Title: Effect of the deletion of *qmoABC* and the promoter distal gene encoding a hypothetical protein on sulfate-reduction in *Desulfovibrio vulgaris* Hildenborough

Running Title: Δ*qmoABC*-DVU0851 mutant of *D. vulgaris* Hildenborough

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Abstract

The pathway of electrons required for the reduction of sulfate in sulfate-reducing bacteria (SRB) is not yet fully characterized. In order to determine the role of a transmembrane protein complex suggested to be involved in this process, a deletion of *Desulfovibrio vulgaris* Hildenborough was created by marker exchange mutagenesis that eliminated four genes putatively encoding the QmoABC complex and a hypothetical protein (DVU0851). The Qmo complex (quinone-interacting membrane-bound oxidoreductase) is proposed to be responsible for transporting electrons to the dissimilatory adenosine-5'phosphosulfate (APS) reductase in SRB. In support of the predicted role of this complex, the deletion mutant was unable to grow using sulfate as its sole electron acceptor with a range of electron donors. To explore a possible role for the hypothetical protein in sulfate reduction, a second mutant was constructed that had lost only the gene that codes for DVU0851. The second constructed mutant grew with sulfate as the sole electron acceptor; however, there was a lag that was not present with the wild-type or complemented strain. Neither deletion strain was significantly impaired for growth with sulfite or thiosulfate as terminal electron acceptor. Complementation of the Δ(*qmoABC*-DVU0851*)* mutant with all four genes or only the *qmoABC* genes restored its ability to grow by sulfate respiration. These results confirmed the prediction that the Qmo complex is in the electron pathway for sulfate-reduction and revealed that no other transmembrane complex could compensate when Qmo was lacking.

Introduction

The sulfate-reducing bacteria (SRB) are a diverse group of organisms with the common ability to gain energy through the delivery of electrons to sulfate in an anaerobic respiration. The in vivo mechanism of sulfate reduction has not been fully elucidated, but the biochemistry of several of the reductases has been studied in detail (23, 31, 32) and a crystal structure determined for the APS reductase (24). The reduction of sulfate has been observed to follow at least a three-step process: activation of intracellular sulfate to adenosine phosphosulfate (APS) through consumption of the equivalent of two ATPs, reduction of APS to sulfite, and reduction of sulfite to sulfide (28).

Genes for the proteins involved in each of the three primary steps have been annotated in sequenced genomes of sulfate reducers (1). Also a number of transmembrane complexes have been predicted to be involved in the sulfate-reduction pathway (29, 31). QmoABC seemed the most likely to be linked to sulfate reduction given the proximity of *qmoABC* to the *apsBA* genes (Fig. 1). The existence of a conduit for electrons from the periplasm to sulfate was a prediction of the hydrogen cycling model proposed by Odom and Peck (26). In this elegant but controversial model, electrons and protons from substrate oxidation were proposed to be used by cytoplasmic hydrogenases to make hydrogen in the cytoplasm. The hydrogen would diffuse through the cytoplasmic membrane to the periplasm, where it would be oxidized by the periplasmic hydrogenases. The protons generated would contribute to the gradient that drives the ATP-synthase, generating ATP. The electrons would then be channeled through the periplasmic *c*-type cytochrome matrix to transmembrane complexes that deliver the electrons to cytoplasmic enzymes able to reduce APS to sulfite or sulfite to sulfide.

Several transmembrane complexes in *D. vulgaris* have been proposed to have involvement in the sulfate-reduction pathway: the high-molecular mass cytochrome *c* complex (Hmc, encoded by DVU0531-36) (10, 19), the Type II-cytochrome c_3 complex (Tmc or TpII-c_3, DVU0263-64) (38), the heterodisulfide reductase-like menaquinol-oxidizing complex (DsrMKJOP or HmeCDEAB, DVU1290-86) (13, 14, 22, 25, 32), the NADH:quinone oxidoreductase complex (RnfCDGEAB, DVU2792-97) (30), and the quinone-interacting membrane-bound oxidoreductase complex (Qmo, DVU0848-50) (13, 14, 25, 31). The Hmc and Qmo complexes have received the most attention as conduits for electrons used in the reduction of APS to sulfite (31). Because of the proximity of the *qmo* genes to the *aps* genes (shown in Fig. 1) in twelve sequenced SRB (*Desulfotalea psychrophila*, *D. vulgaris* (Hildenborough, DP4, and Miyazaki), *Desulfovibrio desulfuricans* (27774 and ND132), *Desulfovibrio* strain G20, *Desulforudis audaxviator*, *Desulfotomaculum reducens*, *Desulfovibrio africanus*, *Desulfococcus oleovorans*, and *Chlorobium tepidum*) (1, 25, 30, S. Brown, personal communication), the Qmo proteins have been suggested to provide the primary pathway of electrons to the APS reductase. By comparison, the *hmc* operon is located more than 300 kb from the *aps* genes. However, *D. vulgaris* strains deleted for the *hmc* genes had a ca. 50% decrease in growth rate and yield with hydrogen as the electron donor and sulfate as the electron acceptor (10). An increase of about 50% in the expression of *hmc* resulting from a mutation in a regulator (19) increased *D. vulgaris* growth with hydrogen by a similar amount but caused a slight decrease in growth rate with lactate. Therefore, the Hmc complex might serve as a component of the electron transport system from hydrogen to sulfate.

Three subunits of the Qmo complex from *Desulfovibrio desulfuricans* 27774 were the first Qmo proteins biochemically studied (31). Homologs of the encoding genes, *qmoABC,* were

recognized in the *D. vulgaris* Hildenborough genome (31), DVU0848-0850*,* respectively (15). QmoC was identified as the likely transmembrane subunit that interacts with the menaquinone pool and with QmoA and/or QmoB located in the cytoplasm (31). Additionally, genome sequence availability showed that an ORF encoding a hypothetical protein (DVU0851) was present at the 3' end of the putative operon containing the *aps* and *qmo* genes (15). Homologs of that predicted gene are found in all genomes of the *Desulfovibrio* strains available (*D. vulgaris* (Hildenborough, DP4, and Miyazaki), *D. desulfuricans* (27774 and ND132), *Desulfovibrio africanus*, *Desulfovibrio* strain G20, and *Desulfovibrio salexigens* (1, S. Brown, personal communication)) and in two additional SRB (*Desulfohalobium retbaense* and *Desulfomicrobium baculatum*) (JGI). However, a homolog to DVU0851 was not identified in other related SRB: *Desulfotalea psychrophila*, *Desulfobacterium autotrophicum* HRM2, *Desulforudis audaxviator* MP104C, and *Desulfococcus oleovorans* HxD3. DVU0851 was identified on a 2-D protein gel when *D. vulgaris* was grown on lactate/sulfate medium (12) but putative functions have not been suggested.

In order to establish the roles of the Qmo complex and DVU0851 in the reduction of sulfate, we constructed two deletion mutants: 1) a single deletion of the *qmoABC* and DVU0851 genes and 2) a deletion of the gene coding for DVU0851. The mutant lacking all four genes was unable to grow on defined medium with sulfate as the sole electron acceptor. Additionally, the mutant lacking only DVU0851 was fully capable of growth by sulfate respiration. From these observations, we infer that the transmembrane QmoABC complex is the unique channel for electron delivery to APS reductase.

Methods and Materials

Strains and media

Strains used in this study are listed in Table 1 (see also Table S1). *Escherichia coli* strains were cultured in SOC medium (components per liter of medium: 5 g yeast extract, 9 g tryptone, 0.5 g sodium chloride, 0.19 g potassium chloride, 3.6 g glucose, 10 ml of 1 M magnesium chloride, and 10 ml of 1 M magnesium sulfate) or LC medium (components per liter of medium: 10 g tryptone, 5 g sodium chloride, and 5 g yeast extract). Where indicated, kanamycin (kan) or spectinomycin (spec) were added to LC medium to a final concentration of 50 µg/ml or 100 µg/ml, respectively. Chemicals and antibiotics were obtained from Fisher Scientific (Pittsburg, PA), Sigma-Aldrich (St. Louis, MO), or RPI corp. (Mt. Prospect, IL).

All *D. vulgaris* strains were grown at 30 ^{o}C in an anaerobic growth chamber (Coy Laboratory Products, Inc., Grass Lake, MI) in MO media. MO basal medium was pH adjusted with 5 M HCl to 7.2 after addition of all medium components: 8 mM magnesium chloride, 20 mM ammonium chloride, 0.6 mM calcium chloride, 2 mM potassium phosphate (dibasic), 60 µM ferrous chloride, 120 µM EDTA, 30 mM Tris (pH 7.4), and 1 ml Thauer vitamin solution (4) and 6 ml trace element solution per liter. Trace element solution contains 2.5 mM manganese chloride, 1.26 mM cobaltous chloride, 1.47 mM zinc chloride, 210 µM sodium molybdate, 320 µM boric acid, 380 µM nickel sulfate, 11.7 µM cupric chloride, 35 µM sodium selenite, and 24 µM sodium tungstate. Where noted for MO media, sodium lactate (60 mM), sodium pyruvate (60 mM), sodium formate (60 mM), ethanol (60 mM), or hydrogen (23 mM) were added as electron donors and sodium sulfate (30 mM), sodium thiosulfate (30 mM), or sodium sulfite (40 mM) were added as terminal electron acceptors. Sodium acetate (10 mM or 20 mM, respectively) was included in formate and hydrogen media and cysteine hydrochloride (1 mM) was included in pyruvate fermentation medium. Antibiotics were added to the MO media as

noted: G418 (RPI corp.) at 400 µg/ml or spectinomycin at 100 µg/ml. G418 was routinely used in place of kanamycin because it proved to be more effective for selection of the kanamycin resistance marker (*aph(3')-II*) in *D. vulgaris*. Because G418 and kanamycin are similar to one another, the same antibiotic resistance gene provided resistance to both antibiotics. For solidified MO media, 15 g agar per liter were added and the sterile molten media were amended with sodium thioglycolate (1.2 mM final concentration) and titanium citrate (380 µM) as reductants. Yeast extract (1 g/l) was added where noted and the medium designated as MOY medium.

Protein yield determination

Protein was determined with the Bradford assay (3) and bovine serum albumin (Sigma, St. Louis, MO) was used as standard.

Plasmid construction

The pMO9020 plasmid (Fig. 2A) for the *qmoABC*-DVU0851 deletion was constructed by splicing by overlap extension (SOEing) PCR (16). Three regions were amplified (Table S2): 977 bp upstream of *qmoA*, 951 bp downstream of DVU0851, and the Tn*5* kanamycin resistance gene with its cognate promoter. The primers for amplifying the kanamycin resistance gene each contained a common sequence (TAGATTCGAGGTACGGCTACAGTCTT and CGGTCTATGAACTTAGTGAGCGGATT, 5' forward and 5' reverse, respectively) external to unique barcodes (CTCTTTCTAAGTGAGTCGAG and CACCTGAGAAGACGTAGTAC) that placed these sequences on each side of the antibiotic resistance gene. The barcodes were included for future experimentation (for example, 11). Amplification of the kanamycin resistance gene was from the plasmid pSC27 (34).

148 The three regions were PCR amplified with the Herculase polymerase (Stratagene, La
149 Jolla, CA) and the primers found in Table S2 (obtained from IDT, Coralville, IA). Herculase
150 polymerase was used for all PCR reactions unless otherwise stated.

151 The resulting mutagenic PCR product (consisting of the upstream region, the kanamycin
152 resistance gene, and the downstream region) was captured in the cloning vector
153 pCR8/GW/TOPO (Invitrogen, Carlsbad, CA) by the manufacturer's suggested protocol,
154 generating the plasmid pMO9020 (Fig. 2A). The recombinant plasmid was transformed via heat
155 shock into the accompanying chemically competent TOP10 cells (Invitrogen). Plasmid was
156 harvested from 1.5 ml of a transformant culture using the Qiaprep Spin Miniprep kit (Qiagen,
157 Valencia, CA). Sequencing of both strands of the mutagenic cassette was performed at the
158 University of Missouri DNA core facilities (http://www.biotech.missouri.edu/dnacore/). The
159 sequences obtained were aligned with the published *D. vulgaris* sequence
160 (http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&val=AE017285.1) to verify
161 that no mutations were introduced during PCR amplifications (data not shown). Following
162 verification, a single construct was designated as pMO9020 (Fig. 2A) and used for subsequent
163 experiments.

164 Construction of the deletion cassette for DVU0851 encoding a hypothetical protein was
165 similar to that for the deletion of *qmoABC*-DVU0851. The three PCR products were combined
166 in a single SOEing PCR reaction, captured in the pCR8/GW/TOPO vector, and transformed into
167 chemically competent TOP10 cells. The captured product was sequenced and compared with the
168 published sequence (data not shown). One base change was observed resulting in a silent
169 mutation in the gene coding for DVU0852 at the 684th base of that gene, a G to an A, converting
170 CGG to CGA both coding for arginine. Sequence data from the deletion cassette for DVU0851

could not be obtained on both strands for the last 53 bases of *qmoC* to the first base of the common sequence barcode apparently because of a predicted stable hairpin structure located between *qmoC* and DVU0851 (Fig. S1C). A single mutagenic plasmid construct was designated pMO9062 and used for subsequent experiments.

To complement the deletion strains, three plasmids able to stably replicate in SRB were constructed that used the promoter from the kanamycin resistance gene *aph(3')-II* (*aph(3')-IIp*, as identified in the pCR4 Zero Blunt TOPO manual (Invitrogen)) to drive the expression of complementing genes: *qmoABC*-DVU0851 (pMO9042), *qmoABC* alone (pMO9040), or DVU0851 alone (pMO9074). For the construction of pMO9040 and pMO9042, an amplicon containing all four genes was obtained by PCR amplification of *qmoABC*-DVU0851. An A-overhang was added to the 5.5 kb product that was then captured in pCR-XL-TOPO (Invitrogen) (according to the manufacturer's recommendations with the exception that the gel extracted fragment was not exposed to UV irradiation). The plasmid was transformed into TOP10 cells via electroporation. One of the transformants was grown, plasmid designated pMO9021 isolated, and digested with either EcoRV (to yield a blunt fragment containing *aph(3')-IIp*:*qmoABC*-DVU0851) or EcoRV and PshAI (to yield a blunt fragment containing only *aph(3')-IIp*:*qmoABC*). Each fragment was then ligated into an EcoRV-digested and dephosophorylated pMO719 to generate pMO9042 and pMO9040, respectively. A ribosomal binding site, TGCAGTCCCAGGAGGTACCAT (9), was introduced to each plasmid via the sequence and ligation independent cloning (SLIC) (21) method with pMO9040 and pMO9042 as template DNAs for PCR amplification (primers: qmoA-SLIC-RBS-F and pMO9075-R2). The products from these amplifications were transformed into *E. coli* α-select (Bioline) and successful transformants isolated on spectinomycin-containing agar plates. Plasmids pMO9116

194 and pMO9117 were isolated from each transformation, respectively. The promoter and
195 complementing genes from each plasmid were sequenced.

196 For complementation of ΔDVU0851, the gene for DVU0851 was amplified and ligated
197 into a SnaBI-digested and dephosphorylated pMO9072 (see construction below), for constitutive
198 expression from *aph(3')-IIp*, yielding pMO9074. A ribosomal binding site was added via the
199 SLIC method with pMO9074 as the template DNA in a PCR amplification (primers: DVU0851-
200 SLIC-RBS-F and pMO9075-SLIC-R2). The product from the PCR reaction was transformed
201 into *E. coli* α-select and transformants isolated on spectinomycin-containing agar plates.
202 Plasmid isolated from one of these colonies was designated pMO9118 and the promoter and
203 DVU0851 were sequenced.

204 **Construction of pMO9072 for complementation studies**

205 To create a vector containing *aph(3')-IIp* followed by cloning sites for insertion of a
206 complementing gene, *aph(3')-IIp* and aminoglycoside phosphotransferase (*aph(3')-II*, Kan^R)
207 were amplified from pCR-XL-TOPO, an A-overhang added with Taq DNA polymerase, and
208 captured in pCR8/GW/TOPO to produce pMO9070. *aph(3')-IIp* and the *aph(3')-II* gene were
209 released from pMO9070 by digestion with HpaI and EcoRV and ligated into an EcoRV-digested
210 and dephosphorylated pMO719, generating plasmid pMO9071. The plasmid pMO9071 was
211 then transformed into chemically competent *dam*$^-$ *E. coli* GM272 (35), to allow the use of a
212 restriction enzyme that is sensitive to *dam* methylation. The plasmid was purified from GM272
213 and the *aph(3')-II* gene (not including *aph(3')-IIp*) was removed with the restriction enzyme
214 BsaBI (NEB). The remaining plasmid was religated, thereby leaving *aph(3')-IIp* and eight
215 unique restriction enzyme recognition sites (XcmI, BsaBI, FspI, ScaI, SnaBI, PmeI, SphI, and

NspI) to create pMO9072 (Fig. 2B). However, it should be noted that this plasmid does not contain a ribosomal binding site that must be included with the gene to be expressed.

Transformation of *D. vulgaris* strains

To prepare *D. vulgaris* for electroporation, a freezer stock was used to inoculate 5 ml MO medium containing lactate/sulfite and 0.1% (wt/vol) yeast extract (MOYLS3) and grown overnight at 30 ^{o}C. The 5 ml overnight culture was diluted to 50 ml of the same medium and grown to an OD_{600} of ca. 0.35 at 30 ^{o}C. The culture was harvested by centrifugation at 4 ^{o}C for 12 min at 3,000 x *g*, and washed with 50 ml of chilled, sterile wash buffer (30 mM Tris-HCl buffer, pH 7.2, not anaerobic). The cells were spun at 4 ^{o}C for another 12 min at 3,000 x *g*, the pellet resuspended in 0.5 ml wash buffer, and 50 µl aliquots used for each electroporation. Approximately 700 ng of plasmid DNA was added to the cells, mixed, and the mixture transferred to a 1-mm gapped electroporation cuvette (Molecular BioProducts, San Diego, CA). The cuvette and the safety stand were transferred into the anaerobic chamber and electroporation was carried out at 1750 V, 250 Ω, and 25 µF with an ECM 630 electroporator (BTX, Holliston, MA). The electroporated cells were diluted into 1 ml MOYLS3 medium and allowed to recover overnight at 30 ^{o}C. Transformants were selected as G418 or spectinomycin resistant colonies from aliquots of electroporated cells mixed into molten MOYLS3 medium and poured into empty Petri dishes to solidify. Colonies were seen after ca. 4 days of incubation at 30 ^{o}C in the anaerobic chamber.

One of the plasmids for complementation (pMO9117) did not yield spectinomycin-resistant colonies when transformed into the deletion strain JW9021. Therefore it was first transformed into wild-type *D. vulgaris*, isolated, and then used to transform JW9021. Transformation efficiency of pMO9117 into wild-type *D. vulgaris* was 155 transformant

239 CFU/µg. The plasmid was isolated and then used to transform JW9021 with increased efficiency
240 of 9.9 x 10^3 CFU/µg.

241 **Storage of *D. vulgaris* mutants**

242 For freezer stocks, sterile glycerol was added to fully grown cultures to a final
243 concentration of 10% (vol/vol), samples were aliquoted into cryogen vials, and filled vials were
244 stored at -80 ^{o}C.

245 **Southern blots**

246 In order to verify that *qmoABC*-DVU0851 was deleted from putative JW9021 isolates, a
247 Southern blot was performed by standard procedures (5). Gels of NcoI-digested genomic DNA
248 were probed with a PCR fragment of *apsA*, which was located on the *D. vulgaris* genome
249 immediately upstream of the *qmoA* gene. A DNA band of 8450 bp showed hybridization in the
250 wild-type sample, in contrast to a fragment of 2760 bp from a correctly constructed marker
251 exchange deletion, JW9021 (data not shown).

252 For confirmation of JW9063 deleted for DVU0851, genomic DNA was digested with
253 BsrBI and probed with a PCR product from *qmoC*. Fragment sizes of 2613 bp for the wild-type
254 strain and 1666 bp for JW9063 were confirmed by Southern analysis (data not shown).

255 **Northern blots**

256 RNA was isolated from *D. vulgaris* strains (wild-type and JW9021) by a protocol
257 supplied with the RNAwiz reagent (Ambion, Austin, TX). Cells were grown in either 50 ml of
258 MO medium containing lactate/sulfate (wild-type) or lactate/sulfite (wild-type and JW9021) and
259 harvested at mid-exponential growth (OD_{600} ~0.3). An RNA ladder (Promega) and equal masses
260 of RNA samples (~3.5 µg each) were prepared, subjected to electrophoresis, transferred and
261 probed similarly as described in Current Protocols in Molecular Biology (6).

Making cDNA

A 10 µg sample of RNA was DNase treated with the Turbo DNA-free kit (Ambion). To determine whether genomic DNA was eliminated, PCR amplification in the absence of reverse transcriptase was performed. The resulting sample was freeze dried overnight and resuspended in RNase-free deionized water to a final concentration of 0.5 µg/µl. A 2 µg aliquot of DNase-treated RNA was used to make cDNA with the ImPromII kit (Promega), according to manufacturer's suggestions.

Growth curves

In an anaerobic growth chamber, 5 ml aliquots of media were added to 15 ml culture tubes (path length 15 mm), inoculated with a 2% volume of stationary phase *D. vulgaris* strains (OD_{600} ~0.8), and sealed with a rubber stopper, leaving 10 ml of headspace. The cultures were grown at 37 ^{o}C and the optical density (600 nm) was read at various time points with a Genesys 20 spectrophotometer (Thermo Spectronic, Waltham, MA).

Sulfide determination

Culture samples were taken approximately every 50 hours (at time of inoculation, 50 h, 100 h, and 150 h) and a colorimetric assay was performed to determine the amount of dissolved sulfide generated (8). We used a slightly modified version of the protocol by Cord-Ruwisch (8). Samples of 100 µl were taken instead of 50 µl and 4 ml of copper reagent were used instead of 1.95 ml.

RESULTS

Construction of deletion strains in sulfate-reducing bacteria

284 QmoABC proteins are hypothesized to function as a transmembrane electron conduit
285 providing reductant for APS reductase encoded by *apsBA* (32). The *qmoABC* genes are
286 downstream of *apsBA* (Fig. 1) and have been predicted to be a part of the same operon
287 (http://www.microbesonline.org/). By deleting *qmoABC*, we sought to test the hypothesis that
288 these genes encode a complex essential for sulfate reduction. These three genes and DVU0851,
289 predicted to be the promoter distal gene of the putative operon (33), were selected for deletion
290 (Fig. 1). As a standard procedure when deleting genes encoding transmembrane complexes, we
291 have initially chosen not to leave orphan genes that might produce proteins that could
292 interact/interfere with other transmembrane complexes.

293 The deletion strategy used in this study was adapted from a marker-exchange strategy
294 developed for *Saccharomyces cerevisiae* (11) and has been previously used for making other
295 deletions in *D. vulgaris* (2). In short, regions upstream and downstream of the genes to be
296 deleted were cloned on either side of a kanamycin resistance marker, *aph(3')-II*. The antibiotic
297 resistance cassette contained two sets of flanking oligonucleotides referred to as barcodes, first
298 those unique to this deletion (20 bp each) and then outermost barcodes (26 bp each) common to
299 most mutants constructed in this lab (2, 11). For deleting *qmoABC*-DVU0851, the constructed
300 segment of DNA - upstream, downstream, and kanamycin cassette regions - was cloned into a
301 $Spec^r$ plasmid creating pMO9020 (Fig. 2A) and confirmed by sequencing. This mutagenic
302 plasmid can not be stably maintained in *D. vulgaris* as a separate replicon. Two homologous
303 recombination events were necessary for marker replacement with elimination of the vector
304 sequences in the transformants. In *D. vulgaris,* double recombination events following the
305 introduction of mutagenic plasmids by the electroporation procedure described were at least ten-
306 fold more frequent than simple plasmid integration into the chromosome. pMO9020 was

307 transformed into wild-type *D. vulgaris* by electroporation (transformation efficiency with
308 pMO9020 was 7.8 x 10^3 CFU/µg plasmid). Successful marker replacement was first tested by
309 screening of JW9021 G418^r isolates for sensitivity to spectinomycin, the antibiotic resistance
310 encoded on the vector. Putative deletion isolates were confirmed by Southern blot (data not
311 shown). In order to determine if DVU0851 contributed to sulfate respiration in *D. vulgaris*, a
312 deletion of this gene alone was also constructed and designated JW9063. The construction,
313 transformation, colony screening, and Southern verification of this strain was similar to that of
314 JW9021 (transformation efficiency with pMO9063 was 5.3 x 10^3 CFU/µg plasmid).

315 **Growth characteristics of deletion and complementation strains**

316 Growths of JW9021, JW9063, and wild-type *D. vulgaris* were compared in defined MO
317 medium with various electron donor and acceptor combinations. Fig. 3 shows the results of
318 these tests with lactate (Fig. 3A, 3B) or pyruvate (Fig. 3C, 3D) as the electron donor with sulfate
319 (Fig. 3A, 3C) or sulfite (Fig. 3B, 3D) as the electron acceptor. Growth of JW9021, lacking
320 *qmoABC*-DVU0851, was undetectable with sulfate as terminal electron acceptor regardless of
321 the electron donor when growth was measured either as a change in optical density (Fig. 3A,
322 3C), whole cell protein concentration increases (data not shown), or dissolved sulfide production
323 (Table 2). In contrast when the electron acceptor was sulfite with either lactate or pyruvate as
324 electron donor, growth of JW9021 was comparable to that of the wild-type (Fig. 3B, 3D). Wild-
325 type and JW9021 were also tested for growth in defined lactate/thiosulfate medium and no
326 significant differences were observed between the two strains (data not shown). Additionally,
327 growth tests on formate/sulfate, formate/sulfite, ethanol/sulfate, ethanol/sulfite, hydrogen/sulfate,
328 and hydrogen/sulfite confirmed that sulfate could not be respired by JW9021 regardless of the
329 electron donor but that all donors were used with sulfite as electron acceptor (data not shown).

To establish that the growth phenotype of the deletion strain JW9021 (Δ(*qmoABC*-DVU0851)) resulted from the absence of the Qmo complex, complementation analyses were performed. JW9021 containing a stable plasmid encoding the four deleted genes or the *qmoABC* genes alone was found to grow at about the same rate and with a similar protein yield to that of wild-type (Fig. 4A). Controls showed that complementing plasmids did not alter yields of protein or rates of growth during sulfite respiration (data not shown).

A possible role for the hypothetical protein DVU0851 in sulfate reduction was then further explored through examination of JW9063, lacking only DVU0851. Growth of JW9063 with sulfite as terminal electron acceptor was comparable to that of the wild-type (179 ± 24.7 µg protein/ml for the mutant versus 227.2 ± 23.2 µg protein/ml for the wild-type)). However, when sulfate was electron acceptor, JW9063 grew well and to cell densities equivalent to the wild-type following a slight lag when lactate was electron donor (Fig 3A). The lag was observed when growth was monitored by optical density (Fig. 3A) or by dissolved sulfide production (Table 2); nevertheless, the final protein (110 µg/ml) and soluble sulfide (12.3 mM) produced were comparable to those obtained with the wild-type strain (132 µg/ml and 11.5 mM at 150 h). Thus, deletion of DVU0851 alone did not inhibit growth with sulfate.

Addition of the plasmid pMO9118, supplying only DVU0851 to JW9021, did not correct the growth defect with sulfate (Fig. 4) demonstrating that this protein alone was not sufficient to restore the missing function of the QmoABC complex. Complementation of JW9021 with *qmoABC* (pMO9117) did restore respiration of sulfate. DVU0851 is apparently not essential but may play a secondary role in formation or stabilization of the Qmo complex. Additional support for a minor role for DVU0851 was the observation that the complemented deletion of DVU0851 no longer exhibited a growth lag on lactate/sulfate medium (Fig. 4C).

353 **Measuring hydrogen sulfide**

354 To confirm that JW9021 was unable to reduce sulfate, the mutant was grown in sulfate-
355 or sulfite-containing medium and dissolved sulfide accumulated at stationary phase was
356 measured (Table 2). After 150 h of incubation, dissolved sulfide concentrations above those
357 carried over from the inocula were not detected in JW9021 or JW9021 containing the DVU0851
358 complementing plasmid with sulfate as terminal electron acceptor. By comparison, all other
359 constructs in lactate/sulfate and all strains in lactate/sulfite contained detectable levels of sulfide.

360 **Northern blot for *apsA* expression**

361 The proximity of *apsBA* encoding the APS reductase to *qmoABC* DVU0851 and the
362 prediction that these genes could form a single operon raised a question regarding the stability of
363 mRNA for *apsBA* in the deletion strain. To ensure that transcripts from *apsBA* could be observed
364 in the Δ(QmoABC-DVU0851) strain, JW9021, a Northern blot was performed. Wild-type cells
365 cultured in MO medium containing lactate/sulfate or lactate/sulfite as well as JW9021 cells
366 cultured with lactate/sulfite were grown to mid-exponential phase and the RNA from each was
367 isolated and probed for *apsA* (Fig. 5). The *apsA*-containing transcripts in both the wild-type and
368 JW9021 in lactate/sulfite medium were present at low levels but were quite similar in
369 concentration. It was noted that *apsA* expression in wild-type in the presence of sulfate was
370 much higher than in the presence of sulfite. Additionally, the transcript size obtained with the
371 *apsA* probe, from all three samples, was about 2.5 kb (Fig. 5). This is shorter than 8.2 kb, the
372 size expected if *apsBA* and *qmoABC* DVU0851 were transcribed as a single mRNA
373 (http://www.microbesonline.org/) (1). Even in the mRNA from wild-type cells that had abundant
374 *apsA* transcripts, no messages longer than ca. 2.5 kb were detected when probed with *apsA* (Fig.

375 5), suggesting that *apsBA* may be transcribed independently of *qmoABC* DVU0851 or that any
376 longer transcripts are readily processed.

377 To further investigate the transcript size found for *apsA* in the Northern blot, we
378 attempted to find evidence for longer transcripts derived from the predicted six gene operon (Fig.
379 1). Genomic DNA and cDNA prepared from RNA of wild-type cells grown with lactate/sulfate
380 were used as templates for PCR with primers specific for six regions: an internal region of the
381 *apsA* gene, an internal portion of *qmoA* gene, a region spanning the 3' end of *apsA* through the 5'
382 end of *qmoA*, an internal portion of qmoC, an internal portion of DVU0851, and a region
383 spanning the 3' end of *qmoC* through the 5' end of DVU0851 (Fig. 1). The genomic DNA was
384 expected to show all six products. The cDNA was also expected to show the six PCR products if
385 the predicted single operon structure were correct and unprocessed message were accumulated.
386 If *apsBA*, *qmoABC*, and DVU0851 are each transcribed separately, the cDNA would yield PCR
387 products for the internal products of *apsA*, *qmoA*, *qmoC*, and DVU0851, but not that containing
388 the intergenic regions. Bands were observed for all primer sets with the exception of PCR
389 products spanning the intergenic regions between *apsA*-*qmoA* and *qmoC*-DVU0851 with cDNA
390 as template (Fig. 1, 6). These results suggest that *apsBA qmoABC* DUV0851 are transcribed as
391 three operons or that any inclusive transcripts are readily processed.

392 Two possible strong hairpin structures were observed between the *apsA* and *qmoA* genes,
393 a predicted intergenic region of 145 bp. One hairpin is a 30-base sequence with a perfect 13-bp
394 intrastrand stem (bold) and 4-base loop (**AGGGCGGTTGCGG**GGTA**CCGCAACCGCCCT**,
395 Fig. S1A) with a melting temperature of 84.2 ^{o}C and the second is a 51-base sequence with a
396 perfect 12-base-pair stem (bold) and an imperfect 10-base stem (underlined)
397 (**ACGGCCTAAGCCG**<u>GGGCAGTAAG</u>CACGC<u>CTTATTGTCTG</u>**GGCTTAGGCCGT**, Fig.

S1B) with a melting temperatures 64.6 ^{o}C (predictions from mFold, http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/). In addition, in the 89 bp intergenic region between *qmoC* and DVU0851, there is potentially a 10 base-pair stem (bold) with a four base loop (**GTCGGCGCCGC**TGCG**GCGCGCCGAC**) that could form a transcription terminator with a melting temperature of 84.2 °C as well (Fig. S1C). These highly stable hairpin structures support the possibility that multiple transcripts are generated from this region.

DISCUSSION

Role of *qmoABC* in sulfate reduction

The exact pathway of electron flow for reduction of sulfate to sulfide in sulfate-reducing bacteria has not yet been completely elucidated. The evidence identifying specific proteins involved in electron transfer to APS reductase or sulfite reductase has been considerable but it is often circumstantial. This evidence includes the conservation of genes in the genomes of sequenced sulfate-reducing bacteria and their absence in organisms not able to respire sulfate (25), frequent co-localization of genes implicated or known to be involved in sulfate reduction (13, 25), homology to existing transmembrane electron-transporting complexes involved in energy generation (22, 23, 31, 32, 38), observations of correlated changes in expression of such genes in different media (13, 14, 30, 36), and reduced growth of mutants deleted for particular genes (10, 19, 27). In those studies where growth was altered on sulfate, the results were dependent upon the electron donor (10, 19, 27) which might indicate that the step affected was the one producing the electrons rather than the step of delivery of electrons to sulfate or sulfite. However, in this study, we observed the complete cessation of growth on media containing

421 sulfate as the sole electron acceptor, with lactate, pyruvate, formate, ethanol, or hydrogen as
422 reductant source in a mutant of *D. vulgaris*, JW9021, lacking *qmoABC*-DVU0851. Secondly, we
423 showed restoration of growth on lactate/sulfate and pyruvate/sulfate media when the deletion
424 strain was complemented with these genes. The role of the *qmoABC*-DVU0851 genes is
425 undoubtedly involved in the reduction of sulfate to sulfite since growth with sulfite was not
426 substantially altered in JW9021 (Fig. 3B, 3D, Table 3). It should also be pointed out that during
427 extended incubation of JW9021 with sulfate available (>150h), growth of suppressors was not
428 observed. We interpret this to mean that other membrane-bound, electron accepting/donating
429 protein complexes (Hmc, DsrMKJOP, Rnf, Tmc, nor Hdr), despite their structural similarity
430 (31), could not readily compensate for the loss of QmoABC. In addition, although NADH
431 oxidase was reported to deliver electrons to APS reductase from NADH in vitro (7), this enzyme
432 was apparently not able to compensate for the lack of a Qmo complex in vivo. Experiments to
433 evaluate possible compensation of loss of individual components of the Qmo complex, i.e. only
434 the soluble components (*qmoAB*) or only the membrane-spanning component (*qmoC*) have not
435 been carried out.

436 Both the reduction of thiosulfate to sulfite and sulfide and its disproportionation to sulfate
437 and sulfide have been proposed as mechanisms for thiosulfate metabolism in sulfate-reducing
438 organisms (20). However, the disproportionation of thiosulfate would then make its respiration
439 dependent on the same enzymes necessary for sulfate reduction, ATP sulfurylase and APS
440 reductase (20). The growth of JW9021 on thiosulfate-containing medium (data not shown) was
441 not significantly different compared to that of wild-type. Therefore, we infer that *D. vulgaris* has
442 the capacity to grow on thiosulfate by reduction to sulfite plus sulfide, bypassing the need for
443 sulfate reduction.

Role of DVU0851 in sulfate reduction

The presence of DVU0851 was not required for sulfate reduction, as observed by the growth of the JW9063 mutant on lactate/sulfate and pyruvate/sulfate (Fig. 3A, 3C, Table 3). However, when JW9063 was transferred to sulfate-containing medium, there was a detectable lag when the medium contained lactate as the electron donor compared with that of the wild-type and JW9063(pMO9118) strains (Fig. 4C). Even though the gene coding for DVU0851 is predicted to be in the same operon as the *aps* and *qmo* genes in *Desulfovibrio* strains, it was not a universally conserved gene among the predicted sulfate-reduction genes (25) nor was it found in several other sequenced sulfate-reducing organisms.

Expression of *apsBA* and *qmoABC*

The data presented here support the possibility of 3 separate operons *apsBA*, *qmoABC*, and DVU0851, unlike the prediction suggested elsewhere (1). However, we cannot yet eliminate the possibility that a single transcript may be synthesized and rapidly processed. We determined that deletion of the *qmoABC* DVU0851 genes did not apparently alter the level of transcription of the *apsBA* genes. As previously reported (12, 13, 14, 30) our data confirmed that the genes encoding the enzymatic machinery for sulfate reduction are not constitutively expressed in *D. vulgaris*. Pereira et al. (30) reported that *apsBA* and *qmoABC* transcripts were decreased in cells with thiosulfate as electron acceptor but increased during pyruvate fermentation. A possible regulatory motif and a transcriptional regulatory protein have been proposed (33). Regulation of these genes would appear to be complex and is under investigation.

CONCLUSION

This study shows that the Qmo complex is essential for sulfate respiration in *D. vulgaris*. Further experiments are now possible to determine whether all subunits of the Qmo complex are necessary for sulfate-reduction, to establish the function of the hypothetical protein DVU0851, and to elucidate the regulation of this process.

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TABLE 1: Strains and plasmids used in this study.

Strain or plasmid	Genotype or relevant characteristics	Source
***E. coli* strains**		
TOP10 (both chemically competent and electrocompetent)	F$^-$, *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*Δ*M15* Δ*lacX74 recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL* (Str^r) *endA1 nupG*	Invitrogen (Cat. nos. C4040-10, C4040-50)
α-select (Bronze efficiency)	F$^-$ *deoR endA1 recA1 relA1 gyrA96 hsdR17*(r_k^-, m_k^+) *phoA supE44 thi*-1 Δ(*lac*ZYA-*arg*F)*U169* ϕ80δ*lacZ*Δ*M15* λ$^-$	Bioline
GM272	F-, *fhuA2 or fhuA31*, *lacY1 or lacZ4*, *tsx-1 or tsx-78*, *glnV44*(AS), *galK2*(Oc), *LAM-*, *dcm-6*, *dam-3*, *mtlA2*, *metB1*, *thi-1*?, *hsdS21*	CGSC[a], #6478; 17
***D. vulgaris* strains**		
ATCC 29579	Wild-type (WT), *D. vulgaris* Hildenborough	ATCC
JW801	WT ΔpDV1	37
JW9021	WT Δ(*qmoABC*-DVU0851); Kan^r	This study
JW9063	WT ΔDVU0851; Kan^r	This study
Plasmids		
pCR8/GW/TOPO	TOPO cloning vector; Spec^r	Invitrogen
pCR-XL-TOPO	TOPO cloning vector; Kan^r	Invitrogen
pSC27	*Desulfovibrio* shuttle vector; source of *aph(3')-II*, Kan^r	34
pMO719	pCR8/GW/TOPO containing SRB replicon (pBG1); Spec^r	18
pMO9020	pCR8/GW/TOPO with 977 bp upstream and 951 bp downstream of *aph(3')-II* cassette to delete *qmoABC*-DVU0851; Spec^r, Kan^r	This study
pMO9021	pCR-XL-TOPO containing *aph(3')-IIp*[b]:*qmoABC*-DVU0851; Kan^r	This study
pMO9040	pMO719 with *aph(3')-IIp*:*qmoABC* under; Spec^r	This study
pMO9042	pMO719 with *aph(3')-IIp*:*qmoABC*-DVU0851; Spec^r	This study

pMO9062	pCR8/GW/TOPO with 964 bp upstream and 951 bp downstream of *aph(3')-II* cassette to delete DVU0851; Kan^r, Spec^r	This study
pMO9070	pCR8/GW/TOPO; Kan^r, Spec^r	This study
pMO9071	pMO719 with *aph(3')-II*; Kan^r, Spec^r	This study
pMO9072	pMO719 with *aph(3')-IIp* and multi-cloning site (MCS); Spec^r; for complementation constructs.	This study
pMO9074	pMO9072 with DVU0851 in MCS; Spec^r	This study
pMO9116	pMO9040 with *aph(3')-IIp*:RBS[c]:*qmoABC*	This study
pMO9117	pMO9042 with *aph(3')-IIp*:RBS:*qmoABC*-DVU0851	This study
pMO9118	pMO9074 with *aph(3')-IIp*:RBS:DVU0851	This study

[a] – CGSC: Coli Genetic Stock Center

[b] – *aph(3')-IIp* – promoter from kanamycin resistance gene *aph(3')-II*.

[c] – RBS, ribosomal binding site (TGCAGTCCCAGGAGGTACCAT)

TABLE 2: Dissolved sulfide generated by wild-type *D. vulgaris*, mutants, and complemented mutants when grown on lactate/sulfate (Lac/SO_4; 60mM/30mM) or lactate/sulfite (Lac/SO_3; 60mM/40mM)[a].

Strain (genotype)	**Plasmid (complementing genes)**	**Medium**	**0 h**	**100 h**
WT	No plasmid	Lac/SO_4	-0.4 ± 0.2	14.0 ± 2.5
		Lac/SO_3	-1.1 ± 1.5	24.7 ± 0.5
JW9021 (Δ*qmoABC*-DVU0851)	No plasmid	Lac/SO_4	-0.3 ± 0.2	-0.1 ± 0.2
		Lac/SO_3	-0.2 ± 0.2	20.9 ± 1.1
	pMO9116 (*qmoABC*)	Lac/SO_4	-0.4 ± 0.1	13.8 ± 1.2
		Lac/SO_3	-0.3 ± 0.1	22.0 ± 0.2
	pMO9117 (*qmoABC*-DVU0851)	Lac/SO_4	-0.3 ± 0.1	10.4 ± 7.7
		Lac/SO_3	-0.3 ± 0.1	23.6 ± 1.0
	pMO9118 (DVU0851)	Lac/SO_4	0.0 ± 0.0	0.0 ± 0.1
		Lac/SO_3	0.0 ± 0.1	21.0 ± 3.7
JW9063 (ΔDVU0851)	No plasmid	Lac/SO_4	0.2 ± 0.1	14.7 ± 0.4
		Lac/SO_3	0.5 ± 0.4	22.6 ± 0.8
	pMO9118 (DVU0851)	Lac/SO_4	0.2 ± 0.1	13.6 ± 0.2
		Lac/SO_3	0.3 ± 0.1	23.1 ± 1.8

[a] Values are mM sulfide and are the average of three biological replicates. The standard deviations are shown. Lac is lactate.

Figure 1: Diagram of the genome region of *D. vulgaris* containing the predicted six gene operon, *apsBA-qmoABC*-DVU0851. The locations of A) the probe used for Northern blots; B), C) & D) amplified regions from *D. vulgaris* genomic DNA and cDNA; E) & F) location of deleted segments; and G) & H) template regions for probes for Southern confirmation of deletions are shown. Arrows represent ORFs and the arrowheads indicate the direction of transcription. Genes in grey are predicted to be in neighboring operons.

Figure 2: Plasmids used in this study. A. Diagram of mutagenic plasmid pMO9020. The region from 683 – 3715 nt is the mutagenic cassette for deletion of *qmoABC* and DVU0851 genes by marker exchange mutagenesis. NcoI sites were used for Southern confirmation of the deletion. B. Vector constructed for complementation studies in SRB, pMO9072. The promoter *aph(3')-IIp* is constitutively expressed and drives expression of a gene placed in the multicloning site. The pBG1 segment is an endogenous cryptic plasmid from *Desulfovibrio* G20 (Rousset, et al. 1998) that allows stable replication of plasmids in SRB. Unique restriction sites that can be used for the introduction of complementing DNA are shown.

Figure 3: Growth of *D. vulgaris* and two deletion mutants on sulfate- and sulfite-containing media. Growth comparisons of *D. vulgaris* Hildenborough and deletion constructs JW9021 (Δ*qmoABC*-DVU0851) and JW9063 (ΔDVU0851) on (A) lactate/sulfate (60mM/30mM), (B) lactate/sulfite (60mM/40mM), (C) pyruvate/sulfate (60mM/30mM), and (D) pyruvate/sulfite (60mM/40mM). Wild-type (*Dv*H, ○), JW9021 (●), and JW9063 (●). Readings reflect an average of three samples and error bars are provided showing the standard deviation.

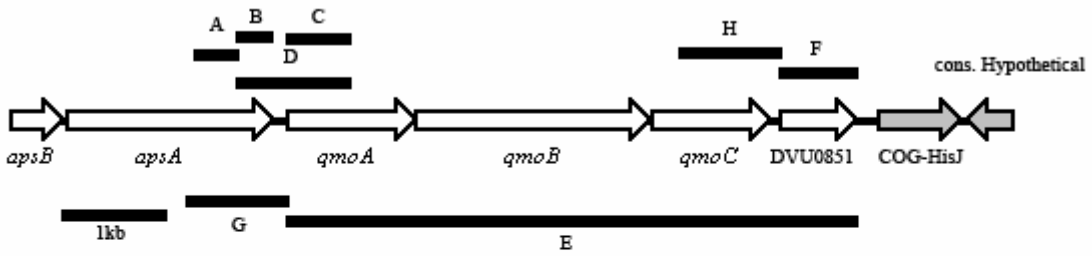
Figure 4: Growth of *D. vulgaris* deletion and complementation strains on sulfate-containing media. Growth on (A) defined lactate/sulfate (60mM/30mM) medium of wild-type *D. vulgaris* (○), JW9021 (Δ(*qmoABC*-DVU0851), ●), and three complemented strains (JW9021(pMO9116) Δ(*qmoABC*-DVU0851) complemented with *qmoABC* (□), JW9021(pMO9117) Δ(*qmoABC*-DVU0851) complemented with *qmoABC*-DVU0851 (■), and JW9021(pMO9118) Δ(*qmoABC*-DVU0851) complemented with DVU0851) (■)). (B) Growth of these strains on defined pyruvate/sulfate (60mM/30mM) medium. (C) Growth on defined lactate/sulfate medium of wild-type *D. vulgaris* (○), JW9063 (ΔDVU0851, ●), and JW9063(pMO9118) ΔDVU0851 strain complemented with DVU0851 (△). Optical density readings are an average of three samples and error bars are provided showing the standard deviation.

Figure 5: Northern blot of *D. vulgaris* and Δ*qmoABC*-DVU0851 mutant probed for *apsA*-containing transcript. Determination of *apsA*-containing transcript from RNA samples of *D. vulgaris* (lanes 1, 2, 4, 5) and deletion mutant JW9021 (Δ*qmoABC*-DVU0851) (lanes 3, 6) grown on lactate/sulfate (60mM/30mM) (lanes 1, 4) or lactate/sulfite (60mM/40mM) (lanes 2, 3, 5, 6). The RNA was probed with an internal fragment of *apsA*. The agarose gel (lanes 4-7) and developed film (lanes 1-3) are shown. An RNA ladder (Promega) was also included (lane 7).

Figure 6: PCR amplification of *apsA*, *qmoA, qmoC*, and DVU0851 and the region spanning the junctions between *apsA*-*qmoA* and *qmoC*-DVU0851 from *D. vulgaris* genomic DNA and cDNA. PCR amplification was performed on *D. vulgaris* genomic DNA (lanes 1-6) and cDNA from *D. vulgaris* grown in lactate/sulfate (lanes 8-13) for an internal fragment of *apsA* (lanes 1,

8; expected size: 440 bp), an internal fragment of *qmoA* (lanes 2, 9; expected size: 610 bp), a region spanning the intergenic region that includes coding sequences for the C-terminus of ApsA and the N-terminus of QmoA (lanes 3, 10; expected size: 1510 bp), *qmoC* (lanes 4, 11; expected size: 875 bp), DVU0851 (lanes 5, 12; expected size:747 bp), and a region spanning the intergenic region that includes coding sequences for the C-terminus of QmoC and the N-terminus of DVU0851 (lanes 6, 13; expected size: 1711 bp). A 1kb Plus DNA ladder (Fermentas) is in lanes 7 and 14. Sizes of observed bands are labeled on left-hand side of gel.

Figure 1.

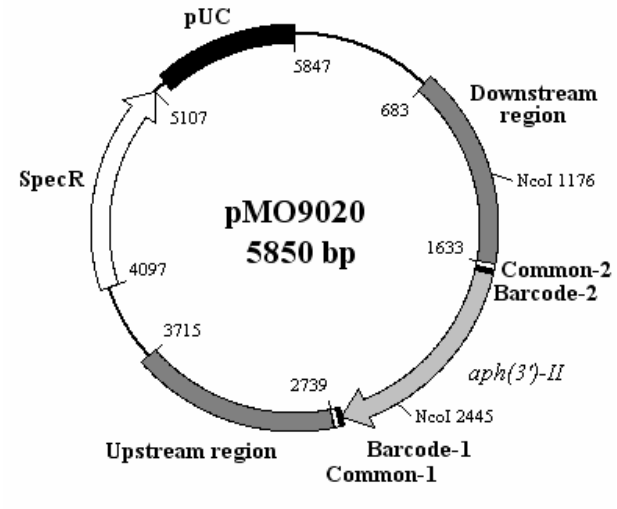


apsBA-qmoABC- DVU0851

9722 bp

A: Northern probe for apsA
B: *apsA* fragment amplified from cDNA and genomic DNA
C: *qmoA* fragment amplified from cDNA and genomic DNA
D: *apsA-qmoA* fragment amplified from genomic DNA
E: section deleted in JW9021
F: section deleted in JW9063
G: Southern probe for JW9021 verification
H: Southern probe for JW9063 verification

Figure 2.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Common-2 Barcode-2
aph(3')-II
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Barcode-1 Common-1
Upstream region
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4097
SpecR
5107
pMO9020
5850 bp

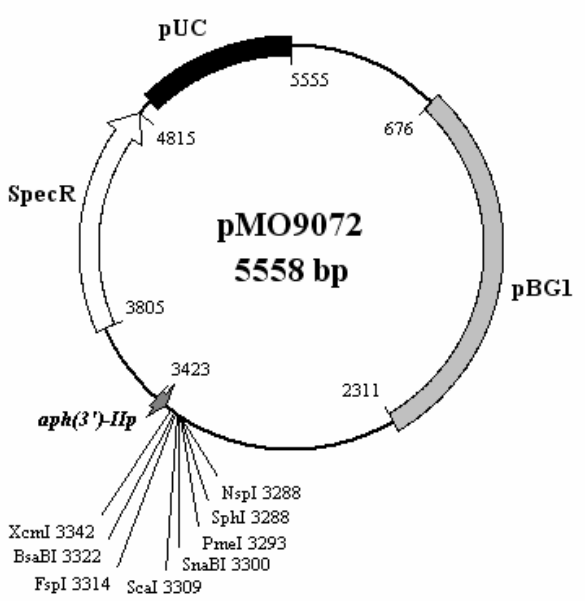
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Figure 3.

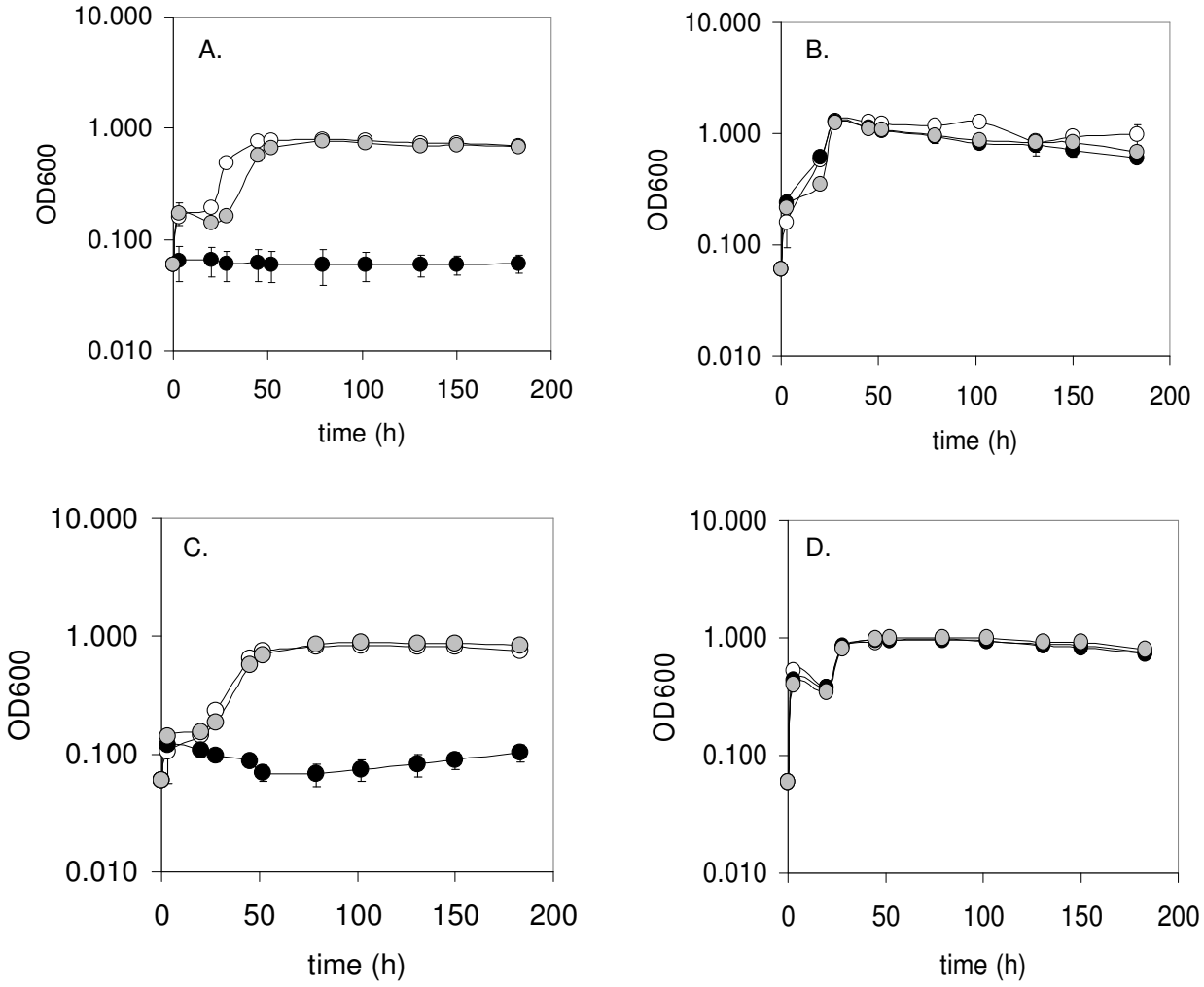


Figure 4.

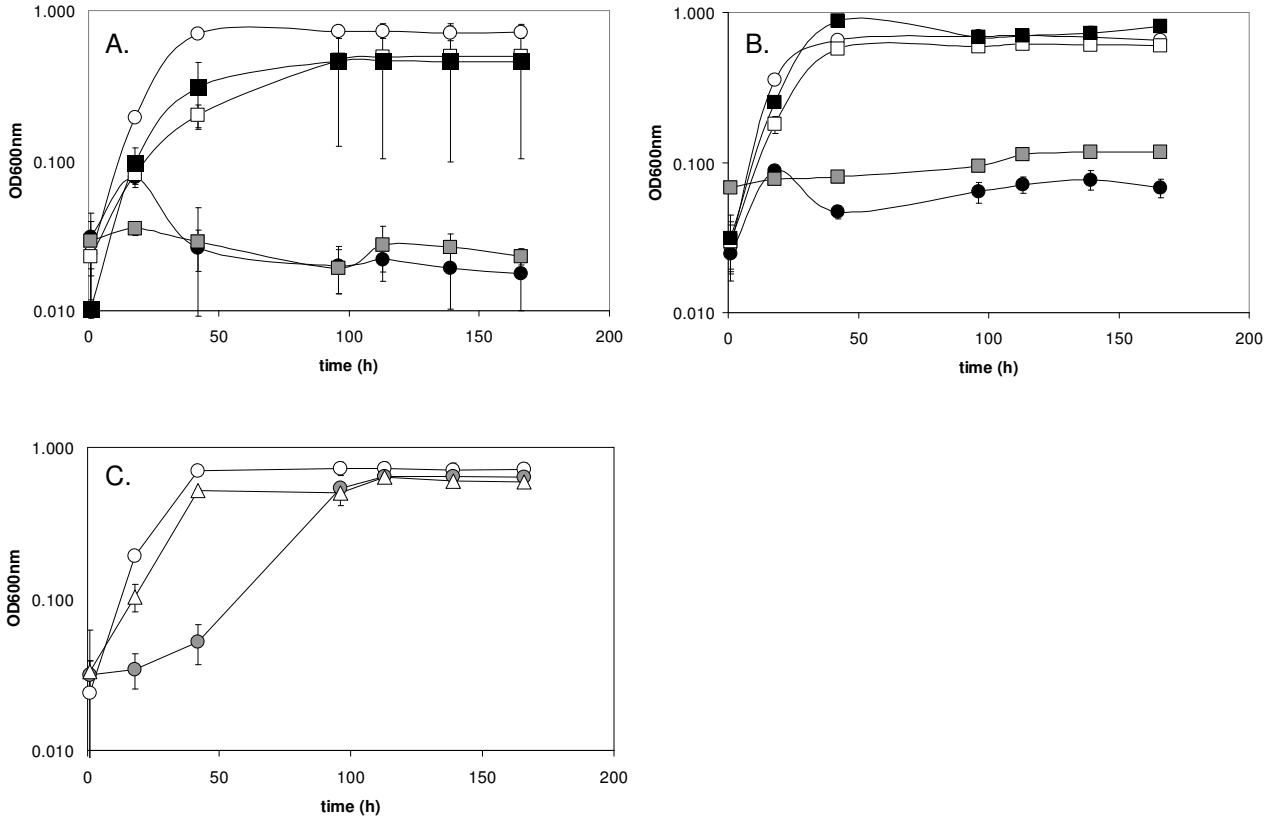
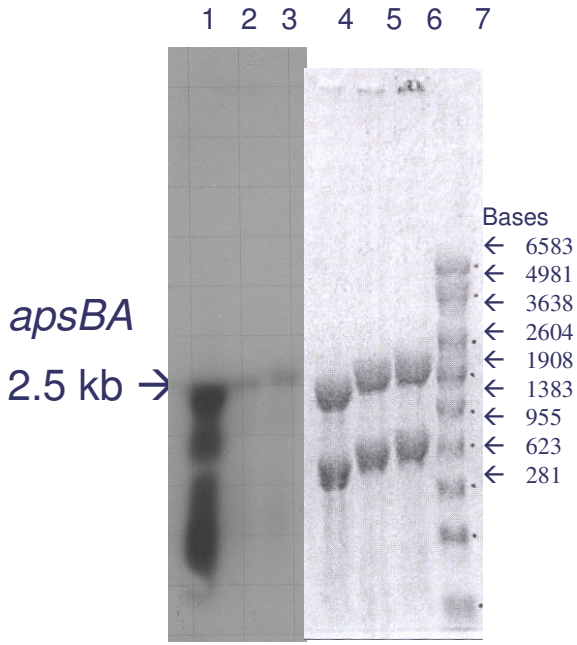


Figure 5.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2.5 kb
Bases
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3638
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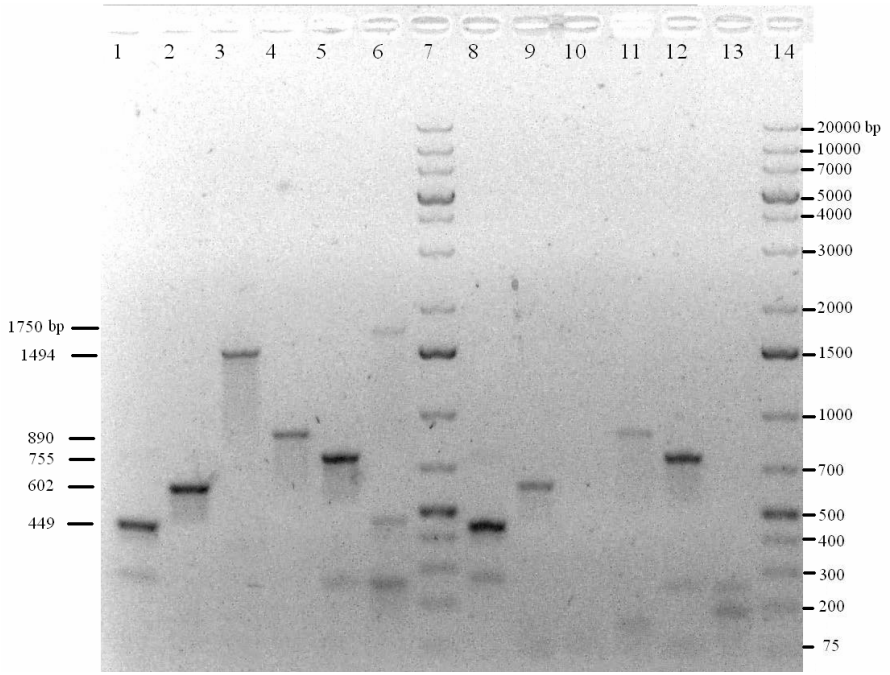
1908
1383
955
623
281

Figure 6.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602
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449
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300
200
75

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