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Functional profiling in *Streptococcus mutans*: construction and examination of a genomic collection of gene deletion mutants

Robert G. Quivey Jr.[b,c,*], **Elizabeth J. Grayhack**[a,*], **Roberta C. Faustoferri**[c], **Cory J. Hubbard**[c,†], **Jeremiah D. Baldeck**[b,§], **Andrew S. Wolf**[a], **Matthew E. MacGilvray**[c,††], **Pedro L. Rosalen**[c,¶], **Kathy Scott-Anne**[c], **Brendaliz Santiago**[c], **Shuba Gopal**[d,¶¶], **James Payne**[a,§§], and **Robert E. Marquis**[b,**]

[a]Department of Biochemistry and Biophysics, University of Rochester, Rochester, NY, USA

[b]Department of Microbiology and Immunology, University of Rochester, Rochester, NY, USA

[c]Center for Oral Biology, University of Rochester; Rochester, NY, USA

[d]Department of Biological Sciences, Rochester Institute of Technology, Rochester, NY, USA

Summary

A collection of tagged deletion mutant strains was created in *Streptococcus mutans* UA159 to facilitate investigation of the aciduric capability of this oral pathogen. Gene-specific barcoded deletions were attempted in 1432 open reading frames (representing 73% of the genome), and resulted in the isolation of 1112 strains (56% coverage) carrying deletions in distinct non-essential genes. Since *S. mutans* virulence is predicated upon the ability of the organism to survive an acidic

[*]Corresponding Authors: Robert G. Quivey, Jr. Robert_Quivey@urmc.rochester.edu, Elizabeth J. Grayhack Elizabeth_Grayhack@urmc.rochester.edu.

Current Contact information for each of the authors

Robert G. Quivey, Jr., University of Rochester School of Medicine and Dentistry, Center for Oral Biology, Box 611, Dept. of Microbiology and Immunology, Rochester, NY 14642, Telephone: (585) 275-0382, Fax: (585) 276-0190, Robert_Quivey@urmc.rochester.edu

Elizabeth Grayhack, University of Rochester School of Medicine and Dentistry, Dept. of Biochemistry and Biophysics, Box 712, Rochester, NY 14642, Telephone: (585) 275-2765, Fax: (585) 275-6007, Elizabeth_Grayhack@urmc.rochester.edu

Roberta C. Faustoferri, University of Rochester School of Medicine and Dentistry, Center for Oral Biology, Box 611, Rochester, NY 14642, Telephone: (585) 275-1435, Roberta_Faustoferri@urmc.rochester.edu

Andrew S. Wolf, University of Rochester School of Medicine and Dentistry, Dept. of Biochemistry and Biophysics, Box 712, Rochester, NY 14642, Telephone: (585) 275-5554, andrews_wolf@urmc.rochester.edu

Kathy-Scott Anne, University of Rochester School of Medicine and Dentistry, Center for Oral Biology, Box 611, Rochester, NY 14642, Telephone: (585) 275-1436, Kathy_ScottAnne@urmc.rochester.edu

Brendaliz Santiago, University of Rochester School of Medicine and Dentistry, Center for Oral Biology, Box 611, Rochester, NY 14642, Telephone: (585) 275-1435, Brendaliz_Santiago@urmc.rochester.edu

[†]Cory J. Hubbard, GWA Gregory Wentworth Advertising, 356 Laurens Road, Montoursville, PA 17754, Telephone: (585) 880-3452, cory.j.hubbard@gmail.com

[§]Jeremiah D. Baldeck, 3399 W Henrietta Rd. Rochester, NY 14623, Telephone: (585) 750-3548, jeremiah.baldeck@gmail.com

[¶]Pedro L. Rosalen, University of Campinas (UNICAMP), Piracicaba Dentistry School, Dept. of Physiological Sciences, Area of Pharmacology, Ave. Limeira, 901, Piracicaba, SP Brazil, 13414-903, Telephone: +55-19-2106-5313, rosalen@fop.unicamp.br

[††]Matthew E. MacGilvray, University of Wisconsin-Madison, Laboratory of Genetics, 3456 Biotechnology Center, 425G Henry Mall, Madison, WI 53706, mmacgilvray18@gmail.com

[¶¶]Shuba Gopal, The Broad Institute of Harvard and M.I.T., 7 Cambridge Center, Cambridge, MA 02142, Telephone: 617-714-7239, shuba.gopal@gmail.com

[**]Robert E. Marquis, Deceased

[§§]James Payne, WEMOCO (Monroe 2-Orleans BOCES), 3589 Big Ridge Road, Spencerport, NY 14550, 585-352-0764, jpayne@monroe2boces.org

SUPPORTING INFORMATION

Table S1. *S. mutans* fitness database table.

Table S2. Primers used in *in vitro* and *in vivo* fitness competition experiments.

pH environment, form biofilms on tooth surfaces, and out-compete other oral microflora, we assayed individual mutant strains for the relative fitness of the deletion strain, compared to the parent strain, under acidic and oxidative stress conditions, as well as for their ability to form biofilms in glucose- or sucrose-containing medium. Our studies revealed a total of 51 deletion strains with defects in both aciduricity and biofilm formation. We have also identified 49 strains whose gene deletion confers sensitivity to oxidative damage and deficiencies in biofilm formation. We demonstrate the ability to examine competitive fitness of mutant organisms using the barcode tags incorporated into each deletion strain to examine the representation of a particular strain in a population. Co-cultures of deletion strains were grown either *in vitro* in a chemostat to steady-state values of pH 7 and 5 or *in vivo* in an animal model for oral infection. Taken together, this data represents a mechanism for assessing the virulence capacity of this pathogenic microorganism and a resource for identifying future targets for drug intervention to promote healthy oral microflora.

Keywords

genomics; pathogenesis; physiology

Introduction

Streptococcus mutans is found in the vast majority of humans living in industrialized nations in which refined sugar consumption is high, with a correspondingly high prevalence of dental caries (Nyvad, *et al.* 2013; Takahashi & Nyvad 2008; Takahashi & Nyvad 2011). *S. mutans* uses sugars in the human diet to produce extracellular glucans that act to irreversibly bind the organism to tooth surfaces. *S. mutans* also ferments sugars to produce organic acids, which accumulate at the tooth surface, resulting in a localized low pH environment, dissolution of enamel, and, eventually, the initiation of dental caries. Metagenomic sequencing projects have readily identified the organism in dental plaque samples from humans, and demonstrated that its relative abundance appears to increase with increasing severity of the disease, concomitant with decreased species diversity (Gross, *et al.* 2012; Nyvad, *et al.* 2013).

Streptococcus mutans has evolved specific mechanisms to cope with the acidic and oxidative stress it faces in the oral cavity (Lemos, *et al.* 2013). While the vast majority of genetic studies involving stress-adaptive pathways in *S. mutans* have involved the explorations of one to several genes in a given study, it is clear from experiments involving genome-wide transcriptional reporting that the loss of a single gene frequently affects mRNA levels from hundreds of additional genes (Hughes, *et al.* 2000). Comparison of the relative contribution of each gene to the overall fitness, or virulence potential, of *S. mutans* would profit from the availability of a collection of strains carrying unique mutations in the genetic complement of the organism.

The elucidation of the genomic sequence for *Streptococcus mutans* (Ajdic, *et al.* 2002) provided the information necessary to attempt precise genetic deletion of single open-reading frames of *S. mutans* on a genome-wide scale. Such a collection of strains could greatly facilitate analysis of the role of each gene in the organism's competition with other

members of the oral flora, as well as the contribution of the gene product to colonization and persistence (Aas, *et al.* 2008; Becker, *et al.* 2002; Costalonga & Herzberg 2014; Gross, *et al.* 2012; Kanasi, *et al.* 2010; Liu, *et al.* 2012). The genomic type strain of *S. mutans*, UA159 (Murchison, *et al.* 1986), has been extensively utilized in studies of the physiological, genetic, and virulence attributes of the organism. *S. mutans* UA159 has a malleable genetic system facilitating its transformation with either linear or circular DNA (Murchison, *et al.* 1986). In the constellation of modern microbiological studies, the number of bacterial mutant collections, or libraries, is still relatively small, and in general, has favored the use of transposon mutant libraries that offer a rapid means by which to construct mutant strains. However, the transposons may exert effects on genes downstream of the insertion site and may fail to reveal particular mutations due to preferred transposition sites (Bose, *et al.* 2013; Fey, *et al.* 2013; Ge, *et al.* 2008; Klein, *et al.* 2012; Valentino, *et al.* 2014). The use of DNA-barcoded deletion libraries, pioneered in yeast (Giaever, *et al.* 2002; Pierce, *et al.* 2007; Smith, *et al.* 2010), requires considerably more effort, but offers the advantages of defined mutations and the ability to control deletions in places such as over-lapping reading frames or the in-frame deletion of genes located in polycistronic messages.

Here, we report the construction of a DNA barcoded genomic collection of 1112 strains deleted for non-essential genes and the identification of 320 putative essential genes, together constituting 73% of the *S. mutans* UA159 genome. The remaining 27% of the genome was refractory to our approach for constructing PCR primers to attempt gene deletion. We have tested the surviving strains for their ability to survive in specific *in vitro* tests, as predictors of their ability to survive *in vivo* models. These assessments were chosen to examine the effect of the loss of each gene on the ability of the organism to withstand stresses encountered in the oral cavity: acid, oxidative damage (here, by chemical agents, particularly H_2O_2), and the presence or absence of atmospheric levels of oxygen. Further, we have tested two different subsets of deletion mutants in mixed-strain experiments to determine their abilities to compete with one another *in vitro*, and also to maintain themselves *in vivo*, in the oral cavity of rats, as a model of human oral infection. The collection is a powerful tool to study the ecology of *S. mutans* and to direct systems approaches to define key mechanisms involved in the pathogenesis of this organism. Our working hypothesis is that potential targets for the therapeutic intervention of dental disease might be more quickly identified by the elucidation of the set of essential genes, and genes that contribute to the overall fitness of *S. mutans.*

Methods

Bacterial strains

Streptococcus mutans UA159 (Murchison, *et al.* 1986), the genomic type strain (Ajdic, *et al.* 2002), was used in this study. All of the deletion strains described were derived from UA159. Deletion of each open reading frame (ORF) was performed essentially as previously detailed (Lau, *et al.* 2002; Sheng, *et al.* 2010) and the reading frame was replaced with an erythromycin resistance marker (Erm^R) flanked by common, as well as unique, barcode sequences, selected from the barcodes used to create the yeast deletion library (detailed in Fig. 1) (Giaever, *et al.* 2002). Strains were maintained on Brain Heart Infusion (BHI) agar

medium (BD/Difco, Franklin Lakes, NJ) supplemented with erythromycin (Erm) at 5 μg/mL. BHI medium was used to prepare overnight cultures for frozen stocks of the deletion strains.

Primer design

The primer design algorithm was based on Primer3 software (Rozen & Skaletsky 2000). Primers were, in general, designed to have a T_m= 62–68°C, no predicted secondary structure, an inability to form primer-dimers, and no primers could have a 10 base pair homology in the *S. mutans* genome. Oligonucleotide primers were designated P1 through P6, as shown in Fig. 1. The P1, P2, P5, and P6 primers were constructed to have a predicted annealing temperature of 62–64°C and did not contain an ATG. The P3 and P4 primers were constructed to have a predicted annealing temperature of 62–68°C, and without an ATG.

The P3 and P4 primers were designed to amplify the erythromycin-resistance (Erm^R) cassette (Aoki, *et al.* 1986) used in the study, and to add unique 20 bp upstream and downstream barcodes. Each barcode was flanked by 18 bp conserved DNA sequences that were common to all of the constructs (UpUp (red), UpDn (blue), DnUp (brown), DnDn (pink) in Fig. 1).

The P1/P2 primer pair functioned to amplify the region upstream of the ORF to be deleted, and via P2, added DNA sequence homologous to the 5' end of P3 (red striped region) (Fig. 1). The P5/P6 primer pair amplified the region downstream of the gene to be deleted, where the P5 primer contained DNA sequence homologous to P4 (purple striped region). The sequences flanking the target genes were approx. 400 bp in length, depending on the local sequence and the ability of the primer-calling algorithm to design oligonucleotides within our parameters.

In addition to the 6 primers synthesized for assembly of the deletion construct, 2 additional primers, P7 and P8, were created to screen the resulting transformants and verify proper integration of the Erm^R cassette (Fig. 1). P7 and P8 were designed to bind to sequences outside the region amplified by the P1/P2 and P5/P6 primer pairs, designated the recombination zone (400bp both upstream and downstream of the ORF to be deleted, respectively). These screening primers were designed to work with primers inside the Erm^R cassette, Erm Left (Erm L) and Erm Right (Erm R) (Table S2), and result in amplicons that would possess a T_m of approx. 50°C. In addition, the primers were designed such that they were 16–21bp long, containing no secondary structure, and preferably not forming a self-duplex.

Assembly of deletion constructs

The linear, double-stranded DNA fragment used to replace each open reading frame (ORF) was constructed via a six-step process, illustrated in Fig. 1 and described below. Amplicons arising from the three PCR reactions were purified and treated with T4 DNA polymerase in the presence of dGTP for the P1/P2 product; in the presence of dCTP for the P3/P4 product; and in the presence of dGTP for the P5/P6 product. Thus, a G (coding strand) was placed at the 5' end of the unique barcode and a C (non-coding strand) was placed at the 3' end of the

unique barcode. Treatment of the PCR products with T4 DNA polymerase would result in a PCR product with 18–20 bp single-strand overhangs at the 5' and 3' ends that would be homologous, respectively, to the upstream and downstream fragments that were similarly prepared.

The fragments were then allowed to anneal to complementary overhangs and precipitated. The annealed product was reconstituted and a portion was ligated. Finally, primer pair P1/P6 was used to PCR amplify across the ligated, double-stranded DNA. Purified PCR products were analyzed by gel electrophoresis and quantitated by ethidium bromide staining.

Deletion mutant construction

DNA deletion constructs, as detailed above, were transformed into *S. mutans* UA159 as previously described (Perry & Kuramitsu 1981). Positive transformants were selected on BHI agar medium containing erythromycin (Erm) at 5 μg/mL. Colonies arising after 2 days were picked to fresh agar plates and then at least 2 isolates were chosen for each construct transformation to be analyzed by colony PCR.

A second attempt was made to delete ORFs that were not successfully deleted in the first attempt. If no colonies were obtained following the 2-day incubation, the transformation was performed again, with the addition of 1 μg/mL competence stimulating peptide (CSP) (Li, *et al.* 2002). If this subsequent transformation with CSP failed to result in Erm^R colonies, deletion of that particular ORF was categorized as a potential lethal mutation and coded red in our database. Of the 1432 ORFs attempted, this occurred in 301 cases (21%).

For some ORFs, the deletion construct could not be produced in sufficient abundance for transformation or was not verified by PCR prior to transformation. Therefore, these ORFs (5%) were characterized as having technical issues and coded purple in our database.

Screening and verification of deletion mutant strains of *S. mutans*

The procedure we employed to verify the deletion constructs in the collection is depicted in the flowchart in Fig. 2. Colony PCR was used to screen Erm^R colonies arising from the transformation of *S. mutans* UA159 with each of the deletion constructs. The P7 and P8 primers (described above) were used in conjunction with Erm Left and Erm Right primers, respectively, to verify that the DNA construct containing the Erm^R cassette, in place of the coding region for the ORF, had recombined into the proper site. Colony PCR was performed essentially as previously described (Buckley, *et al.* 2014; Santiago, *et al.* 2012). Briefly, a colony resulting from the transformation of *S. mutans* was boiled for 10 minutes in dH_2O. Primers (P7/Erm Left, "left" reactions, or P8/Erm Right, "right" reactions; final concentration 0.5μM) and 1 μL from the lysed colony mixture were added to Platinum PCR SuperMix 96 tubes (Life Technologies, Carlsbad, CA) and PCR amplification was performed using the following conditions: 94°C 1 min; 55°C 1 min.; 72°C 1 min. for a total of 35 cycles. The PCR products were analyzed by gel electrophoresis. If both the "left" and "right" reactions yielded products of the expected size, the isolate was grown up for frozen stock preparation. If the products obtained were the incorrect size, colony PCR was repeated a second time to test additional isolates. A second failure to obtain amplicons of the

appropriate size constituted a failure and the transformation step was repeated as detailed above to include CSP. If colonies isolated from this subsequent transformation also failed to yield the proper PCR fragments, the strain was categorized as a potential lethal mutation.

In addition to colony PCR validation of the strains containing deletions, we also performed colony blot analysis for some of the ORFs (25 deletion strains were analyzed in this manner) (Quivey, *et al.* 1991), or sequenced PCR-amplified DNA containing the deletion region via Sanger sequencing. Strains of *S. mutans* that had been verified for the proper integration of the deletion construct, 77% of the attempted ORFs, were stored frozen. In approx. 1% of the verified deletions, cultures for frozen stock preparation did not grow. These were coded orange in the database to indicate a potential nutritional deficiency.

Physiological characterization of deletion strains

Each of the deletion strains of *S. mutans* was categorized with respect to several growth characteristics. Specifically, strains were grown in BHI medium + 44mM glucose, either aerobically or micro-aerobically (with a mineral oil overlay), in the following conditions: (i) unbuffered medium, pH 7.4; (ii) medium titrated to pH 5.4 with HCl; (iii) medium titrated to pH 5.6 with HCl; (iv) medium containing 0.5 mM H_2O_2; and (v) medium containing 5 μM 8-hydroxyquinoline (8-HQ). Conditions chosen for testing the relative fitness of the deletion strains were based on results from empirically-derived pilot studies for this study. Growth rate and yield were assessed using a Bioscreen C automated growth reading system (Growth Curves USA, Piscataway, NJ) essentially as described elsewhere (Buckley, *et al.* 2014; Derr, *et al.* 2012; Kajfasz, *et al.* 2010). Growth studies for each deletion strain in each growth condition were repeated twice with 3 replicates each time.

The deletion strains were also characterized with respect to their ability to form model biofilms on a solid, plastic surface using a modification of an assay previously described (Ahn, *et al.* 2005). Deletion strains, and the parent strain, UA159, were grown in a microtiter dish in 0.5× tryptone-yeast extract (TY) medium with the addition of either 56 mM glucose (TYG) or 29 mM sucrose (TYS). Quantitation of biofilm formation was assessed by measuring the optical density (OD_{575}) of wells stained with crystal violet using a BenchMark Plus microplate reader (BioRad, Hercules, CA). The ability of the parent strain, UA159, to form biofilms was normalized to a value of 1.0.

Finally, the endpoint, minimum glycolytic pH for each of the deletion strains was determined using a modification of a previously established method (Belli & Marquis 1991). The value obtained was normalized to the minimum glycolytic pH of the parent strain.

For each of the physiological characterizations performed above, the parent strain, *S. mutans* UA159, was grown and assessed each time a group of the mutant strains was analyzed. Values for relative doubling time, final yield (indicated by optical density of the culture), and biofilm (BF) formation were compared to the parent strain, UA159, grown under the same conditions.

Establishing the relative fitness score for each deletion strain of *S. mutans*

A scoring system was devised to give each of the deletion strains a phenotypic rating based on the ability of the strain to grow under the various conditions described above, as compared to the parent strain, UA159 (Table S1, Columns J-S). A rating of 0 signifies that there was no growth under the specified condition (red cell). A score of 1 indicates poor growth where the yield of the culture is <50% of control and/or the growth rate is 76–550% of control and/or a lag time greater than control of 4 hours (orange cell). A score of 2 indicates impaired growth where the yield and/or growth rate are 26–50% that of control and/or lag time is between 2.1 to 3.9 hr of control (yellow-orange cell). A rating of 3 signifies reduced growth, as compared to the parent strain, with a yield and/or growth rate 51–75% that of control and/or lag time was between 1.1 to 2 hr of control (yellow cell). A score of 4 indicates normal growth, i.e., similar to parent strain where the yield was 76–110% of control and/or the doubling time was −25 to 25% of control and/or lag time was between −1 to 1 hr of control (green cell). In three instances, deletion of the gene resulted in enhanced growth, as compared to UA159, which was given a score of 5 (blue cell). Yields were greater than 110% of control and/or doubling time was >25% lower and/or a lag time < −1 hr of control. For all 3 cases, this occurred when the cultures were grown aerobically in the presence of 8-HQ. The average of the scores determined for each growth condition was calculated and each deletion strain was assigned a mean growth score (Table S1, Column U). Cells colored grey indicate the inability to obtain a deletion mutant and cells colored white indicate that the construct was not attempted due to inability of our primer-calling algorithm to design primers for the deletion construct

The relative ability of the deletion strains to form single-strain model biofilms in medium containing glucose or sucrose, described above, was scored on a different scale, based on the magnitude and significance (*p*-value determined by Student's *t*-test) of the difference between biofilm formation by the parent strain, UA159 (control), and the specific deletion mutant strain. No statistical difference between control and deletion strain earned a score of 4 (green). For significant variations from control (p-value < 0.05), the score was assigned as follows: relative biofilm formation 0–10% versus control = 0 (red); relative biofilm formation 11–25% versus control = 1 (orange); relative biofilm formation 26–50% versus control = 2 (yellow-orange); relative biofilm formation 51–75% versus control = 3 (yellow); and relative biofilm formation greater than 110% of control = 5 (blue).

Competition experiments using pools of deletion strains

(i) Survival of selected strains in chemostat culture—Selected strains carrying deletions in genes involved in fatty acid biosynthesis and cell wall assembly were used to examine relative fitness in steady-state culture. The strains used were MX804 (Erm^R knock-in strain), MU0022 (Δ*plsX*; SMU.26), MU0023 (Δ*acpP*; SMU.27), MU0898 (Δ*cls*; SMU.988), MU1020 (Δ*nox*; SMU.1117), MU1591 (Δ*fabT*; SMU.1745c), MU1592 (Δ*fabM*; SMU.1746c), and MU1593 (Δ*pgmB*; SMU.1747c). Strains were grown overnight in batch culture in TY medium containing 1% glucose. Cell density was measured at OD_{600} and cultures were normalized such that 25 mL would be equivalent to an OD_{600} = 1. Equal volumes (25 mL) of each normalized culture were used to inoculate the vessel of a BioFlo 2000 fermentor (New Brunswick Scientific, Edison, NJ). The mixed culture was grown in TY

medium with limiting glucose to steady-state (10 generations) at pH values of 7 and 5 at a dilution rate of 0.144 hr^{-1}, as previously described (Fozo, *et al.* 2004). Samples were removed from the mixed culture at various time points (as diagrammed in Fig. 3A), pelleted, and stored frozen.

DNA was prepared from samples taken from the continuous culture of the mixed pool using the Promega Wizard Genomic DNA Purification Kit (Promega, Madison, WI) and used in PCR with primers listed in Table S2 for each of the deletion mutant strains.

(ii) Survival of selected strains in the rat model of oral infection—Two approaches were used to examine transmissibility, between rats, of tagged strains of *S. mutans* carrying deletions in genes of the malolactate fermentation pathway. A pilot study was employed to determine the ability of the strains to implant and be transmitted from an infected pup to an uninfected cagemate. The methodology used was identical to that used in a previous study from our group (Fozo, *et al.* 2007). The following *S. mutans* deletion strains created in this study were utilized: MX804 (Erm^R knock-in strain), MU0121 (Δ*mleR*; SMU.135), MU0123 (Δ*mleS*; SMU.137), MU0124 (Δ*mleP*; SMU.138), MU0125 (Δ*oxdC*; SMU.139), MU1592 (Δ*fabM*; SMU.1746c), and MU1450 (Δ*cah*; SMU.1595) (Sheng, *et al.* 2010). The overall scheme for the experiment is detailed in Fig. 4A.

Strains used in the experiment were grown overnight in BHI medium. The overnight cultures were diluted 1:40 (or 1:20 for MU1592) into a total volume of 20 mL fresh BHI medium the following morning and allowed to grow to mid-logarithmic phase (OD_{600} ~ 0.6–0.8). Aliquots of 1 mL cells were harvested and pellets were saved as “pre-infection pools” from each of the 3 days of infection (Day 1, 2, and 3 for infection of the dams; Day 6, 7, 8 for infection of the pups). Another aliquot of the pooled culture was serially diluted and plated on BHI agar medium to enumerate input bacteria.

Two dams were infected with an actively growing, mid-logarithmic pooled culture by oral swabbing on experimental days 1, 2, 3 (pups were aged 16, 17, 18 days). Two additional dams were not infected. Infection in the dams was confirmed by streaking oral swabs on MSE agar on days 2, 3 and 4 of the experiment (pups aged 17, 18, 19 days). Infected dams were given Diet-2000 and drinking water sweetened with 5% (w/v) sucrose containing 50 mM L-malate, *ad libitum*; the uninfected dams were fed lab chow and water *ad libitum*.

On day 6 of the experiment, pups (aged 21 days) associated with the infected dams (Group I) were weaned, then screened for infection from dams by oral swabbing and plating on MSE agar medium. The pups fed by uninfected dams (Group II) were also weaned and screened for absence of infection by plating oral swabs on MSE agar medium. The pups from Group I were then infected with the actively growing pooled culture of 7 strains over 3 consecutive days (experimental Day 6, 7, and 8). Infection was confirmed in the Group I pups by Day 10. The dams were sacrificed and the lower left jaws were transferred to a saline solution and sonicated to free bacteria. The jaws were removed and the sonicate solution centrifuged with resulting pellets stored at −20°C for use in DNA purification.

On experimental day 10, infected pups from Group I (aged 25 days), referred to as donors, were paired with uninfected pups, and referred to as recipients, from Group II. The new group, Group III, consisted of 10 pairs of donor and recipient animals. All animals were given Diet-2000 containing 5% sucrose water supplemented with 50 mM L-malate, *ad libitum*. The previously uninfected pups, or recipients, were screened for *S. mutans* infection by oral swabbing on day 11, 15, 17 (pups aged 26, 30, 32 days), respectively. Animals were sacrificed by CO_2 asphyxiation on Day 21 (animals aged 36 days).

The lower jaw was removed and separated into 2 hemi-jaws. The left lower hemi-jaw was placed into 5 mL sterile saline and sonicated three times for 10 seconds each. Jaw sonicates were then serially diluted and plated onto BHI agar medium containing Erm (5μg/mL) to determine streptococcal colony forming units (CFUs), as established in previous studies. The remaining sonicate for each of the 20 rat pups was centrifuged and resulting pellets were stored at −20°C until used for DNA purification. The right lower hemi-jaw was also placed in 5 mL sterile saline and sonicated. The sonicates were passed through a 0.8μm syringe filter to clarify the particulate matter, centrifuged, and the resulting pellets were stored at −20°C as a back up, if needed.

DNA was isolated from frozen samples using the Promega Wizard Genomic DNA Purification Kit (Promega, Madison, WI) and used in PCR with primers listed in Table S2 to ascertain presence or absence of a given deletion mutant in the mixed population.

Results and Discussion

Construction of the collection

The methodology that we employed to create deletion strains, shown in Fig. 1 and detailed in METHODS, is generally similar to the approach used to create the *Saccharomyces cerevisiae* deletion library (Giaever, *et al.* 2002) and to create single genetic deletion mutations in *S. mutans* (Lau, *et al.* 2002). Precise gene deletions in which Erm^R replaces the coding sequence were targeted to particular chromosomal locations by homologous recombination between sequences that flank the coding region and sequences that flank the Erm^R gene. Using PCR to amplify the regions upstream and downstream of each gene also provided the opportunity to create two unique DNA tags for each deletion, one upstream of the coding region of a given gene and the other downstream, that could be used as primers for assaying the relative abundance of each deletion strain in mixed populations (Fig. 1). Furthermore, the constructs also contained upstream and downstream common sequences that could be utilized to PCR amplify the *S. mutans*-specific DNA sequences from a co-culture.

One challenge in creating the collection in *S. mutans* was the requirement for relatively long flanking sequences to correctly target the Erm^R gene to specific locations. This was solved by independently amplifying ~400 base pairs of the upstream and downstream sequences, then using ligation independent (LIC) cloning methods to link the three fragments, followed by ligation and PCR amplification. Using this protocol, we generated 1432 constructs of the expected size at sufficient DNA concentrations to transform *S. mutans*. We were unable to generate constructs for 530 genes.

The methodology and decision-making processes used to create the deletion strain collection are illustrated in the flow chart in Fig. 2. From this approach, 1112 strains were recovered that were capable of growth in suspension culture; 301 genes were identified as putative essential genes; and 19 genes exhibited difficulties in the recovery of a stable strain; i.e., no growth. These 19 strains that we were unable to cultivate in liquid culture could represent a nutritional requirement that was not met in our high-throughput approach.

The distribution of essential and non-essential genes is shown in the heat map in Fig. S1. It can be immediately seen that the putative essential genes are non-randomly distributed throughout the chromosome. As can also be seen in Fig. S1, the gaps in deletion coverage from the lack of deletion cassettes to begin the process are distributed around the chromosome. Therefore, we presume that inability to produce a deletion cassette for a particular ORF is due to the A-T rich nature of the organism and not a consequence of the methodology. Current availability of economical synthetic gene constructs would preclude this obstacle.

Each deletion was designed to be non-polar, although we did not test downstream effects of specific deletions on all genes. However, for example, the deletion of *fabT* (SMU.1745c) did not affect transcription of *fabH* (SMU.1744c), which it overlaps by 1 bp (Faustoferri, *et al.* 2014). Further, we have verified in separate experiments that the phenotype that resulted from the deletion of a particular ORF was attributable to loss of that gene product via genetic complementation of the ORF either in an ectopic locus in the genome (Buckley, *et al.* 2014; Derr, *et al.* 2012; Faustoferri, *et al.* 2014; Santiago, *et al.* 2012; Santiago, *et al.* 2013) or *in situ* (MacGilvray, *et al.* 2012).

In vitro characterization of the collection of genome-wide deletion mutant strains of *S. mutans*

The ability of *S. mutans* to respond to stress in its environment represents a key element of the organism's virulence potential. Thus, our primary interest was to assay the ability of each deletion strain to grow in conditions thought to affect virulence. We measured growth rates in the following conditions: in acidic medium, in medium containing compounds known to inhibit growth of the organism, and under reduced oxygen tensions. The deletion strains were grown, individually, to assess doubling times vis-à-vis the parent strain grown under the same conditions. The strains were then assigned overall scores, using these data, to indicate the relative fitness of each strain, compared to the parent, UA159, which earned a score of 4 (see further details in METHODS). We also created an Erm^R cassette knock-in in a non-coding region upstream of *gtfA* (SMU.881), designated MX804, to use as a bar-coded "parent" strain in mixed culture experiments. This strain displayed survival capacity similar to *S. mutans* UA159 in all conditions tested for the single deletion strain assays (data not shown).

The summary statistics deriving from *in vitro* growth experiments for 1059 surviving strains are shown in Table S1. These strains contain deletions in putative non-essential genes that were tested for their ability to grow in suspension culture under specific conditions. Of these, the majority (84%) (861) grew as well as the parent strain in rich medium. The remaining 16% of the strains (168) were statistically different from wild-type in terms of

growth rates and yields in rich medium, with 40 strains improving to the level of the parent strain when provided a reduced oxygen environment.

Growth of the strains under oil-overlay, to create a microaerobic environment, resulted in improved growth for some strains under the conditions tested, but in general, did not result in dramatic movement of strains from one phenotypic rating level to another (Table S1, compare columns J-K, L-M, N-O, P-Q, R-S). Reduced oxygen tensions did appear to make a difference in growth of strains in medium containing the metal chelator 8-hydroxyquinoline (8-HQ, Table S1, columns R-S). The most dramatic effect of reducing oxygen tensions in 8-HQ-containing cultures was in elevating 212 strains to a phenotypic rating comparable to the parent strain (a rating of 4), suggesting that chelation of metals represented a strongly oxidative environment for *S. mutans*, and that reduction of oxygen levels alleviated, to some extent, that stress.

Mutants were identified with impaired growth in two or more conditions relevant to virulence

With the establishment of the growth rates for the strains in rich medium, we began our assessment of the fitness of the individual strains in conditions known to be involved with the virulence potential of the organism: aciduricity (Fozo, *et al.* 2007), ability to form an anaerobic environment (Marquis 1995), and biofilm formation (Bowen & Koo 2011). We examined the ability of the deletion strains to grow in medium titrated to an acidic pH value or in the presence of hydrogen peroxide or 8-HQ. We also determined the ability of deletion strains to produce model biofilms in mono-organism culture, in the presence of glucose or sucrose. The majority of the deletion strains produced quantities of biofilm comparable to the parent strain, in cultures growing in the presence of glucose or sucrose. However, we identified 101 deletion strains that were defective in biofilm formation in the presence of glucose and 64 strains defective in the presence of sucrose.

One of the valuable assets of the deletion collection was that we are able to identify mutants with defects in two or more growth characteristics. In this case, we were interested in mutations that cause defects in multiple aspects of virulence. We found 51 strains (Table 1A) that were both acid-sensitive (a phenotypic rating of ≤2) and biofilm-defective (a biofilm formation rating of ≤2) in the presence of sucrose. We found 49 strains were both defective in oxidative-stress resistance (a phenotypic rating of ≤2) and biofilm-defective (a biofilm formation rating of ≤2) (Table 1B). These two subsets represent mutants of *S. mutans* carrying deletions in genes that are likely involved in the pathogenicity of the organism. Furthermore, there are 39 genes whose deletion caused a defect in all 3 of the conditions; of these, only 6 have established functions, 24 have been annotated and have been assigned a putative function, and 9 are completely unknown or even hypothetical, representing potential targets for future investigations into their role in virulence (Table 1C).

Recently, Cornejo, *et al*. published a pan-genome sequence for *mutans* streptococci based on sequences from 57 strains (Cornejo, *et al.* 2013). The core genome contained 1490 genes common to all strains and 73 conserved hypothetical genes, most of unknown function, though some with an assigned putative function. Examination of these genes in the present database shows that we attempted to delete 55 of the 73 genes, resulting in the isolation of

39 surviving strains, 4 strains that could not be sub-cultured following transformation, and 12 genes that could not be deleted (Table 2). Of the surviving strains, few of them exhibited strong phenotypic differences from the parent strain, though the greatest number of altered phenotypes were seen when the strains were grown in the presence of 8-hydroxyquinoline and oxygen (13 strains), the majority of which reverted to parental rates of growth in the micro-aerobic condition (11 strains), suggesting a role for metals and/or oxygen by these gene products. These results provide evidence toward the utility of the deletion collection for the identification and characterization of hypothetical proteins that would otherwise be missed.

In vitro growth and testing of deletion mutant strains

The primary purpose of creating the *S. mutans* deletion collection was to investigate which of the deletions would indicate presumptive "essential" genes, and which of the surviving "non-essential" genes would be affected in the ability to grow in competition with other deletion strains. We conducted a pilot experiment to evaluate the ability of specific deletion strains to survive growth in a culture consisting of a mixed population of deletion strains under fixed-pH chemostat conditions (Fozo & Quivey 2004b; Quivey, *et al.* 1995). We chose the following strains: MX804 (Erm^R knock-in strain), MU0022 (Δ*plsX*; SMU.26), MU0023 (Δ*acpP*; SMU.27), MU0898 (Δ*cls*; SMU.988), MU1020 (Δ*nox*; SMU.1117), MU1591 (Δ*fabT*; SMU.1745c), MU1592 (Δ*fabM*; SMU.1746c), and MU1593 (Δ*pgmB*; SMU.1747c) (Buckley, *et al.* 2014; Derr, *et al.* 2012; Faustoferri, *et al.* 2014; MacGilvray, *et al.* 2012). This subset of strains was chosen to examine the effects of deletion of particular genes involved in cell membrane homeostasis. The experimental plan for the chemostat run is detailed in Fig. 3. Samples were removed from the chemostat culture at various times during the run to ascertain which strains were still able to thrive under the test conditions: steady-state pH values of 7 and 5 in the presence of other strains carrying deletions in gene products involved in cell wall synthesis (Δ*plsX*, Δ*acpP*, Δ*cls*, Δ*fabT*, Δ*fabM*), or in transport or breakdown of metabolic intermediates (Δ*nox*, Δ*pgmB*). PCR performed with primers specific to the Erm^R cassette contained in the control strain, MX804, yielded an amplicon in all samples removed at every time point (Fig. 3B). Similarly, gene-replacement-specific primers for *pgmB* (SMU.1747c) were able to amplify a product at all time points, representing the presence of the MU1593 strain (Fig. 3C). However, in the case of *plsX*, the amplicon decreased in predominance as the culture pH was reduced to 5. In fact, as the *plsX*-Erm^R cassette became less abundant, another band appeared on the gel representing the Erm^R cassette in the adjacent ORF, *acpP* (Fig. 3D, F). This finding was mirrored in the results obtained with the *fabM*-Erm^R cassette: as the culture pH decreased, the strain lacking *fabM* was lost in the mixed culture (Fig. 3E, G). We have previously demonstrated that loss of *fabM* results in severe acid-sensitivity (Fozo & Quivey 2004a); therefore, the outcome here was anticipated.

Results from this experiment demonstrate the potential uses for the deletion collection in mixed culture experiments to examine the relative fitness of individual deletion strains; for example, the fluctuating environmental conditions mimicking the oral cavity.

In vivo growth and testing of deletion mutant strains

Examination of the pathophysiology of *S. mutans* should, by necessity, include determination of the fitness of a particular strain in the rat model of oral infection. Consequently, we conducted a pilot study to test infection and transmission of a subset of deletion strains, particularly those associated with the malolactic acid pathway, in the rat model (Lemme, *et al.* 2010; Sheng, *et al.* 2010; Sheng & Marquis 2007). The malolactate pathway is controlled by MleR (SMU.135) (Sheng, *et al.* 2010), and consists of a malate permease (*mleP*; SMU.138) and the malolactic synthase (*mleS*; SMU.137), which splits malate into carbon dioxide and lactic acid. The resulting carbon dioxide is available to carbonic anhydrase (*cah*; SMU.1595) for conversion to bicarbonate, which would buffer the cytoplasm. The malolactate fermentation system has been shown to be an important aspect of acid tolerance in *S. mutans* and, indeed, it has been demonstrated that the enzymatic activity is induced in low pH growth conditions (Sheng & Marquis 2007). The experimental design is detailed in Fig. 4A and is essentially similar to published work from our laboratory (Fozo, *et al.* 2007). Here, we were interested in examining whether the presence of other deletion strains in the inoculum used to infect rats would have an effect on the ability of competing members of the pool to infect or to be transmitted to non-infected cage mates. Presence or absence of a particular deletion strain at the completion of the experimental period was assayed by PCR using gene-deletion-specific primers for the individual strains in the inoculum pool, similar to the method used above for the mixed chemostat culture experiment. As shown in Fig. 4B, all the strains were identified in the inoculum pool at the start of the experiment; however, after 19 days *in vivo* on rat jaws, 2 members of the pool were no longer detected by PCR amplification (Fig. 4C), the *fabM* (SMU.1746c; MU1592) and *mleR* (SMU.135; MU0121) strains. These data indicate the further utility of the collection: to investigate differences in the competitive fitness, *in vivo*, of specific members of the deletion collection.

Conclusions

Fitness profiling of genomic deletion collections has provided a wealth of information on everything from responses to chemicals or drugs (Lopez, *et al.* 2008; Nichols, *et al.* 2011; Rooney, *et al.* 2008; Xu, *et al.* 2007), to genes involved in particular processes (Valentino, *et al.* 2014; Korte, *et al.* 2014) (Muller-Herbst, *et al.* 2014), to interactions between gene networks (Gagarinova, *et al.* 2012; Kuzmin, *et al.* 2014; Wang, *et al.* 2013). We report here the application of these principles to *S. mutans*, an organism with a critical role in dental caries. We have constructed barcoded deletions of 1112 genes in *S. mutans* and demonstrated that these can be used to assess defects in growth in conditions relevant to virulence. The value of the mutant collection is immediately obvious in the ability to identify mutations that cause defects in several conditions related to virulence. Moreover, the ability to mix mutant strains and then assess growth effects either *in vitro* or in the rat model, more accurately mimics the consequences of drug targeting in which only a portion of the population may be affected. Mutants that are not rescued by the presence of other wild-type strains are likely to represent the best effective drug targets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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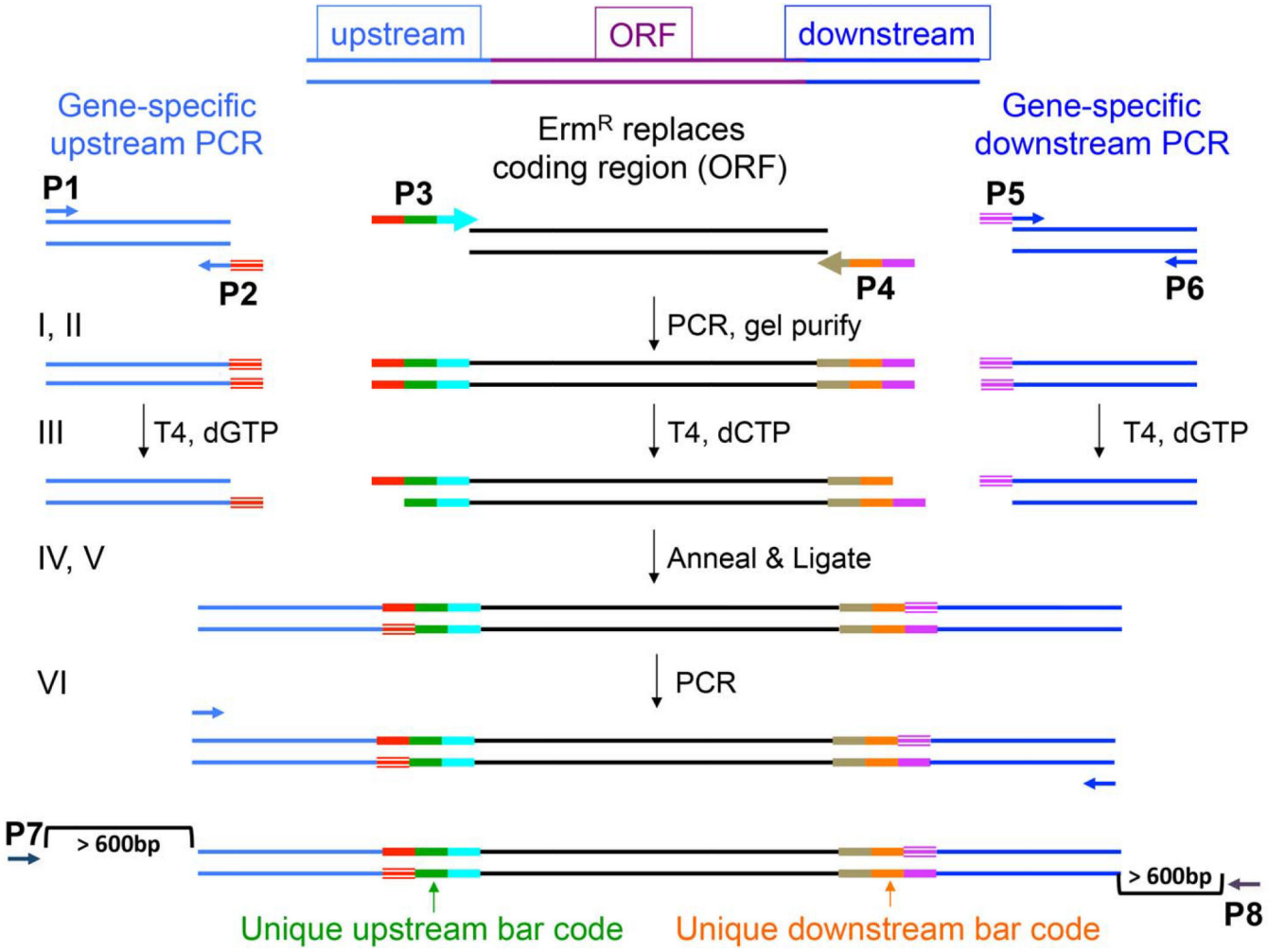


Fig. 1. Scheme for generation of deletion constructs by ligation-independent cloning (LIC)

Panel A. Oligonucleotide primers P1, P2, P3, P4, P5, and P6 were designed according to specifications outlined in **Methods**. The method involves 6 sequential steps, depicted in the figure by I – VI. (I) The designed primers are utilized in 3 separate PCR reactions. (II) PCR products are gel purified. (III) The amplicons from the sequences upstream and downstream of the target gene are treated with T4 DNA Polymerase in the presence of dGTP. The amplicon arising from the amplification of the erythromycin resistance cassette (Erm^R) is treated with T4 DNA Polymerase in the presence of dCTP. This enzymatic treatment allows the digestion of the overhanging ends of the double-stranded DNA fragment to a G residue on the 5' end of the Erm^R cassette and to a C residue on the 3' end. The result is a single-stranded overhang at the 5' and 3' ends homologous to the ends created at the 5' and 3' ends of the Erm^R. (IV) The 3 double-stranded DNA fragments are allowed to anneal their complementary overhangs. (V) The annealed DNA is treated with T4 DNA Ligase to close the gaps. (VI) Primer pair P1/P6 was used to amplify across the linear double-stranded DNA fragment. This final product was called the deletion construct and used in subsequent transformation into *Streptococcus mutans*. The lowermost diagram depicts the approx.

location of the P7 and P8 primers used to screen the integrated deletion construct in positive transformant colonies.

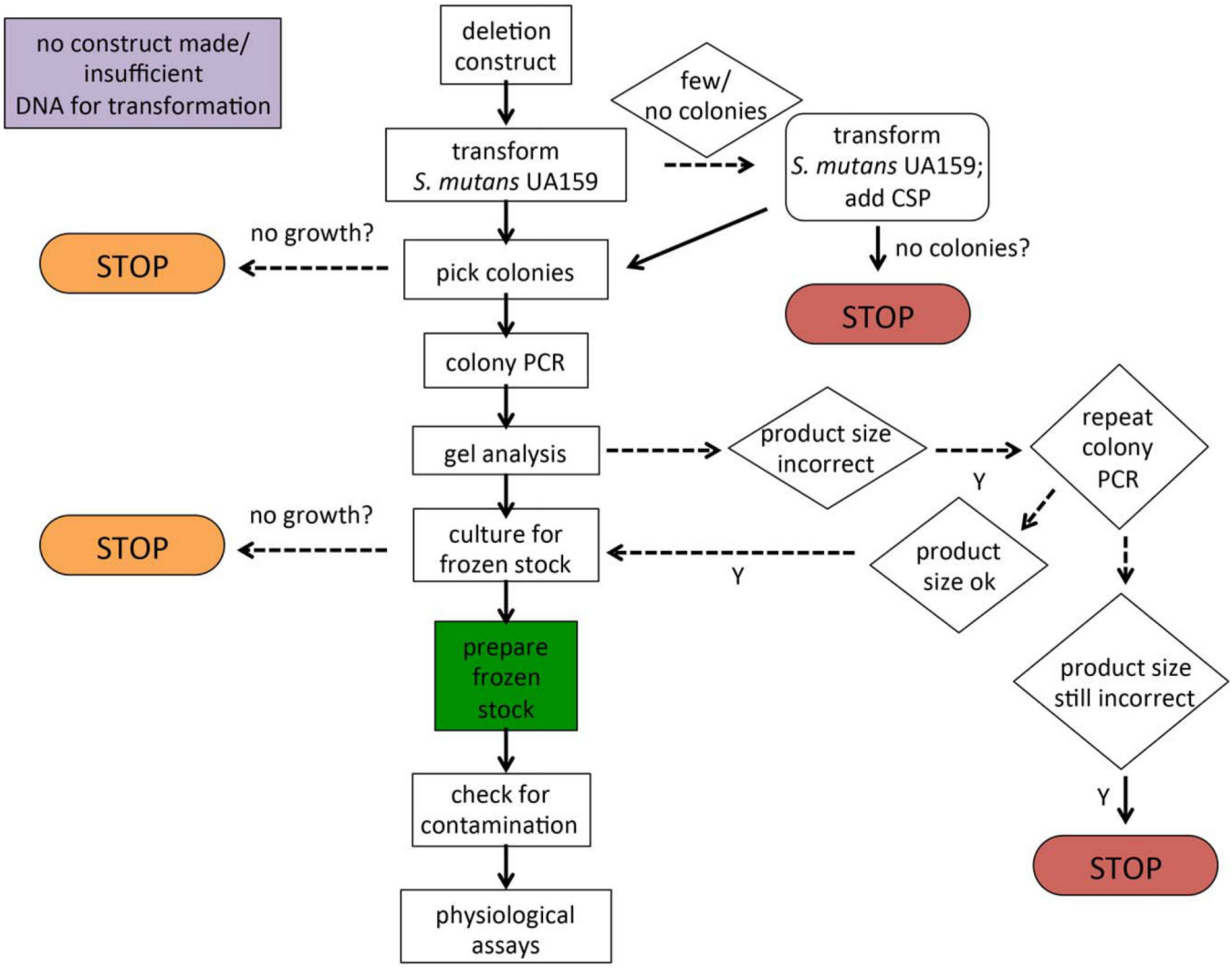
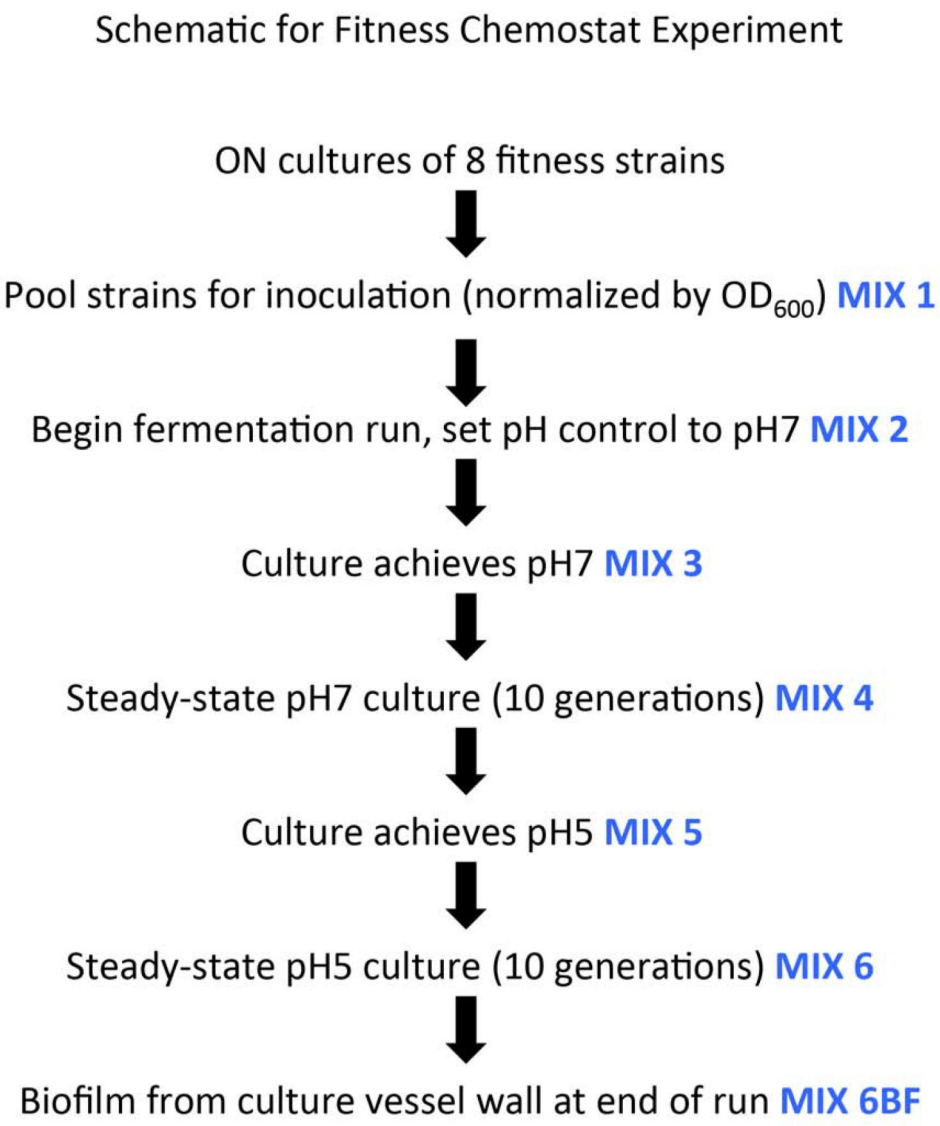


Fig. 2. Decision flow chart for deletion mutant construction

Linear, double-stranded DNA fragments were created as described in Fig. 1. If the construct was not produced in sufficient abundance for transformation or there was a technical problem with the preparation of the DNA, these open reading frames (ORFs) are coded purple in Table S1. After transformation, if there were no colonies, the transformation was repeated with the addition of competence stimulating peptide (CSP). If no colonies arose from this attempt, then the deletion was presumed to be lethal and coded red in Table S1. If after transformation, the picked colonies did not grow on the BHI agar medium supplemented with Erm, then the gene was coded orange in Table S1 for presumed nutritional requirement. Likewise, if the resulting transformants did not grow in liquid culture for the preparation of a frozen stock, then the gene was also coded orange in Table S1. Finally, during PCR verification of the transformants, if the resulting amplicons were incorrect in size or did not appear after 2 attempts, then the gene was coded red in Table S1 to indicate a putative lethal mutation.



A

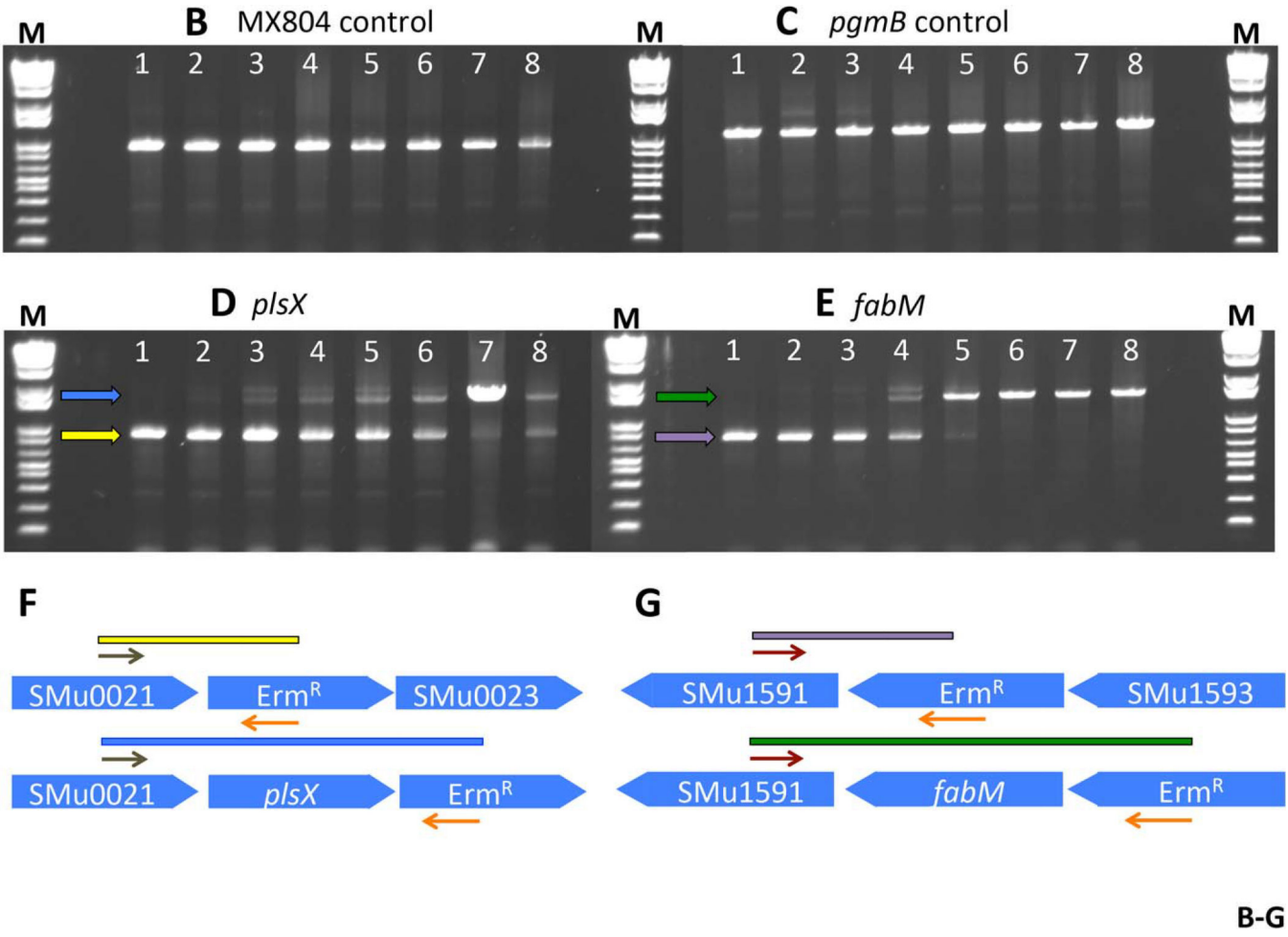
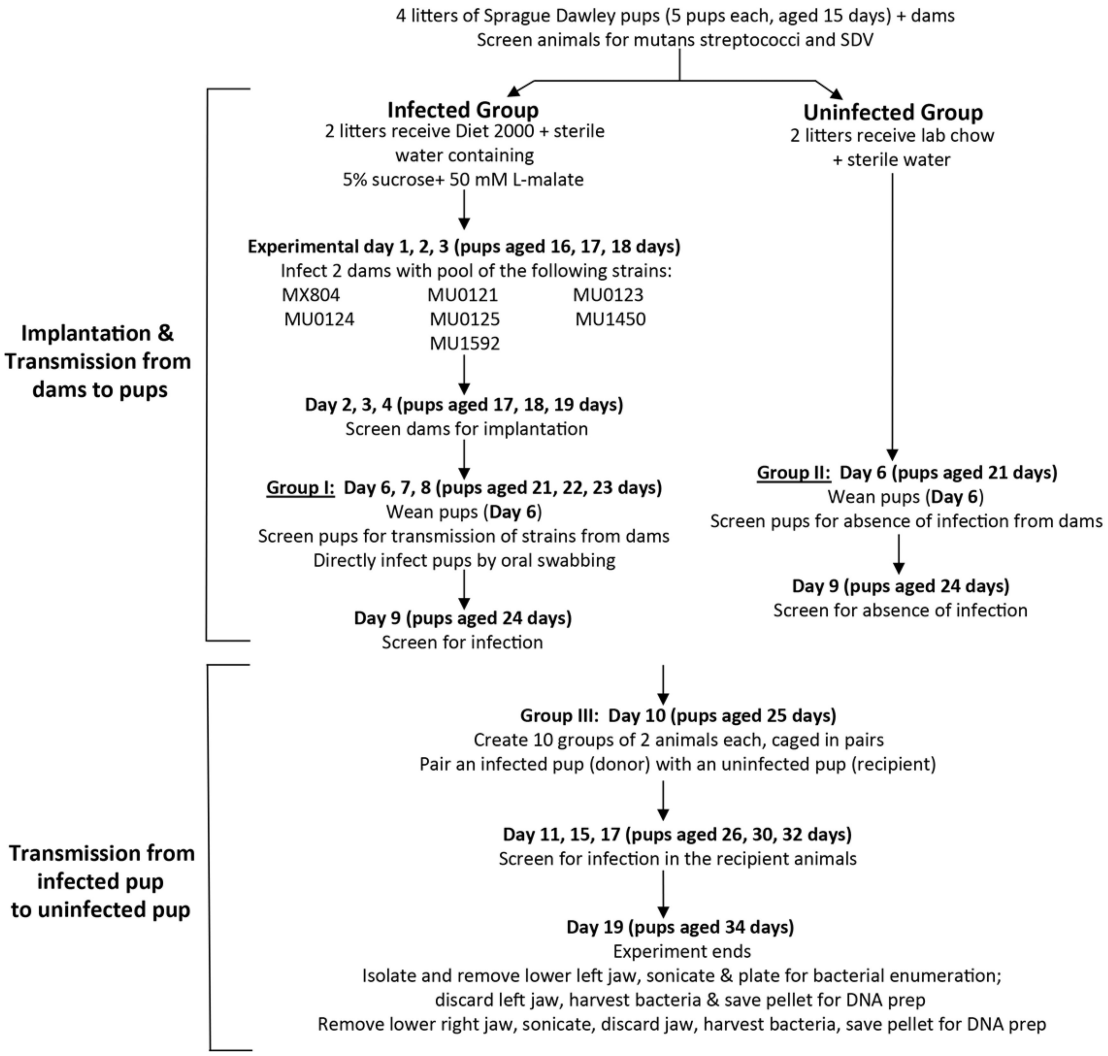


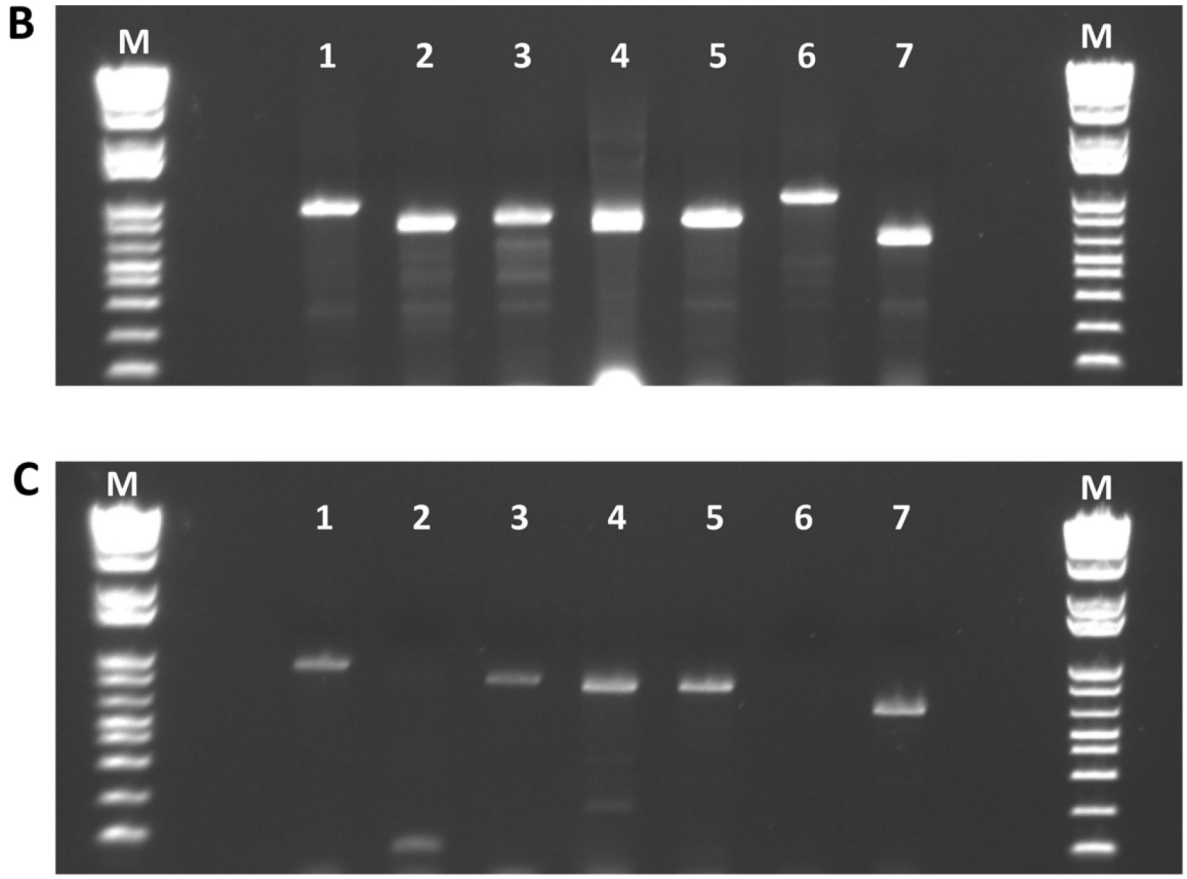
Fig. 3. Fitness testing of a subset of deletion mutant strains in chemostat culture

Panel A. Experimental design and sampling for the chemostat culture. Eight deletion strains were individually cultured, normalized, and used to inoculate a fermenter vessel. This co-culture was sampled and designated Mix 1. The culture was allowed to grow in batch culture before turning on the pH control. At the point that the pH control was activated, and set to pH 7, another sample was taken (Mix 2). Once the culture had reached pH 7, a sample was removed and designated Mix 3. A sample from the steady-state pH 7 culture was called Mix 4. Glucose was added to the culture vessel to reduce the pH to 5, and another sample was removed (Mix 5). A sample from the steady-state pH 5 culture was called Mix 6. Finally, a sample of the biofilm that formed on the wall of the fermenter vessel was removed and called Mix 6BF. **Panel B**. PCR amplification of DNA representing MX804 (ErmR knock-in strain) in the samples derived from the chemostat culture. DNA prepared from the samples recovered at each step described in Panel A (above) was used as a template in a reaction with primer pair MX0804P7UPFG and Erm L to determine the relative amount of the MX804 strain at each time point. Lane order is as follows: lane 1: Genomic DNA control reaction; lane 2: sample from Mix 1; lane 3: sample from Mix 2; lane 4: sample from Mix 3; lane 5: sample from Mix 4; lane 6: sample from Mix 5; lane 7: sample from Mix 6; lane 8: sample from Mix 6 BF. **Panel C**. PCR amplification of DNA representing the MU1593

deletion strain (Δ*pgmB*) (Buckley, *et al.* 2014) in the samples derived from the chemostat culture. DNA prepared from the samples recovered at each step described in Panel A (above) was used as a template in a reaction with primer pair MU1593P7UPFG and Erm L to determine the relative amount of the MU1593 strain at each time point. Lane order is as detailed in Panel B above. **Panel D**. PCR amplification of DNA representing the MU0022 deletion strain (Δ*plsX*) in the samples derived from the chemostat culture. DNA prepared from the samples recovered at each step described in Panel A (above) was used as a template in a reaction with primer pair MU0022P7UPFG and Erm L to determine the relative amount of the MU0022 strain at each time point. Lane order is as detailed in Panel B above. **Panel E**. PCR amplification of DNA representing the MU1592 deletion strain (Δ*fabM*) in the samples derived from the chemostat culture. DNA prepared from the samples recovered at each step described in Panel A (above) was used as a template in a reaction with primer pair MU1592P7UPFG and Erm L to determine the relative amount of the MU1592 strain at each time point. Lane order is as detailed in Panel B above. **Panel F**. Graphical representation of the origin of the bands seen in the gel photo in Panel D. The brown arrows represent the MU0022P7UPFG screening oligonucleotide and the orange arrows represent the Erm L oligonucleotide. If MU0022 DNA is present in the pooled sample, then the yellow bar and yellow arrow in the gel photograph indicate the size and migration of that specific amplicon. However, if MU0022 DNA is no longer present in the pooled sample, then we would expect to obtain the amplicon represented by the blue bar (and the blue arrow on the gel photograph), indicating DNA derived from the presence of MU0023 would arise. **Panel G**. Graphical representation of the origin of the bands seen in the gel photo in Panel E. The red arrows represent the MU1592P7UPFG screening oligonucleotide and the orange arrows represent the Erm L oligonucleotide. If MU1592 DNA is present in the pooled sample, then the purple bar and purple arrow in the gel photograph indicate the size and migration of that specific amplicon. However, if MU1592 DNA is no longer present in the pooled sample, then we would expect that the amplicon represented by the green bar (and the green arrow on the gel photograph), indicating DNA derived from MU1593 would arise.



A



B, C

Fig. 4. Fitness testing of a subset of deletion mutant strains in a rat model for oral infection

Panel A. Transmission experiment outline for 7 deletion mutant strains. SDV = sialoacroadenitis virus. **Panel B**. Gel photograph showing the presence of each of the deletion strains in the pre-infection pool used to inoculate rat pup jaws (from Day 8; see Panel A). Lane contents are as follows: lane 1: MX804-specific primers (MX0804P7UPFG/ Erm L); lane 2: MU0121-specific primers (MU0121P7UPFG/ Erm L); lane 3: MU0123-specific primers (MU0123P7UPFG/ Erm L); lane 4: MU0124-specific primers (MU0124P7UPFG/ Erm L); lane 5: MU0125-specific primers (MU0125P7UPFG/ Erm L); lane 6: MU1592-specific primers (MU1592P8DNRH/ Erm L); lane 7: MU1450-specific primers (MU1450P7UPFG/ Erm L). **Panel C**. Representative gel photograph showing amplified DNAs isolated from strains surviving after 19 days in the rat model. The left hemi-jaw from rat #13 (in Group II uninfected pups) was sonicated and DNA prepared for use as a template in a PCR reaction with the gene-specific primers for each of the 7 deletion strains utilized in the experiment. Lane order and primer pairs are as detailed in Panel B (above).

Table 1

A. Deletion strains exhibiting poor acid survival and deficiencies in biofilm formation				
Oralgen ID	**Locus**	**Gene Name(s)**	**Common Name**	**Functional Class**
0022	SMU.26	plsX ylpD	putative fatty acid/phospholipid synthesis protein	Fatty acid and phospholipid metabolism: Biosynthesis
0072	SMU.80	hrcA	transcriptional regulator repressor (HrcA) of class I	Regulatory functions: DNA interactions
0085	SMU.94c	tpn	hypothetical protein putative transposase fragment	Mobile and extrachromosomal element functions: Transposon functions
0137	SMU.154	rpsO	30S ribosomal protein S15	Protein synthesis: Ribosomal proteins: synthesis and modification
0153	SMU.170	rpsl	30S ribosomal protein S9	Protein synthesis: Ribosomal proteins: synthesis and modification
0223	SMU.246	rgpG	putative glycosyl transferase N-acetylglucosaminyltransferase), RgpG	Cell envelope: Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides
0275	SMU.304	cdd	putative deaminase	Unknown function: Enzymes of unknown specificity
0315	SMU.350		hypothetical protein	Hypothetical proteins
0317	SMU.352	rpe	putative ribulose-phosphate-3-epimerase	Energy metabolism: Pantose phosphate pathway
0318	SMU.353		conserved hypothetical protein	Biosynthesis of cofactors, prosthetic groups, and carriers: Thiamine
0431	SMU.475		conserved hypothetical protein	Unknown function: General
0434	SMU.480	priA	primosomal replication factor Y (primosomal protein N)	DNA metabolism: DNA replication, recombination, and repair
0437	SMU.483	pphA ppL	putative phosphoprotein phosphatase (pppL protein)	Regulatory functions: Protein interactions
0500	SMU.550	divlB ftsQ	putative cell division protein FtsQ (DivlB)	Cellular processes: Cell division
0510	SMU.561c	mutT	putative hydrolase (MutT family)	DNA metabolism: DNA replication, recombination, and repair
0521	SMU.572	folD	putative tetrahydrofolate dehydrogenase/cyclohydrolase	Biosynthesis of cofactors, prosthetic groups, and carriers: Folic acid
0545	SMU.598	recR	putative recombination protein RecM	DNA metabolism: DNA replication, recombination, and repair
0571	SMU.629	sod sodA	putative manganese-type superoxide dismutase, Fe/Mn-SOD	Cellular processes: Detoxification
0645	SMU.706c		conserved hypothetical protein	Transport and binding proteins: Other
0666	SMU.731		putative ABC transporter, ATP-binding protein	Transport and binding proteins: Unknown substrate
0672	SMU.739c		hypothetical protein	Hypothetical proteins: Conserved

A. Deletion strains exhibiting poor acid survival and deficiencies in biofilm formation				
Oralgen ID	**Locus**	**Gene Name(s)**	**Common Name**	**Functional Class**
0698	SMU.770c	hitA	putative manganese transporter	Transport and binding proteins: Cations and iron carrying compounds
0705	SMU.777	aroD	putative 3-dehydroquinate dehydratase	Amino acid biosynthesis: Aromatic amino acid family
0706	SMU.778	aroE	putative shikimate 5-dehydrogenase	Amino acid biosynthesis: Aromatic amino acid family
0708	SMU.780	aroC	putative chorismate aynthase	Amino acid biosynthesis: Aromatic amino acid family
0740	SMU.816	arcT	putative aminotransferase	Amino acid biosynthesis: Aspartate family
0749	SMU.825	rgpAc	putative RgpAc glycosyltransferase	Unclassified: Role category not yet assigned
0750	SMU.826	rgpBc	rhamnosyltransferase	Cell envelope: Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides
0754	SMU.830	rgpFc	RgpFc protein	Unclassified: Role category not yet assigned
0792	SMU.869	trxB	putative thioredoxin reductase	Unknown function: General
0985	SMU.1077	pgmA	putative phosphoglucomutase	Cell envelope: Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides
0988	SMU 1080c		conserved hypothetical protein possible transposon-related protein	Hypothetical proteins: Domain
0991	SMU 1083c		conserved hypothetical protein	Unknown function: General
1016	SMU.1113	srtA	putative sortase	Cell envelope: Other
1035	SMU.1133	phoU	putative phosphate transport system regulatory protein	Regulatory functions: Other
1092	SMU.1193	yhcF	putative transcriptional regulator	Regulatory functions: DNA interactions
1166	SMU.1276c	ezrA	putative septation ring formation regulator	Cellular processes: Cell division
1209	SMU.1324	ftsX	putative cell-division protein FtsX	Cellular processes: Cell division
1210	SMU. 1325	ftsE	putative ABC transporter, ATP-binding component	Cellular processes: Cell division
1329	SMU.1460	rmlC	putative dTDP-4-keto-L-rhamnose reductase	Cell envelope. Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides
1330	SMU.1461	rfbA rmlA	putative glucose-1-phosphate thymidyltransferase	Cell envelope: Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides
1444	SMU.15B9c	cpoA ywaF	putative hexosyltransferase	Cell envelope: Biosynthenis and degradation of surface polysaccharides and lipopolysaccharides
1483	SMU 1629c		putative cell division protein DNA segregation ATPaae	Cellular processes: Cell division
1538	SMU.1688	dlt4 dltD	putative extramembranal protein, DltD protein	Cell envelope: Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides

A. Deletion strains exhibiting poor acid survival and deficiencies in biofilm formation				
Oralgen ID	**Locus**	**Gene Name(s)**	**Common Name**	**Functional Class**
1541	SMU.1691	dlt1 dltA	putative D-alanine-D-alanyl carrier protein ligase	Cell envelope: Biosynthesis and degradation of murein sacculus and peptidoglycan
1551	SMU.1703c		conserved hypothetical protein	Hypothetical proteins
1587	SMU.1741	fabD	putative malonyl-CoA) acyl-carrier-protein) transacylase	Fatty acid end phospholipid metabolism: Biosynthesis
1592	SMU.1746c	fabM phaB	putative enoyl-CoA hydratase	Fatty acid and phospholipid metabolism: Degradation
1662	SMU.1824c	codY	putative transcriptional regulator	Regulatory functions: DNA interactions
1823	SMU.2007	rl15 rplO	50S ribosomal protein L15	Protein synthesis: Ribosomal proteins: synthesis and modification
1886	SMU.2078c		conserved hypothetical protein	Hypothetical proteins: Conserved

B. Deletion strains exhibiting poor survival in oxidative stress conditions and in biofilm formation				
Oralgen ID	**Locus**	**Gene Name(s)**	**Common Name**	**Functional Class**
0012	SMU.14	hgt hprT	putative hypoxanthine-guanine phosphoribosyltransferase	Purines. pyrimidines, nucleosides, and nucleotides: Salvage of nucleosides and nucleotides
0022	SMU 26	plsX ylpD	putative fatty acid/phospholipid synthesis protein	Fatty and and phospholipid metabolism Biosynthesis
0137	SMU.154	rpsO	30S ribosomal protein S15	Protein synthesis: Ribosomal proteins: synthesis and modification
0153	SMU 170	rpsl	30S ribosomal proton S9	Protein synthesis: Ribosomal proteins: synthesis and modification
0223	SMU.246	rgpG	putative glycosyl transferase N-acetylglucosaminyltransferase), RgpG	Cell envelope Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides
0275	SMU 304	cdd	putative deaminease	Unknown function: Enzymes of unknown specificity
0317	SMU.352	rpe	putative ribulose-phosphate-3-epimerase	Energy metabolism Pentose phosphate pathway
0318	SMU.353		conserved hypothetical protein	Biosynthesis of cofactors, prosthetic groups, and carriers: Thiamine
0378	SMU 417		conserved hypothetical protein	Unknown function General
0431	SMU.475		conserved hypothetical protein	Unknown function: General
0434	SMU.480	priA	primosomal replication factor Y (primosomal protein N)	DNA metabolism: DNA replication, recombination, and repair
0437	SMU.483	pphA ppL	putative phosphoprotein phosphatase (pppL protein)	Regulatory functions. Protein interactions
0491	SMU 540	dpr	peroxide resistance protein Dpr	Transport and binding proteins: Cations and iron carrying compounds
0500	SMU.550	divlB ftsQ	putative cell division protein FtsQ (DivlB)	Cellular processes: Cell division

B. Deletion strains exhibiting poor survival in oxidative stress conditions and in biofilm formation				
Oralgen ID	**Locus**	**Gene Name(s)**	**Common Name**	**Functional Class**
0521	SMU.572	folD	putative tetrahydrofolate dehydrogenase/cyclohydrolase	Biosynthesis of cofactors, prosthetic groups, and carriers: Folic acid
0555	SMU.609	bsp	putative 40K cell wall protein precursor	Unknown function: General
0571	SMU 629	sod sodA	putative manganese type superoxide dismutase Fe/Mn-SOD	Cellular processes: Detoxification
0645	SMU.706c		conserved hypothetical protein	Transport and binding proteins: Other
0672	SMU 739c		hypothetical protein	Hypothetical proteins: Conserved
0685	SMU.754	hprK ptsK	HPr(serine) kinase/phosphatase	Regulatory functions: Protein interactions
0705	SMU 777	aroD	putative 3-dehydroquinate dehydratase	Amino acid biosynthesis: Aromatic amino and family
0706	SMU.778	aroE	putative shikimate 5-dehydrogenase	Amino acid biosynthesis: Aromatic amino and family
0708	SMU.780	aroC	putative chorismate synthase	Amino acid biosynthesis: Aromatic amino and family
0749	SMU.825	rgpAc	putative RgpAc glycosyltransferase	Unclassified: Roe category not yet assigned
0750	SMU.826	rgpBc	rhamnosyltransferase	Cell envelope: Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides
0753	SMU.829		putative glycosyltransferase	cell envelope: Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides
0754	SMU.830	rgpFc	RgpFc protein	Unclassified: Role category not yet assigned
0792	SMU.889	trxB	putative thioredoxin reductase	Unknown function: General
0985	SMU.1077	pgmA	putative phosphoglucomutase	Cell envelope: Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides
0988	SMU.1080c		conserved hypothetical protein possible transposon-related protein	Hypothetical proteins: Domain
0991	SMU.1083c		conserved hypothetical protein	Unknown function: General
1035	SMU.1133	phoU	putative phosphate transport system regulatory protein	Regulatory functions: Other
1092	SMU.1193	yhcF	putative transcriptional regulator	Regulatory functions: DNA interactions
1166	SMU.1276c	ezrA	putative septation ring formation regulator	Cellular processes: Cell division
1209	SMU.1324	ftsX	putative cell-division protein FtsX	Cellular processes: Cell division
1210	SMU.1325	ftsE	putative ABC transporter ATP-binding component	Cellular processes: Cell division
1329	SMU.1460	rmlC	putative dTDP-4-keto-L-rhamnose reductase	Cell envelope: Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides
1330	SMU.1461	rfbA rmlA	putative glucose-1-phosphate thymidyltransferase	Cell envelope Biosynthesis and degradation of surface polysaccharides and lipoyolysaccharides
1444	SMU.1589c	cpoA ywaF	putative hexosyltransferase	Cell envelope: Biosynthesis and degradation of surface

B. Deletion strains exhibiting poor survival in oxidative stress conditions and in biofilm formation				
Oralgen ID	**Locus**	**Gene Name(s)**	**Common Name**	**Functional Class**
				polysaccharides and lipopolysaccharides
1468	SMG.1614	fpg mutM	putative formamidopyrimidine-DNA glycosylase	DNA metabolism: DNA replication, recombination, and repair
1483	SMU.1629c		putative cell division DNA segregation ATPase	Cellular processes: Cell division
1551	SMU.1703c		conserved hypothetical protein	Hypothetical proteins
1587	SMU.1741	fabD	putative malonyl-COA (acyl-carrier-protein) transacylase	Fatty acid and phospholipid metabolism: Biosynthesis
1592	SMU.1746c	fabM phaB	putative enoyl-CoA hydratase	Fatty acid and phospholipid metabolism: Degradation
1769	SMU.1947	nusG	putative transcription antitermination factor	Transcription: Transcription factors
1771	SMU.1949	pbp2a	putative membrane carboxypeptidase, penicillin-binding protein 2a	Cell envelope: Biosynthesis and degradation of murein sacculus and peptidoglycan
1812	SMU.1995c	adcR zitR	putative transcriptional regulator	Regulatory functions. Other
1823	SMU.2007	rl15 rplO	50S ribosomal protein L15	Protein synthesis: Ribosomal proteins: synthesis and modification
1086	SMU.2078c		conserved hypothetical protein	Hypothetical: Conserved

C. Deletion strains exhibiting poor survival in acid, peroxide and poor biofilm formation				
ID	**Locus**	**Gene Name(s)**	**Description**	**Functional Class**
0022	SMU.26	plsX ylpD	putative fatty acid/phospholipid synthesis protein	Fatty acid and phospholipid metabolism: Biosynthesis
0137	SMU.154	rpsO	30S ribosomal protein S15	Protein synthesis: Ribosomal proteins: synthesis and modification
0153	SMU.170	rpsl	30S ribosomal protein S9	Protein synthesis: Ribosomal proteins: synthesis and modification
0223	SMU.246	rgpG	putative glycosyl transferase N-acetylglucosaminyltransferase), RgpG	Cell envelope: Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides
0275	SMU 304	cdd	putative deaminase	Unknown function: Enzymes of unknown specificity
0317	SMU.352	rpe	putative ribulose-phosphate-3-epimerase	Energy metabolism: Pentose phosphate pathway
0318	SMU.353		conserved hypothetical protein	Biosynthesis of cofactors, prosthetic groups, and carriers: Thiamine
0431	SMU.475		conserved hypothetical protein	Unknown function: General
0434	SMU.480	priA	primosomal replication factor Y (primosomal protein N)	DNA metabolism: DNA replication, recombination, and repair
0437	SMU.483	pphA pppL	putative phosphoprotein phosphatase (pppL protein)	Regulatory functions. Protein interactions
0500	SMU.550	diviB ftsQ	putative cell division protein FtsQ (DivlB)	Cellular processes Cell division
0521	SMU 572	folD	putative tetrahydrofolate dehydrogenase/cyclohydrolase	Biosynthesis of cofactors, prosthetic groups, and carriers: Folic acid
0571	SMU 629	sod sodA	putative manganese-type superoxide dismutase, Fe/Mn SOD	Cellular processes Detoxification
0645	SMU.706c		conserved hypothetical protein	Transport and binding proteins: Other

C. Deletion strains exhibiting poor survival in acid, peroxide and poor biofilm formation				
ID	**Locus**	**Gene Name(s)**	**Description**	**Functional Class**
0672	SMU.739c		hypothetical protein	Hypothetical proteins: Conserved
0705	SMU.777	aroD	putative 3-dehydroquinate dehydratase	Amino acid biosynthesis Aromatic amino acid family
0706	SMU.778	aroE	putative shikimate 5-dehydrogenase	Amino acid biosynthesis: Aromatic amino acid family
0708	SMU.780	aroC	putative chorismate synthase	Amino acid biosynthesis: Aromatic amino acid family
0749	SMU.825	rgpAc	putative RgpAc glycosyltransferase	Unclassified: Role category not yet assigned
0750	SMU.826	rgpBc	rhamnosyltransferase	Cell envelope: Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides
0754	SMU 830	rgpFc	RgpFc protein	Unclassified: Role category not yet assigned
0792	SMU.869	trxB	putative thioredoxin reductase	Unknown function: General
0985	SMU.1077	pgmA	putative phosphoglucomutase	Cell envelope: Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides
0988	SMU.1080c		conserved hypothetical protein possible transposon-related protein	Hypothetical proteins: Domain
0991	SMU.1083c		conserved hypothetical protein	Unknown function General
1035	SMU.1133	phoU	putative phosphate transport system regalatory protein	Regulatory functions: Other
1092	SMU.1193	yhcF	putative transcriptional regulator	Regulatory functions: DNA interactions
1166	SMU.1276c	ezrA	putative septation ring formation regulator	Cellular processes: Cell division
1209	SMU.1324	ftsX	putative cell-division protein FtsX	Cellular processes: Cell division
1210	SMU 1325	ftsE	putative ABC transporter, ATP-binding component	Cellular processes: Cell division
1329	SMU.1460	rmlC	putative dTDP-4-keto-L-rhamnose reductase	Cell envelope: Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides
1330	SMU.1461	rfbA rmlA	putative glucose 1-phosphate thymidyltransferase	Cell envelope: Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides
1444	SMU.1589c	cpoA ywaF	putative hexosytransterase	Cell envelope: Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides
1483	SMU.1629c		putative cell division protein DNA segregation ATPase	Cellular processes: Cal division
1551	SMU 1703c		conserved hypothetical protein	Hypothetical proteins
1587	SMU.1741	fabD	putative malonyl-CoA (acyl-carrier-protein) transacylase	Fatty acid and phospholipid metabolism: Biosynthesis
1592	SMU.1746c	fabM phaB	putative enoyl-CoA hydratase	Fatty acid and phospholipid metabolism: Biosynthesis
1823	SMU 2007	rl15 rplO	50S ribosomal protein L15	Protein synthesis: Ribosomal proteins: synthesis and modification
1886	SMU.2078c		conserved hypothetical protein	Hypothetical proteins: Conserved

51 deletion mutant strains of *S. mutans* that displayed relative fitness score values of 2 or less for both aerobic growth at pH 5.4 and biofilm formation in 0.5× tryptone-yeast extract (TY) medium with 29 mM sucrose (TYS).

49 deletion mutant strains of *S. mutans* that displayed relative fitness score values of 2 or less for aerobic growth in medium containing 0.5 mM H_2O_2 or medium containing 5 μM 8-hydroxyquinoline (8-HQ) and also for biofilm formation in 0.5× tryptone-yeast extract (TY) medium with 29 mM sucrose (TYS).

49 deletion mutant strains of *S. mutans* that displayed relative fitness score values of 2 or less for aerobic growth in either medium titrated to pH 5.4, medium containing 0.5 mM H_2O_2, or medium containing 5 μM 8-hydroxyquinoline (8-HQ) and also for biofilm formation in 0.5× tryptone-yeast extract (TY) medium with 29 mM sucrose (TYS).

TABLE 2

Unique core gene from *S. mutans* identified in the deletion strain collection

In a study by Cornejo, *et al*., 73 genes were identified from a core *S. mutans* genome that had been annotated as encoding conserved hypothetical proteins (Cornejo, *et al*. 2013). Table 2 contains these 73 genes and the results from our deletion mutant study. In the far left column, red (n) cells indicate a putative lethal mutation, green (y) cells indicate successful mutation, purple (ti) cells indicate a technical issue that precluded the making of a deletion strain, orange (wg) cells indicate that resulting transformants were unable to grow in liquid medium for the preparation of a frozen stock, and white (na) cells indicate that the construct was not attempted due to inability of our primer calling algorithm to design primers for the deletion construct. Scoring for each physiological screen was performed as described in **METHODS**, on a scale from 0 – 5; ND = not determined.

Mutant?	Oralgen ID	Locus Tag (from UA159)	Product	BHI + Gluc (aerobic)	BHI + oil (anaerobic)	pH 5.4 (anaerobic)	pH 5.6 (aerobic)	pH 5.6 (anaerobic)	H_2O_2 (aerobic)*	H_2O_2 (anaerobic)*	BHQ (aerobic)**	BHQ (anaerobic) **	Mean Growth Score
na	0015	SMU.18	hypothetical protein										
na	0027	SMU.31	hypothetical protein										
y	0062	SMU.68	hypothetical protein	4	4	4	ND	4	ND	4	4	4	4
y	0071	SMU.79	fructan hydrolase; exo-beta-Dfructosidase; FruB	4	4	4	4	4	ND	4	4	4	4
na	0109	SMU.121	putative DinF, damage-inducible protein; cation efflux pump (multidrug resistance protein)										
y	0144	SMU.161	putative transcriptional regulator	4	4	4	4	4	4	4	4	4	4
y	0145	SMU.162c	hypothetical protein	4	4	4	ND	4	ND	4	ND	2	4
n	0164	SMU.182	iron/manganese ABC transporter ATPbinding protein										
n	0165	SMU.183	putative Mn/Zn ABC transporter										
y	0166	SMU.184	ABC transporter	1	4	ND	ND	2	ND	4	ND	0	4
y	0167	SMU.185	hypothetical protein	4	4	4	4	4	4	4	4	4	4
na	0171	SMU.189	hypothetical protein										
na	0353	SMU.390	hypothetical protein										
na	0397	SMU.438c	putative (R)-2-hydroxyglutaryl-CoA dehydratase activator-related protein										
y	0402	SMU.444	hypothetical protein	4	4	3	4	2	4	4	4	3	4
y	0406	SMU.448	hypothetical protein	4	4	3	4	4	4	4	4	4	4
y	0409	SMU.451	hypothetical protein	4	4	4	4	4	4	4	4	4	4
na	0456	SMU.503c	hypothetical protein										
n	0480	SMU.529	hypothetical protein										
y	0495	SMU.545	hypothetical protein	4	3	4	4	4	4	4	4	4	4
y	0541	SMU.594	hypothetical protein	4	4	4	4	4	4	4	4	4	4
y	0564	SMU.622c	hypothetical protein	4	4	4	4	4	4	4	4	4	4
na	0573	SMU.631	hypothetical protein										
y	0659	SMU.722	hypothetical protein	4	4	3	4	4	4	4	4	4	4
na	0680	SMU.748	hypothetical protein										
wg	0771	SMU.847c	hypothetical protein										
y	0775	SMU.851	hypothetical protein	4	4	4	4	4	4	4	4	2	4
y	0830	SMU.914c	hypothetical protein	4	4	4	4	4	4	4	4	4	4
n	0870	SMU.958	hypothetical protein										
n	0871	SMU.959c	hypothetical protein										
na	0896	SMU.986c	hypothetical protein										
na	0906	SMU.996	ferrichrome ABC transporter permease protein										
na	0956	SMU.1047c	hypothetical protein										
n	0964	SMU.1056	hypothetical protein										
n	1033	SMU.1131c	hypothetical protein										
ti	1049	SMU.1147c	hypothetical protein										
y	1131	SMU.1236c	hypothetical protein	4	4	4	4	4	4	4	4	4	4
y	1139	SMU.1246c	putative transcriptional regulator	4	4	4	4	4	4	4	4	1	3
y	1159	SMU.1267c	hypothetical protein	4	4	4	ND	4	ND	2	4	2	4
y	1270	SMU.1393c	hypothetical protein	4	4	3	4	4	4	4	4	4	4
y	1272	SMU.1395c	hypothetical protein	4	4	4	4	4	4	4	4	4	4
na	1285	SMU.1409c	putative transcriptional regulator										
n	1286	SMU.1410	putative reductase										
y	1326	SMU.1456c	hypothetical protein	4	3	3	ND	4	ND	4	4	3	3
y	1365	SMU.1502c	hypothetical protein	4	4	4	4	4	4	4	4	4	4
ti	1366	SMU.1504c	hypothetical protein										
y	1419	SMU.1561	putative potassium uptake system protein TrkB	4	4	4	4	4	4	4	4	4	4
y	1420	SMU.1562	putative potassium uptake protein TrkA	4	4	4	4	4	4	4	4	4	4
na	1436	SMU.1579	hypothetical protein										
y	1470	SMU.1616c	hypothetical protein	4	4	4	4	4	4	4	4	4	4
y	1493	SMU.1641c	hypothetical protein	4	4	4	4	4	4	4	4	4	4
n	1495	SMU.1643c	hypothetical protein										
na	1497	SMU.1645	tellurite resistance protein TehB										
ti	1500	SMU.1648c	hypothetical protein										
y	1507	SMU.1655c	hypothetical protein	4	4	4	ND	4	ND	1	4	3	4
n	1643	SMU.1805	putative transcriptional regulator										
y	1689	SMU.1854	hypothetical protein	4	4	4	ND	4	ND	4	4	3	4
y	1691	SMU.1856c	hypothetical protein	4	4	4	4	4	4	4	4	4	4
y	1695	SMU.1861c	hypothetical protein	4	4	4	4	4	4	4	4	4	4
na	1696	SMU.1862	hypothetical protein										
y	1750	SMU.1926	putative transcriptional regulator	4	4	4	4	4	4	4	4	4	4
y	1752	SMU.1928	putative ABC transporter, permease protein	4	4	4	4	4	4	4	4	4	4
na	1794	SMU.1976c	hypothetical protein										
y	1848	SMU.2033c	hypothetical protein	4	4	4	4	4	4	4	4	4	4
n	1859	SMU.2048	hypothetical protein										
y	1868	SMU.2058	putative transcriptional regulator	4	4	4	4	4	4	4	4	2	4
y	1869	SMU.2059c	putative integral membrane protein	4	4	4	4	4	4	4	4	1	4
na	1870	SMU.2060	LysR family transcriptional regulator										
y	1871	SMU.2061	hypothetical protein	4	4	4	4	4	4	4	4	3	4
y	1897	SMU.2090c	hypothetical protein	4	4	4	4	4	4	4	4	2	4
y	1917	SMU.2113c	hypothetical protein	ND	ND								
y	1929	SMU.2126c	putative purine-nucleoside phosphorylase	ND	ND								
n	1938	SMU.2136c	hypothetical protein										

y	surviving deletion strain
n	presumptive lethal mutation
ti	technical issue
wg	nutrition-defective mutation
na	not attempted
ND	not determined