A counterselectable sucrose sensitivity marker permits efficient and flexible mutagenesis in *Streptococcus agalactiae*

Thomas A. Hooven[a], Maryam Bonakdar[b], Anna B. Chamby[c], Adam J. Ratner[c,d*]

Affiliations:

a. Columbia University Vagelos College of Physicians and Surgeons, Department of Pediatrics, New York, NY
b. Brown University, Pathobiology Graduate Program, Providence, RI
c. New York University School of Medicine, Department of Pediatrics, New York, NY
d. New York University School of Medicine, Department of Microbiology, New York, NY

* Corresponding author: Adam J. Ratner
Departments of Pediatrics and Microbiology
Division of Pediatric Infectious Diseases
New York University School of Medicine
430 East 29th Street, Room 505
New York, NY 10016
Tel: (646) 501-0044
Adam.Ratner@nyulangone.org

Running title: Rapid GBS mutagenesis with sucrose counterselection

Abstract

Streptococcus agalactiae (group B *Streptococcus*; GBS) is a cause of severe infections, particularly during the newborn period. While methods exist for generating chromosomal mutations in GBS, they are cumbersome and inefficient and present significant challenges if the goal is to study subtle mutations such as single base pair polymorphisms. To address this problem, we have developed an efficient and flexible GBS mutagenesis protocol based on sucrose counterselection against levansucrase (SacB) expressed from a temperature-selective shuttle vector. GBS containing the SacB expression cassette demonstrate lethal sensitivity to supplemental sucrose whether the plasmid DNA is replicating outside of the chromosome or has been integrated during a crossover event. Transmission electron microscopy shows that SacB-mediated lethal sucrose sensitivity results from accumulation of inclusion bodies that eventually lead to complete degradation of normal cellular architecture and subsequent lysis. We used this new mutagenesis technique to generate an in-frame, allelic exchange knockout of the GBS sortase gene *srtA*, demonstrating that >99% of colonies that emerge from our protocol had the expected knockout phenotype and that among a subset tested by sequencing, 100% had the correct genotype. We also generated barcoded nonsense mutations in the *cylE* gene in two GBS strains, showing that the approach can be used to make small, precise chromosomal mutations.

23

24 Importance

25

26 The ability to generate chromosomal mutations is fundamental to microbiology. Historically,
27 however, GBS pathogenesis research has been made challenging by the relative genetic
28 intractability of the organism. Generating a single knockout in GBS using traditional techniques
29 can take many months, with highly variable success rates. Furthermore, traditional methods do
30 not offer a straightforward way to generate single base pair polymorphisms or other subtle
31 changes, especially to noncoding regions of the chromosome. We have developed a new
32 sucrose counterselection-based method that permits rapid, efficient, and flexible GBS
33 mutagenesis. Our technique requires no additional equipment beyond what is needed for
34 traditional approaches. We believe that it will catalyze rapid advances in GBS genetics research
35 by significantly easing the path to generating mutants.

36

37 Introduction

38

39 *Streptococcus agalactiae* (group B *Streptococcus*; GBS) is the most common cause of neonatal
40 sepsis and meningitis (1-3). It can also cause serious infections in adults (4, 5) and in several
41 animal species, including fish, which can be a source of zoonotic transmission (6-9).

42

43 GBS is not naturally competent under laboratory conditions (10) and exhibits low rates of
44 spontaneous genetic recombination (11). Genetic studies of GBS have mostly relied on

generation of allelic exchange knockouts using mutagenesis cassettes cloned into a temperature-sensitive shuttle vector (12-14). The mutagenesis cassette typically consists of an antibiotic resistance marker with upstream and downstream homology arms matching the chromosomal regions adjacent to the target gene. A second antibiotic resistance marker on the plasmid, outside of the mutagenesis cassette, confers dual resistance to transformed cells.

After electroporation and transformation of competent GBS with the mutagenesis vector, transformed clones are initially grown at a permissive temperature, which allows extrachromosomal replication of the plasmid. A subsequent shift to a higher, non-permissive temperature selects against extrachromosomal plasmid replication, leaving only cells where a crossover event at one of the homology arms has resulted in plasmid integration into the chromosome.

In order to achieve allelic exchange, a second crossover event must then occur at the other homology arm, followed by plasmid expulsion from the cell. Successful completion of these steps is detected by screening individual colonies for a specific antibiotic resistance phenotype: retained antibiotic resistance from the mutagenesis cassette marker with sensitivity to the second antibiotic due to loss of the plasmid during growth at the non-permissive temperature (12).

Without effective counterselection against the plasmid, however, detection of the second crossover event—a stochastic and often rare occurrence—is inefficient. Particularly if the

67 desired mutant has a fitness disadvantage compared to wild type, identification of an allelic
68 exchange mutant may require manual screening of hundreds or thousands of individual
69 colonies (15).
70
71 Furthermore, the existing approach limits the types of mutants that can be generated. Since the
72 final screen depends on persistence of one antibiotic resistance phenotype with loss of a
73 second, mutants produced using traditional techniques must include an antibiotic resistance
74 marker on the chromosome. Small-scale and unmarked alterations, such as single nucleotide
75 polymorphisms (SNP) or subtle mutations to noncoding regions, are very difficult to obtain.
76
77 Levansucrase (sucrose: 2,6-β-D-fructan 2,6-β-D-fructosyltransferase) is an enzyme present in
78 multiple bacterial species and has been extensively studied in *Bacillus subtilis* (16-21). Encoded
79 by the *sacB* gene, secreted *B. subtilis* levansucrase polymerizes sucrose into the branched
80 fructan polymer known as levan (16, 19). While the exact function of levansucrase in *B. subtilis*
81 is unknown, the levan that it generates is believed to serve a structural or nutrient role for the
82 cell (17).
83
84 In other bacterial species, expression of *B. subtilis sacB* confers lethal sensitivity to sucrose (22,
85 23). The mechanism of sucrose toxicity is believed to be from either intracellular or extracellular
86 accumulation of levan, with resultant disruption of normal cellular processes. Sucrose
87 sensitivity from *sacB* expression has been used as counterselection in conjunction with plasmid-

based mutagenesis systems to isolate mutants in several bacterial species (22, 23). To date, however, the technique has not been described in GBS.

Here we report development and validation of a flexible and efficient counterselection system to make targeted mutations in GBS using a *sacB*-containing, temperature sensitive, broad host range plasmid. A schematic of this new system is presented in **Figure 1**. We show that the system can be used to generate marked and unmarked mutations in multiple GBS strains. In our experience, use of this technique dramatically decreases the labor and time required to generate mutants, allowing accelerated discovery.

Results

Construction of pMBsacB, a temperature-sensitive, sucrose counterselectable mutagenesis shuttle vector

Plasmid pMBsacB is derived from pHY304, a widely used mutagenesis shuttle vector with the temperature-sensitive broad host range origin of replication from pWV01 (12, 24).

The *B. subtilis sacB* coding sequence, complete with signal peptide sequence, was amplified from strain 168 purified genomic DNA and cloned into plasmid pOri23, which placed the *sacB* gene downstream of the p23 promoter. This plasmid is designated pSacB23, and was used in initial experiments to test *sacB* functionality in GBS (see next section).

We subsequently generated pMBsacB by amplifying the p23 promoter and *sacB* coding sequence as a single expression cassette then subcloning it into pHY304. **Figure 2** shows the steps involved in developing pMBsacB.

GBS transformed with sacB-bearing plasmids show lethal sucrose sensitivity

Before transforming GBS with *sacB*-bearing plasmids pSacB23 and pMBsacB, we developed an electroporation protocol that did not include the use of sucrose, which is typically used as an osmoprotectant to prevent bacterial death during transformation (24, 25). We had observed very low rates of successful transformation during early trials with sucrose osmoprotection (data not shown), presumably due to sucrose-mediated toxicity.

We initially tried replacing sucrose with maltose, a structurally related disaccharide that we hoped would not be lethal to cells transformed with *sacB* plasmids. However, there was no significant increase in transformation efficiency with maltose osmoprotection (data not shown), which we attributed to *sacB* nonspecific reactivity with maltose, likely generating maltosylfructose (26, 27).

Our transformation efficiency returned to expected levels (10^{-4}-10^{-5} per μg plasmid DNA for GBS strain CNCTC 10/84) with replacement of sucrose osmoprotectant with 25% (mass:mass) polyethylene-glycol (average MW 6000 daltons; PEG-6000), which we dissolved in rich

132 transformation media for competent cell outgrowth and in the wash solution in which we store
133 and electroporate competent GBS.
134
135 GBS strain 10/84 transformed with pSacB23 or pMBsacB showed significant growth defects on
136 solid media with supplemental 0.75 M sucrose (**Figure 3A**). We also tested the sensitivity of
137 planktonic 10/84:pMBsacB to sucrose added to liquid media, and found significant growth
138 impairment. Planktonic and solid media exposure could also be combined (**Figure 3B**). 10/84
139 transformed with pHY304, by contrast, did not demonstrate significant sucrose sensitivity.
140
141 In order to directly visualize the phenotypic effect of sucrose exposure on GBS expressing SacB,
142 we performed transmission electron microscopy on 10/84 transformed with pMBsacB or
143 pHY304 and exposed to sucrose or control conditions. As shown in **Figure 4A-C**, sucrose
144 exposure of 10/84 with pMBsacB resulted in abnormal cellular morphology, with apparent
145 intracellular accumulation of inclusion bodies, eventually resulting in complete degradation of
146 normal cellular architecture and eventual lysis. Apart from expected cell shrinkage from
147 sucrose-mediated osmosis, GBS transformed with the control plasmid pHY304 showed normal
148 architecture regardless of sucrose exposure (**Figures 4D-E**). SacB expression in the absence of
149 sucrose had no apparent effect on morphology (**Figure 4F**).
150
151 *Sucrose counterselection improves the efficiency of allelic exchange mutagenesis*
152

153 To test whether sucrose counterselection could be used in GBS to produce allelic exchange
154 mutants, we applied our system to deleting a gene that we and others had experience knocking
155 out: the sortase gene *srtA* (28). This provided a benchmark against which efficiency of
156 counterselection-assisted mutagenesis could be measured.
157
158 We used overlap extension PCR to generate a *srtA* mutagenesis cassette (*dSrtA*) in which
159 approximately 800-bp homology arms flank an in-frame chloramphenicol acetyltransferase
160 gene (*cat*), which confers chloramphenicol resistance, replacing the *srtA* coding sequence. This
161 cassette was subcloned into pHY304 and pMBsacB, then used for transformation of 10/84 using
162 PEG-6000 osmoprotection.
163
164 After transformation with either pHY304:*dSrtA* or pMBsacB:*dSrtA*, single-cross intermediate
165 strains were produced by transitioning liquid cultures from 28 °C to 37 °C in the presence of
166 erythromycin selection. Once chromosomal insertion was confirmed by PCR, the pHY304 and
167 pMBsacB single-cross strains were serially passaged at 28 °C with no antibiotics or
168 counterselction. At that point, each of the two cultures were used to seed two new cultures
169 with chloramphenicol at 37 °C, one containing 0.75 M sucrose and the other a non-sucrose
170 control.
171
172 Each of the four cultures (pHY304:*dSrtA* and pMBsacB:*dSrtA*, with and without sucrose) was
173 passaged three times at 37 °C with chloramphenicol selection. Serial dilutions were then plated
174 on chloramphenicol-containing solid media with or without erythromycin selection. While the

overall CFU concentration did not differ significantly between conditions—as indicated by equal growth on non-selective media—there was a dramatic difference between pMBsacB grown in the presence of sucrose and the other three outgrowth conditions. Exposure of the pMBsacB single-cross strain to sucrose eliminated virtually all erythromycin-resistant survival, suggesting successful counterselection (**Figure 5A-B**). PCR of the *cat* gene generated the expected 660-bp gel electrophoresis bands when genomic DNA from colonies that survived counterselection was used as template, but not wild type GBS DNA (**Figure 5C**). Sanger sequencing of DNA amplified using PCR primers that bind outside of the *dSrtA* homology arms confirmed that the *srtA* gene had been replaced by *cat*, as intended (**Figure 5D**). The pHY304 single-cross strain was not responsive to sucrose, and the pMBsacB erythromycin-resistant CFUs survived well in the absence of sucrose exposure.

The pMBsacB mutagenesis system permits efficient generation of unmarked mutations in multiple GBS strains

An effective counterselection system could facilitate generation of unmarked mutations, opening the possibility of performing sequential genome edits to produce complex mutants.

To explore whether pMBsacB could accelerate generation of unmarked mutations, we developed a mutagenesis cassette against the *cylE* gene, which encodes a key biosynthetic enzyme responsible for production of the GBS pigmented hemolytic toxin β-hemolysin/cytolysin (29-31). The mutagenesis cassette features a premature stop codon

197 surrounded by a set of silent mutation SNPs, establishing a unique barcode that would not
198 likely arise through spontaneous mutation. The barcoded stop codon was flanked by 500-bp
199 upstream and downstream homology arms (**Figure 6A**).
200
201 We cloned this cassette into pMBsacB, generating pMBsacB:*dCylE*, which was then transformed
202 into two GBS strains, 10/84 and A909. After generation of the single-cross intermediate, which
203 was non-hemolytic (data not shown), we used sucrose counterselection to isolate putative
204 double-cross GBS, which could be either wild type—due to auto-excision of the plasmid at the
205 same homology arm as crossed over during insertion—or a *cylE* knockout.
206
207 Counterselection against the single-cross strain again resulted in near-complete elimination of
208 erythromycin resistant CFUs (**Figure 6B**).
209
210 When plated on solid media without antibiotic selection, the counterselection culture yielded
211 an equal mix of pigmented and non-pigmented colonies, supporting the concept that the
212 plasmid could auto-excise in one of two ways, only one of which would result in the non-
213 pigmented phenotype expected of the knockout. If not exposed to sucrose, the single-cross
214 intermediate retained erythromycin resistance and the colonies were uniformly nonpigmented,
215 reflecting the fact that the population consisted almost entirely of unchanged, single-cross
216 CFUs.
217

In the 10/84 experiment, we selected four non-pigmented colonies from the non-selective plate on which the sucrose-exposed culture had been grown. These were used for genomic DNA purification, followed by amplification of the *cylE* coding sequence and Sanger sequencing. One of the four had the correct barcoded sequence, whereas the other three did not properly amplify during PCR, suggesting that the plasmid auto-excised in a manner that left a partial sequence deletion (**Figure 6C**).

In the case of A909, 20 out of 24 non-pigmented isolates properly amplified by PCR. We sequenced ten of these, nine of which had the expected barcoded premature stop codon (**Figure 6C**). Both 10/84 and A909 *cylE* knockout strains generated using pMBsacB:*dCylE* showed the anticipated non-pigmented, non-hemolytic phenotype (**Figure 6D**).

Unintended deletions during the final plasmid-excision is a phenomenon that we have subsequently observed in other mutagenesis experiments, suggesting that all mutants generated with pMBsacB must be confirmed by some combination of PCR and sequencing to ensure the desired genotype.

Discussion

Reliable methods for creating specific mutations are central to microbiological discovery. Existing methods for doing so in GBS have been limited in multiple respects. Without counterselection, the final screening step for plasmid auto-excision and curing is unreliable and inefficient, since it depends on random identification of a low-probability biological event.

240 Furthermore, most current methods rely on replacement of a coding sequence with an
241 antibiotic resistance marker; this does not support rapid generation of small changes to the
242 chromosome, such as individual SNPs. An earlier counterselection-based approach to GBS
243 mutagenesis was limited by the fact that it required an already mutated background strain,
244 which is not ideal for pathogenesis work (15). Together, these barriers make isolation of
245 complex mutants with multiple, subtle chromosomal changes infeasible.
246
247 Our *sacB*-mediated counterselection system overcomes these limitations. The method is simple
248 and does not require any additional equipment or experience beyond what is required for
249 traditional approaches.
250
251 We validated our system by generating two knockouts: one (*srtA*) involved allelic exchange with
252 a chloramphenicol resistance marker, while the other (*cylE*) demonstrated the ability of our
253 technique to produce small chromosomal changes at the single nucleotide level. In order to
254 confirm that the system works in multiple GBS strains from different serotypes (which can show
255 phenotypic variability under the same growth conditions), we generated the same *cylE*
256 mutation in A909 (serotype Ia) and 10/84 (serotype V). We noted that A909 grew less robustly
257 on 0.75 M sucrose than 10/84; so for the A909 *cylE* mutation, we used 0.5 M sucrose
258 counterselection. When using this system in different strains, it is important to optimize the
259 counterselection conditions prior to starting a new mutation.
260

As **Figure 5** shows, the *srtA* knockout can be generated at reasonable rates even without counterselection. There was considerable variability from one experimental replicate to the next, but the mean recovery rate of knockouts in the sucrose-negative conditions was 29-57%, regardless of whether the mutagenesis plasmid was pHY304 or pMBsacB. In contrast, the mean recovery rate in the pMBsacB sucrose-positive condition was 99%, with low variability.

In the case of low-fitness mutations, however, rates of recovery without counterselection can be much lower (32). The last step in the mutagenesis workflow—auto-excision and curing of the plasmid—essentially establishes a competition assay between the single-cross strain and the intended mutant (12). If the mutant has a survival defect, the odds of randomly selecting a mutant colony from among the single-cross population is very low. By shrinking the single-cross background, the pMBsacB counterselection system increases the odds of isolating the desired mutant. Particularly when the goal is mutation of high-fitness genes, we have found that it is important to confirm sucrose sensitivity of the transformant and single-cross intermediate, since spontaneous mutations in the *sacB* gene could lead to escape of unmodified high-fitness genes once sucrose counterselection is applied.

In the case of unmarked mutations, where no new antibiotic resistance is introduced into the chromosome, the bacterial population after counterselection is expected to be a mixture of the desired mutant and wild type bacteria that reverted following plasmid auto-excision. This occurred in our *cylE* knockout experiment, where roughly equal fractions of the post-

counterselection population showed knockout and wild type β-hemolysin/cytolysin pigmentation phenotypes.

This means that for mutations that do not cause an easily assayable phenotype (such as pigment expression), sequence-based confirmation will be necessary. There are several possible ways to perform this confirmation. Here, we used PCR followed by Sanger sequencing, but probe- or qPCR-based SNP assays are alternative strategies. Confirmation by some means is important, given that plasmid auto-excision can leave unintended deletions, as was the case in several colonies in our *cylE* experiment.

In summary, we have presented a new, straightforward counterselection-based approach to generating flexible mutations in GBS. Our future plans for this work involve modifications to coding and non-coding sequences, including promoter alterations, addition of fluorescent and affinity tags to natively expressed genes, and generation of multiple knockout strains.

Methods

Bacterial strains and growth conditions

GBS strains CNCTC 10/84 (serotype V, sequence type 26) and A909 (serotype Ia, sequence type 7) and *B. subtilis* strain 168 were maintained as frozen glycerol stocks and were grown on tryptic soy (TS; Fisher Scientific product number DF0370-17-3) agar plates or stationary in TS

304 broth at 37 °C or 28 °C. Chemically competent *Escherichia coli* DH5α were purchased from New
305 England Biolabs (product number C2987H) and were stored and transformed according to
306 manufacturer instructions. *E. coli* growth was at 37 °C unless transformed with a temperature-
307 sensitive plasmid, in which case the growth temperature was 28 °C. Antibiotic concentrations
308 were as follows: erythromycin 5 μg/mL (GBS) or 300 μg/mL (*E. coli*), chloramphenicol 1 μg/mL
309 (GBS) or 10 μg/mL (*E. coli*). 0.5 M and 0.75 M sucrose-containing broth and solid media were
310 prepared by diluting a filter-sterilized 2 M sucrose stock solution in appropriate media and
311 adding any necessary antibiotics to the final mixture.

312 *Cloning technique*

313

314 All shuttle vectors and derivatives used in this study were initially cloned into *E. coli* DH5α, from
315 which plasmid DNA for downstream applications was purified using the Qiagen QIAprep
316 Miniprep kit (product number 27104) according to manufacturer instructions.

317

318 *Isolation of genomic DNA*

319

320 Genomic DNA from GBS and *B. subtilis* was isolated using the Applied Biosystems MagMAX
321 CORE kit (product number A32700) with a KingFisher magnetic bead processing system
322 according to manufacturer instructions, with the following minor modifications. Overnight
323 liquid culture volumes were 1-10 mL. After pelleting by centrifugation at 3200 x g, the bacteria
324 were lysed in a solution containing 100 μL manufacturer-supplied proteinase K and PK buffer,
325 50 μL lysozyme (100 mg/mL in water), and 5 μL mutanolysin (10 kU in 2 mL 0.1 M potassium

326 phosphate buffer pH 6.2). Lysis was performed at 37 °C for 30 minutes, then 55 °C for 30
327 minutes, followed by a 2-minute centrifugation at 3200 x g. The rest of the extraction followed
328 manufacturer instructions, using the MagMAX CORE Flex KingFisher protocol file.
329
330 *Construction of pSacB23 and pMBsacB*
331
332 The *sacB* coding sequence was amplified from *B. subtilis* 168 genomic DNA using primers
333 sacB_pORI23_F and sacB_pORI23_R, which contain Gibson assembly overhang sequences
334 compatible with pORI23 digested with *BamHI*. Successful clones were identified by Sanger
335 sequencing (data not shown), after which the p23 promoter-*sacB* cassette was amplified with
336 p23sacB_cassette_F and p23sacB_cassette_R and subloned into pHY304 digested with *AvaII*
337 (see **Figure 1**). Outgrowth of the pMBsacB cloning reaction was performed at 28 °C and
338 successful clones were identified with Sanger sequencing (data not shown).
339
340 *Transformation of GBS with pSacB23 and pMBsacB*
341
342 Electrocompetent GBS suitable for transformation with *sacB*-containing plasmids were
343 prepared following methods outlined by Holo and Nes (25) and Framson et al. (24) modified to
344 prevent toxicity from sucrose osmoprotectant.
345
346 Single 10/84 or A909 colonies from TS agar plates were used to seed 5 mL M17 (BD Difco
347 218561) + 0.5% glucose liquid cultures, which were grown at 37 °C to stationary phase. 500 µL

348 from these cultures were then used to seed filter sterilized 50 mL M17 + 0.5% glucose, 2.5%
349 (A909) or 0.6% (10/84) glycine, and 25% (mass:mass) PEG-6000.
350
351 Following overnight growth at 37 °C, this culture was diluted in pre-warmed 130 mL of the
352 same media and allowed to grow for 1 hr. The entire volume was pelleted at 3200 x g, then
353 washed twice in ice cold 25% PEG-6000 + 10% glycerol. Following these washes, the samples
354 were resuspended in 1 mL of wash solution and either used immediately for transformation or
355 stored in aliquots at -80 °C.
356
357 Electroporation and transformation of competent GBS was performed as described by Holo and
358 Nes (25), except that sucrose in the outgrowth media was replaced with 25% PEG-6000, and—
359 in the case of pMBsacB—outgrowth was performed at 28 °C instead of 37 °C.
360
361 *Sucrose killing assays*
362
363 Wild type GBS or transformants were grown overnight in appropriate antibiotic selection
364 without supplemental sucrose. For killing assays on agar plates, overnight cultures were serially
365 diluted and plated directly on TS agar with appropriate antibiotic selection, with or without
366 supplemental sucrose. CFU quantification was performed after 1-2 days of growth. For killing
367 assays during planktonic growth, the overnight cultures were diluted 1:50 in broth without
368 sucrose, grown to log phase, then exposed to sucrose supplementation (or control outgrowth

with only sterile water added to the broth) for two hours, at which time the cultures were diluted and plated on appropriate solid media for CFU quantification after 1-2 days of growth.

Transmission electron microscopy

10/84 transformed with pMBsacB or pHY304 was grown to mid-log phase in selective broth. That culture was used to seed a new culture with or without supplemental sucrose. After outgrowth to early-mid log phase, the bacteria were fixed with 2.5% glutaraldehyde and 2% paraformaldehyde and washed with cacodylate buffer (50 mM, pH 7.2), then post fixed with 2% osmium tetroxide. Bacteria were embedded in 2% agar, then cut and stained in the dark with 0.5% (w/v) uranyl acetate. Samples were dehydrated with alcohol, transferred to propylene oxide/Epon mixtures and finally embedded in EMbed 812 (Electron Microscopy Sciences, Hatfield, PA). Thin sections were cut, adsorbed on electron microscope grids, and stained with uranyl acetate and lead citrate. Stained grids were then imaged in a Philips CM12 electron microscope (FEI; Eindhoven, The Netherlands) and photographed with a Gatan (4kx2.7k) digital camera (Gatan Inc., Pleasanton, CA).

Construction of pMBsacB:dSrtA and pMBsacB:dCylE

The *dSrtA* mutagenesis cassette consisted of the *cat* gene with 822-bp upstream and 795-bp downstream homology arms that matched the 10/84 chromosomal regions flanking the *srtA* gene. The three fragments were amplified from template DNA (10/84 genomic DNA or pDC123,

391 a shuttle vector containing *cat*). The primers used to generate the upstream, coding, and
392 downstream cassette regions of the mutagenesis cassette were dSrtA_US_F/R, cat_F/R, and
393 dSrtA_DS_F/R, respectively.
394
395 Next, overlap extension PCR was used to join the three regions (28). The upstream homology
396 arm was first joined to *cat*, after which the downstream homology arm was attached. The two-
397 fragment intermediate and the final cassette were gel extracted and used for TOPO cloning into
398 pCR2.1-TOPO, followed by Sanger sequence confirmation (data not shown). After successful
399 construction, the complete cassette was digested out of pCR2.1-TOPO with *NotI* and *KpnI*, then
400 ligated with T4 ligase overnight at 16 °C into pHY304 double-digested with the same restriction
401 enzymes. For insertion of the *dSrtA* cassette into pMBsacB, the construct was amplified out of
402 pHY304:*dSrtA* using primers dSrtA_GA_F and dSrtA_GA_R, then cloned into pMBsacB at the
403 *NotI* and *XhoI* sites using Gibson assembly.
404
405 The 1500-bp *dCylE* cassette was amplified out of GBS strain AR1598, a derivative of 10/84 in
406 which the *cylE* gene has a barcoded premature stop codon (**Figure 6B**), using primers
407 dCylE_GA_F and dCylE_GA_R. This amplicon was similarly cloned into the *NotI* and *XhoI* sites of
408 pMBsacB using Gibson assembly.
409
410 *Allelic exchange using sucrose counterselection against pMBsacB*
411

412 After transformation of GBS, using the method described above, with pMBsacB (or pHY304
413 control) bearing a mutagenesis cassette (d*SrtA* or *dCylE*), successful transformants were grown
414 in TS broth with appropriate antibiotic selection (erythromycin with or without
415 chloramphenicol) at 28 °C. Sucrose sensitivity of the transformants was confirmed by plating
416 serial dilutions on TS agar with 0.5 M (for A909) or 0.75 M (for 10/84) sucrose and appropriate
417 antibiotic selection at 28 °C.
418
419 To generate single-cross intermediates, transformants were serially passaged three times at 28
420 °C with erythromycin selection. The third passage was then used to seed another culture at 37
421 °C with erythromycin selection, which was grown overnight. Serial dilutions of the final culture
422 were plated on TS agar with erythromycin at 37 °C. Individual colonies were grown and tested
423 for sucrose sensitivity. Genomic DNA was also extracted and tested by PCR for proper vector
424 insertion using either cylE_farout_F or srtA_farout_F, which match chromosomal sites outside
425 of the homology arms, and pMBsacB_MCS_F, which binds pMBsacB and pHY304 upstream of
426 the cloning sites used in this study (data not shown).
427
428 Single-cross intermediate strains with the correct sucrose sensitivity phenotype and PCR-
429 confirmed genotype were then grown in TS broth without antibiotics at 28 °C and passaged
430 three times in order to enrich for spontaneous double-cross events. To counterselect against
431 pMBsacB, the third passage was used to seed TS broth with sucrose at 37 °C. In *srtA* knockout
432 experiments, chloramphenicol was added to the sucrose-containing broth. The sucrose culture
433 was passaged three times at 37 °C, and then serial dilutions were plated on TS agar with or

without chloramphenicol. Simultaneous plating on erythromycin-containing TS agar (with or without chloramphenicol) was used to quantify the effectiveness of counterselection against pMBsacB.

Knockout candidates from the non-erythromycin plate were confirmed to be erythromycin sensitive by patching to a new plate. Genomic DNA extraction followed by PCR and Sanger sequencing confirmed plasmid excision and the correct knockout DNA sequence. For the *srtA* knockout, the region was amplified using primers srtA_farout_F/R and these amplicons were sequenced using srtA_farout_F and cat_F primers. For the *cylE* knockout, the region was amplified using cylE_farout_F/R and the barcoded mutation was confirmed by sequencing with the dCylE_conf_F primer.

pHY304 knockout controls were subjected to the same steps, including sucrose exposure, unless otherwise noted. Non-sucrose exposure control conditions were identical except that the three final passages of the single-cross strain at 37 °C were performed in the absence of sucrose.

Acknowledgements

We thank NYU Langone Health DART Microscopy Laboratory for the consultation and assistance with transmission electron microscopy work.

Funding

This work was supported by NIH/NIAID K08AI132555 to TAH and R56 AI136499 to AJR.

Conflicts of interest

AJR has served as a consultant to Pfizer. The other authors have no financial or other conflicts of interest to disclose.

References

1. **Baker CJ**. 2013. The spectrum of perinatal group B streptococcal disease. Vaccine **31 Suppl 4**:D3–6.

2. **Stoll BJ**, **Hansen NI**, **Sánchez PJ**, **Faix RG**, **Poindexter BB**, **Van Meurs KP**, **Bizzarro MJ**, **Goldberg RN**, **Frantz ID**, **Hale EC**, **Shankaran S**, **Kennedy K**, **Carlo WA**, **Watterberg KL**, **Bell EF**, **Walsh MC**, **Schibler K**, **Laptook AR**, **Shane AL**, **Schrag SJ**, **Das A**, **Higgins RD**, **Eunice Kennedy Shriver National Institute of Child Health and Human Development Neonatal Research Network**. 2011. Early onset neonatal sepsis: the burden of group B Streptococcal and E. coli disease continues. Pediatrics **127**:817–826.

3. **Schrag SJ**, **Farley MM**, **Petit S**, **Reingold A**, **Weston EJ**, **Pondo T**, **Hudson Jain J**, **Lynfield R**. 2016. Epidemiology of Invasive Early-Onset Neonatal Sepsis, 2005 to 2014. Pediatrics **138**:e20162013–e20162013.

4. **Muñoz P**, **Llancaqueo A**, **Rodríguez-Créixems M**, **Peláez T**, **Martin L**, **Bouza E**. 1997. Group B streptococcus bacteremia in nonpregnant adults. Arch Intern Med **157**:213–216.

5. **Reingold A**, **Watt JP**. 2015. Group B streptococcus infections of soft tissue and bone in California adults, 1995-2012. Epidemiol Infect **143**:3343–3350.

6. **Kayansamruaj P**, **Pirarat N**, **Hirono I**, **Rodkhum C**. 2014. Increasing of temperature induces pathogenicity of Streptococcus agalactiae and the up-regulation of inflammatory

related genes in infected Nile tilapia (Oreochromis niloticus). Vet Microbiol **172**:265–271.

7. **Botelho ACN**, **Ferreira AFM**, **Fracalanzza SEL**, **Teixeira LM**, **Pinto TCA**. 2018. A Perspective on the Potential Zoonotic Role of Streptococcus agalactiae: Searching for a Missing Link in Alternative Transmission Routes. Front Microbiol **9**:608.

8. **Kalimuddin S**, **Chen SL**, **Lim CTK**, **Koh TH**, **Tan TY**, **Kam M**, **Wong CW**, **Mehershahi KS**, **Chau ML**, **Ng LC**, **Tang WY**, **Badaruddin H**, **Teo J**, **Apisarnthanarak A**, **Suwantarat N**, **Ip M**, **Holden MTG**, **Hsu LY**, **Barkham T**, **Singapore Group B Streptococcus Consortium**. 2017. 2015 Epidemic of Severe Streptococcus agalactiae Sequence Type 283 Infections in Singapore Associated With the Consumption of Raw Freshwater Fish: A Detailed Analysis of Clinical, Epidemiological, and Bacterial Sequencing Data. Clin Infect Dis **64**:S145–S152.

9. **Tan S**, **Lin Y**, **Foo K**, **Koh HF**, **Tow C**, **Zhang Y**, **Ang LW**, **Cui L**, **Badaruddin H**, **Ooi PL**, **Lin RTP**, **Cutter J**. 2016. Group B Streptococcus Serotype III Sequence Type 283 Bacteremia Associated with Consumption of Raw Fish, Singapore. Emerging Infect Dis **22**:1970–1973.

10. **Håvarstein LS**, **Hakenbeck R**, **Gaustad P**. 1997. Natural competence in the genus Streptococcus: evidence that streptococci can change pherotype by interspecies recombinational exchanges. J Bacteriol **179**:6589–6594.

11. **Brochet M**, **Rusniok C**, **Couve E**, **Dramsi S**, **Poyart C**, **Trieu-Cuot P**, **Kunst F**, **Glaser P**. 2008. Shaping a bacterial genome by large chromosomal replacements, the evolutionary history of Streptococcus agalactiae. Proc Natl Acad Sci USA **105**:15961–15966.

12. **Yim HH**, **Rubens CE**. 1998. Site-specific homologous recombination mutagenesis in group

B streptococci. Methods in Cell Science **20**:13–20.

13. **Sheen TR**, **Jimenez A**, **Wang N-Y**, **Banerjee A**, **van Sorge NM**, **Doran KS**. 2011. Serine-rich repeat proteins and pili promote Streptococcus agalactiae colonization of the vaginal tract. Journal of Bacteriology **193**:6834–6842.

14. **Quach D**, **van Sorge NM**, **Kristian SA**, **Bryan JD**, **Shelver DW**, **Doran KS**. 2009. The CiaR response regulator in group B Streptococcus promotes intracellular survival and resistance to innate immune defenses. Journal of Bacteriology **191**:2023–2032.

15. **Tamura GS**, **Bratt DS**, **Yim HH**, **Nittayajarn A**. 2005. Use of glnQ as a counterselectable marker for creation of allelic exchange mutations in group B streptococci. Appl Environ Microbiol **71**:587–590.

16. **Meng G**, **Fütterer K**. 2003. Structural framework of fructosyl transfer in Bacillus subtilis levansucrase. Nat Struct Biol **10**:935–941.

17. **Marvasi M**, **Visscher PT**, **Casillas Martinez L**. 2010. Exopolymeric substances (EPS) from Bacillus subtilis: polymers and genes encoding their synthesis. FEMS Microbiol Lett **313**:1–9.

18. **Porras-Domínguez JR**, **Ávila-Fernández Á**, **Miranda-Molina A**, **Rodriguez-Alegria ME**, **Munguia AL**. 2015. Bacillus subtilis 168 levansucrase (SacB) activity affects average levan molecular weight. Carbohydr Polym **132**:338–344.

19. **Ortiz-Soto ME**, **Rudiño-Piñera E**, **Rodriguez-Alegria ME**, **Munguia AL**. 2009. Evaluation of

524 cross-linked aggregates from purified Bacillus subtilis levansucrase mutants for
525 transfructosylation reactions. BMC Biotechnol **9**:68.

526 20. **Abdel-Fattah AF**, **Mahmoud DAR**, **Esawy MAT**. 2005. Production of levansucrase from
527 Bacillus subtilis NRC 33a and enzymic synthesis of levan and Fructo-Oligosaccharides.
528 Curr Microbiol **51**:402–407.

529 21. **Ortiz-Soto ME**, **Rivera M**, **Rudiño-Piñera E**, **Olvera C**, **López-Munguía A**. 2008. Selected
530 mutations in Bacillus subtilis levansucrase semi-conserved regions affecting its
531 biochemical properties. Protein Eng Des Sel **21**:589–595.

532 22. **Marx CJ**. 2008. Development of a broad-host-range sacB-based vector for unmarked
533 allelic exchange. BMC Res Notes **1**:1.

534 23. **Li Y**, **Thompson CM**, **Lipsitch M**. 2014. A modified Janus cassette (Sweet Janus) to
535 improve allelic replacement efficiency by high-stringency negative selection in
536 Streptococcus pneumoniae. PLoS ONE **9**:e100510.

537 24. **Framson PE**, **Nittayajarn A**, **Merry J**, **Youngman P**, **Rubens CE**. 1997. New genetic
538 techniques for group B streptococci: high-efficiency transformation, maintenance of
539 temperature-sensitive pWV01 plasmids, and mutagenesis with Tn917. Appl Environ
540 Microbiol **63**:3539–3547.

541 25. **Holo H**, **Nes IF**. 1989. High-Frequency Transformation, by Electroporation, of
542 Lactococcus-Lactis Subsp Cremoris Grown with Glycine in Osmotically Stabilized Media.
543 Appl Environ Microbiol **55**:3119–3123.

544 26. **Canedo M**, **Jimenez-Estrada M**, **Cassani J**, **López-munguía A**. 1999. Production of
545 Maltosylfructose (Erlose) with Levansucrase fromBacillus Subtilis. Biocatalysis and
546 Biotransformation **16**:475–485.

547 27. **Seibel J**, **Moraru R**, **Götze S**, **Buchholz K**, **Na'amnieh S**, **Pawlowski A**, **Hecht H-J**. 2006.
548 Synthesis of sucrose analogues and the mechanism of action of Bacillus subtilis
549 fructosyltransferase (levansucrase). Carbohydr Res **341**:2335–2349.

550 28. **Lalioui L**, **Pellegrini E**, **Dramsi S**, **Baptista M**, **Bourgeois N**, **Doucet-Populaire F**, **Rusniok C**,
551 **Zouine M**, **Glaser P**, **Kunst F**, **Poyart C**, **Trieu-Cuot P**. 2005. The SrtA Sortase of
552 Streptococcus agalactiae is required for cell wall anchoring of proteins containing the
553 LPXTG motif, for adhesion to epithelial cells, and for colonization of the mouse intestine.
554 Infection and Immunity **73**:3342–3350.

555 29. **Whidbey C**, **Harrell MI**, **Burnside K**, **Ngo L**, **Becraft AK**, **Iyer LM**, **Aravind L**, **Hitti J**,
556 **Waldorf KMA**, **Rajagopal L**. 2013. A hemolytic pigment of Group B Streptococcus allows
557 bacterial penetration of human placenta. J Exp Med **210**:1265–1281.

558 30. **Rosa-Fraile M**, **Dramsi S**, **Spellerberg B**. 2014. Group B streptococcal haemolysin and
559 pigment, a tale of twins. FEMS Microbiol Rev **38**:932–946.

560 31. **Randis TM**, **Gelber SE**, **Hooven TA**, **Abellar RG**, **Akabas LH**, **Lewis EL**, **Walker LB**, **Byland**
561 **LM**, **Nizet V**, **Ratner AJ**. 2014. Group B Streptococcus β-hemolysin/cytolysin breaches
562 maternal-fetal barriers to cause preterm birth and intrauterine fetal demise in vivo. J
563 Infect Dis **210**:265–273.

564 32. **Link AJ**, **Jeong KJ**, **Georgiou G**. 2007. Beyond toothpicks: new methods for isolating
565 mutant bacteria. Nat Rev Micro **5**:680–688.

566 33. **Zeigler DR**, **Prágai Z**, **Rodriguez S**, **Chevreux B**, **Muffler A**, **Albert T**, **Bai R**, **Wyss M**,
567 **Perkins JB**. 2008. The origins of 168, W23, and other Bacillus subtilis legacy strains.
568 Journal of Bacteriology **190**:6983–6995.

569 34. **Hooven TA**, **Randis TM**, **Daugherty SC**, **Narechania A**, **Planet PJ**, **Tettelin H**, **Ratner AJ**.
570 2014. Complete Genome Sequence of Streptococcus agalactiae CNCTC 10/84, a
571 Hypervirulent Sequence Type 26 Strain. Genome Announcements **2**:e01338–14–e01338–
572 14.

573 35. **Chaffin DO**, **Rubens CE**. 1998. Blue/white screening of recombinant plasmids in Gram-
574 positive bacteria by interruption of alkaline phosphatase gene (phoZ) expression. Gene
575 **219**:91–99.

576 36. **Que YA**, **Haefliger JA**, **Francioli P**, **Moreillon P**. 2000. Expression of Staphylococcus
577 aureus clumping factor A in Lactococcus lactis subsp cremoris using a new shuttle vector.
578 Infection and Immunity **68**:3516–3522.

579

Table 1: Strains, plasmids, and primers used in this study

Strains		
Strain	Description	Reference
E. coli DH5α	Chemically competent cloning strain	New England Biolabs item # C2987H
B. subtilis 168	Legacy strain derived from *B. subtilis* Marburg	(33)
GBS CNCTC 10/84	Serotype V, sequence type 26	(34)
GBS A909	Serotype Ia, sequence type 7	
GBS AR1598	Serotype V, sequence type 26 10/84 mutant with barcoded premature stop codon in *cylE* gene	This study
Plasmids		
Name	Description	Reference
pDC123	Shuttle vector with the chloramphenicol acetyltransferase gene used as PCR template	(35)
pHY304	Broad host range, temperature sensitive mutagenesis plasmid; pWV01 derivative; erythromycin-resistant	(12)
pMBsacB	pHY304 with p23-*sacB* cassette cloned into *AvaII* site, for sucrose-counterselectable GBS mutagenesis	This study
pOri23	Shuttle vector with *ori*ColE1 and *Lactococcus*-derived Gram-positive origin of replication; erythromycin-resistant; p23 promoter adjacent to *BamHI* restriction site	(36)
pSacB23	pORI23 with *sacB* cloned into *BamHI* site for expression off p23 promoter	This study
Primers		
Name	Sequence (5' to 3')	Description
cat_F	ATGGAGAAAAAAATCACTGGATATACCACC	Amplifies *cat* gene for allelic exchange knockout screening and for overlap extension PCR to make *dSrtA* mutagenesis cassette (from pDC123 template)
cat_R	CCCGCCCTGCCACTCATCGC	
cylE_farout_F	TACACGCGAGATCGGTTAGC	Binds GBS chromosome outside of the *dCylE* mutagenesis cassette region, for confirming single- and double-crossover events at the *cylE* locus
cylE_farout_R	CTGGTGTTCCTGAAGCGAGT	
dCylE_GA_F	ATTGGGTACCGGGCCCCCCCAGATGCTATA AAAGCAGC	Amplifies *dCylE* mutagenesis cassette (from AR1958 template) for Gibson assembly into pMBsacB *NotI*/*XhoI* digest
dCylE_GA_R	TGGAGCTCCACCGCGGTGGCCCTGTTTACTT GTTCCGATAAAAAG	
dCylE_Conf_F	CCAACGAAGCCACTGTCTCT	Sequencing primer to confirm barcoded stop codon in the *cylE* gene

dSrtA_DS_F	GCGATGAGTGGCAGGGCGGGGCGTAAAAGG TAGTTAGAATTATGAAATTAAAGGCTGTTC	Amplifies downstream homology arm for *dSrtA* mutagenesis cassette; overlap extension PCR compatible
dSrtA_DS_R	GCGCGCGCCTCGAGGCGTTTTAACTTTCGTGT CTCTAGATTCATCATAATTCAGAAGT	
dSrtA_GA_F	GCTTCCAAGGAGCTAAAGAGGAATTCGAAAA GCCCTGAC	Amplifies *dSrtA* mutagenesis cassette for Gibson assembly into pMBsacB *NotI*/*XhoI* digest
dSrtA_GA_R	ATTCACTACTTTTAGTTAAGTTATTTGTTAA CTGTTAATTGTCC	
dSrtA_US_F	GCGCGCGCGGATCCAATTTAGGGCGTGTGT ATCGTTTGAAAGCATAT	Amplifies upstream homology arm for *dSrtA* mutagenesis cassette; overlap extension PCR compatible
dSrtA_US_R	GGTGGTATATCCAGTGATTTTTTTCTCCATGA AACCATGTGATTTTTTTTTATT	
p23sacB_cassette_F	GCTTCCAAGGAGCTAAAGAGGTCCTTAACT AAAAGTAGTGAATTTTTGATTTTTG	Amplifies p23 promoter-*sacB* gene from pSacB23 for Gibson assembly into pHY304 *AvaII* digest
p23sacB_cassette_R	AAATTCCCCGTAGGCGCTAGGCATGCGTTA TTTGTTAACTGTTAATTGTCC	
pMBsacB_MCS_F	CAATACGCAAACCGCCTCTC	Binds pMBsacB, for use with "farout" primers for confirmation of single-cross insertion
sacB_pORI23_F	TATGAATGACAATGATGTTGATGAACATC AAAAAGTTTGC	Amplifies *sacB* from *B. subtilis* 168 for Gibson assembly into pORI23 *BamHI* digest
sacB_pORI23_R	AGCTTGGCTGCAGGTCGACGTTATTTGTTA ACTGTTAATTGTCC	
srtA_farout_F	AGAGCACAAAAACGTGGAGG	Binds GBS chromosome outside of the *dSrtA* mutagenesis cassette region, for confirming single- and double-crossover events at the *srtA* locus
srtA_farout_R	ACTGCTAAGGTATCGTTAGACCC	

Figure Captions

Figure 1. Schematic of pMBsacB-mediated GBS mutagenesis: Following insertion of a mutagenesis cassette into pMBsacB and transformation of GBS at 28 °C (1), the transformed strain is used to seed a 37 °C culture, selecting for single-crossover events at one of the homology arms (2). After removal of antibiotic selection and growth at 28 °C to promote a second crossover event and plasmid expulsion (3), sucrose is added as counterselection to isolate the desired allelic exchange clones (4). In the absence of sucrose counterselection, step 4 resembles traditional mutagenesis techniques (red box), in which identification of the allelic exchange depends on chance discovery of a clone with spontaneous loss of plasmid-based antibiotic resistance.

Figure 2. Development of pMBsacB: The *sacB* coding sequence was amplified from *B. subtilis* 168 and inserted at the *BamHI* site in pORI23, placing the gene adjacent to the p23 promoter in pSacB23 (1). The promoter-gene cassette was then amplified from pSacB23 (2) and cloned into the AvaII site of the broad host range, temperature-sensitive plasmid pHY304 (3), generating pMBsacB.

Figure 3. *sacB* confers lethal sucrose sensitivity in GBS: Stationary phase WT GBS 10/84 or 10/84 transformed with plasmids used in this study was serially 10-fold diluted and plated on TS agar plates with appropriate antibiotic selection with or without supplemental 0.75 M sucrose (A). The first dilution shown is 10^{-1}. pMBsacB-based sucrose sensitivity functions in

liquid culture and on solid media, whereas pHY304 does not affect GBS survival in sucrose in either growth condition (B; *** $p < 0.0001$, ANOVA).

Figure 4. Transmission electron microscopy reveals that SacB expression under sucrose counterselection results in destructive intracellular inclusions: Early-log phase GBS strain 10/84 transformed with pMBsacB and exposed to 0.75 M sucrose for 3 hours shows intracellular inclusion bodies that lead to degraded architecture and eventual lysis (A-C). GBS transformed with the control plasmid pHY304 shows normal architecture under baseline growth conditions (D) and only expected osmotic effects when grown with supplemental sucrose (E). SacB expression in the absence of sucrose counterselection has no apparent effect on GBS morphology (F).

Figure 5. Allelic exchange mutagenesis of the *srtA* gene is made more efficient by using pMBsacB: Sucrose exposure of single-cross pMBsacB:*dsrtA* knockout intermediates results in selection against erythromycin-resistant clones, which is not seen with the pHY304:*dsrtA* single-cross strain (A). Recovery of phenotypically correct knockout clones is significantly more efficient using pMBsacB than when mutagenesis is performed with pHY304 (B; * $p < 0.05$, T test). PCR of *cat* results in successful amplification of genomic DNA from 11 knockout (KO) candidates, but not from wild type (WT) or template-negative (Neg) controls (C). Sanger sequences of the *srtA* region amplified using primers outside of the mutagenesis cassette were combined to generate a consensus sequence, which was aligned to the expected knockout template. Erm=erythromycin, Cm=chloramphenicol, suc=sucrose.

Figure 6. pMBsacB permits efficient generation of unmarked mutations in the *cylE* gene: After generation of a *cylE* mutagenesis cassette bearing a premature stop codon barcoded by additional silent SNPs, the cassette was cloned into pMBsacB and used to knockout β-hemolysin/cytolysin expression in GBS 10/84 and A909 (A). Sucrose counterselection resulted in near-complete elimination of erythromycin-resistant CFUs (B). Clones that survived counterselection were screened for the barcoded mutation by performing PCR with cylE_farout_F/R primers and Sanger sequencing of the amplified genomic region of interest with dCylE_Conf_F (C). Representative traces are shown for the bands marked by asterisks. The recovered knockout strains had the expected non-hemolytic phenotype when plated on 5% sheep's blood agar (D). Erm=erythromycin.

Figure 1

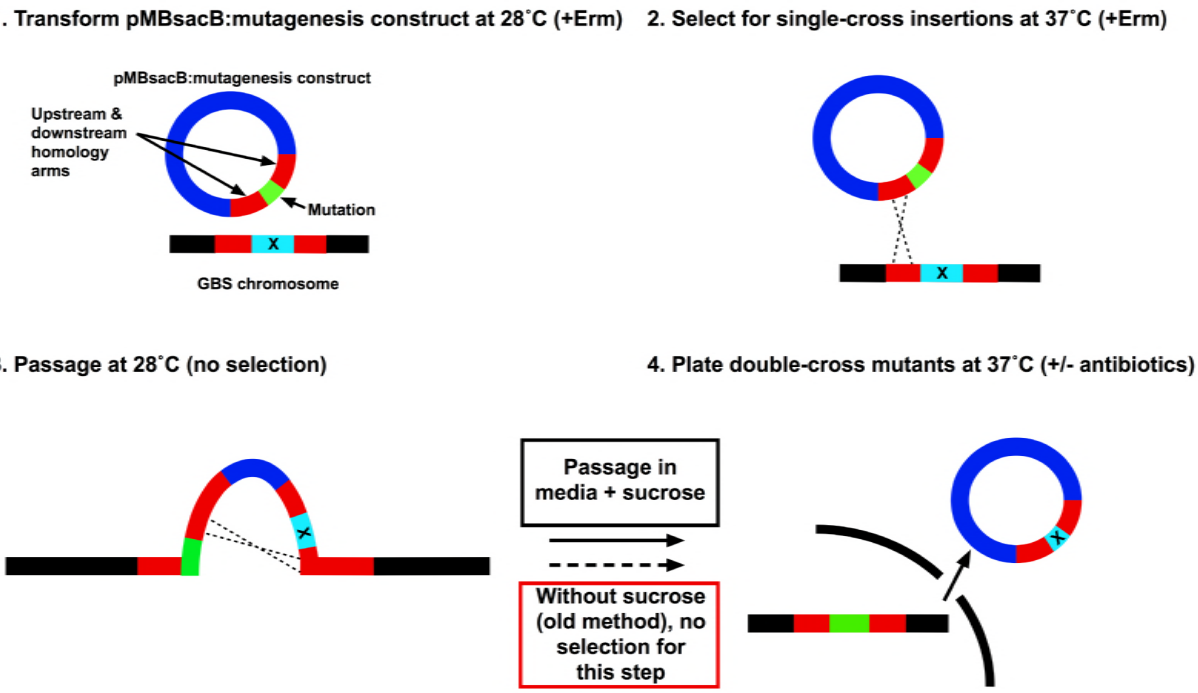

Figure 2

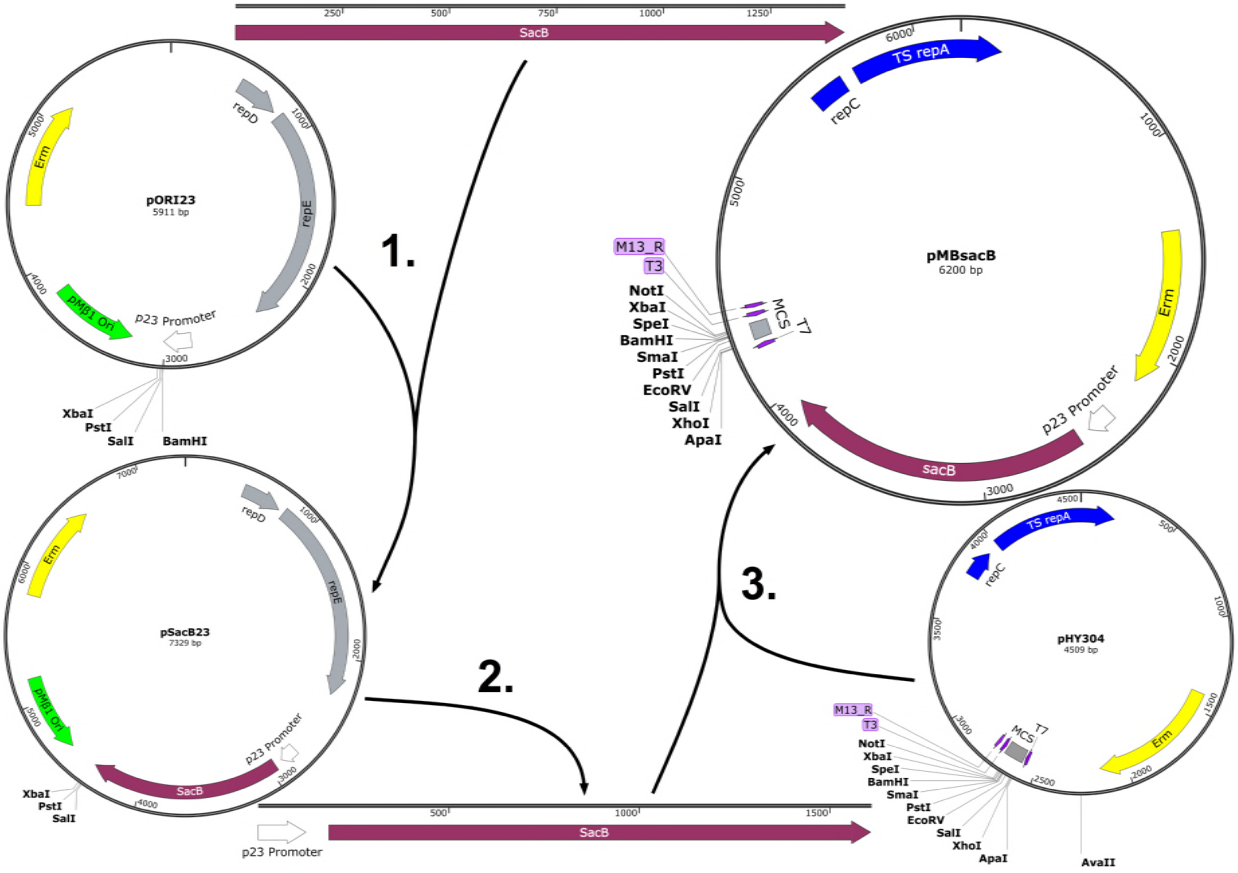

Figure 3

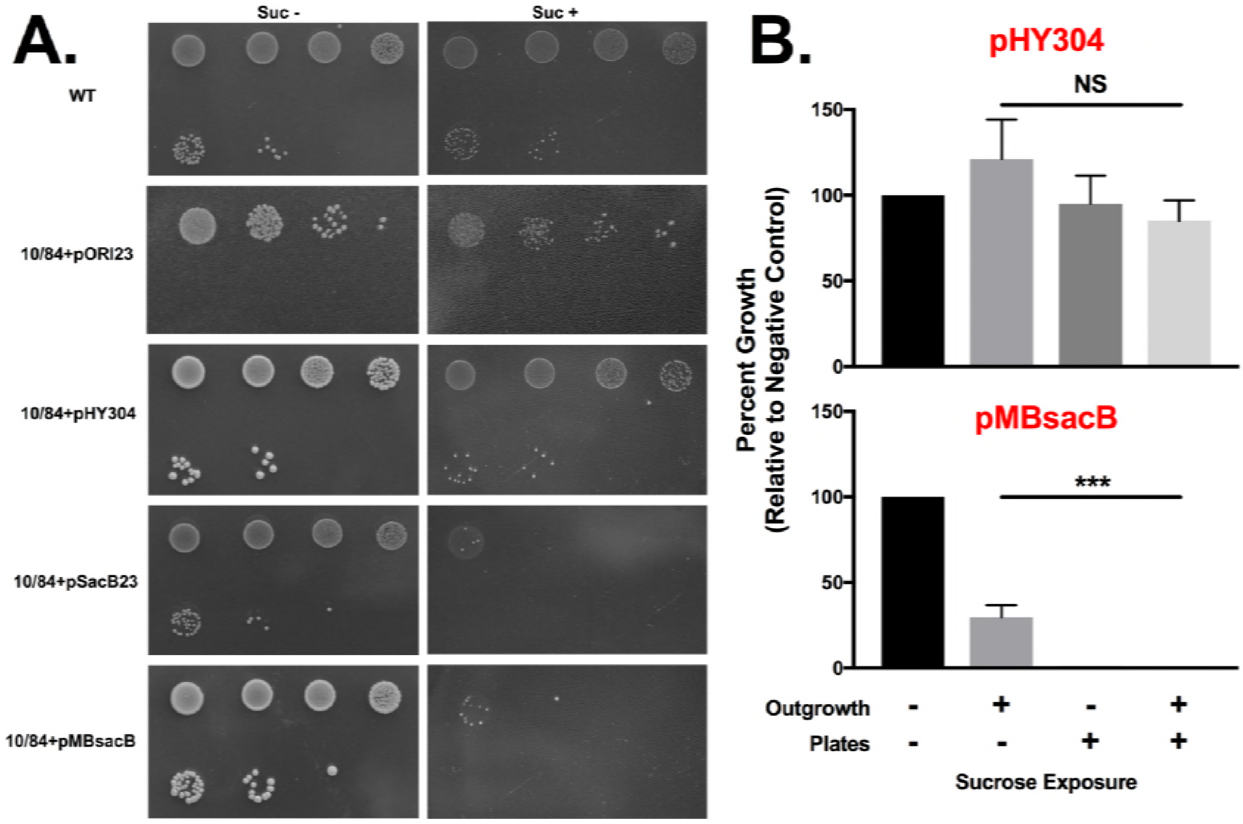

Figure 4

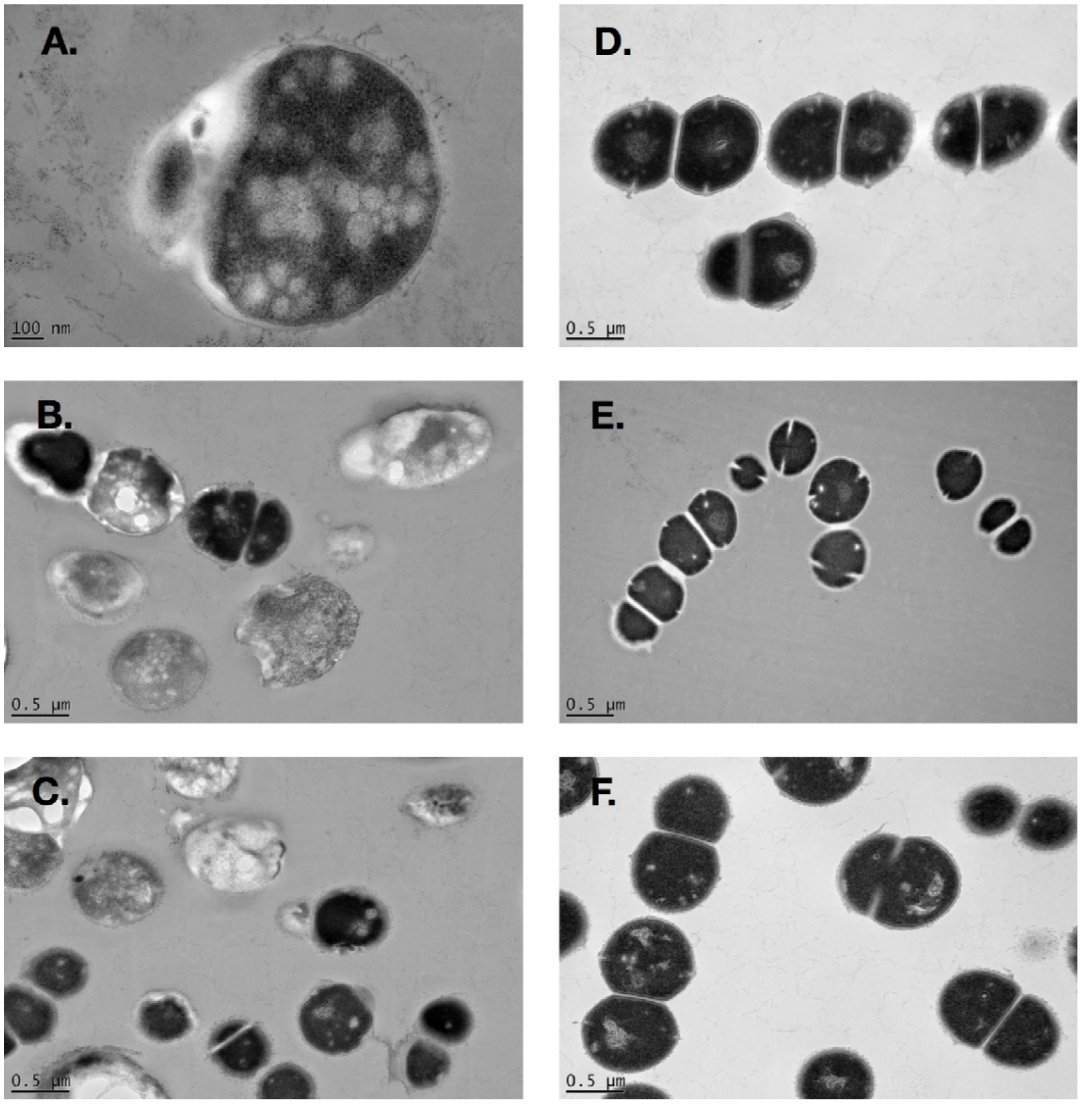

Figure 5

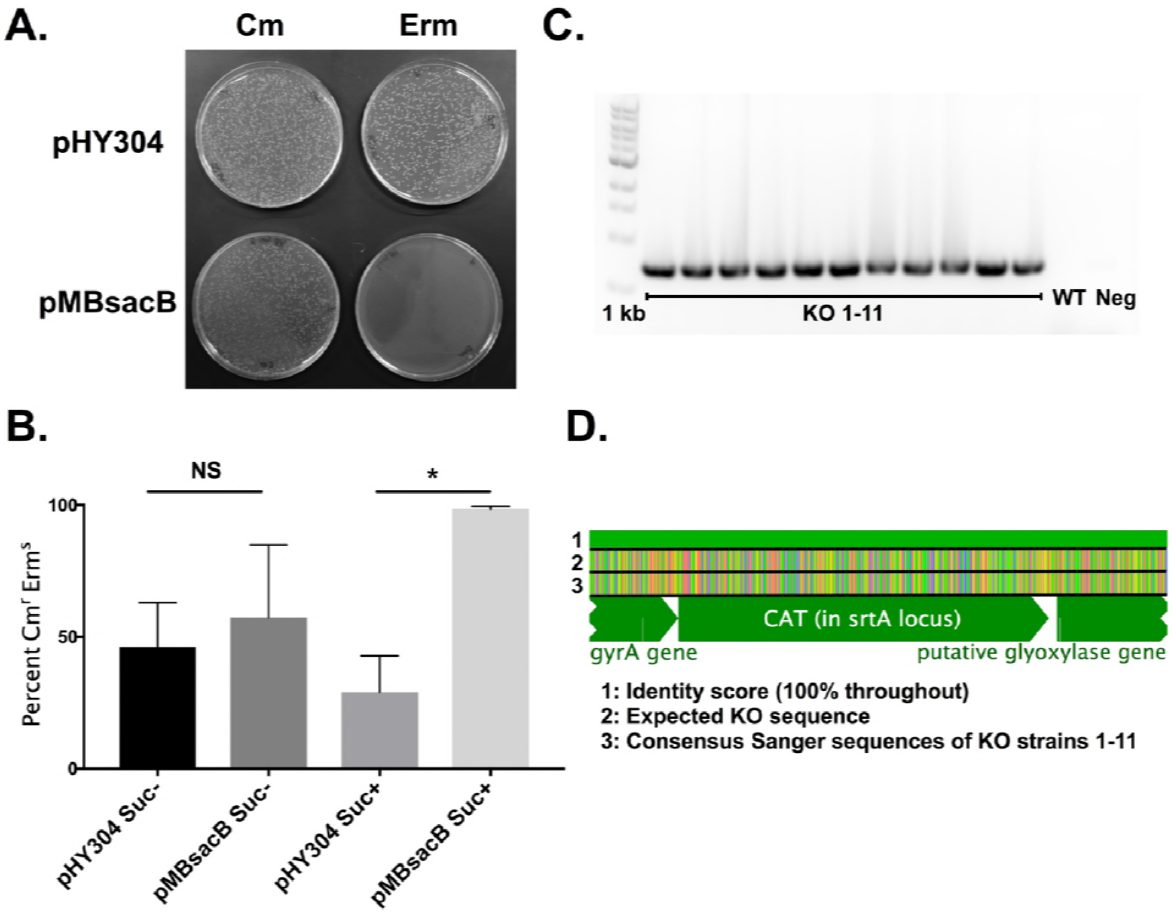

Figure 6