Generalized Schemes for High-Throughput Manipulation of the *Desulfovibrio vulgaris* Genome

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Abstract

The ability to conduct advanced functional genomic studies of the thousands of sequenced bacteria has been hampered by the lack of available tools for making high-throughput chromosomal manipulations in a systematic manner that can be applied across diverse species. In this work, we highlight the use of synthetic biological tools to assemble custom suicide vectors with reusable and interchangeable DNA "parts" to facilitate chromosomal modification at designated loci. These constructs enable an array of downstream applications including gene replacement and creation of gene fusions with affinity purification or localization tags. We employed this approach to engineer chromosomal modifications in a bacterium that has previously proven difficult to manipulate genetically, *Desulfovibrio vulgaris* Hildenborough, to generate a library of over 700 strains. Furthermore, we demonstrate how these modifications can be used for examining metabolic pathways, protein-protein interactions, and protein localization. The ubiquity of suicide constructs in gene replacement throughout biology suggests that this approach can be applied to engineer a broad range of species for a diverse array of systems biological applications and is amenable to high-throughput implementation.

Keywords: Functional Genomics/ Microbial Chromosomal Engineering/ Obligate Anaerobic Bacteria.

Introduction

The rate and depth of characterization of bacterial species has increased over the last few years due to advances in genome sequencing technology and the application of high-throughput functional genomics approaches that identify and quantify mRNA transcripts, expressed proteins, and cellular metabolites. To answer important, far ranging questions in functional genomics [e.g., assessments of gene essentiality or cell-wide genetic interactions (epistasis) and protein-protein interactions (PPI)], experimental validation will require rapid and efficient genetic engineering of the strain of interest. Of the more than 1400 bacterial genomes sequenced so far (3), relatively few transposon-mediated knockout libraries have been reported (15, 20, 22, 24, 25, 30, 31, 34) and systematic, large-scale, single-gene deletion collections exist for only *E. coli* K12 (5) and *Acinetobacter baylyi* ADP1 (20). Furthermore, large-scale Tandem-Affinity-Purification (TAP)-based PPI identified with proteins produced from chromosomally tagged genes have been reported only for *E. coli* K12 (14). Genome-wide genetic interaction screening, which requires an ordered gene knockout library, has also recently been reported, but was only applied in *E. coli* (13, 43). In summary, there is currently no systematic approach for making large-scale, targeted chromosomal manipulations that can be readily applied across a diverse range of bacteria for functional genomics studies.

Here we present a scheme for high-throughput manipulation of bacterial genomes that is both inexpensive and flexible due to the use of interchangeable "parts" for making different kinds of chromosomal modifications, including gene deletions and tagged genes for the study of PPI and protein localization and other applications (Fig. 1). Our goal was to create a systematic approach for chromosomal modification that could be applied to a

wide range of bacteria with minimal need for methodological alteration. For this reason, the direct use of phage-based recombination systems in candidate microbes of interest was deemed unsuitable, as this could require species-specific additional host mutations and extensive development work for individual species. Rather, we chose to leverage a common theme for chromosomal modification: the use of non-replicating gene modification ("suicide") constructs. Suicide constructs have been used successfully for chromosomal modification in a wide range of bacterial species and generally require only host-based RecA-mediated homologous recombination (35). Our approach for high-throughput construction of suicide vectors was based on Sequence and Ligation Independent Cloning (SLIC) (29), heretofore used for plasmid-based (rather than chromosomally based) metabolic engineering and heterologous protein expression studies.

We applied this approach to the sulfate-reducing bacterium (SRB) *Desulfovibrio vulgaris* Hildenborough. *D. vulgaris*, which has been the subject of recent functional genomics studies (7, 16-18, 32, 38). Proposed stress response models of this bacterium are based on gene expression data alone and need to be complemented by other experimental data types. The ability to create targeted gene deletions in a systematic manner in this organism will help fill gaps in metabolic pathways and greatly assist in the functional annotation of unknown genes. In addition, the ability to generate TAP- or visualization-tagged genes will facilitate the development of the corresponding interactome and allow the mapping of protein complex localization within the cell. Here we compare the protocols for facile chromosomal engineering of *D. vulgaris* to achieve

these objectives and provide proof-of-principle data for protein complex isolation, gene deletion and sub-cellular localization (Fig. 4).

Materials and Methods

Mutant Strain Generation. Design principles for the three schemes are shown in Fig. S1. Laboratory Information Management System (LIMS) tools were developed for the Gateway® and SLIC schemes as detailed in the Supplemental Material. The Gateway® scheme involves generation of a library of entry vectors which serve as the source of mobile DNA fragments that are directionally incorporated in destination vectors of choice after the LR reaction. Entry vectors in this study were generated by directional TOPO cloning of desired DNA fragments into pENTR/dTOPO (Invitrogen Inc., Carlsbad, CA) and transformed in One Shot Top10 Chemically Competent *E. coli* (Invitrogen) as per the manufacturer's instructions. The DNA fragments for TOPO cloning were generated by PCR amplification of the respective regions from genomic DNA of wild-type *D. vulgaris* Hildenborough using a proofreading DNA polymerase, Pfu Turbo, (Stratagene Inc., La Jolla, CA). All plasmid extractions were performed using QIAprep Spin Miniprep Kits (Qiagen Inc., Valencia, CA). Design rules for primers used in amplification of desired DNA fragments are described in the Supplemental Material. Amplified targets were visualized on E-gels® (Invitrogen) before proceeding with dTOPO cloning. Up to three colonies were picked from ampicillin (100 μg/ml) containing LB-agar plates bearing the transformed cells for sequence verification of entry vectors. Sequence-verified constructs were used in the subsequent LR recombination step (as per the manufacturer's protocol) along with custom destination vectors. The design strategy for custom destination vectors is described in the Supplemental Material.

Sequence-verified dTOPO constructs were coupled with custom destination vectors through LR recombination to generate Gateway® constructs that carried the desired DNA fragments with the corresponding tag sequence appended at their 3' ends. Products of the LR recombination reaction were transformed into One Shot TOP10 chemically competent *E. coli* and plated on ampicillin (100 μg/ml) and kanamycin (50 μg/ml) containing LB-agar plates. Two colonies were randomly picked from the resulting plates for sequence verification of the Gateway® constructs. Sequence verified suicide constructs were transformed in competent *D. vulgaris* cells for chromosomal integration through homologous recombination as described below.

The λ Red recombination system has been utilized to perform both chromosomal and plasmid modification in *E. coli* (19, 41, 48). Here we utilized *E. coli* strain SW105, which expresses λ Red recombination functions when subjected to heat-shock, to enable the recombination of a PCR product into plasmids carrying fragments of *D. vulgaris* genomic DNA (45). For our desired application, this PCR product encoded a SPA tag and a kanamycin-resistance gene, and was identical to that used to create chromosomal SPA-tagged genes in *E. coli* (51). Five initial *D. vulgaris* target genes present on an 8.7-kb fragment spanning the entire *apsBA-qmoABC-DVU0857* region were provided on pMO9034 (J. Wall, U. Missouri) which is a derivative of pCR8/GW/TOPO (50). A further 18 *D. vulgaris* genes were provided on three genomic DNA fragments each cloned into pUC19 and designated pDVH2-36, pDVH2-37, pDVH2-39. Details on the PCR products employed and subsequent transformation in *E. coli* are described in the Supplemental Material. Transformed cells were plated onto selective LB medium containing kanamycin and colonies isolated. Isolates were then cultured, plasmid DNA

prepared and subjected to restriction analysis to confirm the integration of the PCR product at the correct locus in the plasmid target construct.

The SLIC technique was employed as described previously (29). Specifically four DNA fragments were pieced together to form the final suicide constructs. The same Inserted Part (IP, composed of the tag and one selection marker) and Selection Part (SP, composed of a plasmid origin of replication and a second selection marker) were used for a given library of insertion or deletion suicide constructs. The other two variable regions were generated by PCR amplification of the respective regions from genomic DNA of wild-type *D. vulgaris* Hildenborough using a proofreading DNA polymerase, Phusion High Fidelity DNA Polymerase (Finnzymes Inc., Woburn, MA). Design rules for primer design are detailed in the Supplemental Material. Sizes of amplified PCR products were verified using agarose gels and confirmed products were purified using the QIAquick® PCR Purification Kit (Qiagen) and subsequently quantified using a NanoDrop 2000 (NanoDrop products, Wilmington, DE). 1 μg of each part was treated with 1μl of 0.5 U T4 DNA polymerase in 1 x Buffer 2 (NEB) and 1 X BSA Buffer (NEB) in a 20 μl reaction at room temperature for 30 minutes. The reaction was stopped by adding 1/10 volume of 10 mM dCTP followed by an annealing step of all four parts at 37ºC for 30 minutes. The annealed mixture was next chemically transformed in DH10B competent cells and plated on agar plates bearing spectinomycin and kanamycin (both at 50 μg/ml) antibiotic resistance markers. Two colonies were picked after an overnight incubation (37ºC) for sequence verification of suicide constructs.

Transformation of D. vulgaris strains. For transformations, cells were grown in MOYLS4 medium in an anaerobic growth chamber (Coy Laboratory Products, Inc.,

Grass Lake, MI) with an atmosphere composed of nitrogen, <5 % hydrogen, and <2 % oxygen at 33°C. MOYLS4 medium contained 8 mM magnesium chloride, 20 mM ammonium chloride, 0.6 mM calcium chloride, 1 mM potassium phosphate (dibasic), 1 mM sodium phosphate (monobasic), 0.06 mM ferrous chloride, 0.12 mM EDTA, 30 mM TRIS (pH7.4), 60 mM sodium lactate, 30 mM sodium sulfate, 1 ml/L Thauer's vitamin solution (12), 6 ml/L trace element solution and 1.0 g/L yeast extract. The trace element solution was modified by addition of 2.5 mM manganese chloride, 1.26 mM cobaltous chloride, 35 uM sodium selenite, and 24 uM sodium tungstate. MOYLS4 was adjusted to pH7.2 with 12M HCl and 1.2 mM sodium thioglycolate was added as a reductant prior to sterilization in the autoclave. For plates, 15 g/L agar was added to MOYLS4 before sterilization. Immediately prior to pouring plates of molten MOYLS4 0.38 mM titanium citrate, prepared and stored anaerobically, was added along with antibiotics as appropriate. To prepare *D. vulgaris* cells for transformation, 1.0 ml of a freezer stock (early stationary-phase cells in 10% vol glycerol /vol MOYLS4) was added to 10 ml MOYLS4 and allowed to grow overnight. The 10-ml overnight culture was diluted to 100 ml in MOYLS4 and allowed to grow to an OD_{600} between 0.3 and 0.7 at 33 ^{o}C. The culture was harvested by centrifugation for 12 min at 3,000 x *g* at 4 ^{o}C, supernatant was discarded, and the cells resuspended in 50 ml of chilled, sterile wash buffer (30 mM Tris-HCl buffer, pH 7.2, non-degassed). A second centrifugation, under the same conditions, was used to wash the cells and the supernatant discarded. The pellet of electrocompetant cells was resuspended in 1.0 ml wash buffer and a 100-µl aliquot was used for electroporation. About 1 µg (10 µl) of the plasmid was added to the cells, mixed, and 100 µl of the mixture transferred to a 1-mm gapped electroporation cuvette (Molecular

BioProducts, San Diego, CA). The cuvette was transferred to the ECM 630 electroporator (BTX, Holliston, MA) and electroporated at 1500V, 250Ω, and 25μF. The electroporated cells were diluted into 1 ml MOYLS4 medium containing 0.1% wt/vol yeast extract. The putative transformants were transferred to the anaerobic chamber, opened momentarily for headspace exchange, and allowed to recover overnight at 33 ^{o}C. Aliquots (100 μl and 900 μl) of the recovered cells were then added to Petri plates, followed immediately by ~25 ml of reduced, molten MOYLS4 with 15 g/L agar containing the kanamycin analogue G418 (RPI corp., Mt. Prospect, IL; 400 μg/ml) in the anaerobic chamber. Two reductants were used. Sodium thioglycolate (1.2 mM) was added prior to autoclaving, while titanium citrate (1.2 mM, prepared under nitrogen and stored anaerobically) was added just prior to pouring the medium over the cells in the Petri plates. Colonies were typically observed after 5 d incubation. Selection and storage of mutants as well as protocols for Southern blot and IP western analysis to confirm chromosomal manipulations are described in the Supplemental Material.

Protein-Protein Interactions using Tandem Affinity Purification.

Affinity-tagged *D. vulgaris* strains were cultured in 2 x 1L LS4D medium (7) in stationary bottles present in an anaerobic chamber. Sequential peptide affinity purification was performed using tagged *D. vulgaris* strains exactly as previously reported for *E. coli* (51). The alternative Streptavidin-TEV-FLAG (STF) tag purifications were performed using the SPA protocol (14) with the following modifications. The STF purification is identical to the SPA procedure until immediately following TEV incubation and removal of tagged proteins from the anti-FLAG beads. For STF purification, after the TEV eluate was drained into a new column, 100 μl 50 % slurry

Streptactin Superflow beads (IBA GmbH) were added and incubated for 3 hours with rotation at 4 °C (17). The beads were then washed with 1.4 ml wash buffer I (100 mM Tris-Cl pH7.9, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 10 mM 2-mercaptoethanol, Roche Complete protease inhibitor), followed by 400 µl of the wash buffer without the Triton X-100, and proteins eluted using 300 µl of elution buffer containing 2.5 mM desthiobiotin (Sigma). Purified eluates were subsequently digested with trypsin and analyzed mass spectrometry to obtain protein identifications as detailed in the Supplemental Material.

Growth studies on knockout mutants for examining methionine biosynthesis. Growth study manipulations were done in an anaerobic growth chamber (Coy Laboratory Products, Inc.) which contained approximately 95% N_2 and 5% H_2. *D. vulgaris* wild-type and mutant strains were grown at 37ºC from freezer stocks to early stationary phase in 5 ml MO medium supplemented with 60 mM lactate, 30 mM sulfate, and 0.1% (wt./vol.) yeast extract (MOYLS4(60/30)). DVU0171 and DVU1585 gene deletion mutants were grown in MO medium supplemented with 60 mM lactate, 30 mM sulfate, 40 mM sulfite, and 0.2% (wt./vol.) yeast extract. To select for growth of the mutant strains, the media were supplemented with G418 antibiotic at 400 µg/ml. To impose starvation conditions, two consecutive triplicate 2% subcultures were then grown in 5mL defined medium supplemented with 60 mM lactate and 30mM sulfate [MOLS4(60/30)] (50) plus G418 at 400 µg/ml for mutant selection. As the DVU0890 deletion mutant exhibited little or no growth in subcultures, screening of the DVU0890 deletion mutant was performed without first subculturing in defined medium. To screen for auxotrophic mutant strains, triplicate 15 mL culture tubes containing 5 mL MOLS4(60/30) medium amended with 0.3 mM

amino acids were inoculated with 5% (vol./vol.) of the strains and sealed with rubber stoppers. Controls were prepared by inoculation of MOLS4(60/30) and MOYLS4(60/30) media. For comparison of growth, ODs were monitored at 600 nm and final total protein concentrations were measured using the Bradford Assay (9). LC-glucose plates were streaked to check for aerobic contamination of the initial growth culture and periodically of the subcultures. Gene deletion mutations were verified by PCR prior to and again upon completion of the growth studies.

Labeling of AGT-Tagged Proteins with SNAP Fluorophore.

The AGT tag or the SNAP-tag (New England Biolabs, Ipswich, MA) is a highly engineered modified version of AGT (alkylguanine DNA alkyltransferase), a human DNA repair protein with a molecular weight of 20 kD. It is a visualization tag similar to green fluorescent protein but unlike the latter has been shown to work effectively under anaerobic conditions. It forms a highly stable, covalent thioether bond with fluorophores or other substituted groups when appended to benzylguanine. This reaction is highly specific, i.e., expressed SNAP-tag fusion proteins can be labeled even in the presence of complex protein mixtures such as found in cells or in cleared bacterial lysates. Anaerobically grown cells cultures were harvested at mid-logarithmic phase with an optical density at 600 nm [OD_{600}] of 0.3 to 0.4 and centrifuged under anaerobic conditions at room temperature for 10 minutes at 5000 x *g*. The pellet was resuspended under anaerobic conditions in sterile LS4D medium in the range of 400 µl to 600 µl at OD_{600} of 0.5 (+/- 0.05) in order adjust each sample to the same relative cell density, as determined by initial optical density readings. The SNAP fluorophore reagent (New England Biolabs, Ipswich, MA) in DMSO was added to the cell suspensions to reach a

final reagent concentration of 5 μM. Cell solutions with the fluorophore reagent were protected from light and incubated at 30° C for at least 60 minutes to ensure all cells were exposed to the labeling reagent. Following the incubation period, the cells were centrifuged at room temperature twice at 5000 x *g* for 10 minutes and resuspended in sterile LS4D medium to remove any excess fluorophore reagent from the cells.

SDS-PAGE and In-Gel Fluorescence Detection. Labeled and washed cells were subjected to Pefabloc protease (Sigma-Aldrich Co., St. Louis, MO) and DNase (Sigma-Aldrich Co., St. Louis, MO) treatment, at a concentration of 400 μM and 0.2 mg/mL, respectively and stored on ice. Ice-cold cell suspensions were lyzed using a Branson sonicator (Branson Ultrasonics, Danbury, CT) for 1-2 minutes at 40% duty cycle with output of 3. After lysis, the samples were flash frozen in liquid nitrogen and stored at -20˚C. For SDS PAGE analysis, samples were mixed with 5X SDS loading buffer stock and boiled for 1 minute prior to loading into the pockets of a precast 4-20% Tris-HCL SDS-PAGE well (Thermo Fisher Scientific Inc., Pittsburgh, PA) and run at 140 V for approximately 50 minutes. A Pageruler prestained protein marker (Fermentas Inc., Glen Burnie, MD) was run on the gels, as this marker has a 72 kD protein which emits a fluorescent signal at 488 nm excitation, and therefore allowed easy correlation of in-gel fluorescence detection and Coomassie-stained gels. The gels were imaged for fluorescence using a Bio-Rad Molecular phosphor-imager with an external laser source using Alexa 488 filter settings (Bio-Rad Laboratories, Hercules, CA), followed by Coomassie staining (0.5% Coomassie Brilliant Blue R-250, 30% ethanol, 10% glacial acetic acid in ddH_20), and destaining in 30% ethanol, 10% glacial acetic acid in ddH_20 before imaging on a lightbox with a Canon A540 digital camera. For selected samples,

both intact cells as well as cell lysates were labeled and their in-gel fluorescence values were compared to ensure that the labeling reagent had ready in-vivo access to all tags. Details on cellular imaging using epifluorescence and deconvolution microscopy are provided in the Supplemental Material.

Results and Discussion

Development and application of schemes for high-throughput generation of suicide constructs.

Traditional mutagenesis approaches with suicide constructs have generally been regarded as cumbersome due to difficulties with vector construction (i.e., cloning of large sections of homologous DNA from either side of the locus to be modified) (35). We therefore tested three recombination based approaches – Gateway® (Invitrogen, Carlsbad, CA), 'Recombineering' (19, 47) and Sequence and Ligation Independent Cloning (SLIC) (29), for the generation of suicide constructs in a high-throughput manner (Fig. S1). In all examples, suicide vectors designed for modifying the *D. vulgaris* host chromosome were first generated in *E. coli* and then transformed into *D. vulgaris* resulting in a modified host chromosome through single or double homologous recombination events integrating all or part of the non-replicating delivery vector.

Suicide vector construction via the Gateway® scheme was realized through two steps (Fig. S1-A). In the first step, the sole homology region of the target locus was directionally cloned into a TOPO® entry vector. The second step involved a LR recombination reaction with directional placement of the homology region from the entry clone to a custom-designed destination vector. The destination vector included the sequences for modification of the host cell and a suitable origin of replication.

Destination vectors with different insertion sequences such as TAP tags for elucidation of PPI or visualization tags such as SNAP (O^6-alkylguanine-DNA alkyltransferase, New England Biolabs, Ipswich, MA) that allow protein localization may be used with a given library of entry clones. This powerful attribute of the Gateway® scheme allows facile exchange of the tags once a library of TOPO® entry clones has been constructed.

Importantly, the introduction of a single region of homologous DNA in the construction of the entry clones allows only a single recombination event with the host chromosome that incorporates the entire plasmid. When creating tagging constructs for genes located at the beginning of their operons, we incorporated the native promoter sequence in addition to the target gene to be modified in the suicide vector to allow the expression of downstream genes. Necessary promoter sequences for each gene were assumed to be present within 300 bp upstream of the target gene. From a practical standpoint, this scheme is therefore limited to genes located at terminal ends of their respective operons where downstream polarity effects can be minimized. These caveats render the Gateway scheme useful for rapid modification of a select class of target genes with a range of fusion tags. In order to be able to modify genes in a locus-independent manner, however, we leveraged two other schemes, 'Recombineering' and SLIC, to generate suicide vectors with two homology regions that permitted marker exchange.

In the 'Recombineering' approach, we utilized the bacteriophage lambda general recombination system (λ_{red}) (19) to modify genes carried on recombinant plasmids selected from an ordered genomic library of *D. vulgaris*. Expression of λ_{red} in *E. coli* has been shown to mediate efficient integration of linear DNA molecules into the host chromosome or plasmids through short regions (~40 bp) of sequence homology (47). In

this scheme (Fig. S1-B), a linear DNA molecule was generated by PCR that contained homology regions 1 and 2 (HR1/HR2) flanking the marker to be exchanged or the part to be inserted (insertion part, IP). In the example shown, the IP is an affinity purification tag and a kanamycin-resistance cassette expressed from its own promoter. Plasmid constructs and an HR1-IP-HR2-containing PCR product were transformed together into an *E. coli* strain in which the λ_{red} system was induced. The λ_{red} recombinase, facilitates recombination between the short 40bp homology regions of the target loci flanking the IP and identical regions in the plasmid containing the fragment of chromosomal DNA with the genes to be modified. The length of the chromosomal regions of homology available for double crossover events between modified plasmid constructs and the host chromosome varies with the location of the target gene within the genomic DNA insert in the suicide vector but is generally sufficient for detectable recombination with the genome being modified, with the exception of genes located at the termini of the insert DNA in the suicide vector. During the development of this modification procedure it was noted that many isolated strains contained both modified plasmid, into which the IP fragment had integrated, and unmodified plasmid. Furthermore, when higher plasmid concentrations were used, multimeric plasmids were often isolated, a phenomenon previously reported when using plasmids with the λ_{red} system (40). These factors led to increased processing requirements to generate a pure modified plasmid construct. Therefore, while suicide constructs made by this approach can be used for inserting or deleting sequences through marker exchange by double crossovers, ready availability of a comprehensive ordered genomic library is an essential prerequisite. Due to the lack of such a comprehensive set of library constructs for *D. vulgaris*, and the inefficiencies of

isolating recombineered plasmid constructs (compared to SLIC) this approach was not considered further in this study.

The third approach involves *de novo* assembly of suicide vectors by the SLIC procedure (Li and Elledge, 2007). Vectors assembled by this technique are composed of four parts – two corresponding to the homology regions (HR1/HR2) from the host chromosome, an insertion part (IP) dictated by the application of choice, and a vegetative origin of replication and selection part (RSP). To obtain a suicide vector, the replication origin was functional only in *E. coli* but not in the strain targeted for manipulation ((44); Fig. S1-C). The advantage of this approach lies in the reusability of parts for various applications. The IP and RSP regions remain constant for each specific application; whereas, HR1 and HR2 regions vary. Alternative IP regions such as molecular barcodes, purification tags, antibiotic markers, origins of replication are incorporated into vector construction depending on the downstream application. The RSP region is the most generic part used for suicide vector construction. However modification of the RSP to include an *oriT* (origin of transfer) sequence is possible if the suicide vectors are to be transferred by conjugation from *E. coli* to the target microbe.

Chromosome modifications by suicide vectors may be characterized as 'marked' or 'unmarked' depending on the presence or absence of suitable selection markers in the host chromosome after the modifications have been introduced. In either case, chromosomal modifications at the 3' end of a target gene may alter expression or translation of co-operonic downstream genes. In case of the 'marked' approach, incorporation of a selection marker and its cognate promoter may introduce a second transcription initiation site. For genes located in close proximity to each other, one must

also consider the possibility of a displaced ribosomal binding site (RBS). The SLIC approach, through appropriate design, may be used to correct these problems for both marked and unmarked approaches. Systems for unmarked modifications such as the Cre-lox recombination system (6, 27) or the levansucrase-dependent sucrose sensitivity (11), could easily be implemented by incorporation of the respective parts (loxP sites and *sacB*) into IP or SP regions of SLIC generated suicide vectors. In hosts where sucrose sensitivity is not a strong selection or a residual "scar" is not desired, alternative counter-selection systems are available. For *D. vulgaris,* sensitivity to the toxic pyrimidine analog, 5-fluorouracil, allows selection against the expression of *upp* encoding the salvage enzyme uracil phosphoribosyl transferase. This marker has been successfully used in a number of microbes (26, 28).

We compared 550 distinct target genes for tagging with suicide vectors assembled by the Gateway® and SLIC strategies and generated 297 (54%) and 468 (85%) sequence-verified plasmids, respectively. In general we observed that the SLIC strategy yielded a higher percentage of confirmed *D. vulgaris* mutant strains (304 strains/468 plasmids constructed; ~65%) as compared to the Gateway® scheme (70 strains/ 297 plasmids constructed; ~24%). For the 'Recombineering' strategy we generated 18 sample suicide vectors, which resulted in 9 confirmed mutant strains. Given the apparent superiority of the SLIC approach, it was the method of choice for further manipulation of the *D. vulgaris* chromosome to study effects of gene deletions, to identify physical interactions of proteins, and to localize selected tagged proteins. To date we have generated a library of over 700-engineered strains of *D. vulgaris* using the methodologies described in this work.

Screening for protein-protein interactions by tandem affinity purification.

With plasmids constructed by the SLIC procedure, we introduced sequences into the chromosome-encoding TAP tags in-frame at the 3' end of several genes from *D. vulgaris*. Engineered strains expressing native levels of C-terminally TAP-tagged fusion proteins were used to examine the protein complexes isolated with the tagged baits inferred to represent functional PPI. We validated conserved interactions in several essential complexes, such as the F_1-ATPase the RNA polymerase, the chaperone DnaK and others (Fig. 2A, Table S1).

Next, we examined potential interacting partners of proteins associated with the *D. vulgaris* nucleoid (Fig. 2B). Well-known components of the *E. coli* nucleoid include DNA-binding proteins such as Fis, HNS, Dps, IHF (IhfAB) and HU (HupAB). By the very nature of their inherent DNA-binding capability, these highly abundant proteins are involved in modulation of cellular processes such as transcriptional regulation, maintenance of DNA architecture, replication, recombination and stress protection (1, 2, 10, 21).

Given the common set of functions attributed to these proteins, it is not surprising that they exhibit a high level of interaction with each other. Indeed proteins precipitated with TAP-tagged baits of HU and IHF from *D. vulgaris* suggest a closely knit interaction sub-network comprising many of these DNA-binding proteins. Intriguingly the *D. vulgaris* genome appears to lack the diversity of nucleoid protein domains reported in *E. coli* such as Fis (COG2901), HNS (COG2916), and Dps (COG783) and their corresponding interacting partners (8). In contrast, *D. vulgaris* encodes twice as many proteins with the 'Bacterial nucleoid DNA-binding protein' domain, COG776, as are

found in *E. coli* (3). In order to compare the *E. coli* and *D. vulgaris* sub-networks associated with COG776 family proteins, we identified interacting partners of *D. vulgaris* tagged baits, Hup-3 and IhfB. With the exception of DVUA0004 and DVU1134, all members of the COG776 family appeared to interact with the tagged baits and potentially with each other (Fig. 2B, Table S2).

Unlike topoisomerases from *E. coli*, members of the *D. vulgaris* 'Topoisomerase' family (TopA, TopB) did not appear to co-purify with the tagged HU proteins. This was also confirmed when TopB was used as the bait and none of the COG776 family proteins were observed as interacting partners. In *E. coli,* HU (HupAB) has been reported to introduce negative supercoiling in covalently closed circular DNA in the presence of topoisomerase I (TopA) (37). From these results, it appears that mechanisms of DNA architecture maintenance and global regulatory controls in *D. vulgaris* may differ from those in *E. coli.*

***Gene Deletions: Examining the Methionine Biosynthesis Pathway of* D. vulgaris.** While the genome sequence of *D. vulgaris* was published in 2004, several amino acid biosynthesis pathways in this SRB remain to be elucidated. In this study we examined putative alternative steps in methionine biosynthesis. At least 18 variant methionine pathways have been proposed to originate from the common precursor – homoserine (23). In examining the *D. vulgaris* genome for all known variant genes related to the three major steps of methionine synthesis: (i) homoserine activation; (ii) sulfur incorporation, and (iii) methylation, homologs corresponding only to step (iii) were apparent – B12-dependent methionine synthase (DVU1585, *metH*) and methionine synthase II (cobalamin-independent) (DVU3371, *metE*). We tested these and other genes

putatively involved in the production of the methionine precursor homoserine from L-aspartate. These included a putative aspartate kinase (DVU1913, *lysC*), homoserine dehydrogenase (DVU0890, *hom*) (probable counterparts to bifunctional aspartate kinase II/homoserine dehydrogenase from *E. coli*), a putative beta-cystathionase (DVU0171, similar to *patB* (4)) and a protein with predicted methyltransferase activity (DVU3369, similar to *metW* (20)).

We verified all gene deletion mutations by PCR as well as Southern blot analysis. These gene deletion studies revealed that a majority of the putative methionine biosynthesis pathway knockouts (DVU1585, DVU3371, DVU0890, DVU0171 and DVU3369) did *not* result in methionine auxotrophy. A surprising result of this study was that the mutant deleted for DVU0890, *Δhom*, was found to be auxotrophic for threonine but *not* methionine (Fig. 3). This unexpected phenotype and the difficulty encountered in isolation of a deletion of DVU1913 were interpreted to indicate that an unusual pathway for methionine biosynthesis might be operational in this SRB. Further studies in this direction are currently underway.

Protein Localization with Visualization Tags.

We engineered *D. vulgaris* strains to express proteins bearing a SNAP tag, which is designed for subcellular visualization in anaerobic bacteria. Conventional Green-Fluorescent Protein derivatives require molecular oxygen for proper chromophore formation and hence cannot be utilized under anaerobic culturing conditions. We therefore explored the use of a modified SNAP tag that has a dead–end reaction with a modified O^6-benzylguanine (BG) derivative (33, 36). To validate the use of the AGT tag based method for subcellular localization in anaerobic bacteria, we first compared SNAP

labeling of three AGT-tagged proteins from *D. vulgaris*: DsrC (DVU2776); MreB (DVU0789; data not shown); FtsZ (DVU2499) from the respective engineered strains to the unmodified wild-type strain. We confirmed specific labeling of tagged proteins using two complementary methods: in-gel fluorescence detection SDS PAGE and fluorescence microscopy. SDS PAGE analysis typically yielded single bands at the expected molecular weight, indicating specific labeling of the tag, with little or no non-specific binding. Interestingly, in our fluorescence micrographs we found a robust cell-to-cell variability in labeling signal. To eliminate the possibility that the labeling reagent did not reach all tagged proteins, we compared *in-vivo* labeled intact cells to *in-vitro* labeled whole-cell extracts and observed no difference in the fluorescence signals between the two, as judged by SDS PAGE gel analysis. This suggested efficient reagent access and specific labeling of intracellular AGT-tagged proteins. In case of MreB and FtsZ unlike DsrC, the chromosomal tagging appeared to alter the cellular morphology normally associated with the wild-type strain. Morphological changes included either loss of vibrio-typic cell shape (MreB-AGT; data not shown) or extensive elongation (FtsZ-AGT; Fig. S2), suggesting diminished or altered protein function due to presence of the visualization tag. Our results are comparable to GFP-based protein localization of FtsZ as demonstrated in *E. coli* (35). To our knowledge this is the first account of specific tag-based fluorescence labeling for the purpose of protein localization in an anaerobic bacterium.

Subsequently we expanded the method to fifteen additional proteins (Fig. 4). We were able to decipher localization patterns for each of the fifteen SNAP-tagged proteins presumably reflecting their respective biological roles in this SRB. ParA, MotA-1 and MotA-3 localized exclusively to the poles, a subcellular area that has been referred to as a

"localization hotspot"; whereas, LytR, FtsH, FlgE and UvrB localized at the poles as well as to additional regions in or towards the center of the cells. Hup-3 and PyrB showed a patchy or spotty distribution along the length of the cells. The remaining proteins displayed cytoplasmically uniform distribution. Orthologous counterparts of ParA and FtsH from *Caulobacter crescentus* and *E. coli* have been experimentally visualized previously (42, 46). For the remaining proteins only theoretical *in-silico* localization predictions have been made to date (49). In these localization studies, we consistently noted cell-to-cell variations in fluorescent signals in any given population, which may be attributed to corresponding differences in expression levels (39).

Summary.

In this work, we successfully established the use of a "parts" approach to generate a library of over 700 engineered strains of the model sulfate reducer *Desulfovibrio vulgaris* Hildenborough for advanced systems biology applications. We highlighted three functional genomics tools including (a) gene deletions to study methionine biosynthesis, (b) protein-protein interactions associated with chaperones and nucleoid proteins, and (c) sub-cellular localization of select proteins to demonstrate the utility of our approach in this SRB generally regarded as genetically intractable. One may extend the approach to realize applications such as synthetic genetic arrays (13). *in vivo* expression profiling (39) and others. The ubiquity of suicide constructs in gene replacement throughout biology suggests that our approach may be applied to engineer a broad range of species for a diverse array of systems biological applications and is amenable to high-throughput implementation.

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FIGURES

Figure 1.

A. The SLIC approach (for double recombinations): Suicide vectors are assembled directly from four 'parts' (■,■,■,■) using SLIC. Parts 1 and 2 (■,■) correspond to homology regions (HR1/HR2) of the target loci on the chromosome. Parts 3 and 4 (■,■) correspond to an insertion part (IP) and a vector replication origin plus a selection part (RSP) respectively. Different parts may be mixed and matched depending on the choice of the application.
B. Example of a chromosomal modification in *D. vulgaris* Hildenborough using the SLIC approach: Insertion of the SNAP tag at the 3'-end of DVU0172. The suicide construct is assembled in *E. coli* using the SLIC technique from the following parts: Part 1: 700bp upstream from the 3' end of DVU0172 not including its stop codon; Part 2: the AGT (visualization) tag followed by a Kanamycin resistance gene; Part 3: 700bp downstream from the 5' end of DVU0172; Part 4: The replication origin of the vector (pUC) recognized only in *E. coli* followed by a Spectinomycin resistance gene. The chromosomal modification in *D. vulgaris* Hildenborough after double homologous recombination of the transformed suicide vector is shown.
C. Utilizing the 'parts' based approach for enabling chromosomal modifications in *D. vulgaris* Hildenborough using DVU1585 as the target gene. A set of reusable 'parts' (color coded) were employed for generating suicide constructs in *E. coli* which were then transformed in *D. vulgaris* to examine the role of DVU1585 in this sulfate reducer. Results of gene essentiality, protein-protein interactions and protein localization are discussed in the text.

Figure 2.

A. Conserved protein-protein interactions observed in this study. Chromosomally tagged (STF) baits are shown in orange and prey proteins are shown in brown. The relative sizes of interacting pairs are roughly proportional to their molecular weights and arrows point from tagged baits to the respective prey. Conserved interactions from the following complexes are shown: (1) The chaperonin complex composed of the heat shock proteins DnaK (DVU0811), DnaJ (DVU1876 and DVU3243), DafA (DVU1875), GrpE (DVU0812) and a hypothetical protein (DVU2556); (2) The ATP synthase complex composed of α(AtpA, DVU0777), β(AtpD, DVU0775), γ(AtpG, DVU0776), δ(AtpH, DVU0778) and ε(AtpC, DVU0774) subunits; (3) The RNA polymerase complex composed of α(DVU1329), β(DVU2928), β'(DVU2929) subunits and σ54 factor (DVU1628); (4) The glycyl-tRNA synthetase complex composed of α(GlyQ, DVU1898) and β(GlyS, DVU1897) subunits; and (5) The binary interaction between DNA Topoisomerase III (TopB, DVU2316) and single-strand binding protein (SSB, DVU0222).

B. Comparison of interacting partners of DNA binding proteins (COG766 and COG550) from *E. coli* and *D. vulgaris*. Arrows point from chromosomally tagged baits in each organism to the respective prey. Green colored boxes indicate presence of orthologs in both organisms. Orange colored boxes indicate proteins unique to *E. coli*. Border colors represent proteins from the same COG category.

Figure 3.

Optical density (600 nm) growth curve data for *D. vulgaris* Hildenborough∆DVU0890. Growth of the mutant strain was restored in LS4 minimal medium by supplementation with threonine but not methionine.

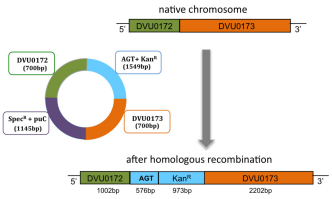
Figure 4.

Predicted and observed localization of AGT tagged proteins in *D. vulgaris*. Each column (L-R) depicts a representative image of an observed localization pattern in ten proteins from *D. vulgaris* Hildenborough bearing chromosomally-inserted visualization tags (AGT) at their respective C-termini. Fluorescently labeled cells were imaged by deconvolution microscopy and images in the table represent an optical section through the middle of the 3D deconvolved image stack (20-30 sections along the z axis). Predicted localizations were obtained from PSORTb (www.psort.org/psortb/). PhsB (DVU0172), a predicted cytoplasmic protein is uniformly distributed intracellularly. Proteins localizing exclusively at both cell poles include MotA (DVU2608) and ParA (DVU 3358). FlgE (DVU1443) and UvrB (DVU1605) proteins localize at four distinct locations along the length of the cell. Hup-3 (DVU1795) and PyrB (DVU2901) proteins show a patchy or spotty distribution. FtsH (DVU1278) localizes to the polar ends in addition to a dispersed cytoplasmic distribution. LytR (DVU0596) displays a bipolar and midband localization. MotA-1 (DVU 0050) has its localization signal restricted to one polar end of the cell. A schematic representation of the observed localization pattern is shown in the inset. Scale bars represent 400 nm for images of PhsB and MotA and 500 nm for the rest.

FIGURE 1

A.

B.



C.

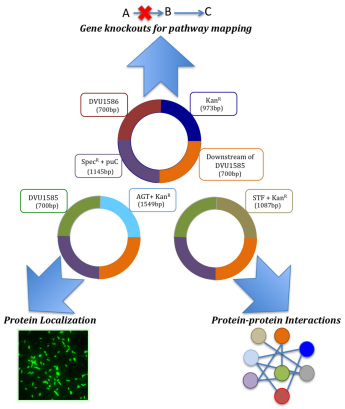
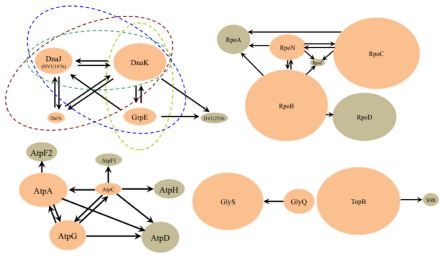
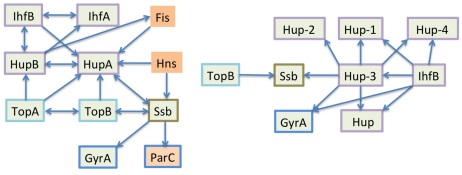


FIGURE 2.

A.



B.



E. coli

D. vulgaris

FIGURE 3.

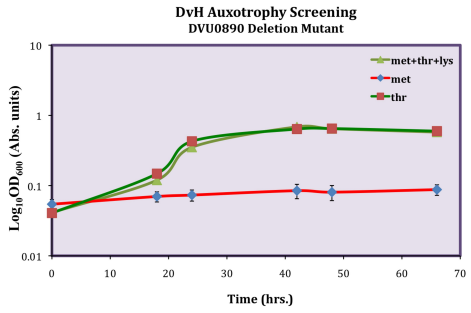
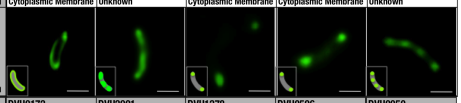


FIGURE 4.

DVU ID	DVU1585	DVU1795	DVU2608	DVU3358	DVU1443
Gene	--	*hup-3*	*motA*	*parA*	*flgE*
Description	Vitamin B-12 dependent Methionine synthase	DNA-binding protein HU	Chemotaxis protein	ParA family protein	Flagellar hook protein
TIGR Role Category	Amino-acid biosynthesis	Chromosome-associated proteins	Chemotaxis and motility	Cell division	Chemotaxis and motility
Predicted Localization	Cytoplasmic Membrane	Unknown	Cytoplasmic Membrane	Cytoplasmic Membrane	Unknown
Observed Localization					
DVU ID	DVU0172	DVU2901	DVU1278	DVU0596	DVU0050
Gene	*phsB*	*pyrB*	*ftsH*	*lytR*	*motA-1*
Description	Thiosulfate reductase	Aspartate carbamoyltransferase	Cell division protein	DNA-binding response regulator	Chemotaxis protein
TIGR Role Category	Electron transport	Pyrimidine ribonucleotide biosynthesis	Cell division	Two-component systems	Chemotaxis and motility
Predicted Localization	Cytoplasmic	Cytoplasmic	Cytoplasmic Membrane	Cytoplasmic	Cytoplasmic membrane
Observed Localization