Title: Development of a Markerless Genetic Exchange System in *Desulfovibrio vulgaris* Hildenborough and Its Use in Generating a Strain with Increased Transformation Efficiency

Running Title: Markerless Genetic Exchange System in *Desulfovibrio*

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Key Words: *Desulfovibrio vulgaris* Hildenborough, markerless deletion, *upp* gene, 5-fluorouracil, type I restriction endonuclease

ABSTRACT:

In recent years, the genetic manipulation of the sulfate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough has seen enormous progress. In spite of this progress, the current marker exchange deletion method does not allow for easy selection of multiple sequential gene deletions in a single strain because of the limited number of selectable markers available in *D. vulgaris*. To broaden the repertoire of genetic tools for manipulation, an in-frame, markerless deletion system has been developed. The counterselectable marker that makes this deletion system possible is the pyrimidine salvage enzyme, uracil phosphoribosyltransferase, encoded by *upp*. In wild-type *D. vulgaris,* growth was shown to be inhibited by the toxic pyrimidine analog 5-fluorouracil (5-FU); whereas, a mutant bearing a deletion of the *upp* gene was resistant to 5-FU. When a plasmid containing the wild-type *upp* gene expressed constitutively from the *aph*(3')-II promoter (promoter for the kanamycin resistance gene in Tn*5*) was introduced into the *upp* deletion strain, sensitivity to 5-FU was restored. This observation allowed us to develop a two-step integration and excision strategy for the deletion of genes of interest. Since this in-frame deletion strategy does not retain an antibiotic cassette, multiple deletions can be generated in a single strain without the accumulation of genes conferring antibiotic resistances. We used this strategy to generate a deletion strain lacking the endonuclease (*hsdR*, DVU1703) of a type I restriction-modification system, that we designated JW7035. The transformation efficiency of the JW7035 strain was found to be 100 to 1000 times greater than that of the wild-type strain when stable plasmids were introduced via electroporation.

INTRODUCTION:

The anaerobic sulfate-reducing bacteria (SRB) are found in a remarkable variety of habitats. These bacteria have received attention recently because they have a potential role in toxic metal bioremediation (23, 26). To fully understand the potential benefits and to maximize opportunities for successful manipulation of the SRB, it would be useful to create deletions in critically important genes. Several activities of particular interest are represented by multiple isozymes, suggesting that compensation may occur upon elimination of one or more of these genes. To fully elucidate alternative pathways, genetic approaches allowing the construction of multiple mutations are needed. The genetic manipulation of the sulfate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough has seen significant improvements in recent years (reviewed in 3). Chloramphenicol and kanamycin marker exchange mutagenesis methods have been developed (2, 10). While gene deletions can be constructed, the necessary retention of antibiotic resistance limits sequential deletions, as each deletion would require an additional antibiotic cassette. To eliminate the necessity of marker retention, an in-frame markerless deletion system has been developed.

A two-step method for marker exchange/deletion that employed the counterselectable marker *sacB* (13) was used by Voordouw and coworkers (10) to generate the first deletion by marker exchange in *D. vulgaris*. The *sacB* gene from *Bacillus subtilis* encodes levansucrase and confers sensitivity to sucrose in many Gram-negative bacteria (7, 32, 35), including *D. vulgaris* (8, 10, 15, 18, 19, 21). In the first step of the process, a suicide plasmid carrying DNA regions from up- and downstream of a target gene flanking a Cm^r cassette was introduced into *D. vulgaris* by conjugation and single recombinants were selected as Cm^r colonies (10). After confirmation of the integration of this plasmid, the double recombination event was selected on

medium containing chloramphenicol and sucrose. This method, with some variation, has been used to make several mutants by the Voordouw group (8, 10, 15, 18, 19, 21). One unexpected complication was the observation that 50% of sucrose resistant colonies were due to events other than the removal of the *sacB* gene and plasmid through a second recombination as desired (11). Also, sensitivity to sucrose is apparently strongly affected by medium composition, initial culture density and time of exposure (11). This method involved a large time investment, but it ultimately resulted in a marker exchange mutant (Cm^r) and established the effectiveness of a two-step recombination process in *D. vulgaris*.

Among alternative counterselectable markers are the purine and pyrimidine salvage enzymes, phosphoribosyl transferases (PRTases). These enzymes allow the recycling of free bases from internal or environmental sources, as well as the incorporation of base analogs into nucleoside monophosphates. Importantly, the incorporation of base analogs can be lethal and are the reason these nucleotide salvage pathways have been widely used as counterselectable markers for gene knockout systems in bacteria, archaea, and eukaryotes (4, 5, 8, 9, 12, 16, 22, 24, 27, 29, 34). Specifically, the incorporation of the pyrimidine analog 5-fluorouracil (5-FU) is lethal in a number of bacteria (8, 16, 22). Mutants deleted for the genes encoding the pertinent PRTases are resistant to the toxic base analogs (4, 5, 8, 9, 12, 16, 22, 24, 27, 29, 34). Reintroduction of these genes restores sensitivity. In order to utilize the genes for PRTases as counterselectable markers, a deletion of the endogenous PRTase gene must be created in the host strain. We have previously shown that wild-type *D. vulgaris* is extremely sensitive to low levels of 5-FU, as little as 0.1 μg/ml (3). In this study, we deleted the *upp* gene (DVU1025) encoding the putative uracil phosphoribosyl transferase in *D. vulgaris* creating strain JW710 and showed it was resistant to 5-FU. When the *upp* gene was reintroduced into JW710 (Δ*upp*), it restored

sensitivity to wild-type levels of 5-FU. These phenotypic observations indicate that the loss of the *upp* provides a selectable marker for a two-step integration and excision strategy for the deletion of target genes without a residual marker exchange. A second advantage of using this markerless method is the facile ability to generate in-frame deletions, eliminating potential polarity.

To test the effectiveness of using the *upp* as a counterselectable marker in *D. vulgaris*, we deleted the *hsdR* gene (DVU1703) the endonuclease of a type I restriction-modification system, creating strain JW7035 (Δ*upp* Δ*hsdR*). The type I restriction-modification system was targeted for deletion in hopes of increasing the transformation efficiency of *D. vulgaris* and facilitating the construction of future deletions. As anticipated, electroporation experiments with stable plasmids revealed an improvement in transformation efficiency for stable plasmids when compared to wild-type *D. vulgaris*. Finally, Gateway Technology (Invitrogen) was applied to generate a destination vector (pMO727) containing the constitutively expressed wild-type *upp* gene. This vector will expedite the process of creating the required suicide deletion vectors for future markerless deletions.

MATERIAL AND METHODS:

Strains, media, and growth conditions

Strains used in this study are listed in Table 1. *Escherichia coli* strains were cultured aerobically in liquid LC medium (components per liter of medium: 10 g tryptone, 5 g NaCl, and 5 g yeast extract) and recovered in SOC medium (components per liter of medium: 5 g yeast extract, 9 g tryptone, 0.5 g NaCl, 0.19 g KCl, 3.6 g glucose, 10 ml of 1M MgCl, and 10 ml of 1M $MgSO_4$) (17) following transformation. Where indicated, ampicillin, kanamycin,

chloramphenicol, and spectinomycin were added to LC medium to final concentrations of 100 µg/ml, 50 µg/ml, 30 µg/ml and 100 µg/ml, respectively. When plating for blue/white selection with pBluescript plasmids (Table 1), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) was added to LC plates at a final concentration of 80 µg/ml. Chemicals and antibiotics were obtained from Fisher Scientific (Pittsburg, PA). For plating, LC medium was solidified with 15 g agar/L.

D. vulgaris strains were manipulated and grown at ~32^{o}C in an anaerobic growth chamber (Coy Laboratory Product, Inc., Grass Lake, MI), unless indicated otherwise, in either LS4D (25) amended to contain 30 mM Tris (pH 7.4) instead of 30 mM PIPES or in Wall medium (8 mM MgCl, 20 mM NH_4Cl, 0.6 mM $CaCl_2$, 2 mM K_2HPO_4, 60 µM $FeCl_2$, 120 µM EDTA, 30 mM Tris (pH 7.4), 0.1% wt/vol yeast extract, 1 ml Thauers vitamin solution per liter (6), and 6 ml trace elements solution per liter, with pH adjusted to 7.2). The trace elements solution contained 2.5 mM $MnCl_2$, 1.26 mM $CoCl_2$, 1.47 mM $ZnCl_2$, 210 µM Na_2MoO_4, 320 µM H_3BO_3, 380 µM NiSO4, 11.7 µM $CuCl_2$, 35 µM Na_2SeO_3, and 24 µM Na_2WO_4. For Wall medium, sodium lactate (60 mM) was used as the electron donor with either 30 mM sodium sulfate (Wall LS4) or 40 mM sodium sulfite (Wall LS3) as the terminal electron acceptor. When necessary and indicated, antibiotics were added to the Wall medium to a final concentration of 100 µg spectinomycin/ml or 400 µg G418/ml. G418 (RPI corp., Mt. Prospect, IL) was used in place of kanamycin because it was found to be more effective for kanamycin resistance selection in *D. vulgaris*. When growing *D. vulgaris* cultures in Wall medium, the 5-FU (Fisher Scientific, Pittsburg, PA) was added to a final concentration of 2.5 µg/ml for liquid cultures or 40 µg /ml in solidified medium. For plating, Wall medium was solidified with 1.5% (wt/vol) agar and two reductants were added: sodium thioglycolate (1.2 mM) and titanium citrate (380 µM). Cells

were dispensed into sterile, empty Petri dishes and then molten Wall LS4 was poured over the cells and swirled. All plating steps were carried out in an anaerobic glove chamber (Coy Laboratory Products Inc., Grass Lake, MI) with an atmosphere of ~95% N_2 and ~5% H_2. Once solidified, the plates were inverted, placed in an airtight container and incubated at ~32^{o}C for 4 – 7 days. *D. vulgaris* cultures were routinely plated on LC plates containing 40 mM glucose and incubated in air to insure that they were free of aerobic contaminants.

Generation of the Δ*upp* strain, JW710

A *upp* deletion cassette was constructed as described by Bender et al. (2), with the differences listed below. Primers (all listed in Table S1 and generated by IDT, Coralville, IA) were designed to amplify approximately 1,000 bp upstream (P1 and P2) and 900 bp downstream (P3 and P4) of the *upp* gene (DVU1025). For future deletion tracking, primers P2 and P3 were designed to contain the following unique barcode ACG TGC CTA AAG TGT ACT ACG ACA CCT CCG CGA TGA GT A, as well as five additional overlapping base pairs on each 5' end to allow the splicing together of the two fragments by sequence overlap extension or SOEing PCR (20). Amplification of the two regions by PCR, the splicing together of those two products, and the capture of that deletion cassette in the EcoRV site of pBluescript SK(+) were performed as previously described (2) and illustrated in Fig. 1. The resulting pMO711 was sent to the DNA Core Facility at the University of Missouri for sequence verification before proceeding.

The preparation of competent *D. vulgaris* and electroporation by pMO711 was performed as described (2), with following modifications. Briefly, the electroporation was carried out anaerobically in a total volume of 75 µl with an ECM 630 electroporator, BTX (Genetronix, San Jose, CA) in 1-mm gapped electroporation cuvettes (Molecular BioProducts, San Diego, CA) at 1750 V, 250 Ω, and 25 µF. The cells were allowed to recover in 1 ml of LS4D medium

overnight at ~32°C. An additional 5 ml of fresh LS4D was then added to the recovered cells which were allowed to grow to early stationary phase. Aliquots of one ml were mixed with 4 ml of molten reduced top agar (1.5 % wt/vol agar, 30 mM PIPES), and poured onto reduced LS4 (LS4D modified to contain 0.1% wt/vol yeast extract) solidified medium containing 40 μg 5-FU/ml and the plates incubated for ~7 days at 32°C in the anaerobic chamber. 5-FU^r colonies were selected and six were grown to stationary phase in liquid LS4D medium with 40 μg 5-FU/ml, gDNA was extracted, and the deletion of the *upp* gene was verified by PCR reactions. Size differences of the PCR amplicons indicated the presence of the wild-type target *upp* gene or the successful deletion (two of the six isolates). One isolate that produced no amplicon from primers P5 and P6 internal to the *upp* gene (Fig. 1) and a band of the predicted size for a successful deletion from primers P7 and P8 was selected and named JW710.

Colony PCR screening

In order to screen *E. coli* colonies for correctly constructed plasmids, colony PCR screens were performed. Individual colonies were picked from a plate and dispersed in 50 μl of a PCR mixture in a 0.2 ml PCR tube. The remaining cells from the colony were streaked on a LC plate containing the appropriate antibiotic for plasmid maintenance. Isolates were identified that produced the expected PCR amplicons from the plasmid constructs. New plasmids were considered verified only after both DNA strands of the construct had been fully sequenced. All sequencing was performed at the DNA Core Facility at the University of Missouri.

Generation of $P_{aph(3')\text{-II}}$-*upp* construct

The pMO715 plasmid (Fig. 2A) was constructed for complementation of the *upp* deletion. Two regions were amplified with Herc polymerase (Stratagene, La Jolla, CA). A 449-bp fragment containing the promoter for the aminoglycoside phosphotransferase II gene,

(*aph(3')*-II from Tn*5*) was amplified with primers P9 and P10, and 697 bp containing the wild-type *upp* gene from *D. vulgaris* was amplified with primers P11 and P12. Primers P10 and P11 contained overlapping sequences to allow splicing of the two products by SOEing PCR and primers P9 and P12 amplified the spliced product. The product was purified with the Wizard® SV Gel and PCR Clean-Up System (Promega) and cloned into pCR4Blunt-TOPO (Invitrogen) (Fig. S1). The recombinant plasmid was transformed into One Shot® TOP10 Competent Cells (Invitrogen) and plated on LC with 100 μg Kan/ml. Individual transformants were screened for the insert by colony PCR with primers P23 and P24. After verification of the sequence, one plasmid isolate was kept as the source of the constitutively expressed *upp* gene and named pMO715.

Generation of plasmids for deletion construction

pMO719 and pMO720: A stable plasmid conferring spectinomycin resistance to *Desulfovibrio* strains was constructed and designated pMO719 (Fig. 2B). The plasmid was built as a shuttle vector. The replicon for *E. coli* and the spectinomycin resistance were provided by the EcoRI-bounded pCR8/GW/TOPO fragment. The *Desulfovibrio desulfuricans* G100A replicon of the cryptic plasmid pBG1 (33) allows for replication in the sulfate-reducing strains and was provided from pSC27 (31) (Fig. S1).

The *upp* gene and the *aph(3')-II* promoter were removed from pMO715 by digestion with the restriction endonucleases PmeI and SnaBI. The gel-purified DNA fragment was ligated into the EcoRV site of pMO719 (Fig. S1). After verification of the sequence, one isolate was named pMO720 (Fig. 2C).

pMO727: In order to convert pMO720 into a Gateway destination vector (Invitrogen) that would allow facile construction of mutagenic vectors for generation of markerless deletions,

the Gateway Vector Conversion System (Invitrogen) was used. The pBG1 replicon and the *att*L sites from pCR8/GW/TOPO were removed from pMO720 by digestion with the restriction endonucleases AclI and BspEI (Fig. S2). The ends of the remaining vector DNA were made blunt with DNA Polymerase I, Large (Klenow) Fragment (NEB). Following dephosphorylation of the vector by Antarctic Phosphatase, the Gateway reading frame cassette B (Invitrogen) was ligated and the recombinant plasmid transformed into One Shot *ccd*B Survival Competent cells (Invitrogen). Transformants were selected on LC medium containing chloramphenicol and screened by colony PCR with primers P29 and P30. After verification of the sequence, one isolate was named pMO727 (Fig. 2D).

pMO728: The plasmid pMO728 (Fig. 2E) is an intermediate in the construction of the plasmid used for deletion of *hsdR,* encoding the type I restriction-modification system endonuclease (DVU1703). The deletion cassette unique to pMO728 was constructed by SOEing PCR of 1079 bp upstream of DVU1702 (primers P13 and P14) directly to 1040 bp of the region downstream of DVU1703 (primers P15 and P16). The amplicon was purified, cloned into pENTR/D-TOPO (Invitrogen), the sequence verified and the construct designated pMO728.

pMO729: The suicide plasmid, pMO729 (Fig. 2F), for the construction of the markerless deletion of the gene encoding the Type I restriction endonuclease *hsdR* (DVU1703) was made from pMO727 and pMO728 with the LR Clonase Reaction as instructed by the manufacturer (Invitrogen) (Fig S2). After verification of the sequence, one isolate was named pMO729.

Electroporation in *D. vulgaris*

For the preparation of *D. vulgaris* cells for electroporation, a 50 ml Wall LS3 culture was grown to an optimal OD_{600} of 0.4 – 0.6. Following centrifugation, cells were washed with 50 ml

of chilled, sterile electroporation wash buffer (30 mM Tris-HCl buffer, pH 7.2, not anaerobic), and centrifuged again. The resulting pellet was resuspended in 0.5 ml of chilled wash buffer. To 50 µl of prepared cells, five µl (~one µg) of the plasmid was added, and the mixture transferred to a cuvette. The electroporation parameters were the same as those described for Δ*upp* construction, except the electroporation was performed aerobically. The transformed cells were allowed to recover in one ml of Wall LS3 medium overnight at ~32^{o}C anaerobically. Various aliquots of the electroporated, recovered cells were plated in Wall LS4 medium with spectinomycin. Plates were incubated for four – seven days at 32^{o}C until Spec^{r} transformant colonies were visible.

Southern blot analysis

Southern blots were performed as previously described (2), with the template for the probe being the PCR amplicon of the upstream region of *hsdR* gene (primers P13 and P14).

RESULTS

Construction of a *Δupp* host strain

The natural resistance of the SRBs to many antibiotics (28) limits the available markers for facile selection in these bacteria. The use of a PRTase as the basis for a counter-selection strategy in *D. vulgaris* would eliminate the necessity of a residual antibiotic resistance for each mutation constructed. A counter selection is possible if: (1) the wild-type strain is sensitive to a toxic base analog; (2) a strain deleted for the gene encoding the PRTase is resistant to the analog; and (3) the reintroduction of the PRTase gene restores sensitivity. The PRTase deletion mutant becomes the host for construction of further deletions without retention of antibiotic resistance markers and each deletion can be made without generating polar mutations.

Analysis of the genome of *D. vulgaris* revealed a gene annotated as uracil PRTase, *upp,* DVU4025 (Fig. 3). Experiments to determine if *D. vulgaris* was inhibited by the toxic pyrimidine analog 5-fluorouracil (5-FU) indicated the bacterium was sensitive to as little as 0.1 μg of 5-FU per ml (3). In order to use 5-FU for counter selection, a host strain with the *upp* gene deleted (5-FU^r) was constructed. An electroporation of *D. vulgaris* was performed with the suicide plasmid vector pMO711 containing 987 bp upstream and 858 bp downstream of the *upp* gene fused together. By selection for 5-FU^r, transformants were obtained that were deleted for the *upp* gene following a double recombination with the mutagenic plasmid. Following PCR verification (data not shown), one isolate was designated JW710 (Fig. 3, Panel B). Resistance of JW710 to 5-FU was confirmed (Fig. 4, Panel B) and this deletion strain serves as the host strain for the markerless deletion system in *D. vulgaris*.

Complementation of Δ*upp*

To complement the *upp* deletion, the *upp* gene was cloned under the control of the *aph*(3')-II promoter known to be constitutively expressed in *D. vulgaris.* The promoter and gene were captured in the pCR4Blunt-TOPO plasmid, creating pMO715 (Fig. 2A). The restriction fragment containing the promoter and *upp* was then cloned into the EcoRV site of pMO719 (Fig. 2B), generating pMO720 (Fig. 2C), a stable plasmid capable of replicating in *D. vulgaris*. Both pMO719 and pMO720 were electroporated into JW710, the strain deleted for *upp*, to explore 5-FU resistance phenotypes.

D. vulgaris and JW710 (Δ*upp*) grown in the presence and absence of 2.5 μg of 5-FU/ml showed that *D. vulgaris* was inhibited at this concentration of the pyrimidine analog (Fig. 4A), while the growth of JW710 was not affected (Fig. 4B). JW710 cells carrying pMO719, the vector without the *upp* gene, were resistant to 5-FU (Fig. 4C); however, JW710 cells carrying

pMO720, constitutively expressing *upp*, were inhibited at the same levels as wild type *D. vulgaris* (Fig. 4). The restoration of 5-FU sensitivity to JW710 demonstrated that the construct was fully functional. Therefore, the deletion of the *upp* gene in *D. vulgaris* created a strain, JW710, to serve as the host for the two step integration and excision markerless deletion procedure.

Generation of the destination dector, pMO727, and its use in deleting the gene for the type 1 restriction-modification endonuclease (DVU1703-1702)

Since a suicide deletion plasmid containing three features -- (1) a fusion of the ~1 kb upstream and ~1 kb downstream of the gene of interest (GOI); (2) an antibiotic resistance gene (spectinomycin resistance); and (3) a functional *upp* gene -- is required for the generation of each markerless mutant, Gateway Technology from Invitrogen was utilized to expedite the mutagenic vector construction process. A universal destination vector, pMO727 (Fig. 2D), was constructed containing the *aph(3')*-II promoter fused to *upp*, a gene conferring spectinomycin resistance, and the *att*R1/*att*R2 sites. DNA fragments flanking the gene to be deleted are captured in pENTR/D-TOPO and the movement of that cassette into pMO727 to create the specific mutagenic vectors is performed in vitro by site specific recombination enzymes from temperate bacteriophage (Invitrogen). The effectiveness of pMO727 as a destination vector was tested by the generation of the deletion vector for a type I restriction endonuclease gene, *hsdR* gene (DVU1703), pMO729 (Fig. 2F). For the deletion of the *hsdR,* a markerless deletion cassette was generated by fusing ~1 kb of the region upstream of *hsdR* (DVU1703) directly to ~1 kb of the region downstream of DVU1702. This markerless deletion cassette was then cloned into pENTR/D-TOPO, producing the entry vector, pMO728 (Fig. 2E). A LR clonase reaction allowed for recombination between the *att*R1/*att*R2 sites in the destination vector pMO727 and the

*att*L1/*att*L2 sites in the entry clone pMO728; which generated the markerless deletion suicide vector pMO729 that was then electroporated into JW710.

Southern blot analysis was performed (data not shown) on several transformants to confirm the expected integration of pMO729 into the chromosome of JW710 (Fig. 5, Step 1). One Spec^r 5-FU^s isolate was confirmed for appropriate integration of this mutagenic plasmid into the JW710 chromosome and was designated JW7034 (Fig. 5).

The second step for creating the Δ*hsdR* mutant was the selection of strains that had lost pMO729 sequences from the chromosome by a second recombination event. Such a recombinational event would render cells 5-FU^r and Spec^s by loss of the *upp* gene and the gene encoding the antibiotic resistance, respectively. The location of that second recombinational event determined whether the 5-FU^r strain had deleted or restored the wild-type *hsdR* gene (Fig. 5, Step 2). JW7034 was grown in Wall LS4 medium (in the absence of spectinomycin) for 24 hours to allow the second recombinational event to occur that would resolve the partial diploid (Fig. 5, Step 2). Sixteen 5-FU^r colonies were picked, grown, and screened by PCR for the deletion of *hsdR* (primer positions shown in Fig. 3). Nine of the 16 transformants had clearly identifiable bands of the expected size for deletion of *hsdR-CHP* (data not shown). Southern blot analysis was performed on six of the nine isolates with a probe corresponding to the DNA upstream of DVU1703 (Fig. 6). DNA from the host strain JW710, *hsdR*$^+$, produced a single BclI band of the predicted size (8100 bp; Fig. 6, lane 1). Strain JW7034 with the integrated mutagenic plasmid produced two bands (7165 bp and 5082 bp) as expected (Fig. 6, lane 2) because it is a merodiploid for the region probed (Fig. 5, Step 1). Genomic DNA from a successful deletion of the *hsdR-CHP* genes was predicted to produce a single 3900 bp BclI band. In contrast to the PCR results, one isolate clearly yielded only the wild type *hsd*R BclI band of

8100 bp (Fig. 6, lane 3). Genomic DNA from five of the transformants had a 3900 bp band (Fig. 6, lanes 4 – 8); however, two of those transformants also contained an 8100 bp band with homology to the probe (Fig. 6, lanes 4 & 5), suggesting that mixed colonies may have been selected. Three isolates appeared to contain a genome structure consistent with a deletion of the *hsdR-CHP* region (Fig. 6). An isolate (Fig. 6, lane 6) was kept as the markerless deletion mutant lacking the *hsdR* and the downstream *CHP* gene and was designated JW7035.

Characterization of transformation efficiency of JW7035

To determine if the deletion of the type I restriction endonuclease *hsdR* gene had an effect on the transformation efficiency, transformation experiments with plasmids that replicate in *D. vulgaris* (containing the endogenous SRB cryptic plasmid pBG1) were performed. Competent cells of wild-type *D. vulgaris* and mutant strains JW710, JW801, and JW7035 were prepared and electroporations performed with either pSC27 or pMO719. Competent cells subjected to electroporation without plasmid DNA served as controls to determine the number of spontaneously antibiotic resistant colonies. Three separate electroporation experiments were performed for each strain and the number of transformant CFUs were counted and compared. While the number of transformants recovered varied among the electroporation experiments, the differences in transformation efficiencies between the strains were consistent (Table 2).

Transformation of wild-type *D. vulgaris* by pSC27, a 9.1-kb plasmid containing a Kan^r determinant, or pMO719, a 5.1-kb plasmid conferring spectinomycin resistance, resulted in low, but similar efficiencies of 2-5 transformants per μg of plasmid DNA. Loss of the *upp* gene did not improve transformation by pSC27, but unexpectedly did appear to increase transformation efficiency of pMO719. In contrast, loss of the *hsdR* gene resulted in 10^2 to 10^3 fold increases in the numbers of transformants obtained for these plasmids when compared to wild type. The loss

of the native plasmid, pDV1, from wild-type *D. vulgaris* that generated JW801 also improved transformation efficiencies by increases similar to JW7035. Annotation of pDV1 revealed a type II restriction modification system and its loss by plasmid curing is a possible cause of the increased transformation observed for JW801. Experiments to confirm this hypothesis are underway.

To verify that the increase in transformation efficiency of JW7035 was due to the deletion of *hsdR* and not due the loss of the native pDV1 plasmid, PCR experiments were performed to amplify genes annotated on either pDV1 or the chromosome. DVUA0015 encoding the *nifH* gene was targeted as a reporter for the presence of pDV1. Chromosomal genes, DVU3152 (encoding a histidine kinase) and DVU0942 (encoding a *fur* homolog), were amplified to confirm the quality of the gDNA templates. The PCR products confirmed that pDV1 was present in wild-type *D. vulgaris,* JW710, and JW7035 (Fig. S3, lanes 2 – 4) and absent in JW801 (Fig. S3, lanes 1). These data were consistent with our interpretation that loss of the type I restriction endonuclease caused by deletion of *hsdR* produced the increase in transformation efficiency in JW7035.

DISCUSSION

Much is still to be learned about the metabolism and electron flow of the SRB, through studies of the model strain *D. vulgaris* Hildenborough. While advances have been made in the ability to generate marker exchange deletion mutants (2), the introduction of multiple deletions into a single strain has been limited (28). In a recent article, Giaever and Nislow (14) discuss the importance of obtaining multiple mutations and knockouts to fully dissect and understand the architecture of cellular pathways, including regulatory networks. As more SRBs are sequenced

and genomes annotated, the need to make multiple deletions to test metabolic pathways is ever increasing.

In this study, we have described the development of a new markerless deletion system in *D. vulgaris* Hildenborough. We have shown that the *upp* gene, in combination with the toxic pyrimidine base analog 5-fluorouracil, is an effective counterselectable marker in *D. vulgaris*. The uracil PRTase salvages pyrimidine bases during nucleotide turnover in the bacterium and permits incorporation of the toxic analog 5-FU. In comparison with most antibiotic resistance determinants whose loss can be detected only by screening a rather small number of cells, the loss of the *upp* gene can be selected as the rare acquisition of resistance to 5-FU. This provides a powerful counterselection. The *upp* gene itself was successfully deleted from wild-type *D. vulgaris* by direct selection of a rare double recombination event, generating the 5-FU^r JW710 (Δ*upp*). JW710 then provided the background for the markerless deletion system (Fig. 5).

To test the mutagenesis system, an unmarked deletion of the gene for a type 1 restriction endonuclease was created. In the final step, two genotypes with identical 5-FU resistance were predicted from the resolution of the partial diploid state. One recombinational event (Fig. 5, Step 2B) generates the desired mutation, while the other restores the wild-type gene and chromosomal structure (Fig. 5, Step 2A). The screening process to distinguish these events can become tedious, especially if the wild-type has any growth advantage over the mutant. Yet, when compared with the widely-used counterselectable *sacB* gene, which confers sucrose sensitivity (8, 10, 15, 18, 19, 21), the selection for 5-FU resistance is more straight forward and cleaner in our hands. From the resolution of the merodiploid, one would expect the theoretical ratio of wild-type gene to desired mutant to be equal, 50% wild-type and 50% mutant, if the relative sizes of homologous DNA available for the recombination events up and downstream of the

target gene are equal. In our initial PCR screening for the *hsdR* deletion, we were able to obtain those theoretical levels, as ~56% of the transformants screened consistent with the desired gene deletion.

Preliminary data indicate JW7035 not only shows an increase in the efficiency of transformation of stable plasmids, but also shows an increase in the number of transformants when the selecting events depend on both transformation and recombination (*e.g.,* Fig. 5, Step 1). By using JW7035 as the host strain for the markerless deletion system for *D. vulgaris,* instead of JW710, we should be able to increase the efficiency of construction of markerless deletion mutants because of the required plasmid integration.

JW801 also had increased transformation efficiency of plasmids when compared to wild-type *D. vulgaris* that may be due to the loss of a type II restriction system encoded on pDV1. We have provided data showing that the increase in efficiency of JW7035 is not due to the loss of pDV1. We are currently in the process of making a markerless deletion of the type II restriction system to test the role of those genes in the transformation efficiency of *D. vulgaris.*

As we begin to make multiple deletions to test the metabolic pathways in *D. vulgaris*, the wild-type may quickly gain a growth advantage over multiple gene deletion mutants. A growth advantage of wild-type over a mutant would make the screening process for a markerless deletion very long and tedious. We are working to modify the procedure described here to eliminate the resolution of the merodiploid resulting in the wild-type gene and drastically reduce screening by a procedure adapted from *Bacillus subtilis* (9) and in *Methanosarcina acetivorans* (30) incorporating the use of the Flp recombinase.

Overall, we have demonstrated the functionality of the *upp* counterselection strategy for markerless deletion in *D. vulgaris* by construction of an in-frame deletion in the *hsdR* gene.

Since the *upp* counterselectable marker is recyclable, many genes can be sequentially deleted in the same strain for study of metabolic pathways. In addition, these markerless deletions can be designed to remain "in-frame" for the deletion of the genes within an operon without having polar effects on the downstream genes. This genetic exchange system can also be used to modify genes for the production of tagged proteins expressed from their native promoters. In addition, site-directed mutagenesis of target genes should be possible. This procedure and future improvements will greatly improve the genetic accessibility of this important group of environmental anaerobes.

ACKNOWLEDGEMENTS

This work was supported by the U. S. Department of Energy, Office of Science, Office of Biological and Environmental Research, through the grants ESPP-ESPP2 (http://VIMSS.lbl.gov) from DOE Office of Science, Genomics: GTL through contract DE-AC02-05CH11231 between Lawrence Berkeley National Laboratory and the U. S. Department of Energy and by the Office of Science (BER), U.S Department of Energy, grant OBER BioHydrogen Production and BioEthanol DE-FG02-083464691.

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Figure 1: Construction of pMO711, the mutagenic plasmid for the deletion of *upp*. Regions upstream and downstream of the *upp* gene were amplified to contain a unique barcode (primers P2 and P3). The two products were joined together using SOEing PCR and cloned into EcoRV site of pBluescript SK(+). Schematic is not drawn to scale.

Figure 2: Plasmids constructed for this study. Illustrations of various plasmids involved in the construction of the markerless deletion suicide plasmid for the type I site-specific restriction-modification system used in the cloning and construction of additional plasmids. Details of the construction of (A) pMO715, (B) pMO719, and (C) pMO720 are illustrated in Fig. S1, while the construction of (D) pMO727, (E) pMO728, and (F) pMO729 are illustrated in Fig. S2.

Figure 3: Illustration of the *upp* (A & B) and *hsd*R (C & D) operons before (A & C) and after (B & D) the deletion of genes within the operons. Shaded boxes and boxed arrows are predicted to be ORFs in the same operon. Arrowheads indicate direction of transcription. Dark outlined ORFs with bold letters were targets for deletions. Operons, gene annotation, and DVU numbers were taken from MicrobesOnline (http://www.microbesonline.org) (1). (Primers are not drawn to scale)

Figure 4: The *aph(3')*-II promoted *upp* gene restores 5-FU sensitivity of JW710 (Δ*upp*) to wild-type *D. vulgaris* levels. Growth of (A) wild-type *D. vulgaris*, (B) JW710 (Δ*upp*), (C) JW710 (Δ*upp*) carrying the SRB stable plasmid pMO719 without *upp*, and (D) JW710 (Δ*upp*) carrying the SRB stable plasmid pMO720 with the *aph(3')*-II promoted *upp* gene. (A) and (B) were grown in Wall LS4 medium with either 2.5 μg of 5-FU/ml (■ square, dashed lines) or

without (♦ diamond, solid line). (C) and (D) were grown in the same medium modified by the inclusion of 100 μg of spectinomycin/ml. Cultures were incubated anaerobically at 37°C for 30 hours and growth was measured as optical density at 600 nm. Each point is the average of triplicate samples, errors bars are within data symbols as the standard deviation of $\leq$ 0.031.

Figure 5: A schematic representation of the multi-step process used for the construction of the markerless deletion mutant JW7035 in *D. vulgaris*. Step 1 shows the integration of the deletion plasmid pMO729 into the chromosome of the Δ*upp* strain, JW710, selected as resistance to spectinomycin. A plasmid integrant achieved in Step 1 (JW7034) was grown in the absence of spectinomycin and plated on Wall LS4 medium containing 40 μg of 5-FU/ml, selecting for 5-FU^R transformants. Depending on the location of the second recombinational event, the 5-FU^R transformants were either wild-type for *hsd*R (A) or deleted for *hsd*R without retention of an antibiotic resistance gene (B). PCR screens were performed on transformants to identify possible markerless deletions. Southern blot analysis was then used to further verify the proper construction of the markerless deletion of *hsd*R, JW7035. Schematic is not drawn to scale.

Figure 6: Southern Blot Verification of Deletion of Type I Restriction Modification, R-subunit (*hsd*R). BclI digestion of gDNA (Lane 1) JW710; (Lane 2) JW7034; and (Lanes 3 – 6) six isolates of JW7035. Homology was predicted to be found in a 8100 bp band for wild type *hsdR* gene, two bands at 7165 bp and 5082 bp for the merodiploid produced by integration of pMO729 into the chromosome, and a 3900 bp fragment for the successful markerless deletion the *hsdR* gene. DNA fragment sizes were determined by comparison with the migration of the GeneRuler™ 1 kb Plus DNA Ladder (Fermentas; Not Shown).

TABLE 1. Bacteria strains and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristics	Source
***E. coli* strains**		
GC5 Compentent cells	F⁻ ϕ80 *lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 λ⁻ *thi*-1 *gyr*A96 *rel*A1 *tonA*	Gene Choice
One Shot® TOP10 Competent Cells	F⁻ ϕ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 mcrA Δ(*mrr-hsd*RMS-*mcr*BC) *ara*D139 Δ(*araleu*) 7697 *gal*U *gal*K *rps*L (Str^r) *nup*G	Invitrogen
One Shot® *ccd*B Survival™ T1 Phage-Resistant Cells	F⁻ *mcr*A Δ*(mrr-hs*dRMS-*mcr*BC) 80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*Δ139 Δ(*ara-le*u)7697 *ga*lU *ga*lK *rps*L (Str^r) *en*dA1 *nu*pG *to*nA::P_{trc} –*ccd*A	Invitrogen
***D. vulgaris* Hildenborough strains**		
Wild-type	*Desulfovibrio vulgaris* Hildenborough, ATCC 29579, (pDV1*), 5-FU^s	ATCC
JW710	Δ*upp,* (pDV1), 5-FU^r	This Study
JW7034	Chromosomal insertion of pMO729 into JW710, (pDV1), Spec^r, 5-FU^s	This Study
JW7035	Δ*upp*, Δ*hsdR CHP***, (pDV1), 5-FU^r	This Study
JW801	*D. vulgaris* Hildenborough, ΔpDV1,5-FU^s	Wall Laboratory
Plasmids		
pBluescript SK(+)	Blue-white cloning vector, Amp^r	Stratagene
pSC27	*Desulfovibrio* shuttle vector; source of SRB replicon pBG1, *mob*, Kan^r	32
pCR4Blunt-TOPO	Cloning vector, Kan^r, Amp^r	Invitrogen
pCR8/GW/TOPO	Cloning vector, Spec^r	Invitrogen
pENTR/D-TOPO	Cloning vector, Kan^r	Invitrogen
pMO711	pBluescript containing the markerless deletion cassette for the *upp* gene, Amp^r	This Study
pMO715	pCR4Blunt-TOPO containing the $P_{aph(3')\text{-II}}$-*upp* (1087 bp); Kan^r, Amp^r	This Study
pMO719	*Desulfovibrio* shuttle vector; SRB replicon pBG1 (EcoRI to EcoRI from pSC27) ligated to the EcoRI bounded pCR8/GW/TOPO fragment, Spec^r	This Study
pMO720	$P_{aph(3')\text{-II}}$-*upp* inserted via PmeI/SnaBI digest from pMO715 into EcoRV site of pMO719, Spec^r	This Study
pMO727	Gateway Destination cassette [reading frame B] into AclI and BspEI of pMO720, Spec^r, Cm^r	This Study
pMO728	pENTR/D containing the DVU1703 (and DVU1702) markerless deletion cassette with TOPO cloning, Kan^r	This Study
pMO729	DVU1703 (and DVU1702) markerless deletion cassette from pMO728 into pMO727 using LR clonase reaction, Spec^r	This Study

* pDV1 is an endogenous 202 kb plasmid in *D. vulgaris* Hildenborough

**CHP = Conserved Hypothetical Protein. The deletion in JW7035 is of both the *hsdR* gene (DVU1703) and the CHP gene (DVU1702); however, the strain will be referred to as Δ*hsdR*

TABLE 2. Characterization of transformation efficiencies[a]		
	Transformants/μg plasmid DNA	
Strain[b]	pSC27 G418^r	pMO719 Spec^r
D. vulgaris	2.1 x 10^0	4.4 x 10^0
JW801	2.3 x 10^2	6.3 x 10^3
JW710 (Δ*upp*)	3.1 x 10^{-1}[c]	3.2 x 10^2
JW7035 (Δ*upp*,Δ*hsdR*)	2.4 x 10^3	2.8 x 10^3

[a]Data are the average from two separate electroporation experiments, with multiple plates counted per experiment.

[b]Spontaneous resistance to G418 or spectinomycin was not detected in the sample sizes of the recipient strains plated. Therefore, fewer than 1 in 10^9 cells of each recipient strain was spontaneously resistant.

[c]The low transformation efficiency for JW710 resulted from a lack of transformation in one of the two experiments used for the averages. Additional experiments showed that 2 – 10 transformants per μg of plasmid DNA was a more typical result.

Figure 1.

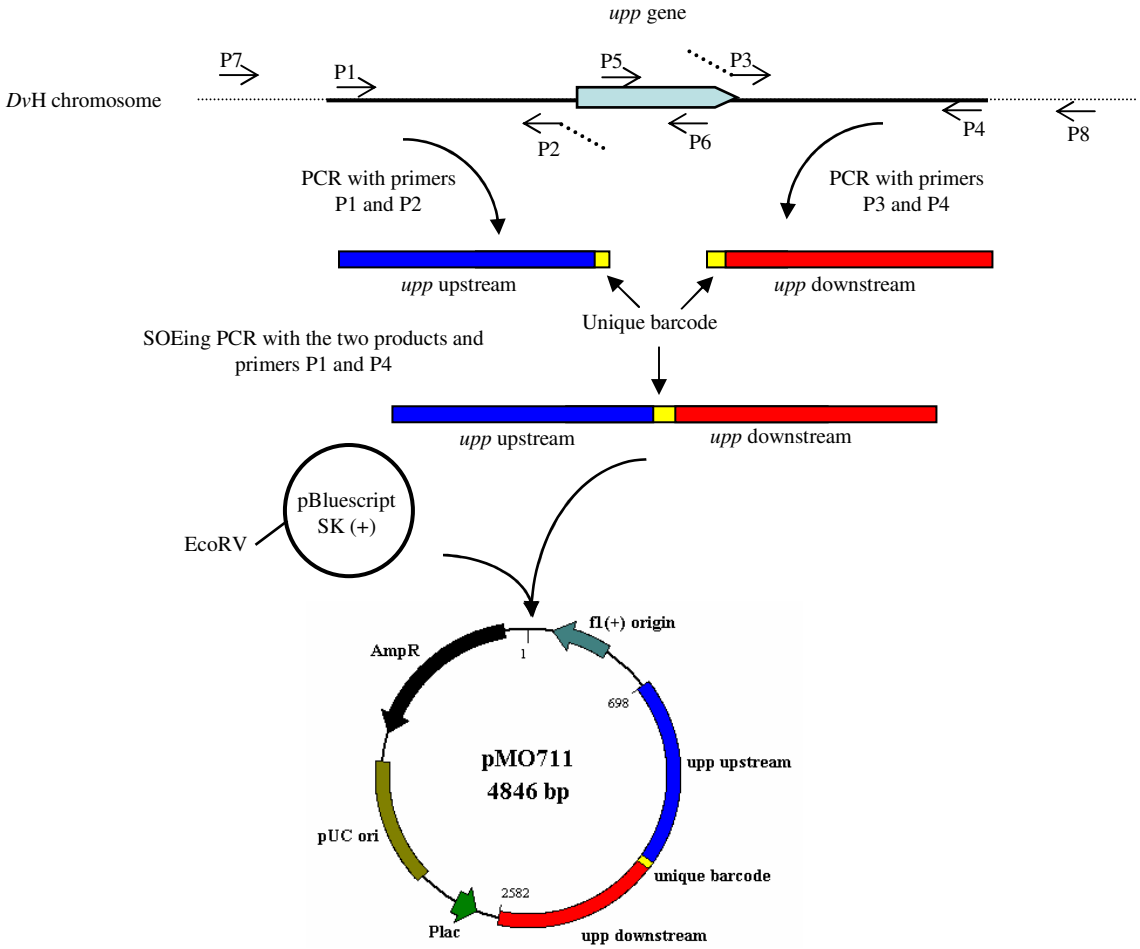

upp gene
P7
P1
P5
P3
*Dv*H chromosome
P2
P6
P4
P8
PCR with primers
P1 and P2
PCR with primers
P3 and P4
upp upstream
upp downstream
Unique barcode
SOEing PCR with the two products and
primers P1 and P4
upp upstream
upp downstream
pBluescript
SK (+)
EcoRV
f1(+) origin
AmpR
1
698
pMO711
4846 bp
upp upstream
pUC ori
unique barcode
2582
Plac
upp downstream

Figure 2.

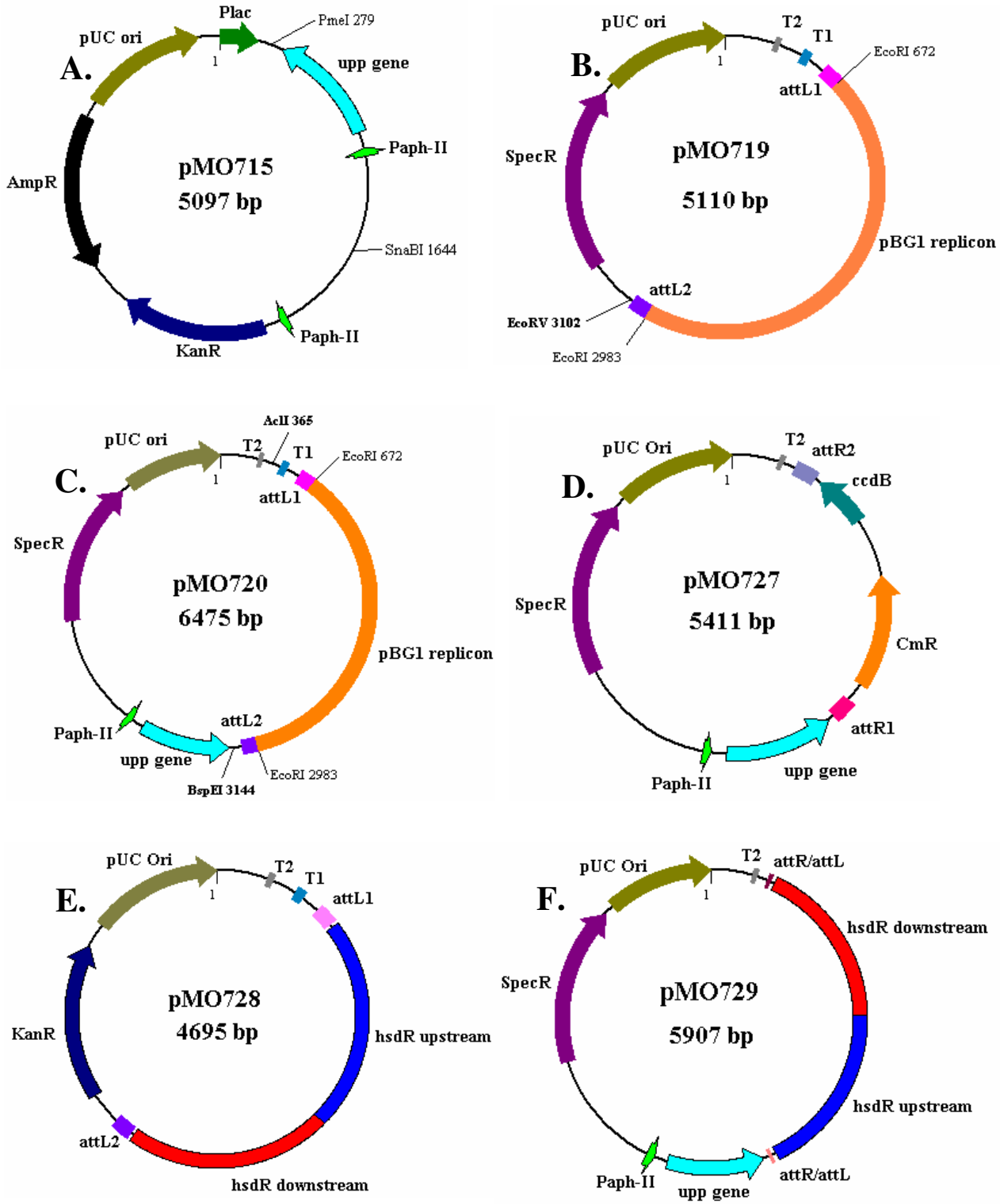

A.
pMO715
5097 bp
pUC ori
Plac
PmeI 279
upp gene
Paph-II
SnaBI 1644
Paph-II
KanR
AmpR
B.
pMO719
5110 bp
pUC ori
T2
T1
EcoRI 672
attL1
pBG1 replicon
attL2
EcoRV 3102
EcoRI 2983
SpecR
C.
pMO720
6475 bp
pUC ori
AclI 365
T2
T1
EcoRI 672
attL1
pBG1 replicon
attL2
Paph-II
upp gene
BspEI 3144
EcoRI 2983
SpecR
D.
pMO727
5411 bp
pUC Ori
T2
attR2
ccdB
CmR
attR1
Paph-II
upp gene
SpecR
E.
pMO728
4695 bp
pUC Ori
T2
T1
attL1
hsdR upstream
attL2
hsdR downstream
KanR
F.
pMO729
5907 bp
pUC Ori
T2
attR/attL
hsdR downstream
hsdR upstream
attR/attL
Paph-II
upp gene
SpecR

Figure 3.

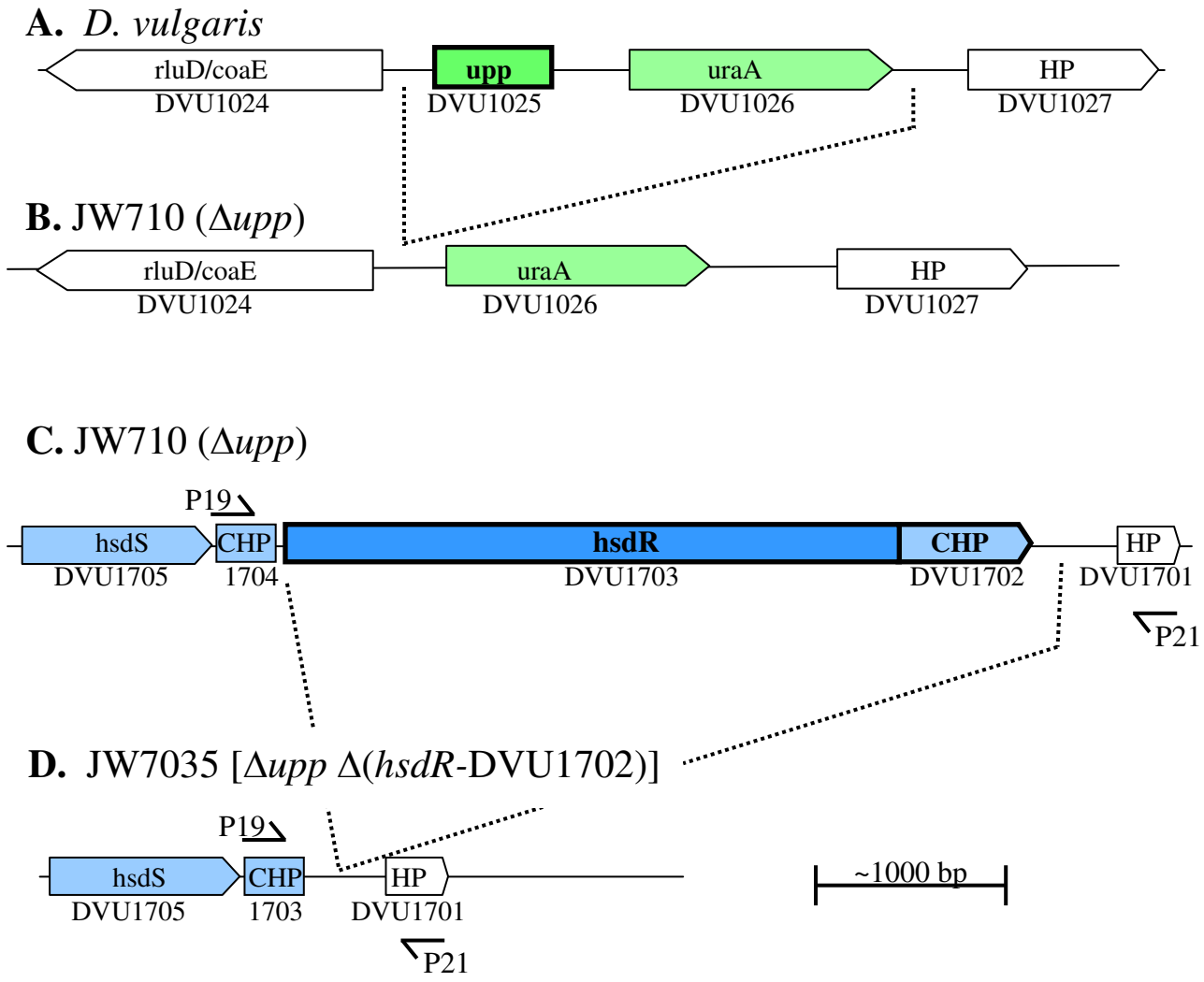

A. D. vulgaris
rluD/coaE
DVU1024
upp
DVU1025
uraA
DVU1026
HP
DVU1027
B. JW710 (Δupp)
rluD/coaE
DVU1024
uraA
DVU1026
HP
DVU1027
C. JW710 (Δupp)
P19
hsdS
DVU1705
CHP
1704
hsdR
DVU1703
CHP
DVU1702
HP
DVU1701
P21
D. JW7035 [Δupp Δ(hsdR-DVU1702)]
P19
hsdS
DVU1705
CHP
1703
HP
DVU1701
P21
~1000 bp

Figure 4.

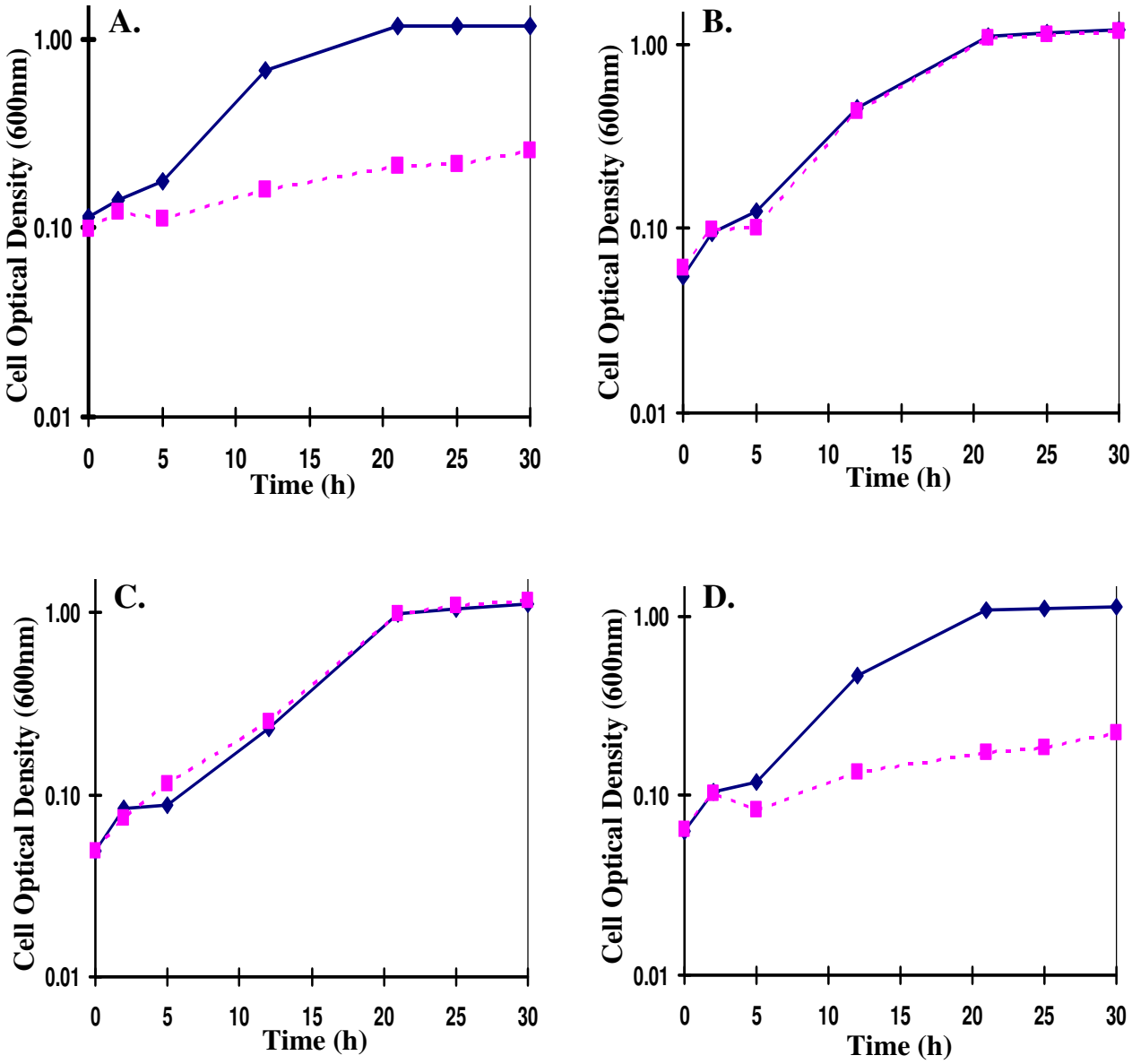

A.
B.
C.
D.
Cell Optical Density (600nm)
1.00
0.10
0.01
0
5
10
15
20
25
30
Time (h)

Figure 5.

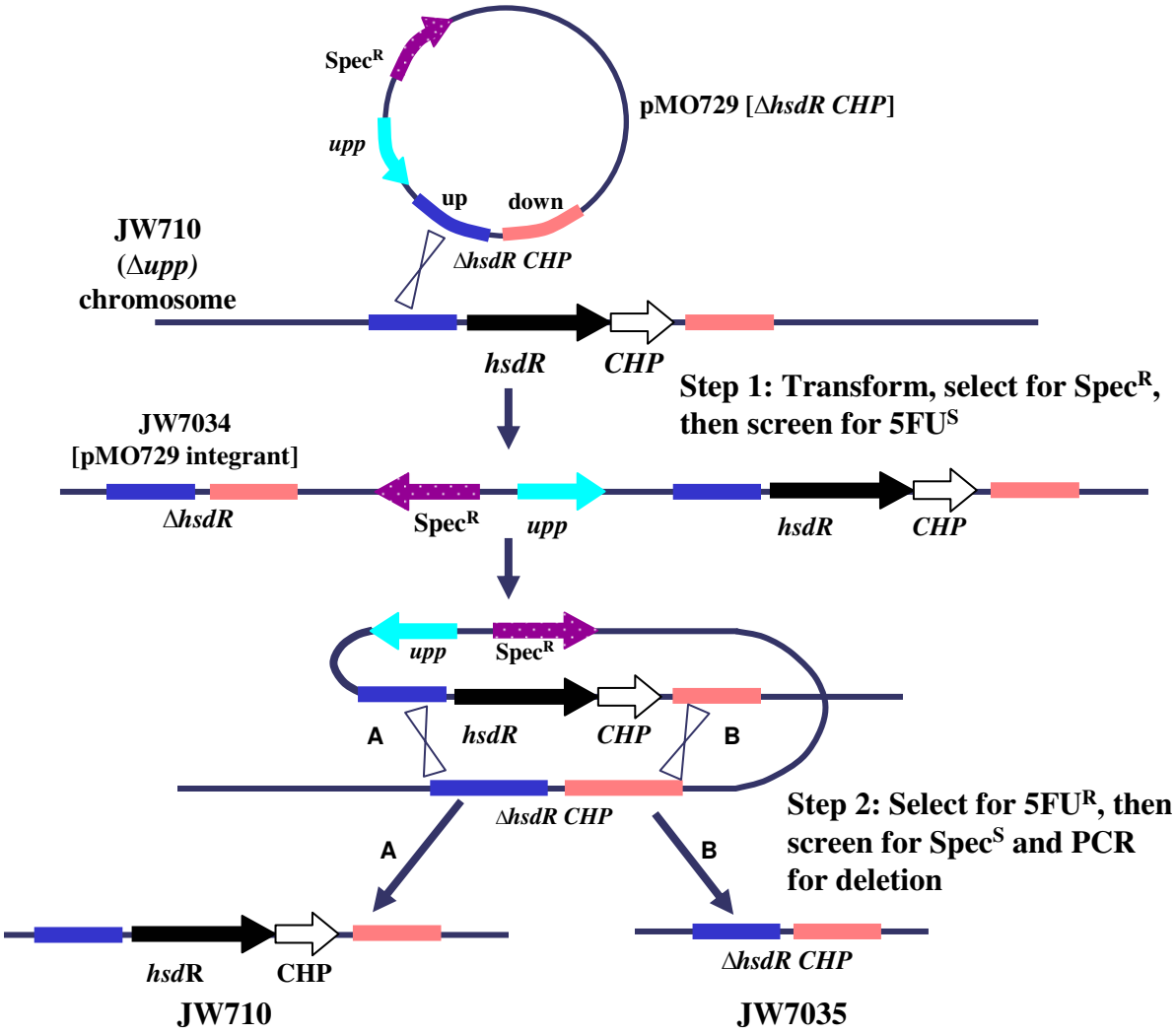
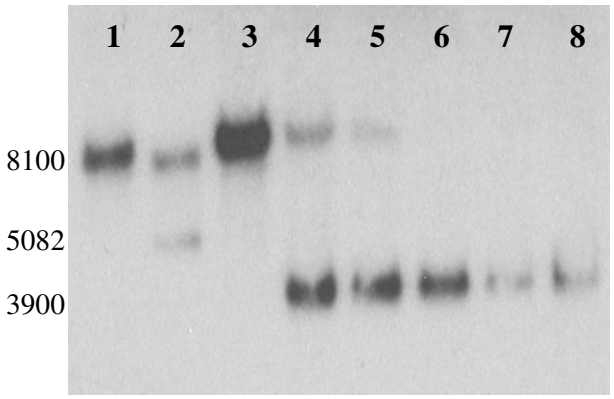

Spec^R
pMO729 [ΔhsdR CHP]
upp
up
down
JW710
(Δupp)
chromosome
ΔhsdR CHP
hsdR
CHP
Step 1: Transform, select for Spec^R, then screen for 5FU^S
JW7034
[pMO729 integrant]
ΔhsdR
Spec^R
upp
hsdR
CHP
upp
Spec^R
A
hsdR
CHP
B
ΔhsdR CHP
Step 2: Select for 5FU^R, then screen for Spec^S and PCR for deletion
A
B
hsdR
CHP
JW710
ΔhsdR CHP
JW7035

Figure 6.



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