colonies screened using PCR with either Primer 1 and Primer 2 or Primer 3 and Primer 4. All ten colonies screened were of proper size for pMO729 as shown. Plasmids from a few isolates were sequenced to verify the proper construction, and one designated as pMO729.

In-Frame Deletion

Figure 5:



CONCLUSION

- **The deletion of the Type I Restriction-Modification enzyme, R subunit (DVU1703), clearly demonstrates the successful development of a two-step integration and excision strategy using *upp* as a counterselectable marker in *Desulfovibrio vulgaris* Hildenborough**
- **Preliminary data show wild-type growth rates for JW7035**
- **The Gateway construction allows for efficient generation of deletion vectors**
- **Because the resulting deletion mutants do not contain antibiotic resistance determinants, multiple genes can be deleted in a single strain**

ACKNOWLEDGEMENT

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