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Genetic Manipulation of *Clostridium difficile*

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Abstract

Clostridium difficile is a Gram-positive, spore forming, anaerobic, intestinal bacterium and is the most common cause of antibiotic-associated colitis. For many years this organism was considered genetically intractable, but in the past 10 years, multiple methods have been developed or adapted for genetic manipulation of *C. difficile*. This unit describes the molecular techniques used for genetic modification of this organism, including methods for gene disruption, complementation, plasmid introduction and integration, and cross-species conjugations.

Keywords

Clostridium difficile; firmicute; anaerobic chamber; plasmid; genomic DNA; RNA; transposon; conjugation; group II intron; mutagenesis

INTRODUCTION

This unit describes methods used for genetic manipulation of *Clostridium difficile*. *C. difficile* is an intestinal pathogen of humans and animals and is the leading cause of antibiotic-associated diarrhea and colitis in humans.

C. difficile is a strict anaerobe and must be maintained and manipulated in an anaerobic environment (for more information, see *UNIT 9A.1* for laboratory maintenance of *Clostridium difficile* and *UNIT 12B.1* for proper use of an anaerobic chamber). However, many of the prerequisite manipulations to the described methods in this unit, such as plasmid construction and transposon modification, can be performed under aerobic conditions in the heterologous hosts *Escherichia coli* or *Bacillus subtilis*.

This unit describes methods for nucleic acid isolation (see Basic Protocol 1, Alternate Protocol, and Basic Protocol 2), Tn*916* transposon conjugation from *B. subtilis* (see Basic Protocol 3), plasmid conjugation from *E. coli* (see Basic Protocol 4), gene disruption using group II introns, such as the ClosTron (see Basic Protocol 5), and site-directed mutagenesis using unstable plasmids (see Basic Protocol 6).

CAUTION: Clostridium difficile is a Biosafety Level 2 (BSL-2) pathogen. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms. See *UNIT 1A.1* and *APPENDIX B* for more information.

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NOTE: All steps involving *C. difficile* should be performed in an anaerobic chamber using sterile technique and pre-reduced medium. For instructions on proper use of an anaerobic chamber, see *UNIT 12B.1*. For proper laboratory maintenance of *C. difficile*, see *UNIT 9A.1*.

BASIC PROTOCOL 1

EXTRACTION OF *C. DIFFICILE* GENOMIC DNA

This protocol describes the extraction of genomic DNA from a *C. difficile* culture. This method yields high-quality genomic DNA (Wren and Tabaqchali, 1987). This DNA is suitable for more sensitive techniques such as Southern blotting.

Materials

Liquid BHIS for *C. difficile* growth (see recipe)

Viable *C. difficile* on a petri plate (*UNIT 9A.1*)

TE buffer (*APPENDIX 2A*)

Genomic DNA solution (see recipe)

Fresh lysozyme solution (see recipe)

20% Sarkosyl (see recipe)

10 mg/ml RNase A (see recipe)

10 mg/ml proteinase K (see recipe)

25:24:1 (v/v/v) phenol/chloroform/isoamyl alcohol (*UNIT 2.1*)

Chloroform

3 M sodium acetate (see recipe)

75% and 95% ethanol

37°C anaerobic chamber

Refrigerated centrifuge

37°C water bath or heating block

Inoculate and prepare culture

1 Inoculate 10 ml BHIS with a colony of *C. difficile* using sterile techniques and incubate overnight in a 37°C anaerobic chamber.

2 Harvest by centrifuging 10 min at 4000 × *g*, 4°C. Discard the supernatant.

This and subsequent steps may be performed outside of the anaerobic chamber.

3 Wash pellet in 1 ml TE buffer by vortexing.

4 Centrifuge 10 min at 4000 × *g*, 4°C. Discard the supernatant.

Lyse bacteria

5 Resuspend cell pellet in 200 µl of genomic DNA solution and add 50 µl lysozyme solution (50 mg/ml).

6 Incubate 1 to 2 hr at 37°C.

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7 Add 100 μl of 20% Sarkosyl and 15 μl RNase A (10 mg/ml) and incubate 30 min at 37°C.

The solution should become viscous as the detergent lyses bacterial cells.

8 Add 15 μl proteinase K (10 mg/ml) and incubate for 30 min at 37°C.

9 Use TE buffer to bring the volume up to 600 μl.

Isolate DNA

10 Add 600 μl of 25:24:1 (v/v/v) phenol/chloroform/isoamyl alcohol and mix.

11 Microcentrifuge 10 min at maximum speed, room temperature.

12 Carefully remove the aqueous phase (upper phase) and transfer into a new clean tube.

Avoid transferring the white interface, which contains proteins and cell debris.

13 Repeat steps 10 to 12 until the white interface is no longer visible.

14 Add 600 μl chloroform, mix, and microcentrifuge 10 min at maximum speed, room temperature.

This step will remove traces of phenol.

15 Transfer the upper phase into a new clean tube.

Precipitate and wash DNA

16 Precipitate DNA with 50 μl of 3 M sodium acetate, pH 5.2, and 3 vol of cold 95% ethanol.

17 Centrifuge DNA 5 min at maximum speed, room temperature.

18 Wash the DNA pellet with 500 μl of 70% ethanol.

19 Resuspend the DNA pellet in 100 to 500 μl TE buffer.

This step may require some time. To reduce this time, incubate at 65°C until completely dissolved.

The concentration of total DNA is evaluated using a spectrophotometer at 260 nm (1 OD at 260 nm = 50 ng/μl DNA). Pure preparations of DNA have a $A_{260/280}$ *ratio of 1.8 to 2. If the extraction is not pure, repeat from step 13.*

For larger volumes of C. difficile culture, scale up volumes accordingly.

ALTERNATE PROTOCOL

QUICK GENOMIC DNA EXTRACTION FROM *C. DIFFICILE* FOR PCR

This protocol describes the quick extraction of DNA from *C. difficile*. This method will quickly and reliably provide DNA for use in multiple PCR reactions. However, the yield and purity of the DNA extracted are relatively low (RNA, protein, and cell debris contaminate the preparation). Should a pure preparation of DNA be required, see Basic Protocol 1.

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Additional Materials (also see Basic Protocol 1)

1× PCR buffer

Sterile inoculating loops or pipet tips

Microcentrifuge tubes

58°C and 90°C water baths or heating blocks

1. Using a sterile inoculating loop or pipet tip, suspend a *C. difficile* colony in a microcentrifuge tube containing 94 μl of 1× PCR buffer.

   IMPORTANT NOTE: *This first step involving C. difficile should be performed in an anaerobic chamber using sterile technique. Subsequent steps may be performed outside the anaerobic chamber.*

   To extract DNA from broth culture, microcentrifuge 250 μ*l C. difficile culture at maximum speed and resuspend the pellet in 1× PCR buffer.*

2. Add 4 μl of fresh lysozyme solution (500 μg/ml final concentration) to the microcentrifuge tube.

3. Incubate 15 min at room temperature.

4. Add 2 μl of 10 mg/ml proteinase K (200 μg/ml final concentration).

5. Incubate 1 hr at 58°C.

   After 1 hr, the tube should have clarified due to the action of proteinase K. If solution does not clarify, a new extraction is recommended.

6. Incubate 15 min at 90°C. After incubation, place on ice.

   This step inactivates the proteinase K and is essential so that the DNA polymerase is not degraded in subsequent steps.

7. Use ~2 to 5 μl for PCR.

BASIC PROTOCOL 2

EXTRACTION OF *C. DIFFICILE* RNA

This protocol describes extraction of RNA from a *C. difficile* culture (Dupuy and Sonenshein, 1998). This method can be used to prepare RNA for microarray analysis, small RNA studies, and RT-PCR.

Materials

Viable *C. difficile* grown on a petri plate (*UNIT 9A.1*)

Liquid medium for *C. difficile* growth (i.e., BHIS or other suitable medium; see recipes)

1:1 (v/v) acetone/ethanol mix

TE buffer (*APPENDIX 2A*)

TRIzol (Invitrogen)

Chloroform

Cold isopropanol

Diethylpyrocarbonate-treated water (DEPC water)

75% ethanol in DEPC water

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37°C anaerobic chamber

Refrigerated centrifuge

Filter tips

2-ml screw-cap tubes (with gaskets)

0.1-mm glass beads

Cell disrupter

Spectrophotometer

Inoculate and harvest C. difficile

1. Inoculate a *C. difficile* culture using sterile techniques in the desired liquid medium.
2. Harvest 1 vol of *C. difficile* culture and add 1 vol of ice-cold 1:1 (v/v) acetone/ethanol, vortex, and store at −80°C until use.

   Volumes are adjusted to collect 100–1 × 10^7 cells.
3. Thaw samples on ice.
4. Centrifuge 10 min at 3000 × *g*, 4°C, remove and discard the supernatant.

   The use of filter tips is recommended for all the RNA purification steps.

Wash pellet

5. Wash the pellet with 500 μl TE buffer.
6. Centrifuge 3 min at 4000 × *g*, 4°C.
7. Air dry the pellet.
8. Add 1 ml of TRIzol and incubate 5 min at room temperature.

Disrupt cells

9. Transfer into a 2-ml screw-cap tube one-fourth to one-third filled with 0.1-mm glass beads.
10. Break the cells using a cell disrupter with two 60-sec rounds on the high setting.
11. Microcentrifuge 10 min at maximum speed, 4°C.
12. Carefully remove the upper phase (60% TRIzol volume) and transfer into a clean tube.
13. Add 1 ml of TRIzol to the upper phase and vortex.
14. Microcentrifuge 10 min at maximum speed, 4°C.

Wash and isolate RNA

15. Carefully remove the upper phase and transfer into a clean tube.
16. Add 200 μl chloroform, vortex.
17. Microcentrifuge 10 min at maximum speed, 4°C.
18. Remove carefully the upper phase, transfer into a clean tube.

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19 Add 500 μl isopropanol, incubate 10 min at room temperature.

20 Microcentrifuge 10 min at maximum speed, 4°C.

21 Discard supernatant and wash pellet with 1 ml of 75% ethanol.

22 Microcentrifuge 5 min at 7500 rpm, 4°C.

23 Air dry the RNA pellet and resuspend in 50 to 100 μl RNase-free water (DEPC water).

The concentration of total RNA is evaluated using a spectrophotometer at 260 nm (1 OD at 260 nm = 40 ng/μl RNA). Pure preparations of RNA have an $A_{260/280}$ *ratio of 1.8 to 2. If the extraction is not pure, repeat from step 13.*

To clean RNA of DNA contamination, treatment with DNaseI (RNase-free) is highly recommended.

Alternatively, a Gram-positive RNA extraction kit may be used (e.g., RNeasy extraction kit available from Qiagen) with modifications; however, extra care must be taken to ensure that cells are sufficiently disrupted for RNA extraction.

BASIC PROTOCOL 3

TRANSFER OF Tn*916* FROM A *BACILLUS SUBTILIS* DONOR STRAIN TO A *C. DIFFICILE* RECIPIENT STRAIN

This protocol describes the transfer of the Tn*916* transposon from a *B. subtilis* donor into *C. difficile*. The use of a Tn*916*-based method to introduce DNA has advantages and disadvantages. While a single copy of a gene can be introduced into *C. difficile* by integration of Tn*916*, the transposon insertion may disrupt genes on the *C. difficile* chromosome, though in some strains the transposon integrates at preferred sites (Mullany et al., 1991).

Materials

DNA of interest

pSMB47 plasmid DNA (Manganelli et al., 1998) or a plasmid that contains homology to Tn*916* to allow homologous recombination into the Tn*916* tetracycline gene

B. subtilis donor that carries Tn*916* on the chromosome, e.g., BS49 (Mullany et al., 1991)

Agar plate with medium containing erythromycin

Petri plate containing viable *C. difficile* colonies (*UNIT 9.1A*)

Reduced BHIS broth medium (see recipe)

Non-reduced BHIS broth medium (see recipe)

Erythromycin (see recipe)

Tetracycline (see recipe)

Reduced BHIS(KNO3) Petri plates (see recipe)

Reduced BHIS(E) Petri plates (see recipe)

Reduced BHIS(ECC) Petri plates (see recipe)

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15-ml culture tubes

Anaerobic chamber at 37°C

Aerobic chamber at 37°C

Additional reagents and equipment for PCR

Construct a modified Tn916 transposon with gene of interest

1 Clone the desired DNA fragment into the suicide vector pSMB47 using standard methods.

2 Transform a *B. subtilis* donor that carries the Tn*916* transposon (e.g., BS49) with the plasmid generated in step 1, using standard methods and select on agar plate with medium containing erythromycin.

Erythromycin resistance is introduced with the suicide vector.

The efficiency of B. subtilis transformation will greatly increase if the DNA to be transformed is isolated from a recA+ strain of E. coli, such as E. coli JM107. Transformation of plasmid DNA into E. coli JM107 results in the formation of plasmid multimers. Plasmid multimers are more efficiently taken up by B. subtilis.

3 Isolate *B. subtilis* colonies and screen for the insertion of the plasmid into the chromosome by PCR.

pSMB47 is not able to replicate in B. subtilis and will integrate via homologous recombination in Tn916.

Introduce the modified transposon into C. difficile

4 Start a 5-ml *C. difficile* overnight culture using 5 ml of the reduced BHIS medium, in a 15-ml culture tube in a 37°C anaerobic chamber.

5 After overnight growth, dilute the *C. difficile* culture 1:20 into fresh reduced BHIS broth medium and allow the culture to grow anaerobically for 6 hr in a 37°C anaerobic chamber.

6 Three hours after the start of the *C. difficile* culture (step 5), start the *B. subtilis* donor from step 3 by picking a single colony and grow in 5 ml non-reduced BHIS medium supplemented with erythromycin (2 μg/ml) and tetracycline (10 μg/ml) and grow aerobically 3 hr at 37°C. After incubation, move the donor to the anaerobic chamber for use in step 7.

Erythromycin is added to the culture to ensure growth of isolates containing pSMB47. Tetracycline is added to the culture to induce the expression of the Tn916 conjugation machinery and increase the efficiency of transfer (Showsh and Andrews, 1992).

7 In the anaerobic chamber, spread 100 μl of each culture (*C. difficile* recipient and *B. subtilis* donor) onto a BHIS(KNO_3) plate and grow overnight in a 37°C anaerobic chamber.

It is essential to pre-reduce the medium to allow C. difficile growth.

The addition of KNO_3 to the BHIS plate allows the B. subtilis donor to grow in the absence of oxygen (Glaser et al., 1995).

8 After overnight growth, suspend the growth on the BHIS(KNO_3) plate in 2 ml reduced BHIS medium by adding the 2 ml of BHIS medium to the plate containing the growth and scraping with an inoculating loop.

Isolate C. difficile containing the transposon

9 Spread 100 μl of the resuspended medium onto a pre-reduced BHIS(E) plate and grow overnight in a 37°C anaerobic chamber.

This step enriches the population of C. difficile cells that have received the transposon. Because KNO_3 is not added to the BHIS(E) plate, B. subtilis growth should be reduced.

10 After overnight growth, suspend the cells from the BHIS(E) plate as in step 8.

11 Spread 100 μl of the resuspended cells onto each of ten pre-reduced BHIS(ECC) plates and grow overnight in a 37°C anaerobic chamber.

Depending on the mating efficiency, the use of ten separate BHIS(ECC) plates may not be needed. However, this should ensure that under low mating efficiencies, colonies do arise.

Cefoxitin and D-cycloserine inhibit the growth of B. subtilis. C. difficile is resistant to both cefoxitin and D-cycloserine.

12 After overnight growth, check the plates for growth. If the mating is efficient, there should be many colonies on all ten plates.

Colonies may take longer than 24 hr to appear. Incubate up to 72 hr if no colonies are present.

13 Screen colonies by PCR, followed by gel electrophoresis, for *C. difficile*-specific gene (e.g., *tcdB*) and for the introduced DNA in pSMB47 using gene-specific primers.

PCR analysis of a specific C. difficile gene will ensure that the colonies are C. difficile and not a B. subtilis contaminant; B. subtilis does not encode tcdB.

Tn916 can be mobilized into C. difficile in the absence of pSMB47. If this is preferred, omit the use of erythromycin from steps 6, 9, and 11, and substitute 10 μg/ml tetracycline. This use of Tn916 can provide a method for random mutagenesis, though Tn916 has been reported to integrate at preferred cites in the C. difficile chromosome.

BASIC PROTOCOL 4

TRANSFER OF PLASMID DNA FROM AN *E. COLI* DONOR TO *C. DIFFICILE* RECIPIENT

This protocol describes the transfer of plasmid DNA from an *E. coli* donor strain to a *C. difficile* recipient strain. Transfer of plasmid DNA from *E. coli* to *C. difficile* provides an excellent tool in the study of the molecular biology of the organism. Plasmids can be used to introduce mutations, to carry genes to complement mutations, or to introduce reporter constructs. Many different plasmid systems have been developed to study *C. difficile* biology. Some systems carry unstable origins of replication that allow for plasmid maintenance, e.g., *C. perfringens* pIP404 origin of replication: pJIR1456 or pJIR1457 plasmids (Lyras and Rood, 1998). Other systems use origins of replication from naturally occurring *C. difficile* plasmids (clostridial origins of replication: pMTL8XXXX vectors; Heap et al., 2009). This protocol describes a general method for the introduction of plasmid DNA into *C. difficile*.

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Materials

DNA of interest

E. coli/C. difficile shuttle plasmid, e.g., thiamphenicol-resistant shuttle plasmid, pJIR1456

E. coli strain carrying a broad host range plasmid RP4 derivative, e.g., *E. coli* HB101 pRK24

LB medium (*APPENDIX 4A*)

Petri plate containing *C. difficile* colonies (see *UNIT 9.1A*)

BHIS medium (see recipe)

Appropriate antibiotics (e.g., Amp, Cm)

Reduced BHIS agar Petri plates (see recipe)

Reduced BHIS(TCC) Petri plates (see recipe)

15-ml culture tubes

37°C anaerobic chamber

Sterile inoculating loops

1. Clone the desired DNA fragment into a *E.coli/C. difficile* conjugation plasmid (e.g., pJIR1456).

   There are many different shuttle plasmids that can be mobilized into C. difficile. This protocol describes the introduction of a thiamphenicol-resistant shuttle plasmid as an example. Use the antibiotic resistance that is encoded on the specific plasmid being used. More information on C. difficile plasmids can be found elsewhere (Lyras and Rood, 1998; Heap et al., 2009).

2. Transform an *E. coli* strain that carries the broad host range plasmid RP4, such as *E. coli* HB101 pRK24, with the plasmid obtained in step 1 in LB medium.

   RP4 is capable of mobilizing IncP oriT plasmids (such as pJIR1456). RP4 is Tra+,Mob+, AmpR, and TcR (Trieu-Cuot et al., 1991).

3. Inoculate a *C. difficile* culture in 5 ml BHIS medium in a 15-ml culture tube and grow overnight in a 37°C anaerobic chamber.

   For proper laboratory maintenance of C. difficile, see UNIT 9A.1.

4. At the same time, start a 5-ml culture of *E. coli* HB101 with pRK24 containing the plasmid (donor) generated in step 2 in BHIS medium with the appropriate antibiotics (Amp, Cm).

5. After overnight growth, centrifuge 1 ml of the *E. coli* donor culture 10 min at 5000 × *g*, room temperature; remove all of the supernatant.

   The E. coli supernatant contains antibiotics to which C. difficile is sensitive. Removal of all the supernatant will minimize their action against C. difficile in subsequent steps.

6. Wash the *E. coli* pellet in 1 ml of BHIS medium (resuspend by gently flicking the tube) and centrifuge as in step 5.

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7. In the anaerobic chamber, suspend the *E. coli* pellet from step 6 in 200 μl of the overnight *C. difficile* culture from step 3.

8. Spot 100-μl aliquots onto a BHIS agar Petri plate and grow overnight in a 37°C anaerobic chamber.

   Take care when spotting the culture on the BHIS Petri plate. The goal is to have four spots on the plate. Careful manipulation of the plate will ensure that the spots remain stationary and not spread.

9. After overnight growth, use a sterile inoculating loop to scrape the growth from the plate and suspend in 2 ml of reduced BHIS medium.

10. Spread 100-μl aliquots of the suspension onto ten BHIS(TCC) plates.

    Thiamphenicol is included in these plates as an example for mating; however other antibiotic selections may be used.

11. Incubate plates 48 to 72 hr anaerobically at 37°C.

    E. coli background is sometimes observed. If the C. difficile strain being used is kanamycin resistant and the E. coli strain being used is kanamycin sensitive, 50 μ*g/ml kanamycin can be substituted for cefoxitin in the BHIS(TCC) plates to reduce E. coli growth.*

12. Screen colonies by PCR for a *C. difficile*–specific gene (e.g., *tcdB*) and for the introduced plasmid.

    PCR analysis of a C. difficile–specific gene will ensure that the colonies are C. difficile and not an E. coli contaminant.

BASIC PROTOCOL 5

GENE DISRUPTION IN *C. DIFFICILE* USING GROUP II INTRON TARGETING

This protocol describes a method to create insertion mutations in the *C. difficile* genome. The ClosTron system is based on the bacterial group II intron intrinsic features. The bacterial group II intron target specificity is determined mainly by base-pairing between the target site DNA and intron RNA. The group II mobility is aided by the presence of an intron-encoded protein (IEP). Expression from pMTL007, or derivative plasmids, of the targeted intron RNA and IEP allows for the formation of an RNA-protein complex that inserts the RNA into the targeted DNA sequence and is reverse transcribed by IEP. This method has been developed for many bacterial species by Sigma and was recently adapted for use in *C. difficile* (Heap et al., 2007, 2010).

Materials

Primers: IBS, EBS2, EBS1d, and EBS universal primer (see recipe)

Intron PCR template DNA (Sigma) or pMTL20IT1 and pMTL20IT2

High-fidelity *Taq* polymerase

25:24:1 (v/v/v) phenol/chloroform/isoamyl alcohol (*UNIT 2.1*)

Cold 75% and 95% ethanol

TE buffer (*APPENDIX 2A*)

*Hind*III and *BsrG*I restriction enzymes (New England Biolabs)

pMTL007 plasmid DNA (Heap et al., 2009)

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Reduced BHIS(TCC) plates (see recipe)

Petri plate containing viable *C. difficile* (*UNIT 9A.1*)

Reduced BHIS(E) plates (see recipe)

37°C anaerobic chamber

Sterile inoculating loops

1. Copy and paste the gene sequence into the algorithm in the intron design site (http://www.clostron.com).

   This algorithm will design primers IBS, EBS2, and EBS1d necessary for targeting the intron in a gene of interest.

   The algorithm identifies potential intron insertion sites. Pick the target site with the best location and the best score. An intron can be inserted in the sense or antisense orientation.

2. Synthesize the four primers (IBS, EBS2, EBS1d, and EBS universal primer) required to retarget the intron.

3. Prepare a primer mix using 2 μl of IBS primer (100 μM), 2 μl of EBS1d (100 μM), 2 μl of EBS2 (20 μM), 2 μl of EBS universal (20 μM), and 12 μl of water.

   The total volume is 20 μ*l.*

4. Set up a PCR reaction using intron PCR template DNA (Sigma) or pMTL20IT1 and pMTL20IT2 plasmids, the primer mix, and a high-fidelity *Taq* polymerase, following polymerase manufacturer's instructions for amplification.

   This PCR reaction will generate a 350-bp fragment. To verify that the intron is correctly targeted, sequence the 350-bp fragment and check that the IBS, EBS2, and EBS1d primers match the sequences.

5. Purify the amplified DNA using 25:24:1 (v/v/v) phenol/chloroform/isoamyl alcohol extraction, followed by ethanol precipitation (see Basic Protocol 1, steps 9 to 18).

   Alternatively, the PCR fragment can be purified using a PCR purification kit (e.g., QIAquick PCR purification kit available from Qiagen)

6. Resuspend the DNA in 30 μl TE buffer.

7. Digest and clone the purified PCR fragment into pMTL007 using *Hind*III and *Bsr*GI restriction sites and standard techniques.

   BsrGI has increased activity at 60°C.

   A ClosTron plasmid ready-targeted to a gene of choice can be ordered directly from http://www.Clostron.com. (see Web site for details).

8. Transfer the resulting plasmid from *E. coli* to *C. difficile* (see Basic Protocol 4).

   pMTL007 provides chloramphenicol/thiamphenicol resistance. Thiamphenicol is typically used for selection of C. difficile and chloramphenicol for E. coli.

9. Streak the transconjugants onto a reduced BHIS(TCC) plate.

   This step allows expression of the intron RNA. Integration of the intron into the chromosome target site activates the erythromycin marker. The erythromycin-resistance gene is interrupted by a group I intron. When the group II intron is transcribed, the group I intron is spliced, allowing

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activation of erythromycin resistance when the group II intron is reverse transcribed at the target site.

10. Harvest growth with an inoculating loop and spread uniformly onto a reduced BHIS(E) (see *UNIT 9A.1* for laboratory maintenance of *C. difficile*).

    Lincomycin (20μg/ml) can be used in place of erythromycin if the target strain is naturally erythromycin resistant.

11. Pick colonies, extract DNA (see Basic Protocol 1 or Alternate Protocol), and perform PCR to verify the integration of the intron.

BASIC PROTOCOL 6

SITE-DIRECTED MUTAGENESIS USING UNSTABLE PLASMIDS

This protocol describes the use of unstable plasmids to introduce site-directed insertions into the *C. difficile* chromosome. This method has been used to introduce mutations into many different genes (O'Connor et al., 2006; Dineen et al., 2007; Lyras et al., 2009) and is based upon the use of *Clostridium perfringens* origins of replication in *C. difficile*. The *C. perfringens* origin of replication (pIP404) is unstable in *C. difficile*, and, in the absence of antibiotic selection, is not maintained. With a low frequency, these plasmids can insert by homologous recombination into the *C. difficile* chromosome.

This method has both advantages and disadvantages. The advantage over the TargeTron method of mutagenesis (see Basic Protocol 5) is that if a target DNA sequence does not have efficient group II intron insertion sites, the protocol described below will allow for insertional inactivation of a gene. The disadvantage is that because the plasmid origin of replication is still recognized by *C. difficile*, albeit poorly, the plasmid can excise from the *C. difficile* chromosome, thereby restoring the wild-type copy of the gene.

Materials

400- to 1000-bp DNA fragment, internal to the target gene

E. coli strain carrying an RP4 plasmid derivative (see Basic Protocol 4)

BHIS Petri plate containing viable *C. difficile* JIR8094 colonies (*UNIT 9.1A*)

Reduced BHIS medium (see recipe)

BHIS(T) plate containing viable *C. difficile* JIR8094 pJIR1456 colonies (see Basic Protocol 3 for introduction of plasmid DNA)

Reduced BHIS(TCC) Petri plates (see recipe)

Reduced BHIS Petri plate (see recipe)

15-ml culture tubes

37°C anaerobic chamber

Sterile inoculating loops

1. Clone a ~400- to 1000-bp DNA fragment, internal to the target gene, into the *E. coli/C. difficile* shuttle vector pJIR1456.

    pJIR1456 will provide thiamphenicol resistance to C. difficile and is used as an example in this protocol. pJIR1457 can also be used and provides erythromycin resistance. If pJIR1457 is used, substitute this plasmid instead of pJIR1456 below.

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2. Introduce this plasmid into *C. difficile* JIR8094 by following Basic Protocol 4.
3. After confirming that the plasmid has been introduced into *C. difficile* JIR8094, inoculate 5 ml of BHIS medium (no antibiotics) with a colony of the newly generated strain and a separate 5 ml with a control strain, *C. difficile* JIR8094 pJIR1456, in 15-ml culture tubes. Grow overnight in a 37°C anaerobic chamber.
4. After overnight growth, spread 100 µl of each strain onto separate BHIS(TCC) Petri plates.
5. Inoculate two culture tubes containing 5 ml BHIS medium with 10 µl of the overnight cultures. Grow overnight in a 37°C anaerobic chamber.
6. After overnight incubation, check for growth of the control and test strains on BHIS(TCC).

   By growing C. difficile in the absence of antibiotic, there is no pressure for the strain to maintain the poorly replicating plasmid. In the absence of antibiotic selection, the plasmid will be lost. At a low frequency, the plasmid will integrate by homologous recombination into the chromosome using the cloned DNA fragment on the plasmid. This single crossover will maintain antibiotic resistance on the chromosome and should not be lost during passage.

7. Repeat from step 4 as necessary.

   After a few passages, the strain carrying the target DNA should yield antibiotic-resistant colonies, due to site-specific integration at the targeted locus within the chromosome.

8. Once a strain engineered with the target DNA yields antibiotic resistant colonies and the control plasmid does not, subculture the colonies on a reduced BHIS(TCC) Petri plate to confirm the antibiotic resistance.
9. After confirmation of the antibiotic resistance, screen individual colonies for the integrated plasmid.

   This procedure can be inefficient. However, it has been used successfully to introduce targeted mutations into the C. difficile chromosome. Thus far, this technique has only been successful in C. difficile JIR8094.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.

BHIS agar

Dissolve powdered brain-heart infusion extract (BD Difco; 37 g/liter or per manufacturer's instructions) and 5 g/liter yeast extract in 800 ml of water. Bring volume to 1 liter with water and add 15 g/liter agar (BD Difco). Autoclave for 20 min, cool to touch, and add 10 ml of filter-sterilized 10% (w/v) L-cysteine. Pour ~25 ml into sterile Petri plates and allow to solidify. For growth of *C. difficile*, medium must be placed in an anaerobic chamber for a minimum of 2 hr to reduce the O_2 content. Store plates up to 3 months at 4°C.

BHIS medium (broth)

Dissolve powdered brain-heart infusion extract (BD Difco; 37 g/liter or per manufacturer's instructions) and 5 g/liter yeast extract in 800 ml of water. Bring volume to 1 liter with

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water. Autoclave for 20 min. Cool medium at room temperature and add 10 ml of filter-sterilized 10% (w/v) L-cysteine to each bottle. For growth of *C. difficile*, bottles must be placed in an anaerobic chamber overnight to reduce the O_2 content. Store indefinitely at room temperature.

BHIS(E) plates

Prepare BHIS agar (see recipe). Prior to pouring plates, add 10 ml of 2.5 mg/ml erythromycin stock solution (see recipe) per liter of medium (final concentration of 2.5 μg/ml). Store up to 1 month at 4°C.

BHIS(ECC) plates

Prepare BHIS agar (see recipe). Prior to pouring plates, add 10 ml of 10 mg/ml erythromycin stock solution (final concentration of 10 μg/ml; see recipe), 10 ml of 8 mg/ml cefoxitin stock solution (final concentration of 8 μg/ml; see recipe), and 10 ml of 25 mg/ml cycloserine stock solution (final concentration of 250 μg/ml; see recipe). Store up to 1 month at 4°C.

BHIS(KNO_3) plates

Prepare BHIS agar (see recipe). Prior to pouring plates, add 10 ml of 500 mM KNO_3 stock solution (see recipe) per liter of medium (final concentration of 5 mM). Store up to 1 year at 4°C.

BHIS(T) plates

Prepare BHIS agar (see recipe). Prior to pouring plates, add 10 ml of 10 mg/ml thiamphenicol stock solution per liter of medium (final concentration of 10 μg/ml). Store up to 1 month at 4°C.

BHIS(TCC) plates

Prepare BHIS agar (see recipe). Prior to pouring plates, add 10 ml of 10 mg/ml thiamphenicol stock solution (final concentration of 10 μg/ml), 10 ml of 8 mg/ml cefoxitin stock solution (final concentration of 8 μg/ml; see recipe), and 10 ml of 25 mg/ml of fresh cycloserine stock solution (final concentration of 250 μg/ml; see recipe). Store up to 1 month at 4°C.

Cefoxitin stock solution, 8 mg/ml

Dissolve 8 mg of cefoxitin (Sigma-Aldrich) per 1 ml water. Filter sterilize using a 0.45-μm filter and store up to 1 year at −20°C.

Cycloserine stock solution, 250 mg/ml

Dissolve 250 mg D-cycloserine (Sigma-Aldrich) per 10 ml water. Filter sterilize using a 0.45-μm filter. Prepare fresh for each use.

EBS universal primer

5′-CGAAATTAGAAACTTGCGTTCAGTAAAC-3′ (Sigma)

Erythromycin, 2.5 mg/ml

Dissolve 2.5 mg of erythromycin per 1 ml ethanol. Store up to 1 year at −20°C.

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Genomic DNA solution

Dissolve 34.23 g of sucrose in TE buffer (*APPENDIX 2A*) to a final volume of 100 ml. Filter sterilize using a 0.45-μm filter and store indefinitely at room temperature.

KNO_3 solution, 500 mM

Dissolve 0.51 g of KNO_3 per 10 ml water. Filter sterilize using a 0.45-μm filter and store up to 1 year at room temperature.

Lysozyme solution

Dissolve 50 mg of lysozyme per 1 ml water. Prepare fresh for each use.

Proteinase K

Dissolve 10 mg of proteinase K per 1 ml of water. Store for 6 months at −20°C.

RNase A, 10 mg/ml

Dissolve 10 mg of RNase A per 1 ml of water. Store for 1 year at −20°C.

Sarkosyl, 20% (w/v)

Dissolve 2 g of sarkosyl (*N*-lauroylsarcosine) per 10 ml of water. Store for 1 year at room temperature.

Sodium acetate, 3 M (pH 5.2)

Dissolve 24.6 g of sodium acetate per 100 ml of water. Adjust pH to 5.2 with acetic acid. Store indefinitely at room temperature.

Tetracycline stock solution, 10 mg/ml

Dissolve 10 mg of tetracycline per 1 ml of ethanol. Store up to 6 months at 4°C protected from light.

COMMENTARY

Background Information

Clostridium difficile is a Gram-positive anaerobic bacterium that causes potentially fatal intestinal disease in humans. Infections in humans can be chronic and incredibly difficult to treat (O'Brien et al., 2007). *C. difficile* infection is often precipitated by antibiotic therapy. Such treatment results in an alteration in the normally protective intestinal flora. *C. difficile* is thought to enter the body as dormant spores via the fecal-oral route of infection and germinate to produce vegetative cells capable of generating toxins that contribute to the inflammation and diarrhea characteristic of infection (Voth and Ballard, 2005; Lyras et al., 2009).

In 1935, Hall and O'Toole reported that a "difficult Clostridium" was present in the intestinal flora of newborns (Hall and O'Toole, 1935). This bacterium, first dubbed *Bacillus difficile*, was initially difficult to isolate and grew slowly in culture. By the 1950s, antibiotic-associated diarrheal diseases had become more common, but other organisms were thought to be the causative agents of disease (Hummel et al., 1964; Khan and Hall, 1966). In 1974, a study of the causation of diarrheal disease in patients treated with the antibiotic clindamycin concluded that pseudomembranous colitis was associated with the administration of antibiotics (Tedesco et al., 1974). However, it was not until 1978 that *C. difficile* was reported as the agent of antibiotic-associated colitis (Bartlett et al., 1978). As a

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result, Bartlett and colleagues investigated the root of *C. difficile*-associated symptoms, which led to the conclusion that toxins produced by the bacteria are directly responsible for pseudo membranous colitis (Bartlett et al., 1977, 1978). On the basis of these results, other investigators were able to identify major risk factors for infection with *C. difficile*, namely, antibiotic exposure, disturbance of the normal colonic flora, advanced age, and hospitalization (Viscidi et al., 1981; McFarland et al., 1989; Fekety and Shah, 1993; Karlstrom et al., 1998).

Progression of *C. difficile* disease is dependent on the ability of the bacteria to reside, multiply, and produce toxins within the intestinal tract. Since 2002, an epidemic strain has spread globally (toxinotype III, PFGE type NAP1/REA type B1/Ribotype 027), and is associated with a significant increase in morbidity and mortality due to the increased production of the virulence factors, toxins A and B (McDonald et al., 2005; Warny et al., 2005; Redelings et al., 2007). These toxins act by glycosylating host Rho GTPases in intestinal epithelial cells, resulting in disruption of the cytoskeleton and cell death (Just et al., 1995). Some strains produce an additional toxin, CDT or binary toxin (Perelle et al., 1997). CDT toxin has been shown to induce microtubule formation and facilitate adherence to intestinal epithelial cells; however, the role of this toxin in disease progression is not clear (Schwan et al., 2009).

To date, research efforts involving the genetic manipulation of *C. difficile* lag far behind the progress made in "model" prokaryotic organisms such as *Escherichia coli* and *Bacillus subtilis*. This lag has largely been due to the lack of reliable genetic tools available for work with this organism. However, in the past decade several tools and protocols have been adapted for use in *C. difficile*, including methods for gene disruption and the introduction of DNA into cells via plasmids and transposons, as described in this unit. The development of these techniques has made it possible to insertionally disrupt genes in *C. difficile*, a feat that had never been successful prior to 2006 (O'Connor et al., 2006). Advancements such as the adaptation of group II introns for gene disruption (TargeTron/ClosTron system) have permitted the disruption of many more *C. difficile* genes (Dineen et al., 2007; Heap et al., 2007, 2010; Emerson et al., 2009; Kirby et al., 2009; Twine et al., 2009; Underwood et al., 2009; Burns et al., 2010). The techniques described herein have the potential to make research of *C. difficile* and its associated diseases much more accessible to genetic study.

Critical Parameters and Troubleshooting

Extraction of C. difficile genomic DNA—Effective lysing of cells is critical for DNA extraction. If no clearing of cell extracts occurs after addition of sarkosyl, the likelihood of the subsequent steps yielding DNA is low. Restart the protocol with fresh culture and increase the incubation times in Basic Protocol 1, steps 5 and 7.

Quick genomic DNA extraction from C. difficile for PCR—If cell extracts do not clear after incubation with proteinase K, a new extraction is recommended. The 90°C incubation post-proteinase K treatment is essential for preventing degradation of enzymes in subsequent reactions (i.e., *Taq*).

Extraction of C. difficile RNA—Special care should be taken to avoid contamination by RNases while working with RNA.

Transfer of Tn916 from a B. subtilis donor strain to C. difficile recipient strain—The addition of KNO_3 to BHIS plates is essential for growth of *B. subtilis* in an anaerobic environment (Glaser et al., 1995). The use of multiple *B. subtilis* transconjugants for mating to *C. difficile* may increase conjugation success.

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Transfer of plasmid DNA from E. coli to C. difficile—Maintaining stable replicating plasmids in *C. difficile* is considerably easier than maintaining plasmids that are not stable, and as a result the transfer of stable plasmids is also more efficient. In addition, *E. coli* used for mating should be gently manipulated to avoid destruction of conjugation machinery, which will decrease mating efficiencies.

Gene disruption in C. difficile using group II intron—The algorithm output will indicate insertion sites with the highest predicted success; however, this process is not perfect. Designing multiple insertion targets for each gene is recommended to increase the chances of achieving successful intron targeting.

Site-directed mutagenesis using unstable plasmids—Maintenance of unstable plasmids can be problematic, and therefore constant selective pressure (antibiotic) is required. It is generally accepted that the probability of homologous integration into the chromosome increases proportionally with the length of homologous target DNA. Single-homologous integration (via replicating plasmid) into genes, for which there is selective pressure to restore the wild-type allele, is likely to result in mutant instability and loss of plasmid integration.

Anticipated Results

Extraction of C. difficile genomic DNA—Yield of DNA from this protocol varies depending on density of cell culture and efficiency of cell lysis, but is typically between 100 and 1000 ng/μl. Purity of the DNA is sufficient for standard molecular biology uses.

Quick genomic DNA extraction from C. difficile for PCR—This protocol generates enough DNA for ~50 standard PCR reactions. However, the DNA purity is low, so procedures requiring greater purity should refer to the genomic DNA extraction protocol (see Basic Protocol 1).

Extraction of C. difficile RNA—This protocol generates high-quality RNA, although the amount will vary by growth condition and density of the starting culture. This protocol has advantages over standard Gram-positive RNA kit extractions (featuring column purifications) because small RNAs are extracted as well as larger mRNA transcripts.

Transfer of Tn916 from a B. subtilis donor strain to C. difficile recipient strain—Efficiency of Tn*916* transfer from *B. subtilis* to *C. difficile* is very good. This protocol is useful for complementation of disrupted genes and introduction of reporter fusion constructs in *C. difficile*. At the completion of this protocol, the Tn*916* should be mobilized into the *C. difficile* chromosome.

Transfer of plasmid DNA from E. coli to C. difficile—Conjugation of plasmids from *E. coli* is the standard method for plasmid introduction into *C. difficile*. However, efficiency of transfer is dependent on the stability of the plasmid in *C. difficile*. Successful completion of this protocol will result in the mobilization of a plasmid into *C. difficile*.

Gene disruption in C. difficile using group II intron—Efficiency of group II intron targeting varies by gene and insertion site. This protocol has been effective for knocking out many *C. difficile* genes; however, some genes may not have good target sites or simply not target well. Assuming an efficient integration site is chosen, this protocol will lead to the inactivation of the target gene with the group II intron.

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Site-directed mutagenesis using unstable plasmids—Though inefficient, at the end of this protocol, a plasmid disruption of the target gene should be observed.

Time Considerations

Extraction of C. difficile genomic DNA (Basic Protocol 1)—Overnight growth is usually required to achieve cell densities sufficient for good DNA yield. The actual DNA extraction protocol may take up to 6 hr depending on the amount of incubation time required to achieve cell lysis, which can vary by strain.

Quick genomic DNA extraction from C. difficile for PCR (Alternate Protocol)—This protocol generally requires <2 hr to complete.

Extraction of C. difficile RNA (Basic Protocol 2)—The time required for preparation of cultures for RNA extraction is dependent on the strain and growth conditions used. The actual RNA extraction procedure takes ~2 hr for a single sample.

Transfer of Tn916 from a B. subtilis donor strain to C. difficile recipient strain (Basic Protocol 3)—Much of the time required for this protocol is spent on culture growth. Actual manipulation time depends on the number of matings performed. Allow ~30 min per day for manipulations in the chamber.

Transfer of plasmid DNA from E. coli to C. difficile (Basic Protocol 4)—Much of the time required for this protocol is spent on culture growth. Actual manipulation time depends on the number of matings done. Allow ~30 min per day for manipulations in the chamber and 10 to 15 min to start the *C. difficile* and *E. coli* cultures.

Gene disruption in C. difficile using group II intron (Basic Protocol 5)—Much of the time required for this protocol is spent on culture growth. Actual manipulation time depends on the number of matings done. Allow ~30 min per day for manipulations in the chamber and 10 to 15 min to start the *C. difficile* and *E. coli* cultures.

Site-directed mutagenesis using unstable plasmids (Basic Protocol 6)—Much of the time required for this protocol is spent on culture growth. Actual manipulation time depends on the number of matings done. Allow ~30 min per day for manipulations in the chamber and 10 to 15 min to start the *C. difficile* and *E. coli* cultures.

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Literature Cited

Bartlett JG, Onderdonk AB, Cisneros RL, Kasper DL. Clindamycin-associated colitis due to a toxin-producing species of Clostridium in hamsters. J. Infect. Dis. 1977; 136:701–705. [PubMed: 915343]

Bartlett JG, Moon N, Chang TW, Taylor N, Onderdonk AB. Role of *Clostridium difficile* in antibiotic-associated pseudomembranous colitis. Gastroenterology. 1978; 75:778–782. [PubMed: 700321]

Burns DA, Heap JT, Minton NP. SleC is essential for germination of *Clostridium difficile* spores in nutrient-rich medium supplemented with the bile salt taurocholate. J. Bacteriol. 2010; 192:657–664. [PubMed: 19933358]

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

Dineen SS, Villapakkam AC, Nordman JT, Sonenshein AL. Repression of *Clostridium difficile* toxin gene expression by CodY. Mol. Microbiol. 2007; 66:206–219. [PubMed: 17725558]

Dupuy B, Sonenshein AL. Regulated transcription of *Clostridium difficile* toxin genes. Mol. Microbiol. 1998; 27:107–120. [PubMed: 9466260]

Emerson JE, Reynolds CB, Fagan RP, Shaw HA, Goulding D, Fairweather NF. A novel genetic switch controls phase variable expression of CwpV, a *Clostridium difficile* cell wall protein. Mol. Microbiol. 2009; 74:541–556. [PubMed: 19656296]

Fekety R, Shah AB. Diagnosis and treatment of *Clostridium difficile* colitis. JAMA. 1993; 269:71–75. [PubMed: 8416409]

Glaser P, Danchin A, Kunst F, Zuber P, Nakano MM. Identification and isolation of a gene required for nitrate assimilation and anaerobic growth of *Bacillus subtilis*. J. Bacteriol. 1995; 177:1112–1115. [PubMed: 7860592]

Hall IC, O'Toole E. Intestinal flora in newborn infants with a description of a new pathogenic anaerobe, *Bacillus difficilis*. Am. J. Dis. Child. 1935; 49:390–402.

Heap JT, Pennington OJ, Cartman ST, Carter GP, Minton NP. The ClosTron: A universal gene knock-out system for the genus *Clostridium*. J. Microbiol. Methods. 2007; 70:452–464. [PubMed: 17658189]

Heap JT, Pennington OJ, Cartman ST, Minton NP. A modular system for *Clostridium* shuttle plasmids. J. Microbiol. Methods. 2009; 78:79–85. [PubMed: 19445976]

Heap JT, Kuehne SA, Ehsaan M, Cartman ST, Cooksley CM, Scott JC, Minton NP. The ClosTron: Mutagenesis in *Clostridium* refined and streamlined. J. Microbiol. Methods. 2010; 80:49–55. [PubMed: 19891996]

Hummel RP, Altemeier WA, Hill EO. Iatrogenic staphylococcal enterocolitis. Ann. Surg. 1964; 160:551–560. [PubMed: 14206857]

Just I, Selzer J, Wilm M, von Eichel-Streiber C, Mann M, Aktories K. Glucosylation of Rho proteins by *Clostridium difficile* toxin B. Nature. 1995; 375:500–503. [PubMed: 7777059]

Karlstrom O, Fryklund B, Tullus K, Burman LG. A prospective nationwide study of *Clostridium difficile*-associated diarrhea in Sweden. The Swedish *C. difficile* Study Group. Clin. Infect. Dis. 1998; 26:141–145. [PubMed: 9455523]

Khan MY, Hall WH. *Staphylococcal enterocolitis*—Treatment with oral vancomycin. Ann. Intern. Med. 1966; 65:1–8. [PubMed: 5936663]

Kirby JM, Ahern H, Roberts AK, Kumar V, Freeman Z, Acharya KR, Shone CC. Cwp84, a surface-associated cysteine protease, plays a role in the maturation of the surface layer of *Clostridium difficile*. J. Biol. Chem. 2009; 284:34666–34673. [PubMed: 19808679]

Lyras D, Rood JI. Conjugative transfer of RP4-oriT shuttle vectors from *Escherichia coli* to *Clostridium perfringens*. Plasmid. 1998; 39:160–164. [PubMed: 9514706]

Lyras D, O'Connor JR, Howarth PM, Sambol SP, Carter GP, Phumoonna T, Poon R, Adams V, Vedantam G, Johnson S, Gerding DN, Rood JI. Toxin B is essential for virulence of *Clostridium difficile*. Nature. 2009; 458:1176–1179. [PubMed: 19252482]

Manganelli R, Provvedi R, Berneri C, Oggioni MR, Pozzi G. Insertion vectors for construction of recombinant conjugative transposons in *Bacillus subtilis* and *Enterococcus faecalis*. FEMS Microbiol. Lett. 1998; 168:259–268. [PubMed: 9835037]

McDonald LC, Killgore GE, Thompson A, Owens RC Jr, Kazakova SV, Sambol SP, Johnson S, Gerding DN. An epidemic, toxin gene-variant strain of *Clostridium difficile*. N. Engl.J. Med. 2005; 353:2433–2441. [PubMed: 16322603]

McFarland LV, Mulligan ME, Kwok RY, Stamm WE. Nosocomial acquisition of *Clostridium difficile* infection. N. Engl. J. Med. 1989; 320:204–210. [PubMed: 2911306]

Mullany P, Wilks M, Tabaqchali S. Transfer of Tn*916* and Tn*916* delta E into *Clostridium difficile*: Demonstration of a hotspot for these elements in the *C. difficile* genome. FEMS Microbiol. Lett. 1991; 63:191–194. [PubMed: 1647998]

O'Brien JA, Lahue BJ, Caro JJ, Davidson DM. The emerging infectious challenge of *Clostridium difficile*–associated disease in Massachusetts hospitals: Clinical and economic consequences. Infect. Control Hosp. Epidemiol. 2007; 28:1219–1227. [PubMed: 17926270]

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

O'Connor JR, Lyras D, Farrow KA, Adams V, Powell DR, Hinds J, Cheung JK, Rood JI. Construction and analysis of chromosomal *Clostridium difficile* mutants. Mol. Microbiol. 2006; 61:1335–1351. [PubMed: 16925561]

Perelle S, Gibert M, Bourlioux P, Corthier G, Popoff MR. Production of a complete binary toxin (actin-specific ADP-ribosyltransferase) by *Clostridium difficile* CD196. Infect. Immun. 1997; 65:1402–1407. [PubMed: 9119480]

Redelings MD, Sorvillo F, andMascola L. Increase in *Clostridium difficile*–related mortality rates, United States, 1999–2004. Emerg. Infect. Dis. 2007; 13:1417–1419. [PubMed: 18252127]

Schwan C, Stecher B, Tzivelekidis T, van Ham M, Rohde M, Hardt WD, Wehland J, Aktories K. *Clostridium difficile* toxin CDT induces formation of microtubule-based protrusions and increases adherence of bacteria. PLoS Pathog. 2009; 5 e1000626.

Showsh SA, Andrews RE Jr. Tetracycline enhances Tn916-mediated conjugal transfer. Plasmid. 1992; 28:213–224. [PubMed: 1334267]

Tedesco FJ, Barton RW, Alpers DH. Clindamycin-associated colitis. A prospective study. Ann. Intern. Med. 1974; 81:429–433. [PubMed: 4412460]

Trieu-Cuot P, Carlier C, Poyart-Salmeron C, Courvalin P. Shuttle vectors containing a multiple cloning site and a lacZ alpha gene for conjugal transfer of DNA from Escherichia coli to Gram-positive bacteria. Gene. 1991; 102:99–104. [PubMed: 1864514]

Twine SM, Reid CW, Aubry A, McMullin DR, Fulton KM, Austin J, Logan SM. Motility and flagellar glycosylation in *Clostridium difficile*. J. Bacteriol. 2009; 191:7050–7062. [PubMed: 19749038]

Underwood S, Guan S, Vijayasubhash V, Baines SD, Graham L, Lewis RJ, Wilcox MH, Stephenson K. Characterization of the sporulation initiation pathway of *Clostridium difficile* and its role in toxin production. J. Bacteriol. 2009; 191:7296–7305. [PubMed: 19783633]

Viscidi R, Willey S, Bartlett JG. Isolation rates and toxigenic potential of *Clostridium difficile* isolates from various patient populations. Gastroenterology. 1981; 81:5–9. [PubMed: 7239125]

Voth DE, Ballard JD. *Clostridium difficile* toxins: Mechanism of action and role in disease. Clin. Microbiol. Rev. 2005; 18:247–263. [PubMed: 15831824]

Warny M, Pepin J, Fang A, Killgore G, Thompson A, Brazier J, Frost E, McDonald LC. Toxin production by an emerging strain of *Clostridium difficile* associated with outbreaks of severe disease in North America and Europe. Lancet. 2005; 366:1079–1084. [PubMed: 16182895]

Wren BW, Tabaqchali S. Restriction endonuclease DNA analysis of *Clostridium difficile*. J. Clin. Microbiol. 1987; 25:2402–2404. [PubMed: 2828418]