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Genetic Manipulation of *Stenotrophomonas maltophilia*

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Abstract

Stenotrophomonas maltophilia is a Gram-negative, aerobic, motile, environmental bacterium that is emerging as an important nosocomial pathogen (Brooke, 2012; Looney, Narita, & Mühlemann, 2009) with high rates of attributable mortality in severely ill patients (Falagas et al., 2009; Paez & Costa, 2008; Sattler, Mason, & Kaplan, 2000; Senol, DesJardin, Stark, Barefoot, & Snyderman, 2002; Weber et al., 2007). *S. maltophilia* is of particular concern to patients suffering from cystic fibrosis (CF) as it has been shown to colonize airway epithelial and establish a chronic infection (Goncalves-Vidigal et al., 2011). Here we describe several molecular techniques for the genetic manipulation of this bacterium, including DNA extraction, RNA extraction, conjugation of plasmids from *E. coli* and allelic exchange.

Keywords

Stenotrophomonas maltophilia; genetic manipulation; nosocomial; hospital acquired infection

INTRODUCTION

Stenotrophomonas maltophilia is a broad-spectrum opportunistic pathogen. A member of the gamma proteobacteria, *Stenotrophomonas maltophilia* is naturally found in the soil, the rhizosphere of plants, as an endoparasite of amoebae, or as a pathogen of vertebrates including fish, reptiles, and mammals (Albini, Abril, Franchini, Hussy, & Filioussis, 2009; Berg, Marten, & Ballin, 1996; Chen, Yang, Hu, & Liu, 2011; Corsaro, Muller, & Michel, 2013; Di Gregorio, Lampis, & Vallini, 2005; Furushita, Okamoto, Maeda, Ohta, & Shiba, 2005; Garbeva, Van Overbeek, Van Vuurde, & Van Elsas, 2001; Harris & Rodgers, 2001; Kralova-Kovarikova, Husnik, Honzak, Kohout, & Fictum, 2012; Petridou, Filioussis, Karavanis, & Kritas, 2010; Rocco, De Gregorio, Colonna, & Di Nocera, 2009; Winther, Andersen, Baptiste, Aalbæk, & Guardabassi, 2010; Zhu et al., 2013). In humans, *Sm* is emerging as a significant cause of concern for clinicians (Gaynes & Edwards, 2005; Jones, 2010; Kim et al., 2014; Pathmanathan & Waterer, 2005; Timsit, Zahar, & Chevret, 2011; Weber et al., 2007). The association of a single microorganism with these diverse pathologies is indicative of a generic virulence program that emerges in opportunistic pathogens due to redeployment of offensive and defensive mechanisms that contribute to

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fitness in the soil environment (Pallen & Wren, 2007). Colonization of implanted medical devices via biofilm production has been noted in many syndromes, but very little is known regarding the genetic mechanisms that control biofilm formation or virulence in this organism.

This unit describes basic techniques for genetic manipulation of *S. maltophilia* in the laboratory. The procedures here are effective with *S. maltophilia* K279a. The reader should consider them a starting point for optimization of procedures for manipulation of other isolates.

CAUTION

Most isolates of *S. maltophilia* are considered to be Biosafety Level 1 (BSL-1) organisms; however, the virulence mechanisms of these organisms are still not well understood. For this reason, it is recommended that BSL-2 handling protocols be followed when working with this organism. For general biosafety information, see *UNIT 1A.1*.

STRATEGIC PLANNING

Strain Selection

Commercial strain collections and most large medical centers contain numerous *S. maltophilia* isolates. Whole genome sequences are available for several isolates, which is convenient for many genetic studies. Since *S. maltophilia* isolates are usually resistant to multiple antibiotics, including those commonly used in the laboratory, investigators are cautioned to check for innate resistances for compatibility with planned selection schemes.

Growth Conditions

Many *S. maltophilia* isolates are sensitive to modest concentrations of salt and the reader is referred to *UNIT 6F.1*.

BASIC PROTOCOL 1

ISOLATION OF *S. MALTOPHILIA* GENOMIC DNA

The method described is modified from (Flamm, Hinrichs, & Thomashow, 1984) and proven effective for isolation of high-quality genomic DNA from a large number of both clinical and environmental isolates of *S. maltophilia*. Genomic DNA extracted with this method is suitable for sensitive techniques such as Southern blots and whole genome sequencing.

Materials

Viable *S. maltophilia* on a petri plate (see *UNIT 6F.1*)

Luria Bertani (LB) Broth (APPENDIX 4A)

SSC, 0.1× (see *REAGENTS AND SOLUTIONS*)

Stock Lysozyme Solution; 50 mg/ml (see *REAGENTS AND SOLUTIONS*)

Stock Proteinase K Solution; 20mg/ml (see *REAGENTS AND SOLUTIONS*)

Phenol; molecular biology grade

Chloroform; molecular biology grade

70% ethanol in nuclease-free ddH₂O, ice-cold

100% (absolute) ethanol, ice-cold

Buffer 1 (see *REAGENTS AND SOLUTIONS*)

Lysis Buffer (see *REAGENTS AND SOLUTIONS*)

TE buffer, pH 8.0 (APPENDIX 2A)

Sodium Chloride (NaCl) Solution, 5M

Microcentrifuge tubes, nuclease free

Orbital shaker set to 37° C, 225 rpm

Centrifuge

Water bath set to 37° C

Inoculating loop or needle, sterile

15 ml and 50 ml conical tubes, sterile

100 ml culture flask, sterile

1. Aseptically, transfer 20 ml sterile LB broth into a 100 ml culture flask and inoculate with a single isolated colony of *S. maltophilia*.
2. Transfer to a shaking incubator set to 225 rpm and incubate at 37° C for 24 hours.
3. Using a sterile pipette, transfer 10 ml of the saturated broth culture to a sterile 15 ml conical tube.
4. Pellet the bacteria by centrifugation at 5000 rpm for 10 minutes at room temperature.
Caution: Ensure that the centrifuge is properly balanced, using a water blank if necessary.
5. Remove the tube from the centrifuge and carefully decant the supernatant into a waste container without disturbing the bacterial pellet.
6. Add 5 ml of 0.1× SSC (see *REAGENTS AND SOLUTIONS*) and suspend cells by vortexing at high power for 1 min.
7. Repeat steps 4–5 to wash the cells.
8. After decanting supernatant, add 900 µl of Buffer 1 (see *REAGENTS AND SOLUTIONS*). Vortex at high power for 1 min to suspend the cells.
9. Add 100 µl of lysozyme solution to the cell suspension. Tightly cap the tube and invert several times to mix.
10. Place the tube in a water bath set to 37° C and incubate for 30 minutes.

11. Remove from the water bath and add 9 ml of Lysis Buffer (see *REAGENTS AND SOLUTIONS*). Tightly cap the tube and invert several times to mix.
12. Immediately add 50 μ l Proteinase K solution. Cap tightly and invert tube several times to mix.
13. Place the tube in a water bath set to 37°C and incubate for 30 minutes.
14. Remove from the water bath, dry the outside of the tube, and carefully pour contents into a sterile 50 ml conical tube.
15. Gently add 10 ml phenol by allowing it to flow down the side of the tube, cap tightly, and mix solution by gently inverting the tube several times. This should produce a homogenous emulsion.

Note: this step should be conducted in a fume hood.

16. Separate the aqueous and organic phases by centrifugation at 4,000 rpm for 10 minutes at room temperature.

Note: Phase-lock gels may help with separation of the aqueous and organic phases. If desired, use them in accordance with the manufacturer's instructions.

17. Using a sterile pipette, carefully transfer the aqueous (top) layer to a new sterile 50 ml conical tube.
18. Gently add 10 ml chloroform by allowing it to flow down the side of the tube, cap tightly, and mix by gently inverting the tube several times.

Note: this step should be conducted in a laminar flow cabinet.

19. Separate the aqueous and organic phases by centrifugation at 4,000 rpm for 5 minutes at room temperature.

Note: Phase-lock gels may help with separation of the aqueous and organic phases. If desired, use them in accordance with the manufacturer's instructions.

20. Wash once more with chloroform by repeating steps 17–19.
21. Remove the tube from the centrifuge and carefully transfer the aqueous (top) layer to a new sterile 50 ml conical tube.

Optional: If precipitate is present or aqueous layer is cloudy, repeat chloroform washes (Steps 17–19) until it is clear.

22. Note the total volume of the aqueous phase obtained at step 21 and carefully add one-tenth that volume of 5 M NaCl. Cap the tube and mix by carefully inverting the tube several times.
23. In order to precipitate the DNA, slowly add 2.5 volumes of ice-cold absolute ethanol, allowing it to gently run down the side of the tube. Mix slowly by gently swirling the tube.
24. Place the tube on ice for 5 minutes to allow for chromosomal DNA to aggregate.

25. Using a sterile pipet tip, transfer the DNA globule to a sterile, labeled microcentrifuge tube.
26. Add 1 ml of ice-cold 70% ethanol to the DNA globule. Gently swirl the tube to mix.
27. Pellet the DNA by centrifuging at 5000 rpm for 5 minutes at room temperature. Carefully decant the supernatant into a waste container.
28. Allow the pellet to air dry for 10 minutes before adding 1 ml of TE buffer.
29. Store the DNA indefinitely at -20°C . If necessary, transfer 50 μl aliquots to sterile, labeled microcentrifuge tubes in order to avoid multiple freeze-thaw cycles, which can damage the DNA.

ALTERNATE PROTOCOL

QUICK EXTRACTION OF *S. MALTOPHILIA* DNA FOR PCR

This method is not suitable for more sensitive techniques, such as direct genomic sequencing, as the yield and purity of the DNA is relatively low. This alternate protocol is considerably more simply compared to Basic Protocol 1 and produces DNA of sufficient purity for most PCR applications.

Materials

Viable *S. maltophilia* on a petri plate (see UNIT 6F.1)

TE buffer, pH 8.0 (APPENDIX 2A)

Inoculating loop or needle, sterile

Sterile 200 μl PCR tubes

Microcentrifuge fitted with 200 μl tube adapter

Two water baths, set to 55°C and 80°C or a thermocycler

1. Using a sterile inoculating loop or needle, transfer a single colony of *S. maltophilia* to 40 μl of TE buffer in a sterile 200 μl PCR tube. Mix by tapping the side of the tube.

Note: To extract *S. maltophilia* DNA from a broth culture, centrifuge 200 μl of broth culture at maximum speed to pellet the cells, discard the supernatant, and suspend the pellet in 40 μl of TE.

2. Place the tube in a 55°C water bath or heat block for 10 minutes.
3. Transfer the tube to an 80°C water bath or heat block for 10 minutes.

Note: A thermocycler can be used to carry out the above heating steps (Steps 2–3), if so desired.

4. Remove the tube from the water bath or heat block and add an additional 80 μl of room temperature TE buffer. Mix by pipetting up and down several times.

5. Centrifuge at 6000 rpm for 5 minutes at room temperature in order to pellet cellular debris.
6. Being careful to avoid disrupting the pellet, transfer the supernatant to a new nuclease-free PCR tube.
7. Store lysates at -20°C . Lysates will remain useful for PCR for several months.

Note: Use 2 to 5 μl of supernatant per PCR reaction.

BASIC PROTOCOL 2

EXTRACTION OF *S. MALTOPHILIA* RNA

This protocol describes the isolation of RNA from a liquid culture of *S. maltophilia*. The RNA obtained is typically of sufficient quality for RT-PCR, microarray, and RNAseq procedures. Keep samples on ice unless otherwise noted.

Materials

Viable *S. maltophilia* on a petri plate (see *UNIT 6F.1*)

Luria Bertani (LB) Broth (APPENDIX 4A)

Acid phenol:chloroform (pH 4.3 to 4.7)

Chloroform

ddH₂O, nuclease free

70% ethanol in nuclease-free ddH₂O, ice-cold

100% (absolute) ethanol, ice-cold

Isoamyl alcohol

RNasin[®] Plus RNase Inhibitor (Promega)

Dithiothreitol (DTT), 0.1 M

RQ1 RNase-Free DNase

RQ1 RNase-Free DNase buffer, 10×

Sample Buffer (see recipe)

Stop Solution (see recipe)

Sodium acetate, 3 M pH 5.2

Sodium dodecyl sulfate (SDS), 20%

TE buffer, pH 8.0 (APPENDIX 2A)

Bead beating tubes, 8 ml

15 ml and 50 ml conical tube, sterile

Acid-washed zirconium beads, 0.1 mm (see recipe)

Beadbeater (Biospec Products)

Microcentrifuge tubes, sterile

Swinging bucket centrifuge, pre-chilled to 4° C

Orbital shaker set to 37° C, 225 rpm

Water bath set to 37° C

Water bath set to 65° C

Ice bath

1. Inoculate a single isolated colony of *S. maltophilia* into 10 ml LB broth and incubate at 37° C until stationary phase is reached, shaking at 225 rpm (*UNIT 6F. I*).
2. Add 1 ml Stop Solution to the culture and briefly vortex for 10 seconds.
3. Transfer the sample to a new pre-chilled (on ice), sterile 50 ml conical tube.
4. Immediately place in a swinging bucket centrifuge pre-chilled to 4° C. Centrifuge at 10,000 rpm for 15 minutes.

Note: Centrifuge for 20 minutes for larger culture volumes. Ensure centrifuge is balanced.
5. Carefully remove the tube from the centrifuge and decant the supernatant without disturbing the pellet.
6. Suspend the cell pellet in 2 mL of Sample Buffer and transfer to bead beating tube.

Note: Preparing bead beating tubes: Add 1 cc of acid-washed 0.1 mm zirconium beads to 8 ml bead-beating tubes.
7. Add 3 ml of room temperature acid phenol and 0.1 ml of 20% SDS to sample in bead beating tube.
8. Homogenize sample in beadbeater on maximum speed for 3 minutes.
9. Place the beadbeating tube in a swinging bucket centrifuge pre-chilled to 4° C and centrifuge at 4,000 rpm for 10 minutes.
10. Remove tube and carefully transfer the top aqueous layer to tube (15ml conical) containing 3 ml acid phenol. Vortex thoroughly for approximately 1 minute.
11. After mixing, incubate the tube for 10 minutes in a waterbath at 65° C, vortexing every 2–3 minutes.
12. Transfer the tube to a swinging bucket centrifuge pre-chilled to 4° C. Centrifuge at 4,000 rpm for 20 minutes.
13. Remove tube and transfer the top aqueous layer (~ 2ml) to a new tube. Add 1.2 ml acid phenol, 0.8 ml chloroform, and 30-µl isoamyl alcohol. Vortex thoroughly to mix (approximately 1 minute).

14. Place the tube in a pre-chilled swinging bucket centrifuge and centrifuge at 4,000 rpm for 20 minutes.
15. Collect top aqueous layer (~2ml) and transfer to a new tube. Add 2 ml chloroform and 0.1 ml isoamyl alcohol and vortex well.
16. Centrifuge tubes at 4,000 rpm for 20 minutes at 4° C in swinging bucket centrifuge.
17. Collect aqueous phase (~1.8ml), transfer to a new 15 ml conical tube and add 5 ml of ice cold absolute ethanol. Mix by inverting the tube several times.
18. Transfer the tube to a –80° C freezer and incubate overnight to precipitate the RNA.
19. Place sample in a pre-chilled (4° C) tabletop centrifuge and pellet RNA by centrifuging at 12,000 rpm for 20 minutes.
20. Without disrupting the pellet, carefully discard the supernatant. Add 6 ml ice-cold 70% ethanol, gently vortex to dislodge pellet, and centrifuge at 12,000 rpm for 20 minutes at 4° C.
21. Without disrupting the pellet, decant supernatant and use a sterile pipette to carefully remove any remaining residual ethanol.
22. Air-dry the pellet for 15 minutes at room temperature in laminar flow cabinet.
23. Hydrate the pellet by adding 100 µl nuclease free ddH₂O, mix by pipetting up and down several times, and transfer the sample to a sterile microcentrifuge tube.

Note: At this point, the RNA can be stored at –80° C and the protocol can be resumed at a later time. Remove the sample and allow it to thaw completely on ice before continuing.
24. To each 100 µl sample, add the following reagents and mix well by vortexing:
 1. 10 µl RNasin
 2. 2 µl 0.1M DTT
 3. 20 µl 10× RQ1 buffer
 4. 70 µl RQ1 DNase
25. Place tubes in a 37° C water bath and incubate for 60 minutes.
26. Remove tube from the water bath and add 120 µl acid phenol:chloroform (5:1) and 80 µl chloroform, for a final ratio of 1:1 phenol: chloroform. Mix well by vortexing for 1 min.
27. Place the tube in a tabletop centrifuge set to 4° C. Centrifuge at 13,000 rpm for 10 minutes.
28. Carefully remove the tube from the centrifuge, being careful to avoid mixing. Carefully transfer the aqueous (top) layer to a new microcentrifuge tube. Add an equal volume of chloroform. Mix well by vortexing for 1 min.

29. Centrifuge at 13,000 rpm for 10 minutes at 4° C.
30. Carefully remove the tube from the centrifuge, being careful to avoid mixing. Transfer the aqueous (top) layer to a new microcentrifuge tube. Add 1/10 volume 3 M sodium acetate, pH 5.5, and 2.5 volumes ice-cold 100% ethanol. Mix well by vortexing for 1 min.
31. Place the tubes at –80° C and incubate overnight.
32. Remove the samples from –80° C and centrifuge at 13,000 rpm for 30 minutes at 4° C.
33. Being careful not to disrupt the pellet, carefully remove and discard the supernatant.
34. Add 1ml 70% ethanol and gently tap the tube to wash the pellet.
35. Centrifuge at 13,000 rpm for 10 minutes at 4° C.
36. Repeat steps 33–35 for a second ethanol wash.
37. Being careful not to disrupt the pellet, remove the supernatant using a sterile pipet. Invert the tube onto a paper towel with the cap open and allow the pellet to dry for approximately 5 minutes at room temperature.
38. Add 100 µl TE buffer to the pellet to hydrate the RNA.
39. Store the RNA sample at –80°C.

BASIC PROTOCOL 3

TRANSFER OF PLASMID DNA TO *S. MALTOPHILIA* RECIPIENT FROM *E. COLI* DONOR

This protocol describes the transfer of plasmid DNA between an *Escherichia coli* donor and *S. maltophilia* recipient via conjugation. The common *E. coli* donor strain S17-1 is used, but others should work equally well.

Materials

Viable *S. maltophilia* on a petri plate (Recipient)(UNIT 6F.1)

Viable *E. coli* on a petri plate (Donor)

Broad-host range shuttle vector

LB Broth (APPENDIX 4A)

LB Agar plates, dry (APPENDIX 4A)

ddH₂O, nuclease-free

15 ml centrifuge tube, sterile

Orbital shaker set to 37° C, 225 rpm

Inoculating loop or needle, sterile

1. Transform *E. coli* S17-1 (Simon, Priefer, & Puhler, 1983) with shuttle vector of choice (e.g., pBHR2 (Schell et al., 2007)). Select on appropriate antibiotic-containing media.
2. Inoculate a 3 ml *S. maltophilia* culture in LB broth in a 15-ml culture tube. Incubate overnight at 37° C shaking at 225 rpm.
3. Inoculate a 3 ml culture of *E. coli* S17-1 in LB broth containing the appropriate antibiotic for maintenance of the shuttle vector used in step 1. Incubate overnight at 37° C shaking at 225 rpm.
4. The next morning, transfer 100 µl of the *S. maltophilia* culture to a 15 ml centrifuge tube containing 5 ml of fresh LB broth grow to mid log phase (usually 3–4 hours) at 37° C shaking at 225 rpm.
5. When mid-log growth is achieved, transfer the culture to a 42° C water bath and incubate for 15 minutes.
6. While the *S. maltophilia* culture is incubating, remove the *E. coli* S17-1 culture from the incubator, transfer 1 ml to a microcentrifuge tube and centrifuge at 5,000 rpm for 5 minutes at room temperature to pellet the cells.
7. Remove the tube and carefully discard the supernatant, being careful not to dislodge the cell pellet.
8. Add 1 ml fresh LB broth (without antibiotics) and centrifuge as in step 6.

Note: This washing the *E. coli* S17-1 cells ensures that antibiotics are removed prior to mixing with *S. maltophilia*.
9. Remove the tube from the centrifuge and carefully discard the pellet, being careful not to dislodge the cell pellet.
10. Remove the *S. maltophilia* culture from the 42° C water bath. Vortex briefly to ensure a consistent suspension of cells throughout the tube.
11. Transfer 200 µl of the *S. maltophilia* culture to the microcentrifuge tube containing the *E. coli* S17-1 pellet and mix gently by slowly pipetting up and down several times.

Note: Wide bore pipette tips can be used here to reduce sheering force, which can damage exposed conjugation machinery on the cell surface.
12. Spot 100 µl aliquots of the cell mixture onto LB agar Petri plates that have been allowed to air dry. Incubate the plates at 37° C overnight.

Note: Excess moisture on the Petri plate negatively impacts conjugation efficiency.
13. After overnight growth, scrape the growth from each spot and suspend in 1 ml LB broth.

14. Spread 50 µl aliquots of the suspension onto LB agar Petri plates containing the antibiotic appropriate for the shuttle vector used and a second antibiotic for counterselection of *E. coli* S17-1.

Note: See UNIT 6F.1 for concentrations typically used with *S. maltophilia*. Norfloxacin and ampicillin are commonly used for counterselection of *E. coli* since most isolates of *S. maltophilia* are resistant to these antibiotics.

15. Incubate the plates for 48 to 72 hours at 37° C and observe for colony formation.
16. Screen colonies by PCR for the presence of the shuttle vector and for *S. maltophilia*-specific genomic content.

Note: Primers SM1 and SM2 (see reagents) amplify from all *S. maltophilia* isolates

BASIC PROTOCOL 4

ALLELIC EXCHANGE IN *S. MALTOPHILIA* USING pEX18TC

This protocol describes the use of pEX18tc to create unmarked allelic exchange mutations in *S. maltophilia*. This method is derived from that first described by Hoang et al. for the use in *Pseudomonas aeruginosa* (Hoang, Karkhoff-Schweizer, Kutchma, & Schweizer, 1998). Similar procedures have been used by Karaba et al. (Karaba, White, & Cianciotto, 2013) and Pompilio et al. (Pompilio et al., 2010) in *S. maltophilia*. An unmarked deletion is achieved through a two-step integration/excision process and requires that 1000-bp fragments homologous to the region directly upstream and directly downstream of the DNA to be deleted be cloned into pEX18tc, maintaining their normal orientation (minus the intervening DNA).

Materials

pEx18tc

LB broth (APPENDIX 4A)

LB agar (APPENDIX 4A)

Sucrose

1. Using PCR, amplify a 1000-bp fragment of DNA from directly upstream of the DNA to be deleted and a second 1000-bp fragment of DNA from directly downstream of the DNA to be deleted. Using standard techniques, clone these fragments into the pEX18tc multiple cloning site, maintaining the directionality of the *S. maltophilia* chromosome.

Note: pEX18tc confers resistance to tetracycline. If the *S. maltophilia* isolate is resistant to tetracycline, another vector should be used or the marker should be exchanged prior to use.

2. Introduce the resulting plasmid into *S. maltophilia* using Basic Protocol 3. Select on LB media containing 20 µg/ml tetracycline.

Note: pEX18tc does not replicate in *S. maltophilia*. However, at low frequency it will integrate into the chromosome by homologous recombination at one of the cloned fragments, resulting in a merodiploid genotype.

3. After verifying integration of pEX18tc via PCR (Basic Protocol 3), inoculate 3 ml of LB broth lacking antibiotics in a 15 ml culture tube and incubate overnight at 37° C, shaking at 225 rpm.

4. Using a sterile inoculation loop or pipette, transfer 10 µl to an LB agar plate containing 10% sucrose. Streak to isolation. Incubate the plates at 37° C overnight.

Note: pEX18tc contains the *sacB* gene for counterselection on sucrose-containing media. Only isolates that have lost the plasmid by excision should form well-isolated colonies.

5. Screen well-isolated colonies for loss of pEX18tc by patching onto LB agar containing 20 µg/ml tetracycline.

6. Using PCR, screen tetracycline sensitive colonies for the wild-type or knock out allele.

Note: Sucrose resistant, tetracycline sensitive clones can arise from recombination at the original site of insertion or at the second region of homology. The first will revert to the wild-type sequence while the second will generate the desired genetic deletion. The ratios of wild-type to knock-out vary widely depending on the region targeted.

REAGENTS AND SOLUTIONS

Deionized, distilled water should be used for all microbiological application. Nuclease-free water should be used for all molecular biology applications. APPENDIX 2A provides recipes for common stock solutions.

Acid washed zirconium beads, 0.1 mm—Add 100 ml of 6N HCl to 75 ml of 0.1 mm zirconium beads (Biospec Products) and mix thoroughly. Let sit for 30 minutes and wash beads with ddH₂O in order to remove HCl (pour out HCl, add water, shake to mix, pour out water, repeat with water). Remove water, autoclave beads at 120° C for 15 minutes. Ensure beads are dry before use.

Buffer 1 (0.01 M sodium phosphate + 20 g sucrose), (pH 7.0)—Dissolve 20 grams of sucrose in 10 ml of 10 mM sodium phosphate. Bring to a final volume of 100 ml in dH₂O. Filter sterilize by using a 0.2 µm filter. Store at room temperature.

Component “A”—Dissolve 13.61 grams of NaH₂PO₄³⁻ (monobasic) into 100 ml of dH₂O. Store at room temperature. (Use to make Buffer 1).

Component “B”—Dissolve 14.2 grams of NaHPO₄³⁻ (dibasic) into 100 ml of dH₂O. Store at room temperature. (Use to make Buffer 1).

Diagnostic Primer—Primers SM1 (5'-CAGCCTGCGAAAAGTA-3') and SM4 (5'-TTAAGCTTGCCACGAACAG-3') are directed to the 23S rRNA gene and can be used as diagnostic primers for *S. maltophilia* (Whitby, 2000).

Ethanol, 70 % in sterile ddH₂O—Combine 70 ml of 95% ethyl alcohol with 30 ml of sterile ddH₂O. Store at room temperature.

Ethylene-diamine-tetraacetic acid (EDTA) Buffer, 1 mM (pH 8.0)—Add 1 ml of 0.5 M EDTA (pH 8.0) to 499 ml of sterile dH₂O. Store at room temperature.

Ethylene-diamine-tetraacetic acid (EDTA) Buffer, 0.5 M (pH 8.0)—Add 90.8 grams of EDTA dehydrate to 10.0 grams of NaOH in 400 ml of dH₂O. While stirring, adjust the pH to 8.0 using NaOH pellets. Bring the final volume to 500 ml with dH₂O. Autoclave at 120° C for 15 minutes before use and store at room temperature.

Lysis Buffer—Combine 1 ml of 1M Tris-Cl (pH 8.0) with 1 gram of Sodium Dodecyl Sulfate (SDS) and 0.2 ml of 0.5 M EDTA (pH 8.0) into 100 ml dH₂O. Filter sterilize by using a 0.2 µm filter. Store at room temperature.

Lysozyme—Dissolve 25 mg of lysozyme into 1 ml of Buffer 1. Filter sterilize by using a 0.2 µm filter. Prepare fresh when needed.

Potassium Acetate (KOAc), 5 M—Dissolve 98.1 grams of KOAc into 160 ml of dH₂O. Adjust pH to 7.0 using acetic acid. Bring final volume to 200 ml using dH₂O. Store at room temperature.

Sample Buffer—Dissolve 200 mM concentration of NaCl in a 20 mM concentration of ethylene-diamine-tetraacetic acid (EDTA) and filter sterilize using a 0.2 µm filter. Store at room temperature.

Sodium Acetate, 3 M (pH 5.2)—Dissolve 24.6 grams of sodium acetate anhydrous (CH₃COONa) or 40.8 grams sodium acetate trihydrate (CH₃COONa 3H₂O) into 100 ml of dH₂O. Adjust the pH to 5.2 using acetic acid. Autoclave at 120° C for 15 minutes before use. Store at room temperature.

Sodium Chloride, 5 M—Dissolve 29.22 grams of NaCl into 100 ml ddH₂O. Autoclave at 120° C for 15 minutes before use. Store at room temperature.

Sodium dodecyl sulfate (SDS), 1%—Dissolve 1 gram of SDS into 50 ml of sterile ddH₂O. Heat to 68° C and adjust final volume to 100 ml in dH₂O. Store at room temperature.

Sodium dodecyl sulfate (SDS), 10%—Dissolve 10 grams of SDS into 80 ml of sterile ddH₂O. Heat to 68° C and adjust final volume to 100 ml in dH₂O. Store at room temperature.

Sodium dodecyl sulfate (SDS), 20%—Dissolve 20 grams of SDS into 70 ml of sterile ddH₂O. Heat to 68° C and adjust final volume to 100 ml in dH₂O. Store at room temperature.

Sodium Hydroxide (NaOH), 10 N—Dissolve 40 grams of NaOH into 50 ml of dH₂O. Bring final volume to 100 ml in dH₂O. Autoclave at 120° C for 15 minutes and store at room temperature.

Sodium Phosphate, 10 mM—Add 19.5 ml of Component “A” with 30.5 ml of Component “B” and bring to final volume of 100 ml dH₂O. Store at room temperature. (Use to make Buffer 1).

SSC, 20×, pH 7.0—Dissolve 175.3 grams of NaCl and 88.2 grams of sodium citrate into 800 ml of dH₂O. While stirring, adjust the pH to 7.0 using HCl. Adjust final volume to 1 liter using dH₂O. Autoclave at 120° C for 15 minutes and store at room temperature.

SSC, 0.1×—Add 1 ml of 20× SSC, pH 7.0, into 199 ml dH₂O. Filter sterilize by using a 0.2 µm filter. Store at room temperature.

Stop Solution (Acid phenol, 10%)—Combine 10 ml of acid phenol chloroform (Ambion #AM9722) in 90 mL of 99% ethanol. Glass containers are required for storing acid phenol chloroform as well as the Stop Solution. Store the working stocks at 4°C and remaining aliquots at –20°C until use.

Tris-Cl, 1 M (pH 8.0)—Dissolve 60.55 grams of Tris in dH₂O and adjust pH to 8.0 using HCl. Bring to a final volume of 500 ml. Autoclave at 120° C for 15 minutes and store at room temperature.

COMMENTARY

Background Information

S. maltophilia receives research interest for a wide variety of reasons. In addition to its recognized significance as an opportunistic pathogen, certain *S. maltophilia* isolates are of biotechnological and agricultural interest due to potentially beneficial phenotypes (Brooke, 2012).

Infections associated with *S. maltophilia* have become a particular concern for patients afflicted with cystic fibrosis and other severe comorbidities (Stanojevic et al., 2013). A recently published sequence of the *S. maltophilia* genome revealed a large capacity for drug and heavy metal resistance and a number of possible mobile regions of DNA that encode for numerous putative biofilm formation proteins (Crossman et al., 2008). This suggests that this organism may act as a reservoir of antimicrobial drug resistances and would explain why it is so pervasive in a clinical setting.

S. maltophilia has undergone taxonomic reclassification several times since its first discovery, as *Bacterium bookeri*, from pleural fluid in 1943. *B. bookeri* was reclassified as a member of

the *Pseudomonas* genus in 1961 and was later reassigned to the genus *Xanthomonas* in 1983. Based on DNA homology, it was finally moved to its current genus *Stenotrophomonas* in 1993 (Palleroni & Bradbury, 1993). The genus *Stenotrophomonas* is currently divided into four distinct species, ranging from the ubiquitous soil organism, *S. nitritireducens*, to the opportunistic human pathogen, *S. maltophilia* (Coenye, Vanlaere, Falsen, & Vandamme, 2004). It should be noted that only *S. maltophilia* has been documented to cause disease in humans. Recent genetic analysis suggests that *S. maltophilia* was able to colonize human epithelial after losing certain plant pathogenic traits and acquiring human virulence factors (Crossman et al., 2008).

Critical Parameters and Troubleshooting

Extraction of *S. maltophilia* DNA—Complete lysis of *S. maltophilia* cells is critical to isolation of high quality genomic DNA. If the solution does not clear after addition of lysis buffer, start again using half the original volume of cells. Most often, lysis is inhibited by the presence of too many cells. Take care to avoid carryover of organic solvents or alcohol as they often inhibit downstream applications. The use of phase-lock gel can aid in separation of organic and aqueous fractions during the protocol.

Isolation of *S. maltophilia* RNA—This protocol should be conducted under strict RNase-free conditions in order to avoid contaminating samples with RNases. As in the DNA extraction, care should be taken to avoid carryover of organic solvents or alcohol during processing.

Transfer of plasmid DNA—Effective counterselection of the *E. coli* donor is critical for this protocol. However, cross-induction of resistance has been observed in *S. maltophilia*. Therefore, it is necessary to test the selection and counterselection strategies in the absence of conjugation in order to verify their effectiveness for the strains and vectors of interest. Some isolates of *S. maltophilia* produce antibiotics during overnight growth that may inactivate the *E. coli* donor strain. If no transconjugate colonies are obtained, wash *S. maltophilia* cells prior to mixing with the donor.

Allelic exchange—It is critical that both the upstream and downstream cloned fragments be equivalent in size. If one is substantially larger than the other, the propensity is for both the insertion and excision reactions to occur at that fragment, thus producing only the wild-type allele at the end of the protocol. High levels of merodiploid cells can, on occasion, survive sucrose counterselection. Thus, it is critical to patch clones to tetracycline-containing media before proceeding further. It is often necessary to patch several hundred clones in order to identify deletion mutants.

Anticipated Results

Extraction of *S. maltophilia* DNA—A number of factors influence the yield and purity of genomic DNA obtained by this protocol. Typical extractions produce between 100 ng and 1 µg/µl.

Isolation of *S. maltophilia* RNA—The total yield from this protocol is heavily dependent on the density of cells in the starting culture. However, the RNA should be pure (as judged by standard methods) and useful for demanding applications.

Transfer of plasmid DNA—The efficiency of transfer varies widely for different vectors and different *S. maltophilia* recipients. However, this protocol should result in transfer of a shuttle vector or suicide vector from *E. coli* into *S. maltophilia*.

Allelic exchange—Efficiency of deletion will vary depending on a number of factors. However, this procedure should allow for the deletion of any non-essential locus within the *S. maltophilia* genome.

Time Considerations

Extraction of *S. maltophilia* DNA—After overnight growth, it can take between 4 and 6 hours to complete this protocol.

Quick extract of DNA for PCR—This protocol can be completed in less than 2 hours.

Isolation of *S. maltophilia* RNA—After harvesting cells, it can take between 4 and 6 hours to complete this protocol.

Transfer of plasmid DNA—Most of the time associated with this protocol involves long incubation periods. Actual hands on time is limited to about an hour.

Allelic exchange—Again, most of the time associated with this protocol involves long incubation and growth periods. However, patching large numbers of clones to screen for loss of tetracycline can be extremely time consuming, often requiring several hours.

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