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Genetic Manipulation of *Campylobacter jejuni*

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Abstract

Molecular manipulation has been a limiting factor in *C. jejuni* research for many years. Recent advances in molecular techniques adapted for *C. jejuni* have furthered our understanding of the organism. This unit is dedicated to common molecular tools in bacterial research specifically tailored for *C. jejuni*. These include colony PCR, DNA isolation, and RNA isolation. The unit also reviews techniques for genetic manipulation, such as the use of plasmids, natural transformation, electroporation, conjugation, and transposition. In addition, a reporter system, the arylsulfatase assay, can be used to study gene expression.

Keywords

natural transformation; arylsulfatase; genomic DNA; RNA isolation

INTRODUCTION

Molecular biology has been a limiting factor in *C. jejuni* research. Indigenous plasmids are commonly used for cloning and expression vectors. *C. jejuni* is most efficiently transformed by plasmid DNA from its own species, thereby limiting the ease of genetic manipulation. Mutations are produced in *C. jejuni* using allelic replacement via suicide plasmids. Because these methods are described elsewhere (see Hendrixson et al., 2001, for a strategy that works well for *C. jejuni*), we will not discuss them in this unit, which will focus on moving plasmids into *C. jejuni* by conjugation (Basic Protocol 1), electroporation (Basic Protocol 2), and natural transformation (Basic Protocol 3). Numerous plasmids have been developed for *C. jejuni* research and may be used in lieu of specific plasmids described here. However, those discussed (see Table 8A.2.1) are commonly used and have proven successful in many studies. An inducible expression plasmid has yet to be developed for *C. jejuni*.

This unit also outlines several common molecular techniques for bacterial studies that have been adapted for *C. jejuni*. Common techniques include transposition (Basic Protocol 4), colony PCR (Basic Protocol 5), isolation of DNA (Basic Protocol 6) and RNA (Basic Protocol 7 and Alternate Protocol), and an arylsulfatase assay used as a reporter for *C. jejuni* gene expression studies (Basic Protocol 8).

The sequencing of genomes of several *C. jejuni* strains, as well as the development of a variety of genetic tools for use in *C. jejuni*, have provided the opportunity for rapid expansion in the knowledge of the biology of this organism. The protocols outlined in this

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unit, combined with genome sequence data, can be used to make and complement in-frame deletions, create libraries of transposon mutants, and study gene expression using gene fusions and techniques such as microarrays and qRT-PCR.

CAUTION: Campylobacter jejuni is a Biosafety Level 2 (BSL-2) pathogen. Follow appropriate guidelines and regulation for the handling of pathogenic microorganisms. Proper hand washing is essential because *C. jejuni* has been shown to cause gastroenteritis at a small dose (10 to 100 organisms). See *UNIT 1A.1* and other pertinent resources for more information.

NOTE: Incubations are performed at 37°C in a tri-gas incubator, as recommended in *UNIT 8A.1*.

BASIC PROTOCOL 1: CONJUGATION OF *C. JEJUNI* AND *E. COLI*

One method of moving DNA into *C. jejuni* is through conjugation. Conjugation is the transfer of DNA between bacteria though direct cell-to-cell contact. *E. coli* strains carrying the mobilization plasmid pRK212.1 are able to conjugate with *C. jejuni*. This technique is useful for incorporating replicating plasmids, such as pECO101, pECO102, pRY468pRY109, and pRY112 into *C. jejuni* (Yao et al., 1993; Wiesner et al., 2003). This protocol is modified from one used by the laboratory of Dr. Pat Guerry, Uniformed Service University (pers. comm.).

Materials

DNA fragment of interest

C. jejuni plasmid (e.g., pRY108 or pECO101; also see Table 8A.2.1)

Donor strain: *E. coli* DH5α [pRK212.1]

LB agar plates with appropriate selective antibiotics (*APPENDIX 4A*)

Recipient strain: *C. jejuni* (*UNIT 8A.1*)

Mueller Hinton (MH) agar plates, with and without appropriate selective antibiotics (BD Biosciences)

LB liquid medium with appropriate selective antibiotics (*APPENDIX 4A*)

Mueller Hinton (MH) broth without antibiotics (BD Biosciences)

Additional reagents and equipment for cloning of DNA (Struhl, 2000), introduction of plasmid DNA into *E. coli* (Seidman et al., 1997), growth of *E. coli* on solid medium (Elbing and Brent, 2002a) and in liquid medium (Elbing and Brent, 2002b), and growth of *C. jejuni* (*UNIT 8A.1*)

Construct *E. coli* donor strain

1 Clone fragment to be maintained in *C. jejuni* plasmid (i.e., pRY108, pECO101).

Struhl (2000) contains protocols for cloning of DNA fragments.

2. Move plasmid into *E. coli* DH5*α* [pRK212.1].

    Seidman et al. (1997) describe methods for introducing plasmid DNA into *E. coli* cells.

3. Grow the transformed *E. coli* (Elbing and Brent, 2002a) on LB plates containing selection antibiotics: ampicillin (100 μg/ml), tetracycline (12.5 μg/ml), and plasmid-specific antibiotic (e.g., kanamycin, chloramphenicol).

    Chloramphenicol would be used with pECO102.

Conjugate *E. coli* donor strain into *C. jejuni*

4. Grow recipient strain on MH agar with appropriate antibiotics.

    C. jejuni can be selected for resistance to streptomycin, which does not alter its ability to colonize natural host models or to cause disease in the ferret model. The streptomycin resistance phenotype provides a counter-selectable marker for use in these conjugation experiments. Concentrations of antibiotics used in *C. jejuni* media are listed in *Table 8A.1.2*. It is also possible to select for *C. jejuni* using trimethoprim (10 μg/ml) or cefoperazone.

5. Streak *C. jejuni* recipient strain onto MH plate with appropriate antibiotics for 16 to 20 hr at 37°C in microaerophilic conditions (*UNIT 8A.2*). Start an overnight culture of the *E. coli* donor strain in LB liquid medium with appropriate antibiotics.

6. Inoculate 5 ml LB liquid medium containing selective antibiotic for the plasmid with 375 μl of the overnight culture of the donor strain prepared in step 5. Incubate at 37°C while shaking until OD_{600} = 0.45 to 0.5.

7. Resuspend recipient *C. jejuni* strain from the 16- to 20-hr plates (step 5) in 1.8 ml MH broth without antibiotics to an OD of ~1.0.

8. Wash 0.5 ml of the donor strain culture from step 6 once by microcentrifuging 5 min at 10,000 × *g*, 4°C, removing the supernatant, adding 10 ml MH broth without antibiotics, then centrifuging again as before and removing the supernatant to eliminate any antibiotics. Resuspend donor pellet with 1 ml of *C. jejuni* recipient strain suspension.

9. Spin bacterial mixture for 2 min at full speed in microcentrifuge, and remove supernatant.

10. Resuspend cells in 100 μl MH broth without antibiotics. Spot onto an MH plate without any antibiotics.

11. Incubate plate organism-side-up at 37°C in microaerophilic conditions (*UNIT 8A.1*) for 5 hr.

12. Resuspend bacteria from plate in 1.8 ml MH broth. Pellet bacteria by centrifuging 5 min at 10,000 × *g*, 4°C, and removing the supernatant.

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13 Spread bacteria on MH agar with 10 μg/ml trimethoprim (TMP), 20 μg/ml cefoperazone, 2 mg/ml streptomycin, and an antibiotic for selection of plasmid.

14 Incubate for 4 to 5 days at 37°C in microaerophilic conditions (*UNIT 8A.1*).

BASIC PROTOCOL 2: ELECTROPORATION OF *C. JEJUNI*

Electroporation of bacteria involves the application of electrical current to the bacterial cells, which permeabilizes the membrane, allowing uptake of DNA from the environment. This technique is primarily used for the incorporation of plasmid DNA. This technique was first described for *C. jejuni* by Miller et al. (1988). Electroporation of *C. jejuni* is primarily done to incorporate DNA from suicide plasmids into the chromosome of *C. jejuni*. Electroporation is useful for this because plasmids derived from *E. coli* will not transform well into *C. jejuni* by natural transformation.

Materials

C. jejuni frozen stock (*UNIT 8A.1*)

Mueller Hinton (MH) agar plates with appropriate selective antibiotics (BD Biosciences)

Mueller Hinton (MH) broth without antibiotics (BD Biosciences)

Wash buffer: 15% (v/v) glycerol/9% (w/v) sucrose

DNA of interest, to be electroporated into *C. jejuni* (15 μg DNA/reaction)

SOC medium (see recipe)

Nitrocellulose membrane (0.025-μm pore size VSWP (Millipore, cat. no. VSWP04700)

Electroporation cuvettes (BioRad Gene Pulser Cuvettes, 0.2-cm electrode; cat. no. 165-2006)

Electroporator (BioRad *E. coli* Pulser)

Additional reagents and equipment for streaking bacteria (*APPENDIX 4A*) and growing *C. jejuni* (*UNIT 8A.1*)

Grow *C. jejuni*

1 Streak strain (*APPENDIX 4A*) from frozen stock onto one MH agar plate containing 10 μg/ml trimethoprim (TMP). Grow for 24 hr at 37°C under microaerophilic conditions (*UNIT 8A.1*).

2 Restreak strain onto one MH agar plate containing 10 μg/ml TMP, using a heavy inoculum. Grow for 16 hr at 37°C under microaerophilic conditions (*UNIT 8A. 1*).

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Prepare electrocompetent *C. jejuni* cells

3 Resuspend bacteria in 1.8 ml MH broth (without antibiotics) using an automatic pipettor and 1-ml pipet tip by pipetting 900 μl of broth up and down across the overnight lawn of bacteria.

4 Pellet bacteria by microcentrifugation for 5 min at maximum speed, 4°C.

5 Gently resuspend pellet in 2 ml ice-cold wash buffer.

6 Pellet bacteria by centrifugation for 5 min at full speed in microcentrifuge at 4°C

7 Repeat steps 5 and 6 three times.

8 Resuspend pellet in 900 μl ice-cold wash buffer. Use immediately (keep cold on ice).

Perform electroporation

9 For large volumes (>5 μl) of DNA that have been prepared in TE buffer, dialyze DNA on top of a nitrocellulose membrane floating in 20 ml distilled deionized water for 20 min.

10 Cool electroporation cuvettes on ice.

11 In a microcentrifuge tube, combine 50 μl electrocompetent bacteria (from step 8) and 15 μg DNA. Keep on ice.

12 Transfer bacteria-DNA mix to an electroporation cuvette. Electroporate the sample at 2.5 kV, 200 Ω, and 25 μF.

The time constant is usually ~5 msec.

13 Flush cuvette with 100 μl SOC medium. Spread bacteria onto MH agar plate containing 10 μg/ml TMP.

14 Incubate plate for 5 hr at 37°C under microaerophilic conditions (*UNIT 8A.1*).

15 Harvest bacteria by resuspending in 1.8 ml of MH broth without antibiotics. Microcentrifuge bacteria for 2 min at maximum speed and remove supernatant. Resuspend bacteria in 100 μl MH broth. Plate onto MH agar containing the appropriate selective antibiotics.

16 Incubate for 2 to 4 days at 37°C in microaerophilic atmosphere (*UNIT 8A.1*).

BASIC PROTOCOL 3: NATURAL TRANSFORMATION OF *C. JEJUNI*

C. jejuni is naturally transformable, meaning that it readily takes up DNA from the environment. Natural transformation can lead to genetic diversity within a population. It is also a useful tool for molecular biology in bacteria. This technique is useful in *C. jejuni* for the uptake of its own DNA, but not foreign DNA (i.e., *E. coli* derived) from the environment (Wang and Taylor, 1990; Wiesner et al., 2003).

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Materials

C. jejuni frozen stock (*UNIT 8A.1*)

Mueller Hinton (MH) agar plates with and without appropriate selective antibiotics (BD Biosciences)

Mueller Hinton (MH) broth without antibiotics (BD Biosciences)

DNA of interest, to be transformed into *C. jejuni* (*C. jejuni* genomic DNA prepared as in Basic Protocol 6)

Additional reagents and equipment for streaking bacteria (*APPENDIX 4A*) and growing *C. jejuni* (*UNIT 8A.1*)

Grow *C. jejuni*

1 From frozen stock, streak strain (*APPENDIX 4A*) onto MH agar containing 10 μg/ml trimethoprim (TMP). Grow for 16 to 24 hr at 37°C under microaerophilic conditions (*UNIT 8A.1*).

2 From the 16 to 24 hr growth plate, streak strain onto MH agar containing 10 μg/ml TMP, using a heavy inoculum. Streak plates ~16 hr before the start of the transformation experiment.

3 Make fresh MH agar (without antibiotics) and pipet 1-ml aliquots into plastic tubes.

4 Resuspend the entire growth from the 16-hr plate (from step 2) in 1 ml MH broth (without antibiotics).

Perform transformation

5 Dilute the bacteria to an OD_{600} of 0.5 (~1×10^9 cfu/ml) with MH broth (without antibiotics).

6 Add 0.5 ml resuspended bacteria to each tube (from step 3) containing 1 ml of solidified MH agar. Incubate tubes for 3 hr at 37°C in microaerophilic conditions (*UNIT 8A.1*).

7 Gently mix tubes.

8 Add 5 to 10 μg of the DNA to be transformed to each tube and pipet up and down.

9 Incubate for 4 hr at 37°C in microaerophilic conditions (*UNIT 8A.1*).

10 Transfer bacteria into a microcentrifuge tube. Microcentrifuge for 2 min at maximum speed, room temperature.

11 Resuspend bacteria and plate onto MH agar containing 10 μg/ml TMP and appropriate selection antibiotics.

12 Incubate plates at 37°C in microaerophilic environment (*UNIT 8A.1*) for 2 to 4 days.

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BASIC PROTOCOL 4: TRANSPOSON MUTAGENESIS OF *C. JEJUNI*

Transposons are DNA elements that can insert themselves relatively randomly throughout the genome. Transposon mutagenesis of *C. jejuni* has been a widely accepted tool for genetic manipulation, and this method has identified a number of *C. jejuni* genes needed for colonization and motility (Golden et al., 2000; Hendrixson et al., 2001) A number of transposons have been developed for *C. jejuni*. This section will outline the protocol for in vitro transposition of *C. jejuni* with a mariner-based transposon (Lampe et al., 1999; Hendrixson, 2001). An alternative, pOTHM, is also a mariner-based transposon method, and specifics on that system can be found in Golden et al. (2000). The delivery plasmids and corresponding transposons used in previous studies are listed in Table 8A.2.2.

Materials

5× salt buffer (see recipe)10 mg/ml bovine serum albumin (BSA)

100 mM dithiothreitol (DTT)

Donor DNA (containing transposon)

Recipient DNA (containing target of mutagenesis *or* total *C. jejuni* genomic DNA prepared as in Basic Protocol 6)

Transposase: (available from Dr. David Lampe (Lampe et al., 1999)

TE buffer, pH 8.0 (*APPENDIX 2A*)

dNTP mix: 1.25 mM each dNTP (*APPENDIX 2A*)

T4 DNA polymerase and T4 DNA polymerase buffer (Invitrogen)

T4 ligase and T4 ligase buffer (Invitrogen)

30° and 70°C water baths and 11° and 16°C recirculating water baths (or thermal cycler)

Nitrocellulose membrane (0.025 µm pore size VSWP (Millipore, cat. no. VSWP04700)

Additional reagents and equipment for phenol:chloroform extraction and ethanol precipitation of DNA (Moore and Dowhan, 2002)

Perform transposition reaction

1 Prepare transposition reaction by combining the following:

16.0 µl 5× salt buffer

2.0 µl 10 mg/ml BSA

1.6 µl 100 mM DTT

1.0 µg donor DNA

2.0 µg recipient DNA

500 ng transposase

Distilled deionized H_2O to 80 μl

Incubate reactions at 30°C for 4 hr.

Purify DNA

2. Extract DNA once with phenol:chloroform, then ethanol precipitate DNA (Moore and Dowhan, 2002).
3. Microcentrifuge DNA 30 min at maximum speed, 4°C.
4. Remove supernatant and resuspend pellet in 40 μl TE buffer, pH 8.0.

Perform repair reactions

The repair reactions are necessary to repair small gaps at the transposon-chromosomal junctions. The first reaction (step 5) fills in the gaps, while the ligase reaction (step 8) completes repair.

5. Prepare first repair reaction by combining the following on ice (total volume, 60 μl):

   40 μl DNA from transposition reaction

   6 μl T4 DNA polymerase buffer

   4.8 μl dNTP mix

   7.7 μl distilled H_2O

   1.5 μl 1 U/μl T4 DNA polymerase.

6. Incubate the reaction at 11°C for 20 min in a recirculating water bath or thermal cycler.
7. Inactivate the polymerase by incubating the reaction at 75°C for 15 min.
8. Prepare second repair reaction by combining the following on ice:

   60 μl reaction mixture from first repair reaction (step 5)

   12 μl T4 DNA ligase buffer

   1.5 μl T4 DNA ligase

   46.5 μl dH_2O.

9. Incubate at 16°C overnight in a recirculating water bath or thermal cycler.

Use DNA for transformation

10. Dialyze DNA on top of a nitrocellulose membrane floating on 10 to 20 ml distilled deionized water for 20 min.
11. Use all of the reaction for one transformation of *C. jejuni* (see Basic Protocol 3).

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BASIC PROTOCOL 5: PREPARATION OF *C. JEJUNI* TEMPLATE FOR COLONY PCR

Colony PCR is a useful tool to screen for newly constructed mutants. Individual colonies take 3 to 5 days to grow on MH agar containing 10 μg/ml TMP in microaerophilic conditions at 37°C. Once individual colonies are visible, colony PCR can be used to confirm any mutations made. The *C. jejuni* DNA preparation for PCR requires boiling the *C. jejuni* strain in K buffer. This is done by spotting the isolated colony onto a fresh plate, then resuspending the colony in K buffer and incubating the sample at 100°C.

Materials

Proteinase K

K buffer (see recipe)

100°C water bath

Additional reagents and equipment for PCR (Kramer and Coen, 2001)

1. Add proteinase K to a final concentration of 100 μl/ml in K buffer immediately before use.
2. Resuspend one or two isolated colonies in this 100 μl of K buffer containing proteinase K, using a toothpick or a similar implement.

   Spot the individual colonies onto a fresh plate of MH agar containing 10 μg/ml TMP before resuspending in K buffer and keep in microaerophilic conditions at 37°C to preserve the colonies. Individual colonies will take 3 to 5 days to grow on MH agar containing 10 μg/ml TMP in a tri-gas incubator. See UNIT 8A.1 for further information.
3. Place in 100°C water bath for 10 min.
4. Microcentrifuge 5 min at 10,000 rpm to remove cell debris.
5. Use 2 to 5 μl of the supernatant for 50-μl PCR reactions.

BASIC PROTOCOL 6: ISOLATION OF *C. JEJUNI* GENOMIC DNA

Isolated bacterial DNA can be used for a multitude of purposes, such as cloning of specific genes by PCR, sequencing, or transposon mutagenesis. Although kits such as Qiagen's Genomic DNA Isolation Kit can be utilized, higher yields of genomic DNA are achieved by using the protocol outlined here.

Materials

C. jejuni frozen stock (*UNIT 8A.1*)

Mueller Hinton (MH) agar plates (BD Biosciences) with 10 μg/ml trimethoprim (TMP)

Phosphate-buffered saline (PBS; *APPENDIX 2A*)

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10 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)/0.1 mM EDTA

10 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)/0.1 mM EDTA containing 1 mg/ml lysozyme

Proteinase K

20% (w/v) SDS (*APPENDIX 2A*)

Buffered phenol (*APPENDIX 2A*)

24:1 chloroform:isoamyl alcohol

7.5 M ammonium acetate

Isopropanol

70% (v/v) ethanol

1 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)/0.1 mM EDTA

Filter paper (Whatman)

Additional reagents and equipment for growing *C. jejuni* (*UNIT 8A.1*) and phenol/chloroform extraction of DNA (Moore and Dowhan, 2002)

Days 1 and 2: Grow *C. jejuni*

1. From a frozen stock, streak strain onto MH with 10 μg/ml trimethoprim (TMP) agar. Grow for 16 to 24 hr at 37°C under microaerophilic conditions (*UNIT 8A.1*).
2. From the 16- to 24-hr growth plate incubated in step 1, restreak strain onto one plate of MH agar containing 10 μg/ml TMP, using a heavy inoculum. Incubate 18 hr at 37°C under microaerophilic conditions (*UNIT 8A.1*).

Day 3: Isolate and purify DNA from *C. jejuni*

3. Resuspend *C. jejuni* from 18-hr growth plate in PBS and microcentrifuge 5 min at 10,000 rpm, 4°C.
4. Remove supernatant and resuspend pellet in 200 μl of 10 mM Tris·Cl, pH 8.0/0.1 mM EDTA.
5. Add 400 μl of 10 mM Tris·Cl, pH 8.0/0.1 mM EDTA, containing 1 mg/ml lysozyme.
6. Incubate on ice for 5 min.
7. Add proteinase K to 20 μg/ml and mix gently.
8. Add 50 μl 20% SDS. Mix gently to clear the lysate.
9. Incubate with gentle rocking at 37°C for 30 min.
10. Add 200 μl buffered phenol. Vortex and microcentrifuge 5 min at 12,000 rpm, room temperature.

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Phenol extraction may have to be repeated a couple more times by transferring the aqueous (upper) phase to a fresh tube and adding more phenol. When the phases separate well and quickly, further phenol extractions are unnecessary.

Additional detail on phenol/chloroform extraction of DNA is found in Moore and Dowhan (2002).

11 Extract twice with 24:1 chloroform:isoamyl alcohol.

12 Add 0.5 vol of 7.5 M ammonium acetate and mix. Fill tube with isopropanol.

13 Mix well. Allow DNA to precipitate at −80°C for at least 1 hr.

14 Collect DNA by microcentrifuging 10 min at 12,000 rpm, 4°C.

15 Carefully decant isopropanol and rinse pellet twice with 70% ethanol. Microcentrifuge 5 min at 12,000 rpm, 4°C.

16 Carefully pour off the last wash, and wick out the remaining ethanol with Whatman filter paper. Dry the pellet under vacuum in a Speedvac evaporator for about 5 min.

17 Resuspend pellet in 300 μl of 1 mM Tris·Cl, pH 7.5/0.1 mM EDTA.

Resuspension should be allowed to take place overnight. If necessary, pipet with a large-orifice genomic tip.

BASIC PROTOCOL 7: ISOLATION OF RNA FROM *C. JEJUNI*

The protocol outlined below is a variation on the RNeasy protocol. It was modified from a procedure recommended by Drs. Nick Dorrell and Brendan Wren, London School of Hygiene and Tropical Medicine (pers. comm.). As with all RNA work, everything must be RNase-free. Clean the bench and pipets with ethanol and RNase-eliminating solutions, such as Eliminase, before starting. Make sure all solutions are prepared using RNase-free reagents and RNase-free water.

Materials

C. jejuni frozen stock (*UNIT 8A.1*)

Mueller Hinton (MH) agar plates (BD Biosciences) with 10 μg/ml trimethoprim (TMP)

Mueller Hinton (MH) broth (BD Biosciences) with 10 μg/ml trimethoprim (TMP)

RNA Protect Bacteria Reagent (Qiagen)

TE buffer, pH 8.0 (*APPENDIX 2A*) containing 1 mg/ml lysozyme

RNeasy kit (Qiagen) including:

Buffer RLT

Buffer RW1

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Buffer RPE

RNA Mini Spin columns

2-ml collection tubes

RNase-free H_2O

2-mercaptoethanol (2-ME)

100% ethanol, ice cold

10× DNase buffer

10 U/μl DNase (Invitrogen)

Buffered phenol (*APPENDIX 2A*)

Chloroform

3 M sodium acetate, pH 5.2 (*APPENDIX 2A*)

70% (v/v) ethanol

75-cm^2 tissue culture flask

15-ml conical centrifuge tubes

Centrifuge

Spectrophotometer

Additional reagents and equipment for growing *C. jejuni* (*UNIT 8A.1*)

Grow *C. jejuni*

1. Grow a full lawn of *C. jejuni* from frozen stock on one MH agar plate with 10 μg/ml trimethoprim for 16 to 20 hr.
2. Prepare a biphasic 75-cm^2 tissue culture flask (*UNIT 8A.1*; see Fig. 8A.1.1) with 20 ml MH agar and 20 ml MH broth with 10 μg/ml trimethoprim (TMP).
3. Resuspend the bacteria from the prepared overnight plate (step 1) to an OD_{600} of 0.4 in MH broth with 10 μg/ml trimethoprim (TMP).
4. Dilute the bacterial suspension 1:10 with MH broth containing 10 μg/ml trimethoprim (TMP) and inoculate 80 μl into the biphasic flask.
5. Incubate for 48 hr at 37°C in microaerophilic atmosphere (*UNIT 8A.1*).
6. Transfer 4-ml aliquots of the MH broth containing the 48-hr bacterial growth into 15 ml conical tubes.

   Due to the limitations of the RNeasy Spin Column, better yield is achieved when 4 ml of the culture is used per spin column. Multiple samples can be performed and combined prior to DNase treatment, for higher yields.

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Lyse bacteria

7 Add 8 ml RNA Protect Bacteria Reagent per 4 ml of the 48 hr culture in the 15-ml conical tube. Vortex for 5 sec and incubate for 5 min at room temperature.

8 Centrifuge tubes at 10 min at 10,000 × *g*, 4°C. Decant supernatant and dab on paper towel.

9 Add 400 μl TE buffer, pH 8.0, with 1 mg/ml lysozyme to each tube and resuspend the bacteria by pipetting.

10 Incubate for 10 min at room temperature, vortexing every 2 min.

Extract and purify RNA

11 Add 1200 μl Buffer RLT and 12 μl 2-mercaptoethanol to each tube, and vortex vigorously.

12 Add 1000 μl of 100% ethanol. Mix by pipetting and transfer all to an RNeasy Mini spin column.

13 Centrifuge for 15 sec at 10,000 rpm and discard the flowthrough.

14 Repeat as required, based on volume.

15 Wash an RNeasy column by adding 350 μl Buffer RW1 onto the column, microcentrifuging 15 sec at 10,000 rpm, and discarding the flowthrough. Repeat this wash a second time.

16 Add 500 μl Buffer RPE onto the RNeasy spin column. Microcentrifuge 2 min at 10,000 rpm and discard the flowthrough.

17 Place column into fresh 2-ml collection tube and microcentrifuge for an additional 1 min at 12,000 rpm, to eliminate any remaining buffer.

18 Transfer RNeasy column into a fresh 1.5-ml microcentrifuge tube.

19 Add 50 μl RNase-free water to the RNeasy membrane in the column and centrifuge for 1 min at 10,000 rpm.

20 Combine any multiple samples done from the original 48 hr overnight culture (see step 6).

21 Dilute sample to 200 μl with RNase-free water and add 20 μl of 10× DNase buffer.

22 Add 1 μl of 10 U/μl DNase to each tube. Incubate at room temperature for 1 hr.

23 Add 220 μl of buffered phenol and vortex. Let sit for 5 min at room temperature.

24 Microcentrifuge 15 min at maximum speed, 4°C.

25 Save top layer and transfer to a new microcentrifuge tube.

26 Add 1 volume chloroform, vortex, and let sit for 5 min.

27 Microcentrifuge 15 min at maximum speed, 4°C.

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28. Save top layer and transfer to a new microcentrifuge tube.
29. Precipitate RNA by adding 0.1 vol of 3 M sodium acetate pH 5.2, and 2.5 vol of ice-cold 100% ethanol.
30. Incubate at −20°C overnight (or 2 to 3 hr, minimum).
31. Spin down RNA by microcentrifuging 15 min at maximum speed, 4°C.
32. Remove supernatant, then wash pellet by adding 1 ml of 70% ethanol and microcentrifuging 7 min at maximum speed, 4°C.
33. Remove supernatant and dry pellet completely.
34. Resuspend pellet in 25 to 50 µl RNase-free water.
35. Quantitate RNA by measuring OD_{260} in a spectrophotometer and determine purification of RNA by comparing OD_{260}/OD_{280} (should be >1.8 to 2.0).

    OD_{260} = 1 is equivalent to 40 µg/ml RNA.

ALTERNATE PROTOCOL: TWO-STEP ISOLATION OF RNA FROM *C. JEJUNI* USING TRIZOL

The protocol outlined below, like Basic Protocol 7, utilizes the Qiagen RNeasy kit. However, it has a Trizol extraction preceding the RNeasy columns. The extraction step is for ~1–4 × 10^9 cfu/ml of *C. jejuni*. The Qiagen columns can easily handle up to 1–2 × 10^{10} cfu/ml of *C. jejuni* RNA as long as it was Trizol-extracted first. This is in contrast to Basic Protocol 7, which dilutes the original 10^9 cfu/ml culture to approximately 10^7 cfu/ml of *C. jejuni*. The protocol outlined below was adapted from one used by the lab of Dr. Erin Gaynor, University of British Columbia (Gaynor et al., 2005).

Additional Materials (also see Basic Protocol 7)

10× stop Solution: 5% (v/v) buffered phenol (*APPENDIX 2A*) in 100% ethanol

Liquid N_2

TE buffer, pH 8.0, containing 0.4 mg/ml lysozyme

Trizol reagent (Invitrogen)

Buffer RDD from RNeasy Kit (Qiagen)

Harvest bacteria

1. Prepare a 48-hr liquid culture of *C. jejuni* as described in Basic Protocol 7, steps 1 to 5.
2. Harvest from liquid culture by centrifuging 10 min at 10,000 × *g*, 4°C, removing the supernatant, and resuspending the pellet in 1× stop solution at 1/10 the original volume.
3. Invert several times to mix.

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4. Microcentrifuge 5 to 10 min at 11,000 × *g*, 4°C. Aspirate supernatant.

5. *Optional:* Wash by adding 1 ml of 1× stop solution, centrifuging again as in step 4, and aspirating the supernatant.

    This optional wash removes any left-over medium and helps ensure a clean isolation.

6. Freeze in liquid nitrogen in 1ml aliquots in 1.5-ml microcentrifuge tubes and store at −80°C until ready to isolate RNA.

    This is a good stopping point if taking multiple samples over a period of time.

Perform Trizol extraction

7. Remove tubes from freezer and place into ice once thawed.

8. Resuspend bacteria in 50 µl TE buffer containing 0.4 mg/ml lysozyme.

9. Let sample sit at room temperature for ~5 min.

10. Add 950 µl Trizol reagent and vortex for 1 min to lyse.

11. Add 200 µl chloroform. Shake for 15 sec and let sit at room temperature for 2 to 3 min.

12. Microcentrifuge 15 min at 12,000 rpm, 2° to 8°C.

13. Transfer top (aqueous) phase to a new microcentrifuge tube.

14. Slowly add an equal volume of 70% ethanol to precipitate RNA.

Perform RNeasy purification/DNase treatment

15. Apply RNA solution to an RNeasy Mini spin column in 700-µl aliquots.

16. Add 350 µl RW1 buffer. Microcentrifuge for 15 sec at maximum speed and discard flowthrough.

17. For each column, prepare a mixture of 10 µl of 10 U/µl DNase in 70 µl RDD buffer.

18. Apply the 80 µl of DNase/RDD mix to the middle of the column membrane. Leave at room temperature for 30 min to 1 hr.

19. Add 350 µl RW1 buffer to the column. Microcentrifuge 15 sec at 12,000 rpm, 4°C.

20. Put the column into a new collection tube.

21. Add 500 µl RPE buffer, microcentrifuge 15 sec at 12,000 rpm, 4°C, and discard flowthrough.

22. Add another 500 µl of RPE buffer. Microcentrifuge 15 sec at 12,000 rpm, 4°C, and discard flowthrough.

23. Microcentrifuge 2 min at 12,000 rpm, 4°C, to remove all of the RPE.

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24 Transfer column to a 1.5-ml collection tube.

25 Add 50 μl RNase-free water to the membrane. Microcentrifuge 15 sec at 12,000 rpm, 4°C, to collect RNA. Store at −80°C.

BASIC PROTOCOL 8: ARYLSULFATASE ASSAY

Similar to the *lacZ* reporter system in *E. coli*, the arylsulfatase assay, described in Hendrixson and DiRita (2003), quantifies the expression of a gene of interest. Arylsulfatase cleaves sulfates from substrates, and is encoded by the *astA* gene in *C. jejuni*. The assay measures arylsulfatase activity colorimetrically, using nitrophenyl sulfate as a substrate. Arylsulfatase liberates the sulfate from nitrophenyl sulfate (a colorless compound), forming nitrophenol. Nitrophenol is yellow in color, and by monitoring a colorimetric change, this assay can be used to determine the amount of arylsulfatase activity. Briefly, a promoterless *astA* gene is cloned into the gene of interest on the chromosome of *C. jejuni*, creating an *astA* transcriptional fusion. Expression of the gene of interest can then be monitored through the arylsulfatase assay, described below.

Materials

p-Nitrophenol (Sigma, cat. no 104-8)

Arylsulfatase buffer 1 (AB1): 0.1 M Tris·Cl, pH 7.2 (*APPENDIX 2A*)

C. jejuni (*UNIT 8A.1*)

Mueller Hinton (MH) agar plates with antibiotics (BD Biosciences)

Phosphate-buffered saline (PBS; *APPENDIX 2A*)

Arylsulfatase buffer 2 (AB2): 2 mM tyramine in 0.1 M Tris·Cl, pH 7.2

Arylsulfatase buffer 3 (AB3): 20 mM nitrophenyl sulfate (potassium 4-nitrophenyl sulfate; Sigma, cat. no. N3877) in 0.1 M Tris·Cl, pH 7.2 (*APPENDIX 2A*)

0.2 N NaOH

Spectrophotometer

Additional reagents and equipment for growing *C. jejuni* (*UNIT 8A.1*)

Prepare standard curve

1 Prepare standard curve using *p*-nitrophenol:

a. Prepare 200 μM nitrophenol in buffer AB1 by dissolving 27.82 mg nitrophenol in 10 ml AB1, then diluting this solution 1:100 with buffer AB1 for a 200 μM solution.

b. Serially dilute the 200 μM nitrophenol 1:2 with buffer AB1 for six dilutions.

c. Read OD_{420} for the dilutions of nitrophenol. Create a standard curve of OD_{420} values versus μM nitrophenol.

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Prepare bacteria

2 Streak *C. jejuni* onto MH agar containing 10 μg/ml trimethoprim and grow at 37°C for 24 hr under microaerophilic conditions (*UNIT 8A.1*).

3 Restreak heavily onto one MH agar plate. Grow at 37°C for 18 hr.

4 Resuspend the growth in PBS to an OD_{600} of 1.0.

5 Divide each sample into two 1-ml aliquots.

6 Wash one aliquot with AB1 by microcentrifuging 5 min at maximum speed, room temperature, and removing the supernatant. Add 1 ml of buffer AB1, repeat microcentrifugation, and remove supernatant. Resuspend in 1 ml AB1.

7 Wash the second aliquot in AB2 using the technique described in step 6. Resuspend in 1 ml AB2.

Perform assay

8 Add 200 μl of each sample to 200 μl of freshly prepared AB3 in microcentrifuge tubes.

9 Incubate for 1 hr at 37°C.

10 Stop reaction by adding 800 μl of 0.2 N NaOH.

11 Read OD_{420}.

Samples in AB1 are blanks for AB2 samples.

12 Compare values to standard curve. Report data as nmol or μM nitrophenol.

REAGENTS AND SOLUTION

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

DNase buffer, 10×

200 mM sodium acetate, pH 4.5

100 mM $MgCl_2$

100 mM NaCl

Store up to 1 year at −20°C

K buffer

50 mM KCl

10 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)

2.5 mM $MgCl_2$

0.5% (v/v) Tween 20

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Filter sterilize

Store up to 1 year at room temperature

Salt buffer, 5×

6.25 ml 80% (w/v) glycerol (50% final)

1.25 ml 1 M HEPES, pH 7.9 (125 mM final)

1.25 ml 4 M NaCl (500 mM final)

0.25 ml 1 M $MgCl_2$ (25 mM final)

1 ml H_2O

Store up to 1 year at room temperature

SOB medium

2 g Bacto tryptone (BD Biosciences)

0.5 g yeast extract (BD Biosciences)

0.05 g NaCl

10 ml 250 mM KCl

Adjust pH to 7.0 using 1 M HCl

Store up to 1 year at room temperature

SOC medium

1 ml SOB medium (see recipe)

10 µl 1 M $MgSO_4$

10 µl 1 M $MgCl_2$

20 µl 20% (w/v) glucose

Store up to 1 year at 4°C

COMMENTARY

Background Information

Since its discovery, work on *Campylobacter* species has been limited due to challenging culture requirements and limited genetic tools. However, work in recent years has contributed much to our understanding of *Campylobacter jejuni*, as well as the tools used to study it.

Critical Parameters and Troubleshooting

Conjugation—The conjugation of plasmids into *C. jejuni* from *E. coli* containing the conjugation plasmid pRK212.1 (Basic Protocol 1) is a fairly robust protocol. The protocol is quite forgiving of variation in OD_{600} of both the donor and recipient strains, as long as both

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are collected during exponential growth and mixed at approximately the indicated ratio. The main problem encountered during conjugation is the outgrowth of the donor *E. coli* strain on the selective plates. In general, use of multiple antibiotics to which the *C. jejuni* strain, but not the *E. coli* strain, is resistant solves this problem. In the protocol given here, trimethoprim, cefoperazone, and streptomycin are used together to eliminate growth of *E. coli*. If the recipient strain is resistant to additional antibiotics, they can be used as well. Obviously, streptomycin should only be used with streptomycin-resistant *C. jejuni* (i.e., DRH212).

Electroporation—Electroporations (Basic Protocol 2) may require more troubleshooting and repetitions to achieve introduction of foreign DNA. If the electrocompetent cells are to be used in the future, aliquots can be frozen at −80°C in 20% glycerol until they are needed. In general, if the protocol has been attempted unsuccessfully, new electrocompetent cells and DNA should be prepared before attempting the procedure again.

Electroporation is often used to make insertional mutants, where an antibiotic cassette is inserted into a gene of interest. It can also be used, with the correct intermediate strain, to replace a dual cassette (i.e., chloramphenicol resistance and streptomycin sensitivity) with an in-frame deletion or point mutation. These unmarked mutants can be much more difficult to make, often due to high background (i.e., streptomycin-resistant colonies that retain the chloramphenicol resistance/streptomycin sensitivity cassettes and have not acquired the unmarked mutation). To combat high background in these cases, the following alterations in the protocol can be tried. The electroporation plates can be incubated in a microaerophilic environment (i.e., in zip-lock bags filled with the appropriate gas mix) for the first few days and then transferred to a more stable microaerophilic environment (i.e., Campy-Pak jars or tri-gas incubator; see *UNIT 8A.1*). Additionally, several different concentrations of the selective antibiotic can be tried. If these changes do not lead to successful electroporation, one can test several colonies of the recipient strain for appropriate antibiotic resistance/ sensitivity, colony purify the strain, and make a new stock for future electroporation. Alternatively, success can sometimes depend on the location of the insertion in the intermediate strain. Construct a new intermediate strain with the insertion at a different location within the gene of interest.

Natural transformation—With regard to Basic Protocol 3, it should be noted that *C. jejuni* does not efficiently take up DNA from *E. coli*.

In vitro transposition—For the transposon mutagenesis protocol (Basic Protocol 4), recipient DNA here must be *C. jejuni* derived, or it will not be taken up efficiently in the succeeding natural transformation step.

Preparation of template for PCR—The concentration of proteinase K in the K buffer is an important parameter in Basic Protocol 5. If too much proteinase K is added, no PCR product is seen, presumably because the proteinase K degrades the *Taq* polymerase. If multiple PCRs are planned, or must be performed at a later time, the supernatant can be frozen at −20°C, if removed from the cell debris after the spin in step 4.

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Genomic DNA isolation—DNA isolated via Basic Protocol 6 is of sufficient quality for cloning, but not for sequencing reactions. If sequence from the chromosome is needed, use of the Qiagen Genomic DNA extraction protocols is recommended.

RNA isolation—Working with RNase-free reagents in an RNase free area is critical to Basic Protocol 7. Additionally, although the Alternate Protocol calls for on-column DNase treatment of the RNA, several labs have indicated that this procedure does not always efficiently eliminate any contaminating DNA. If DNA contamination is a problem, perform DNase treatment as indicated in Basic Protocol 7. DNase treatment can be repeated multiple times, followed by phenol and chloroform extractions and RNA precipitation. RNA can be tested for contaminating DNA by a PCR reaction (without prior reverse transcription) and DNase treatment can be repeated until there is no PCR product.

Arylsulfatase assay—The substrate for the arylsulfatase assays (Basic Protocol 8), nitrophenyl sulfate in solution, can be unstable and degrade after long-term storage at −20°C, resulting in high background readings. If this occurs, a fresh stock of nitrophenyl sulfate should correct the problem.

Time Considerations

Table 8A.2.3 provides time considerations for the protocols in this unit. It should be noted that for protocols that aim to produce new strains of *C. jejuni*, the time indicated does not include the time to verify the identity of the new strain, colony purify the strain, and freeze the strain down into new glycerol stocks.

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Table 8A.2.1

Commonly Used Plasmids

	Stable in *C. jejuni*	Source	Notes	Antibiotic resistance	Mobilizable with pRK212.1?	Source
pUC19	N	*E. coli*	Used for cloning	Ampicillin	N (electroporation only)	NEB
pRY108	Y	pILL550	Replicating plasmid	Kanamycin	Y	Yao et al. (1993)
pRY112	Y	pRY110, pWSK29	Replicating plasmid	Chloramphenicol	Y	Yao et al. (1993)
pECO102	Y	pRY112	Replicating plasmid	Chloramphenicol	Y	Wiesner et al. (2003)

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Table 8A.2.2

Transposons

Plasmid	Transposon	Transposase	Reference
pFD1	HImar1	Himar1	Rubin et al. (1999); Kakuda and DiRita (2006)
pEnterprise	picard	Himar1	Hendrixson et al. (2001) or Hendrixson and DiRita (2004)
pFalcon	Solo	Himar1	Hendrixson et al. (2001) or Hendrixson and DiRita (2004)
pOTHM	Mariner	Himar1	Golden et al. (2000)

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Table 8A.2.3

Time Considerations for Protocols

Protocol	Description	Time required
Basic Protocol 1	Conjugation	7–8 days[a]
Basic Protocol 2	Electroporation	5–7 days[a]
Basic Protocol 3	Natural transformation	5–7 days[a]
Basic Protocol 4	In vitro transposition	2 days[b]
Basic Protocol 5	Preparation of template for PCR	<1 day[c]
Basic Protocol 6	Genomic DNA isolation	3–4 days[a]
Basic Protocol 7 or Alternate Protocol	RNA isolation	4–5 days[a]
Basic Protocol 8	Arylsulfatase assay	3 days[a]

[a]Time indicated includes time from streaking *C. jejuni* strain out from freezer stocks to end of protocol as written. For making new strains via introduction of foreign DNA, this includes time until single colonies will appear on selective media, but not the time required to verify the identity of the new strain, isolate single colonies, and freeze down the new strain.

[b]Time does not include purification of transposase or natural transformation of transposition reaction into *C. jejuni*.

[c]Time indicated does not include time required to grow *C. jejuni* strain before beginning the protocol.