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Genetic Transformation and Complementation

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Abstract

The disciplines of *Borrelia (Borrelia) burgdorferi* microbiology and Lyme disease pathogenesis have come to depend on the genetic manipulation of the spirochete. Generating mutants in these recalcitrant bacteria, while not straightforward, is routinely accomplished in numerous laboratories, although there are several crucial caveats to consider. This chapter describes the design of basic molecular genetic experiments as well as the detailed methodologies to prepare and transform competent cells, select for and isolate transformants, and complement or genetically restore mutants.

Keywords

Borrelia burgdorferi; Lyme disease; Spirochete; Molecular genetics; Electroporation; Transformation; Mutagenesis; Complementation; Shuttle vector; Antibiotic resistance

1 Introduction

The first genetic transformation of the spirochete *Borrelia (Borrelia) burgdorferi* was published in 1994 [1] and a detailed protocol was described in this *Methods in Molecular Biology* series a year later [2]. We came a long way in the subsequent two decades [3–8]. Landmark advances include the genome sequence [9, 10], improved selectable markers [11–13], shuttle vectors [14–16], transposon mutagenesis [17–19] (*see* Chapter 16), inducible promoter systems [20–22] (*see* Chapter 17), gene reporters [14, 20, 23–25] (*see* Chapter 18), and a counter-selectable marker [26]. However, the genetic manipulation of the serpentine bacterium remains rather labyrinthine due to its extraordinarily complex genome composed of a ~950-kb linear chromosome and about 20–25 linear and circular plasmids encoding several paralogs [9, 10] as well as its fastidiousness and the lack of a defined culture medium [27, 28].

The electrotransformation protocol for *B. burgdorferi* [1, 2] is not unlike that of other bacteria [29]: A high-voltage electric pulse produces pores in the membrane and DNA enters the cell [30–32]. This chapter presents an updated, detailed protocol for transformation of *B. burgdorferi* by electroporation in addition to the approach for designing allelic exchange and complementation experiments. The most important parameter for successful electrotransformation is probably the growth phase or culture cell density during competent cell preparation [1, 33]. The other key consideration is maintaining and tracking the profusion of plasmids, many of which can be lost during in vitro passage and genetic manipulation, particularly complementation [34–37]. The methodologies detailed in this chapter have laid a foundation upon which borreliologists have continued to forge a

formidable genetic understanding of the microbial physiology of the spirochete and the molecular pathogenesis of Lyme disease.

2 Materials

1. A few strains of *B. burgdorferi* are available from ATCC (<http://www.atcc.org>), including B31 and 297, although they may not be low-passage, infectious isolates (see Note 1). Several low-passage, infectious isolates have been genetically manipulated, including strain B31 [35, 37, 38], strain 297 [14, 20, 39–41], strain N40 [42, 43], and *Borrelia afzelii* (a closely related genospecies) PKo [44] (see Note 2).
2. Barbour-Stoenner-Kelly (BSK) II medium (without gelatin): 8% (v/v) 10× CMRL-1066 (without L-glutamine and sodium bicarbonate), 4 g/L Neopeptone, 40 g/L bovine serum albumin (BSA; fraction V), 1.6 g/L Yeastolate (TC), 4.8 g/L *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 4 g/L glucose, 0.56 g/L sodium citrate, 0.64 g/L sodium pyruvate, 0.32 g/L *N*-acetyl-D-glucosamine, 1.76 g/L sodium bicarbonate, and 6.6% rabbit serum (non-hemolyzed). Adjust to pH 7.6 with 10 N NaOH, stir slowly for 2–3 h, and sterilize by filtration (0.22-μm filter). Store at –20 °C (see Note 3).
3. Dulbecco's phosphate-buffered saline (dPBS): 8 g/L NaCl, 0.2 g/L KCl, 1.15 g/L Na₂HPO₄, and 0.2 g/L KH₂PO₄. Sterilize by filtration and store at 4 °C.
4. Electroporation solution (EPS): 93 g/L sucrose and 15% (v/v) glycerol. Sterilize by filtration and store at 4 °C.
5. Plating-BSK (P-BSK) medium: Add 83 g BSA, 8.3 g Neopeptone, 10 g HEPES, 1.2 g sodium citrate, 8.3 g glucose, 1.3 g sodium pyruvate, 0.7 g *N*-acetyl-D-glucosamine, 3.7 g sodium bicarbonate, and 4.2 g Yeastolate to 1 L of water (18 MΩ cm). Adjust to pH 7.5 with 1 N NaOH, stir slowly for 2–3 h, and sterilize by filtration (0.22-μm filter). Store at –20 °C.
6. 1.7% agarose: We use LE (low electro-endosmosis) agarose.
7. Antibiotic stock solutions (for selection of transformants): 50 mg/mL streptomycin, 40 mg/mL gentamicin, and 50 mg/mL kanamycin (all dissolved in water and filter-sterilized) or 10 mg/mL erythromycin (dissolved in 95% ethanol) (see Notes 4–6).
8. 5% sodium bicarbonate: prepared fresh and filter-sterilized.

3 Methods

3.1 Designing and Generating Transformation Substrates for Gene Disruption by Allelic Exchange (See Note 7)

1. Screen all relevant DNA sequences for appropriate restriction enzyme sites, including the targeted gene, flanking regions, selectable marker, and cloning vector (see Note 8).

2. Design four primers to amplify two regions flanking the sequence to mutate with convenient restriction sites on the ends (*see* Note 8). The 5' end of the upstream piece will not have any synthetic restriction sites. The 3' end of the upstream piece will have two adjacent restriction sites (we usually use an AatII site on the inside and an AgeI site on the outside) with a suitable spacer sequence between them (three to five nucleotides), incorporated into the primer. The 5' end of the downstream piece will have the inside restriction site of the upstream piece (AatII) and the 3' end will have the outside restriction site of the upstream piece (AgeI), incorporated into the two primers (Fig. 1A).
3. The two pieces are amplified using a high-fidelity DNA polymerase (we use KOD polymerase from Novagen) and cloned (we TA-clone in pCR2.1-TOPO or, for larger pieces, TOPO-XL from Invitrogen). All cloned PCR products are confirmed by sequencing and comparison to the published genome sequence.
4. The downstream piece is restriction digested out of the vector with the two restriction sites (AatII and AgeI), gel-purified (*see* Note 9), and ligated into the vector with the upstream piece, which has also been digested with these two restriction enzymes and gel-purified. This creates a fusion in which the sequence to be mutated has been replaced with a restriction site (AatII) (Fig. 1A).
5. One (or more) of the selectable markers (*see* Note 7), which itself is flanked on both sides with this engineered restriction site (AatII), is cloned into this new, unique site. The result is that the region to be mutated has been swapped with the antibiotic resistance cassette (Fig. 1A).
6. Linearize the DNA by restriction digestion (usually with AhdI, which destroys the ampicillin resistance in pCR2.1 for biosafety reasons) and check by agarose gel electrophoresis to ensure the plasmid is completely linearized (*see* Note 10).
7. Purify and concentrate the linearized transformation substrate by ethanol precipitation.

3.2 Preparation of Competent Cells

1. Inoculate 500 mL of BSK II medium in a 500-mL screw-top bottle with 5 mL of a late-log-phase culture (*see* Note 11). Incubate at 32–34 °C (without agitation) until the culture reaches a density of about 5×10^7 cells/mL (*see* Notes 12 and 13). This requires 36–96 h.
2. Transfer culture to four sterile 250-mL screw-top centrifuge bottles and cap.
3. Centrifuge at $4000 \times g$ for 20 min at 4 °C. Decant the supernatant fraction and resuspend each cell pellet in 15 mL of cold, sterile dPBS (*see* Note 14).
4. Transfer cells to two sterile 50-mL screw-top centrifuge tubes and cap.
5. Centrifuge at $3000 \times g$ for 10 min at 4 °C. Decant the supernatant fraction, and resuspend each cell pellet in 30 mL of cold dPBS.
6. Centrifuge at $3000 \times g$ for 10 min at 4 °C. Decant the supernatant fraction, and resuspend each cell pellet in 10 mL of cold EPS.

7. Transfer cells to two sterile 14-mL polypropylene tubes and cap.
8. Centrifuge at $2000 \times g$ for 10 min at 4 °C. Decant the supernatant fraction, and resuspend each cell pellet in 10 mL of cold EPS. Repeat.
9. Centrifuge at $2000 \times g$ for 10 min at 4 °C. Decant the supernatant fraction, and pool the cell pellets in 0.6 mL of cold EPS (*see* Note 15).
10. Distribute 50- μ L aliquot fractions of the cell suspension into sterile 1.7-mL tubes pre-chilled on ice (*see* Notes 16 and 17).

3.3 Electrotransformation

1. Cool electroporation cuvettes (0.2-cm electrode gap) to 4 °C (*see* Note 18).
2. Transfer 1–10 μ L of a solution containing 5 μ g of DNA in water (*see* Notes 19 and 20) to the cell suspension, mix gently, and incubate on ice for about 5 min.
3. Transfer the cell/DNA mixture to a chilled electroporation cuvette. Cap the cuvette and gently tap the cell/DNA mixture to the bottom of the cuvette or otherwise ensure that the solution spans the two electrodes, being careful not to introduce bubbles.
4. Place the cuvette in the pulse generator and deliver a single exponential decay pulse of 2.5 kV, 25 μ F, and 200 Ω . This should produce a time constant of 4–5 ms (*see* Note 20).
5. Immediately (within 1 min) add 1 mL of BSK II medium (pre-warmed to 34 °C) without antibiotics to gently resuspend the cells.
6. Transfer the entire mixture to a sterile 14-mL tube that contains an additional 9 mL of BSK II medium (at 34 °C) and incubate (without agitation) at 32–34 °C for 20 h (*see* Note 21).

3.4 Selection of Transformants by Limiting Dilution (See Note 22)

1. Recovered transformants are diluted into 90 mL of liquid BSK II medium with antibiotics (*see* Note 4) and 250 μ L of culture is distributed with an 8-channel repeat pipettor into each well of four 96-well plates (*see* Note 22). Antibiotic final concentrations are 50 μ g/mL streptomycin (*see* Note 5), 40 μ g/mL gentamicin (*see* Note 6), 400 μ g/mL kanamycin, or 0.06 μ g/mL erythromycin.
2. The plates are incubated at 32–34 °C in humidified atmosphere containing 1.5% to 5% CO₂ and positive wells containing putative mutants are detected by the change in color of the medium from red to yellow (*see* Note 23).
3. Isolate clones by transferring the putative mutants in the well to 10 mL of BSK II in the presence of antibiotics. Cultures will reach late-log phase in 3–5 days of growth at ~35 °C.
4. Screen clones for desired mutation and plasmid profile by PCR (*see* Note 24).

3.5 Selection of Transformants by Plating (See Note 22)

1. Mix 240 mL of P-BSK medium, 38 mL of 10× CMRL-1066, and 12 mL of rabbit serum. Equilibrate the mixture at 42 °C in a water bath. Autoclave 200 mL of 1.7% agarose, equilibrate to 42 °C, and combine with the medium mixture. Add 20 mL of fresh 5% sodium bicarbonate with antibiotics (the final volume is 510 mL, which is sufficient to pour 12–14 plates) (*see Note 4*). Antibiotic final concentrations are 50 µg/mL streptomycin (*see Note 5*), 40 µg/mL gentamicin (*see Note 6*), 400 µg/mL kanamycin, or 0.06 µg/mL erythromycin.
2. Transfer 0.1 mL of BSK II medium containing the electroporated cells to a 50-mL tube. Add 35 mL of the molten medium (at 42 °C), and mix by pipetting up and down once. Transfer the mixture to the 100-mm culture dishes and allow to solidify at room temperature.
3. Centrifuge the remaining 9.9 mL of culture at $8000 \times g$ for 5 min, resuspend in 1 mL of supernatant fraction, and plate as above.
4. Incubate the plates at 32–34 °C in a humidified 5% CO₂ atmosphere. Colonies will appear in about 7–14 days.
5. Isolate single colonies by picking with a plugged 15-cm sterile Pasteur pipette (with bulb). Transfer to 10 mL of BSK II in the presence of antibiotics. Cultures will reach late-log phase in 6–9 days of growth at ~35 °C.
6. Screen clones for desired mutation and plasmid profile by PCR (*see Note 24*).

3.6 Trans-Complementation Using a Shuttle Vector (See Note 25)

1. Screen all relevant DNA sequences for appropriate restriction enzyme sites, including the gene to be complemented, the relevant flanking regions to be cloned, and the shuttle vector (*see Note 8*).
2. Choose a shuttle vector containing a selectable marker different from what was used to generate the mutant. We typically use pKFSS1 [13], which carries spectinomycin/streptomycin resistance, and pBSV2 [16], which carries kanamycin resistance. Other choices include pBSV2G [12], which carries gentamicin resistance, pCE323, which carries kanamycin resistance [45], and pGK12 [15], which confers erythromycin resistance.
3. Design two primers to amplify the complementing gene and incorporate an appropriate restriction site at both ends of the product (again, we typically use AatII). The amplification product should include the promoter if the gene is a singleton or the first gene in an operon (*see Note 26*). Fuse a promoter to the ORF if one is not directly upstream (*see Note 27*).
4. Clone the gene or gene fusion into the shuttle vector and electrotransform (*see Note 19*).

3.7 *Cis*-Complementation by Genetic Reconstitution (See Note 25)

1. Screen all relevant DNA sequences for appropriate restriction enzyme sites, including the gene to be complemented, the flanking regions, the selectable marker, and the cloning vector (*see* Note 8).
2. Design four primers to amplify two regions, one of which will include the gene to be complemented with convenient restriction sites on the ends (we use AatII and AgeI, as described above for gene disruption) (*see* Notes 8 and 28). The strategy is similar to the mutagenesis described above, except the synthetic restriction site should be located between two genes outside of the operon. The two pieces are amplified and cloned as described above (Fig. 2).
3. The downstream piece is restriction digested out of the vector with the two restriction sites (AatII and AgeI), gel-purified, and ligated into the vector with the upstream piece, which has also been digested with these two restriction enzymes (AatII and AgeI) and gel-purified. This creates a new restriction site (AatII).
4. One (or more) of the selectable markers (*see* Note 7), which itself is flanked on both sides with this new restriction site (AatII), is cloned into this new, unique site. The result is that a marker has been inserted either upstream or downstream from the wild-type sequence.
5. Linearize the DNA by restriction digestion (usually with AhdI, which destroys the ampicillin resistance in pCR2.1 for biosafety reasons) (*see* Note 10).
6. Purify and concentrate the transformation substrate by ethanol precipitation.
7. Electrotransform into the mutant.

4 Notes

1. B31 is the type strain [46] and its genome was the first to be completely sequenced [9, 10]; however, genomes of many other *B. burgdorferi* strains and related genospecies (as well as other *Borrelia* species) are available [47]. A complete genome, including all plasmids, is useful, particularly for transcriptome studies of mutants (*see* Chapter 12). Note that there is conserved synteny among chromosomes of different strains, but the plasmids have considerable variability in gene composition between strains [48]. We have used strains B31-A3 [38] and 297 [40], but now rely on strain B31-5A4 [37].
2. Low-passage, infectious strains are more difficult to transform than high-passage, attenuated strains. The transformation barrier is likely to be restriction-modification systems [34, 36, 49, 50]. Two linear plasmids, lp25 and lp56 (in strain B31), carry genes that are predicted to encode type IV restriction-modification enzymes and are correlated with low transformation efficiency; transformants often lose lp25, which carries *bbe02*, encoding one of these putative enzymes [36]. Transformation efficiency increases about fortyfold in *bbe02* mutants [34], although our laboratory has not observed this level of

enhancement [21]. Alas, lp25 also carries the essential gene *pncA* [51] that is required for infectivity [35, 37]. Transformation efficiency can be increased by either first transforming the DNA into a strain with only one of the putative restriction-modification systems [50, 52] or modifying shuttle vectors in vitro with the methylase M. SssI [49]. However, the principal procedure for overcoming the transformation barrier is the use of large amounts (10 µg or more, up to ~50 µg) of DNA [14, 16, 33, 38, 40].

3. The quality of BSA varies by source and lot. We have found Gemini Bio-Products to be a reliable source. We reserve 5- or 10-kg lots and test samples for the ability to support growth, transformation, and temperature-induced *ospC* gene induction. Pretested BSK II medium without gelatin can be purchased from Sigma (BSK-H), but it is expensive (and homemade is always best).
4. The original antibiotic for selection of resistant mutants was coumermycin A₁ [53, 54]; however, the coumermycin A₁-resistant *gyrB* marker has not been routinely used in many years because it requires extensive screening of transformants, due to homologous recombination into the chromosomal *gyrB* locus [55, 56], and it causes pleiotropic effects, due to anomalous DNA supercoiling [57, 58]. The *ermC* gene confers erythromycin resistance in *B. burgdorferi* [15], but it is not widely used. There are both biosafety and physiological limitations on the use of many other antibiotics. Some antibiotic resistance genes do not function in *B. burgdorferi*: The *cat* and *pac* genes do not confer resistance to chloramphenicol and puromycin, respectively [12], probably because of esterases in the medium [59].
5. When selecting for the *aadA* gene, which confers resistance to spectinomycin and streptomycin [13], use 50 µg/mL spectinomycin in *E. coli* (when constructing transformation substrates) and 50 µg/mL streptomycin in *B. burgdorferi*. Selection with spectinomycin fails in *B. burgdorferi* because of the high frequency of background-resistant mutants [60] and the vast majority of *E. coli* laboratory strains are resistant to streptomycin.
6. The *aacC1* gene, which confers resistance to gentamicin [38], does not confer sufficient resistance in *E. coli* in our hands: we first select *E. coli* transformants with another antibiotic (typically kanamycin because we usually clone DNA into pCR2.1-TOPO, which carries kanamycin resistance) and then replicaplate to gentamicin.
7. Our approach to generating mutants is to replace the entire open reading frame (ORF) with an antibiotic resistance gene or cassette [61–64]; we believe this is cleaner and more rigorous than an insertional mutation as no remnant of the gene product can be produced. A salient caveat to heed is polar effects on adjacent genes. Ensure that there are no overlapping genes: these could be other ORFs or RNA-encoding genes. We recommend using a promoterless antibiotic resistance gene to disrupt genes in operons [65] (Fig. 1B) or a transcriptional terminator fused to antibiotic resistance cassettes transcribed by strong promoters for singleton genes and terminal genes in operons [61]. For the latter, we prefer

antibiotic resistance cassettes transcribed from the *B. burgdorferi* *flgB* promoter [11, 66] with the *Bacillus subtilis* *trpL* terminator [67]: *flgBp-aadA-trpLt* conferring streptomycin resistance, *flgBp-aacC1-trpLt* conferring gentamicin resistance, and *flgBp-aphI-trpLt* conferring kanamycin resistance. We typically design two different constructs with two different antibiotic resistance markers for two reasons: there is a backup if one marker does not work and there are two independent mutants if both markers work. For site-directed mutations of the genome, the antibiotic resistance cassette is inserted adjacent to the gene targeted for mutation and outside of the operon, preferably between two divergently or two convergently transcribed genes [55, 68–70]; this will require screening for the mutation(s) following selection and confirmation by DNA sequencing. We usually use overlap extension PCR to generate mutated transformation substrates [1, 21, 55, 68, 69], but QuikChange is also effective [51, 64, 70, 71].

8. We use MacVector software (although many other applications are suitable) to analyze DNA sequences, including finding suitable restriction enzymes; the “List non-cutters” option is particularly useful. The restriction enzyme sites that we have typically used are AatII and AgeI; we use NdeI (CATATG) for fusions of an ORF to a promoter. Again, ensure that these restriction sites are not present elsewhere in the flanking sequences to be amplified, the cloning vector, or the selectable marker.
9. Large fragments of DNA (>1.5 kb) are more easily cloned using crystal violet to stain the DNA in the gel instead of ethidium bromide.
10. Following restriction enzyme digestion of the transformation substrate for allelic exchange, analyze a small amount next to the uncut vector by agarose gel electrophoresis to confirm that the DNA is completely linearized. A circular transformation substrate can recombine into the genome and result in a merodiploid rather than a gene disruption.
11. *B. burgdorferi* is a class 2 human pathogen and therefore should be handled at BSL-2 (biosafety level 2) in a class II BSC (biological safety cabinet). In addition, BSK II is a rich medium and all procedures should be performed aseptically. Introduction of recombinant DNA into a class 2 pathogen requires approval from the IBC (Institutional Biosafety Committee) before initiation of the experiments according to Section III-D of the *NIH Guidelines* (<https://osp.od.nih.gov/biotechnology/biosafety-and-recombinant-dna-activities/>).
12. The cell density (or growth phase) is a significant factor for successful electrotransformation [1, 33]. The cells will not transform efficiently if the cell density is too high (when the color of the medium is yellow). We have had success in electrotransforming cultures harvested at $1-7 \times 10^7$ cells/mL, although a low cell density ($1-2 \times 10^7$ cells/mL) requires pelleting the cells at a higher *g* force (up to $5000 \times g$) and adjusting the final volume of the cell suspension (see Note 15). Cell density should be determined using a Petroff-Hausser Counting Chamber (Hausser Scientific Partnership). Dilute 0.1 mL of the culture with 0.9 mL of cold dPBS and place in the counting chamber. Count cells over all 25

groups of 16 small squares in all planes using a dark-field microscope. Multiply the number of cells counted by 5×10^5 to calculate cells/mL. Alternatively, cell density can be determined by spectrophotometry [53]. Centrifuge 10 mL of the culture at $5000 \times g$ for 10 min. Decant the supernatant fraction, and resuspend the cell pellet in 1 mL of dPBS. Centrifuge at $8000 \times g$ for 5 min. Decant the supernatant fraction, resuspend the cell pellet in 1 mL of dPBS, and measure the OD at 600 nm. Multiply the OD by 1.4×10^8 to calculate cells/mL in the culture.

13. Cells can be cultured at 23 °C (or ambient temperature) to slow growth until the appropriate density is reached at a convenient time.
14. Thorough washing is important to remove components of the medium (*see* Note 20). Cell pellets are resuspended in both dPBS and EPS by pipetting up and down followed by vortex mixing. The two PBS washes can be eliminated [33, 38], although we find that decreases transformation efficiency (unpublished data).
15. The final cell concentration should be $1\text{--}5 \times 10^{10}$ cells/mL (with a final volume of about 0.9 mL). The volume of EPS used to resuspend the final cell pellet may have to be adjusted to account for initial cell number and efficiency of decanting.
16. We use presterilized aerosol-resistant pipette tips (with aerosol barriers) to help maintain sterility when handling small volumes of liquid.
17. Competent cells can be stored on ice for a few hours or at -80 °C for a long time. Transformation efficiency peaks (up to threefold) between 2 and 6 h of incubation on ice before transformation (unpublished data). Freezing cells only slightly decreases transformation efficiency (~50% of peak), but is better than leaving the cells on ice overnight (unpublished data).
18. We suggest using Electroporation Cuvettes Plus, Model 620, from BTX (part number 45-0125), which includes a disposable sterile pipette that allows for easier transfer of the fragile electroporated cells from the cuvette to the culture tube.
19. We routinely obtain about five transformants/ μ g of DNA in allelic exchange experiments, but this depends on the strain and the structure of the electrotransformation substrate. The transformation efficiency of shuttle vectors is higher than allelic exchange with linear DNA [12] (unpublished data). We often use less DNA (1–2 μ g) when transforming shuttle vectors than when transforming linear allelic exchange substrates (otherwise, there are too many colonies or positive wells). DNA ranging in size from 27-mer oligonucleotides [30] to 28-kb linear plasmids [72] has been used to transform *B. burgdorferi*.
20. Electroporation in the presence of high-ionic-strength solutions causes arcing (and a lower time constant). Two arcs will kill all of the *B. burgdorferi* cells. We use the Wizard DNA purification system (Promega), ethanol-precipitate eluted DNA, wash with 70% ethanol, and resuspend in a small amount of water: ~10 μ g DNA in 10 μ L water and electroporate 9 μ L for allelic exchange experiments. The ethanol precipitation is important to remove traces of salt from the column

and we use no more than 10 μ L DNA solution in water. Again, transformation of low-passage, infectious isolates requires large amounts of DNA [14, 16, 33, 38, 40].

21. Do not vortex or even gently mix the electroporated cells after they have been transferred to the culture medium; vortexing will kill the electroporated cells.
22. Isolating *B. burgdorferi* mutants by selection as single colonies in a semi-solid medium [73–75] was an experimental breakthrough. However, we have almost exclusively switched to cloning by limiting dilution [41] primarily because of the convenience as the protocol requires considerably less time and effort. In addition, at least one mutant was not able to be isolated in semi-solid medium despite exhaustive efforts [41].
23. The color change in the medium is due to the production of lactic acid by the bacteria during growth as detected by phenol red. Incubating the 96-well plates at ambient atmosphere for 1–4 h increases the color difference by shifting the bicarbonate-CO₂ equilibrium, which results in more basic (pink) wells where there is no growth. Transformants are isolated from 96-well plates that have fewer than ten positive wells because the probability that a well is inoculated with a single cell is greater than 0.94 [26].
24. Plasmid content is determined by PCR screening [14, 35, 51]; there is a multiplex primer set [76] that some laboratories find convenient (*see* Note 2).
25. Complementation in *trans* from a shuttle vector is the most convenient approach; however, complementation in *cis* via either genetic reconstitution or insertion of the wild-type gene into a second genomic site is preferred for three reasons: (1) shuttle vectors can be lost in vivo in the absence of selection [77]; (2) the expression of genes in *trans* from shuttle vectors can be different than in *cis* [68]; and (3) the copy number of shuttle vectors is higher than the other genomic elements [77].
26. Promoters should be empirically mapped by 5'-RACE [64] or primer extension [78]; they are hard to discern by manually examining the sequence due to the low GC content and they can be far upstream from the ORF.
27. To fuse a promoter to the ORF, amplify a promoter (either the native promoter for the operon or a heterologous promoter, such as the strong constitutive *flgB* promoter or the inducible *flac* promoter) with an NdeI site on the 3' end and amplify the ORF with an NdeI site (CATATG) on the 5' end, overlapping the start codon. The PCR products are cloned into pCR2.1-TOPO. After the cloned DNA is confirmed to be the correct sequence, the clones are restriction digested with NdeI and a restriction enzyme that only cuts at the multiple cloning site of the vector on the 3' end of the ORF. The promoter needs to be in the same orientation of the ORF in pCR2.1-TOPO so NdeI and the other restriction enzyme cut adjacent to each other. Thus, the promoter remains with pCR2.1-TOPO and the ORF is excised. The ORF is then ligated to the promoter in pCR2.1-TOPO.

28. Instead of *cis*-complementation by genetic reconstitution, the complementing gene can be inserted into the genome at another location. This strategy would entail amplifying the region surrounding the site of insertion and inserting the complementing gene.

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Fig. 1.

Molecular cloning strategies for generating transformation substrates used to disrupt genes by allelic exchange. **(a)** Mutagenesis by replacing all or most of a gene of interest with an antibiotic resistance cassette transcriptionally driven by a constitutive *B. burgdorferi* promoter (P). **(b)** Mutagenesis by replacing all or most of a gene of interest with a promoterless antibiotic resistance gene fused to the promoter for the gene of interest at a synthetic NdeI site. Small arrows represent primers with the relevant restriction enzyme sites (AatII, AgeI, and NdeI); the *large black arrow* represents the gene of interest to be mutated; the *large white arrows* represent the genes conferring antibiotic resistance; and the *large gray boxes* represent the flanking upstream (*up*) and downstream (*down*) sequences for recombination.

Fig. 2. Molecular cloning strategy for generating transformation substrates used to *cis*-complement mutated genes by genetic reconstitution. *Small arrows* represent primers with the relevant restriction enzyme sites (AatII and AgeI); the *large black arrow* represents the gene of interest to be complemented; the *large dark gray arrow* represents the gene conferring antibiotic resistance, which must be different than the antibiotic resistance gene used to mutate the gene of interest, although the promoter (P) driving expression can be the same; and the *large light gray boxes* represent the flanking upstream (*up*) and downstream (*down*) sequences for recombination.