

Protocol for Detection of *Dickeya* and *Pectobacterium* in Potato Tubers, Stems or Irrigation Water

22 Nov 2017

The majority of this protocol was derived from Humphries et al. (1). Updates are from results of Secor (NDSU), Hao (University of Maine), Ishimaru (University of Minnesota) and Charkowski (CSU) based on work funded by USDA-ARS and multiple grower organizations.

1. Sample Collection

Samples should be shipped in insulated containers to protect them from temperature extremes during shipment. In tubers, the bacteria will be present in lenticels the periderm, around the eyes, and in the stem end. Periderm and stem end samples may be collected separately from each tuber or the stem end may be sliced off and processed with the periderm intact.

Symptomatic Tubers, Minitubers, Stems, or Micropropagated Plants

- Collect symptomatic tubers or stems individually from different lots/locations and place in separate labeled plastic bags to avoid cross contamination. Micropropagated plants can be shipped in the vessel used to grow the plant.
- To reduce shipping components, a 2-3 inch stem section that contains the intersection between diseased and healthy stem tissue (edge of lesions) should be collected.
- Decontaminate hand tools between samples if tools are used during collection.
- Ship samples overnight.

Asymptomatic Tubers, Minitubers, Stems, or Micropropagated Plants

- For seed lot screening, collect random tuber or stem samples. The number of samples per seed lot will determine the probability of finding the pathogen (2).
 - Collecting at least 200 tuber samples from a single seed lot is recommended if growers are interested in determining if the seeds lot contains *Dickeya* or *Pectobacterium*. Larger sample sizes will enable detection of lower incidences in the seed lots. Many growers use 400 tuber samples, which provides a 95% confidence that pathogen incidence is less than 1% if no pathogen is found during testing. ***Epidemiological research is still needed to determine appropriate Dickeya and Pectobacterium thresholds for seed potatoes, so this protocol cannot provide growers with recommendations on incidence levels for seed potatoes.***
 - The stem ends of tubers can be sliced off and shipped for processing to save on shipping costs and decrease processing time. Be sure to include tuber periderm in samples on the stem ends.
 - Healthy-appearing tuber samples collected from a single seed lot do not need to be separated from each other.
- For stems, collect approximately 2-3 inch sections of stems at ground level. If *Dickeya* or *Pectobacterium* are present it will be at the highest concentration at this location.
- Micropropagated plants can be shipped in the vessel used to grow the plant.

Water Samples

- Collect 250 ml water samples in sterile containers. If possible collect the sample approx. 10-12 inches under the water surface.
- Pack samples in shipping box with ice packets surrounding containers to keep samples cool. Ship overnight as samples should be processed within 24 of collection.

2. Sample Processing

Symptomatic Samples do not require enrichment to detect *Dickeya* or *Pectobacterium*.

- If necessary, wash tuber or stem samples to remove excess soil.
- Extract several small portion(s) of plant tissue (tuber or stem) from the intersection of the diseased and healthy tissue (edge of lesion). Note if more than one tuber or stem is submitted as a sample, the material from all tubers or stems can be combined to make one sample. However, it is common to find both *Dickeya* and *Pectobacterium* in the same field. Processing the samples individually will provide some information on which pathogen is more prevalent.
- Place diseased tissue pieces in a 2 ml centrifuge tube. Sample from diseased tissue can be stored at -80°C prior to DNA isolation if necessary.
- If planning to isolate living cells from the extracts, the antioxidant diethyldithiocarbamic acid (DIECA) may be added to water or enrichment medium at 5% vol:vol to protect cells during isolation and enrichment.
- At this point 100 µl of enrichment medium can be placed on semi-selective media if interested in obtaining *Dickeya* or *Pectobacterium* isolates. Importantly, even if multiple species are detected with PCR assays, it may be difficult to isolate multiple species from a sample because the different species may be present in very disparate ratios.
- Sterilize the surface with 70% alcohol between samples to ensure no cross contamination occurs. Tools should be submerged in 95% alcohol and then exposed to a flame to burn excess alcohol. Change gloves between samples. Samples can be cut on paper towels, which should be disposed of between samples.

Asymptomatic Samples typically require a culture-based enrichment step to detect *Dickeya* or *Pectobacterium*.

- If necessary, wash tuber or stem samples to remove soil.

Tubers or Minitubers

- A tuber sample may be processed in groups 25-200 tubers/samples. Smaller batches, such as 25 tubers, allow for estimates of incidence, while larger tuber batches aid in determining if the pathogen is present. Many growers use 400 tubers/seed lot sample. (In the Netherlands, seed lots are tested in 50-tuber batches and lots with 3 or more batches positive for *Dickeya* or *Pectobacterium* are considered at high risk for disease in the subsequent crop year. Similar data are not yet available for North America.)
- Tuber samples may be covered in soil and can be washed in tap water prior to processing. If rotten tubers are present in a lot submitted for testing, bulk washing of

the tubers will result in cross-contamination of the tubers (3). Either wash the tubers individually or wash them in mesh bags in the same composites that will be used for testing.

- Remove a sample from the tuber that includes both the stem end and a portion of tuber peel. These bacterial pathogens can reach a very high concentration in the stem end of the tuber, but may be found at higher incidence (although low concentration) on tuber periderms. Both tuber stem end sections containing the core and peel (4, 5) and tuber peels (3) have been used to detect soft rot bacteria on tuber samples and both have been shown to correlate with field incidence.
- It is not necessary to sanitize cutting tools between tubers in a single batch (ex: 200 tuber batch), but tools and cutting surfaces should be sanitized between batches of tubers.
- Sterilize tools by submerging in 95% alcohol and then flaming to burn excess alcohol.
- Place tuber batches into separate universal extraction bags, sterile tubes or flasks.
- Add quarter-strength sterile Ringer's buffer to cover the sample. Buffer can be made from scratch (below) or tablets can be ordered from Sigma (96724-100tab).
 - ¼ strength Ringer's buffer: per liter: 2.25 g NaCl, 0.11 g KCl, 0.12 g $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ and 0.05 g of sodium bicarbonate; adjust to pH 7.0; autoclave.
- Place samples on a shaker (100 rpm) for at least 2 hours to allow for bacteria to stream out of samples or smash tuber samples.
- Remove 5 ml of solution and place in 12 ml sterile centrifuge tube.
- Add 5 ml of D-PEM (Appendix A) which is double strength pectate enrichment medium that selects for growth of *Dickeya* and *Pectobacterium*
- Loosen lids of the 12 ml centrifuge tubes a quarter of a turn to allow for gas exchange and place in disposable anaerobic chambers with indicator (Fisher B260683).
- Place samples at 36-37°C for 48 hrs under anaerobic (low oxygen) conditions to promote growth of *Dickeya* sp. Using an incubation temperatures above 33°C will kill or inhibit growth of many other plant-associated bacteria.
- At this point 100 µl of supernatant liquid can be placed on semi-selective media if interested in obtaining *Dickeya* or *Pectobacterium* isolates. Importantly, even if multiple species are detected with PCR assays, it may be difficult to isolate multiple species from a sample because the different species may be present in very disparate ratios.

Stems

- If present in field or greenhouse grown stems or in micropropagated plants, the bacteria will be at the highest concentration at the base of the stem.
- Use a sterile blade to extract plant tissue from the base of the stem.
- Sterilize tools (submerge in 95% alcohol and flame excess alcohol) and work surface (wipe down with 70% alcohol between samples).
- Place samples into separate universal extraction bags, sterile tubes or flasks. 12 ml sterile centrifuge tubes work well.
- Add quarter-strength sterile Ringer's buffer to cover the sample. Buffer can be made from scratch (below) or tablets can be ordered from Sigma (96724-100tab).

- ¼ strength Ringer's buffer: per liter: 2.25 g NaCl, 0.105 g KCl, 0.12 g CaCl₂·6H₂O and 0.05 g of sodium bicarbonate; adjust to pH 7.0; autoclave.
- Soak samples overnight at room temperature to allow the bacteria to stream out of the samples. Samples may be placed on a shaker to aid in recovery of bacteria from the samples.
- Remove supernatant liquid (approx. 1.5 ml) and dispense into 2 separate sterile 1.7 ml tubes.
- At this point 100 µl of supernatant liquid can be placed on semi-selective media if interested in obtaining *Dickeya* or *Pectobacterium* isolates. Importantly, even if multiple species are detected with PCR assays, it may be difficult to isolate multiple species from a sample because the different species may be present in very disparate ratios.
- Spin both 1.7 tubes in a centrifuge at 14,000 rpm for 2-5 min until a pellet forms.
- Remove supernatant liquid from both tubes.
- Not all supernatant needs to be removed as the pellet is delicate.
- Designate one tube as 'use for PCR reaction'. Note the pellet can be frozen at -80°C and retrieved at a later date for processing.
- Add 500 µl of filter-sterilized 20% glycerol (vol:vol) to the second tube, resuspend the pellet and designate as back-up storage. Store at -80°C. Cells from this suspension can either be plated onto CVP medium or used for PCR.

Water samples typically require an enrichment step to detect *Dickeya* and *Pectobacterium*.

- Subdivide into aliquots of 40 ml and clarify by centrifugation at a low speed (180 × g) for 10 min.
- Remove 20 ml of supernatant and mix with an equal volume of D-PEM (appendix A) in a 50 ml centrifuge tube.
- Incubate anaerobically at 36-37°C for 48 hrs.
- Centrifuge at high speed (10,000 × g) to concentrate the bacterial cells
- Resuspend the pellet in 1 ml sterile water.
- At this point serial dilutions can be made and samples plated onto CVP to isolate single colonies or DNA can be extracted from the resuspended pellet.

3. Strain Isolation

Culture-based assays can be used to confirm the presence of *Dickeya* or *Pectobacterium* in samples.

- 100 µl of bacterial suspension obtained from the tuber, stem or water extracts can be used to inoculate pectate enrichment broth or plated directly onto CVP.
- Pectate enrichment broth can be incubated at 23-28°C for 24 hours prior to plating onto CVP.
- Alternatively if plating directly onto CVP; store the plates at 23-28°C until growth is observed.
- If *Dickeya* or *Pectobacterium* are present, pits will form in the medium within 2 days. *Dickeya* grows more slowly and *Dickeya* colonies are smaller than *Pectobacterium*, so plates should be evaluated daily and any new pitting colonies should be removed to a

new plate. Some *Pectobacterium* strains produce copious amounts of plant cell wall degrading enzymes and can liquefy CVP plates. If this occurs, other media, such as Nutrient Agar+X-gal+IPTG or NGM can be used to attempt isolation.

Note- Only semi-selective media for *Dickeya* and *Pectobacterium* isolations are available and secondary invaders compete well with *Dickeya* and *Pectobacterium* in diseased plants. Therefore, *Dickeya* or *Pectobacterium* colonies may not be the prevalent colony type on growth media. Pure cultures can generally be obtained by streaking bacteria from pits that form on CVP onto a general medium, such as nutrient agar. *Dickeya* does not survive well on some agar medium, such as LB, and it survives poorly on media stored at cool temperatures (4-10°C). On LB agar or at cool temperatures, cells die or become non-culturable within a few days. Therefore, isolates should be stored as soon as a pure culture is obtained. Isolates may be stored indefinitely at -80°C by suspending cells in cryovials filled with filter-sterilized 20% vol:vol glycerol or in cryovials containing ceramic beads (Copan Diagnostics). It is crucial that vigorous cells are stored, so cells from freshly streaked plates that are incubated for no more than one day should be used for stored cultures.

4. DNA Extraction

Symptomatic Samples

- If stored samples are used, remove the frozen sample tissue (tubers or stems) from the 1.7 centrifuge stored at -80°C and leave at room temp to warm.
- Add 1 ml of sterile water to the tube, vortex and allow the tissue to soak for 10-20 min.
- Remove 400 µl of liquid and extract DNA using the MP Biomedical Soil Extraction Kit
- DNA extraction kits such as MP Biomedical Soil Extraction Kit as higher quality DNA product than the boiling method and are recommend for consistent results.
- Unused tissue can be refrozen.

Asymptomatic Samples

- If stored sample pellets are used, remove the 1.7 ml tube selected for PCR stored at -80°C and leave at room temp to warm.
- Add 1 ml of sterile water to tube, vortex and allow the tissue to soak for 10-20 min.
- Remove 400 µl of liquid and extract DNA using the MP Biomedical Soil Extraction Kit.
- DNA extraction kits such as MP Biomedical Soil Extraction Kit as higher quality DNA product than the boiling method.
- DNA extraction kits such as MP Biomedical Soil Extraction Kit as higher quality DNA product than the boiling method and are recommend for consistent results.

5. PCR Assays

Dickeya and *Pectobacterium* strains with a validated identification should be included in every assay.

Notes on primer efficacy from testing in Wisconsin: Of approximately 250 samples tested, the Df/Dr primer set has had 4 false negatives and the DIA-A probe has had 11 false negatives, which indicates that positive samples may be missed.

Based on these observations we recommend using the ADE primer along with the DIA-C probe to test samples as they seem to be the most accurate. Some *D. dianthicola* strains found in the US lack the target site for the DIA-A primers, so this set should not be used as the sole method for *D. dianthicola* detection. The ECH primer has also been working fairly well but results are not as clear for tuber samples and there were some questionable results from stem samples so this primer set should be used with caution.

Multiplex PCR Protocol (6)

This protocol used 3 primer sets to detect *Dickeya* and *Pectobacterium*. The primer sets can also be used individually to detect particular taxa. The primers are: Dsp (Df and Dr) which distinguishes *Dickeya* sp. with a band at ~130 bp, Pba (Y45 and Y46) which distinguishes *Pectobacterium atrosepticum* with a band at ~420 bp and Pcc (ExpccF and ExpccR) which distinguishes *Pectobacterium carotovorum* with a band at ~550 bps.

| Primer | Sequence |
|--------|---------------------------------|
| Df | 5'-AGAGTCAAAAAGCGTCTTG-3' |
| Dr | 5'-TTTACCCACCGTCAGTC-3' |
| Y45 | 5'-TCACCGGACGCCGAAGTGTGGCGT-3' |
| Y46 | 5'-TCGCCAACGTTTCAGCAGAACAAGT-3' |
| ExpccF | 5'-GAACTTCGCACCCGCCGACCTTCTA-3' |
| ExpccR | 5'-GCCGTAATTGCCTACCTGCTTAAG-3' |

To prepare the primers upon arrival:

- A 100 µM stock solution - determining the nmoles in the vial and multiplying that by 10 and add that amount of Low TE buffer pH 8.
- Example: if sent 29.4 nmoles of Df then add 294 µl of Low TE buffer pH 8.
- A 10 µM working solution-dilute the stock solution 1:10 with 10 mM Tris buffer pH 8.
- Example: add 90 µl of 10 mM Tris buffer pH 8 to 10 µl of forward primer stock solution.

Prepare a known positive and negative sample to include when running reaction.

Master Mix for this reaction:

| Master Mix Component | Volume per Rxn |
|---------------------------------|----------------|
| Promega 2X Master Mix | 12.5 µl |
| 10 µM Pcc Forward Primer (ExpF) | 3.0 µl |
| 10 µM Pcc Reverse Primer (ExpR) | 3.0 µl |
| 10 µM Dsp Forward Primer (Df) | 0.8 µl |
| 10 µM Dsp Reverse Primer (Dr) | 0.8 µl |
| 10 µM Pba Forward Primer (Y45) | 0.25 µl |
| 10 µM Pba Reverse Primer (Y46) | 0.25 µl |
| Water (molecular grade) | 2.4 µl |
| Extracted sample DNA | 2.0 µl |
| Total Rxn Volume | 25.0 µl |

The following PCR parameters should be used:

| Step 1 | Step 2 | Step 3 |
|---|---|-----------------|
| Preheat lid to 105.5°C; 95°C for 4 min | 30 cycles: 94°C for 45s, 62°C for 90s, 72 °C for 90s | 72 °C for 5 min |

Samples should be run in 2% agarose gel to determine if bands are present.

PeIADE PCR assay

Two protocols below are used on a single primer set to amplify a ~420 bp fragment from *Dickeya*.

| Primer | Sequence |
|---------|--|
| peIADE1 | 5'- GAT CAG AAA GCC CGC AGC CAG AT -3' 5'-ADE2 CTG TGG CCG ATC AGG ATG GTT TTG TCG TGC-3' |

To prepare the primers upon arrival:

A 100 µM stock solution - determining the nmoles in the vial and multiplying that by 10 and add that amount of Low TE buffer pH 8.

Example: if sent 29.4 nmoles of primer then add 294 µl of Low TE buffer pH 8.

A 10 µM working solution-dilute the stock solution 1:10 with 10 mM Tris buffer pH 8.

Example: add 90 µl of 10 mM Tris buffer pH 8 to 10 µl of forward primer stock solution.

Prepare a known positive and negative sample to include when running reaction.

Master Mix for this reaction:

| Master Mix Component | Volume per Rxn |
|--------------------------|----------------|
| Promega 2X Master Mix | 12.5 µl |
| 10 µM ADE Forward Primer | 1.0 µl |
| 10 µM ADE Reverse Primer | 1.0 µl |
| Water (molecular grade) | 8.5 µl |
| Extracted sample DNA | 2.0 µl |
| Total Rxn Volume | 25 µl |

The following PCR parameters should be used:

| Step 1 | Step 2 | Step 3 |
|---|---|-----------------|
| Preheat lid to 105.5°C; 94°C for 5 min | 30 cycles: 94°C for 60s, 72°C for 120s | 72 °C for 5 min |

Samples should be run in a 2% agarose gel to determine if bands are present.

Dia-C Real-time PCR (7)

The protocol uses one primer set to specifically detect *D. dianthicola* via real-time PCR.

The Dia-A primers reported in this reference failed to detect *D. dianthicola* isolates from Wisconsin and Texas.

| | |
|---------------------------------------|--|
| Primer | Sequence |
| DIA-C | 5'- CCA ACG ATT AGT CGG ATC T -3' 5'- TAG TTG GTG CCA GGT TGG TA - 3' |
| Probe | Sequence |
| CY-5' HPLC channel 4 Iowa black RQ-3' | 5' – TCG ACG TAT GGG ACG GTC GC – 3' |

To prepare the primers upon arrival:

A 100 µM stock solution - determining the nmoles in the vial and multiplying that by 10 and add that amount of Low TE buffer pH 8.

Example: if sent 34.5 nmoles of primer then add 345 µl of Low TE buffer pH 8.

A 10 µM working solution-dilute the stock solution 1:10 with 10 mM Tris buffer pH 8.

Example: add 90 µl of 10 mM Tris buffer pH 8 to 10 µl of forward primer stock solution.

Prepare a known positive and negative sample to include when running reaction.

Master Mix for this reaction:

| Master Mix Component | Volume per Rxn |
|--|----------------|
| BioRad SsoAdvanced Universal Probes Supermix | 12.5 µl |
| 10 µM DIA-C Forward Primer | 0.75 µl |
| 10 µM DIA-C Reverse Primer | 0.75 µl |
| 10 µM Probe | 0.25 µl |
| Water (molecular grade) | 8.75 µl |
| Extracted sample DNA | 2.0 µl |
| Total Rxn Volume | 25 µl |

The following PCR parameters should be used:

| Step 1 | Step 2 | Step 3 |
|---|------------------|--|
| Preheat lid to 105.5°C; 48°C for 30s | 95 °C for 10 min | 40 cycles: 95°C for 15s, 50°C for 1 min |

Currently, there is no multiplex assay suitable for detection and differentiation of all *Dickeya* species from plant samples. Primers reported for *Dickeya* species other than *D. dianthicola* and *D. solani* have not been validated with field samples.

6. Interpretation of Results

Epidemiological research is still needed to determine appropriate *Dickeya* and *Pectobacterium* thresholds for seed potatoes, so this protocol cannot provide growers with recommendations on appropriate incidence levels for seed potatoes. At the present time, growers will need to determine themselves which threshold they feel is appropriate for their own farming operation.

Appendix A

Semi-Selective Media Recipes Suitable pectin for CVP is available from Agdia (Elkhart, IN). The recipe is here: <https://orders.agdia.com/Documents/m336.pdf>

Single-layer CVP (SL-CVP) medium (8)

| Mix A | In 500 ml of distilled water |
|-------------------------------------|------------------------------|
| CaCl ₂ ·H ₂ O | 1.02 g |
| Tryptone | 1.0 g |
| Tri-sodium citrate | 5.0 g |
| Na NO ₃ | 2.0 g |
| 0.1% crystal violet* | 1.5 ml |
| Agar | 4 g |
| Mix B | In 500 ml of distilled water |
| 5 M NaOH | 2.8 ml |
| Pectin | 18.0 g |

*corrected from error in original publication

Add each ingredient in the order listed and dissolve each ingredient before adding the subsequent one on the list. For mix B, add the pectin slowly and heat the solution to 80-100°C while stirring to dissolve the pectin before autoclaving. Autoclave the two solutions at 121°C for 15 min. Carefully pour mix A into mix B while the solutions are still hot and swirl to mix the solutions together. Pour the medium into Petri dishes. Dry the medium in a biosafety hood or laminar flow hood to eliminate condensation prior to using the CVP. This medium should be stored at 4°C prior to use. CVP medium will be grey when properly prepared.

Double-layer CVP (DL-CVP) medium (8)

| Basal Layer | In 1000 ml of distilled water |
|-------------------------------------|-------------------------------|
| CaCl ₂ ·H ₂ O | 5.5 g |
| Tryptone | 1.0 g |
| Na NO ₃ | 1.6 g |
| 0.1% crystal violet* | 1.5 ml |
| Agar | 15 g |
| Top Layer | In 800 ml of distilled water |
| 5% EDTA pH 8.0 | 2 ml |
| 5 M NaOH | 4.8 ml |
| Pectin | 20.0 g |

*corrected from error in original publication

Add each ingredient in the order listed and dissolve each ingredient before adding the subsequent one on the list. For the top layer, add the pectin slowly and heat the solution to 80-100°C while stirring to dissolve the pectin before autoclaving. Autoclave the two solutions at 121°C for 15 min. Pour the basal layer into Petri dishes (approximately 15 ml per dish in 20 mM dishes) and allow the medium to set. Then pour the top layer (approximately 7 ml per dish in 20 mM dishes). Dry the medium in a biosafety hood or laminar flow hood to eliminate condensation prior to using the CVP. This medium should be stored at 4°C prior to use. CVP medium will be grey when properly prepared.

Nutrient Glycerol Manganese (NGM) medium (9)

| | Per L |
|--------------------------------------|-------|
| Nutrient Agar (Becton Dickinson) | 23 g |
| Glycerol | 10 ml |
| MnCl ₂ ·4H ₂ O | 0.4 g |

Dickeya will form colonies with light purple centers (indigoidine production) on this medium.

Pectobacterium does not produce indigoidine, so will not be purple on this medium.

PEM medium (10) (11)

| | Per L |
|---|-------|
| MgSO ₄ ·7H ₂ O | 0.3 g |
| (NH ₄) ₂ SO ₄ | 1.0 g |
| K ₂ HPO ₄ | 1.3 g |
| Sigma Pectin | 1.5 g |

*Sigma- pectin from citrus peel

1. Add ingredients and heat the solution until the pectate is dissolved. Adjust the pH to 7.2 once all ingredients are added and dissolved.
2. Autoclave the solution in small batches at 120°C for 15 min. Do not re-use individual batches after opening the aliquot.

D-PEM (1)

In 300 ml distilled water, dissolve the following components in order; heat if necessary.

| | Per L |
|---|--------|
| MgSO ₄ ·7H ₂ O | 0.64 g |
| (NH ₄) ₂ SO ₄ | 2.16 g |
| K ₂ HPO ₄ | 2.16 |
| Sigma Pectin | 1.5 g |

1. Bring volume up to 1,000 ml.
2. Suspend 3.4 g of sodium polypectate in 5 ml of absolute ethanol and add to solution. Mix.
3. Adjust pH to 7.2; dispense solution into small aliquots (50 ml).
4. Autoclave at 120°C for 15 min. Store at 4°C until ready to use.

Appendix B: *Dickeya* and *Pectobacterium* species identification – biochemical phenotypes and MLSA recommendations

Dickeya is a member of the Enterobacteriaceae and used to be referred to as *Erwinia chrysanthemi*. *Dickeya* cells are Gram-negative rod shaped cells with peritrichous flagella. These species cause soft rot, blackleg, and wilt diseases in potato and other vegetable and ornamental crops. Recent reviews on isolation and identification of *Dickeya* species are available (1, 12).

Biochemical assays used to differentiate *Dickeya* and *Pectobacterium*

| Test | <i>Dickeya</i> | <i>Pectobacterium</i> |
|----------------------------|----------------|-----------------------|
| Phosphatase activity | + | - |
| Indole production | + | v ^{-A} |
| Erythromycin sensitivity | + | v- |
| NaCl sensitivity | + | - |
| Pellicle formation in SOBG | + | - |
| Indigoidine production | + | - |

^Av- = variable, but most strains are negative for this phenotype

Dickeya species names and phenotypes^A

| Species name | Ref. | Growth at 39°C | D-Arabinose | Melibiose | Raffinose | Mannitol | β-Gentiobiose | Casein |
|---|------|----------------|-------------|-----------|-----------|----------|---------------|------------|
| <i>D. aquatica</i> | (13) | + | - | + | + | - | - | + |
| <i>D. chrysanthemi</i> | (14) | + | - | + | + | + | - | + |
| <i>D. dadantii</i> <i>subsp.dadantii</i> | (14) | + | + | + | + | + | +(Variable) | + |
| <i>D. dadantii subsp. dieffenbachiae</i> | (14) | + | + | - | - | + | + | variable |
| <i>D. dianthicola</i> | (14) | - | - | variable | variable | + | - | variable |
| <i>D. fangzhongdai</i> | (15) | + | + | + | + | + | - | - |
| <i>D. paradisiaca</i> | (14) | + | + | variable | variable | - | + | - |
| <i>D. solani</i> | (16) | weak | + | + | + | + | weak | not tested |
| <i>D. zeae</i> | (14) | + | + | + | + | + | - | + |

^A Phenotype information was compiled by (13) and (15) and includes data from other references (14, 16, 17)

Table 2. *Pectobacterium* species names and phenotypes^A

| Species | <i>P. aroide-arum</i> | <i>P. atro-septicum</i> | <i>P. carotovorum</i> | | | <i>P. betavas-culorum</i> | <i>P. parmentieri</i> | <i>P. wasa-biae</i> |
|------------------------------|-----------------------|-------------------------|-----------------------|--------------------|-------------------|---------------------------|-----------------------|---------------------|
| | | | <i>brasiliense</i> | <i>carotovorum</i> | <i>odoriferum</i> | | | |
| Subspecies | | | | | | | | |
| Growth at 36°C | + | - | + | + | + | + | - | - |
| Reducing sugars from sucrose | + | + | + | - | + | + | ND | - |
| Phosphatase activity | ND | - | ^w - | ^w - | ^w - | - | ND | + |
| ONPG test | ND | - | + | + | + | + | ND | ND |
| Utilization of: | | | | | | | | |
| Keto-methyl glucoside | ND | + | - | - | + | + | ND | ND |
| Cellobiose | + | + | ND | + | + | - | ND | + |
| Melibiose | + | + | ND | + | + | - | + | - |
| Raffinose | + | + | ND | + | + | + | + | - |
| Galactose | ND | + | ND | + | ND | + | + | - |
| Acid produced from: | | | | | | | | |
| Sorbitol | + | + | + | + | + | - | + | - |
| Alpha-methyl glucoside | - | + | + | - | + | + | ND | - |
| Melibiose | + | + | + | + | + | - | + | - |
| Citrate | + | + | + | + | + | - | + | + |
| Raffinose | + | + | + | + | + | - | + | - |
| Arabitol | + | - | - | - | + | - | + | - |

^A Phenotype information was compiled from (18-22). ^wSome strains weakly positive

Although there are phenotypic characteristics that differ among *Dickeya* and *Pectobacterium* species, classification by genome sequence is more accurate than classification by phenotype. Multiple primer sets for multilocus sequence analysis (MLSA) have been described and all of the reported MLSA of *Dickeya* and *Pectobacterium* appear to work well. A more general set of MLSA primers than shown here can be found in Ma et al. (23)

MLSA Primers (24)

| Target | Primer Code | Sequence (5'-3') | PCR Cycling Conditions | T _m ^a (°C) | Fragment size (nt) |
|-------------|------------------------------|---|---|----------------------------------|--------------------|
| <i>dnaA</i> | <i>dnaAF</i> <i>dnaAR</i> | CCTATCGYTCTGAACGTGAA CTGCTCGATTTTGCGGCAG | 4 min 94 °C, 35 x (30 s 94 °C, 1 min 59 °C, 30 s 72 °C), 10 min 72 °C | 59 | 720 |
| <i>dnaJ</i> | <i>dnaJF</i> <i>dnaJR</i> | GATTTACGCTACAMCATGGA TTCACGCCRTCRAARAARY | 3 min 94 °C, 35 x (30 s 94 °C, 30 s 55 °C, 1 min 72 °C), 10 min 72 °C | 55 | 672 |
| <i>dnaX</i> | <i>dnaXF</i> <i>dnaXR</i> | TATCAGGTYCTTGCCCGTAAGTGG TCGACATCCARCGCYTTGAGATG | 3 min 94 °C, 35 x (1 min 94 °C, 1 min 59 °C, 2 min 72 °C), 5 min 72 °C | 59 | 450 |
| <i>gyrB</i> | <i>gyrBF</i> <i>gyrBR</i> | TTAGTTYGACGAYAACTCSTAYAAR GT CCCCTTCCACCAGGTASAGTTC | 4 min 94 °C, 35 x (1 min 94 °C, 1 min 56 °C, 2 min 72 °C), 10 min 72 °C | 56 | 822 |
| <i>recN</i> | <i>recNF</i> <i>recNR</i> | AGTAACTTCGGCATYGTGCGCGA AYRTGRTGYTTGCGTGCCA | 4 min 94 °C, 30 x (1 min 94 °C, 1 min 58 °C, 1 min 72 °C), 10 min 72 °C | 58 | 762 |

PCR Reaction Reagents: 25 µl Reactions

0.5 µl dNTPS

5µl Gotaq Buffer

1 µl Forward Primer

1 µl Reverse Primer

0.5 µl Template DNA

0.125 µl taq Polymerase

16.875 µl Milli-Q H₂O

References

1. **Humphris SN, Cahill G, Elphinstone JG, Kelly R, Parkinson NM, Pritchard L, Toth IK, Saddler GS.** 2015. Detection of the bacterial potato pathogens *Pectobacterium* and *Dickeya* spp. using conventional and real-time PCR. *Methods in Molecular Biology* **1302**:1-16.
2. **Clayton MK, Slack SA.** 1988. Sample-Size Determination in Zero Tolerance Circumstances and the Implications of Stepwise Sampling - Bacterial Ring Rot as a Special Case. *American Potato Journal* **65**:711-723.
3. **Degefu Y, Virtanen E, Vayrynen T.** 2009. Pre-PCR processes in the molecular detection of blackleg and soft rot *Erwinia* in seed potatoes. *J Phytopathol* **157**:370-378.
4. **Tsrer L, Erlich O, Hazanovsky M, Ben Daniel B, Zig U, Lebiush S.** 2012. Detection of *Dickeya* spp. latent infection in potato seed tubers using PCR or ELISA and correlation with disease incidence in commercial field crops under hot-climate conditions. *Plant Pathology* **61**:161-168.
5. **Czajkowski R, Grabe GJ, van der Wolf JM.** 2009. Distribution of *Dickeya* spp. and *Pectobacterium carotovorum* subsp *carotovorum* in naturally infected seed potatoes. *European Journal of Plant Pathology* **125**:263-275.
6. **Potrykus M, Sledz W, Golanowska A, Slawiak M, Binek A, Motyka A, Zoledowska S, Czajkowski R, Lojkowska E.** 2014. Simultaneous detection of major blackleg and soft rot bacterial pathogens in potato by multiplex polymerase chain reaction. *Annals of Applied Biology* **165**:474-487.
7. **Pritchard L, Humphris S, Saddler G, Parkinson NM, Bertrand V, Elphinstone JG, Toth IK.** 2013. Detection of phytopathogens of the genus *Dickeya* using a PCR primer prediction pipeline for draft bacterial genome sequences. *Plant Pathol* **62**:587-596.
8. **Hélias V, Hamon P, Huchet E, van der Wolf J, Andrivon D.** 2012. Two new effective semiselective crystal violet pectate media for isolation of *Pectobacterium* and *Dickeya*. *Plant Pathology* **61**:339-345.
9. **Lee Y-A, Yu C-P.** 2006. A differential medium for the isolation and rapid identification of a plant soft rot pathogen, *Erwinia chrysanthemi*. *Journal of Microbiological Methods* **64**:200-206.
10. **Meneley JC, E. SM.** 1975. Establishment of an inactive population of *Erwinia carotovora* in healthy cucumber fruit. *Phytopathology* **65**:670-673.
11. **Perombelon MCM, van der Wolf J.** 2002. Methods for the detection and quantification of *Erwinia carotovora* subsp. *atroseptica* (*Pectobacterium carotovorum* subsp. *atrosepticum*). *Scottish Crop Research Institute Occasional Publication* **10**:1-82.
12. **Czajkowski R, Perombelon MCM, Jafra S, Lojkowska E, Potrykus M, van der Wolf JM, Sledz W.** 2015. Detection, identification and differentiation of *Pectobacterium* and *Dickeya* species causing potato blackleg and tuber soft rot: a review. *Annals of Applied Biology* **166**:18-38.
13. **Parkinson N, DeVos P, Pirhonen M, Elphinstone J.** 2014. *Dickeya aquatica* sp. nov., isolated from waterways. *International Journal of Systematic and Evolutionary Microbiology* **64**:2264-2266.
14. **Samson R, Legendre JB, Christen R, Fischer-Le Saux M, Achouak W, Gardan L.** 2005. Transfer of *Pectobacterium chrysanthemi* (Burkholder et al. 1953) Brenner et al. 1973 and *Brenneria paradisiaca* to the genus *Dickeya* gen. nov as *Dickeya chrysanthemi* comb. nov and *Dickeya paradisiaca* comb. nov and delineation of four novel species, *Dickeya dadantii* sp nov., *Dickeya dianthicola* sp nov., *Dickeya dieffenbachiae* sp nov and *Dickeya zae* sp nov. *Intern J Syst and Evol Microbiol* **55**:1415-1427.
15. **Tian Y, Zhao Y, Yuan X, Yi J, Fan J, Xu Z, Hu B, De Boer SH, Xiang Li X.** 2016. *Dickeya fangzhongdai* sp. nov., a plantpathogenic bacterium isolated from pear trees (*Pyrus pyrifolia*). *International Journal of Systematic and Evolutionary Microbiology* **66**:2831-2835.

16. **van der Wolf JM, Nijhuis EH, Kowalewska MJ, Saddler GS, Parkinson N, Elphinstone JG, Pritchard L, Toth IK, Lojkowska E, Potrykus M, Waleron M, de Vos P, Cleenwerck I, Pirhonen M, Garlant L, Helias V, Pothier JF, Pfluger V, Duffy B, Tsror L, Manulis S.** 2014. *Dickeya solani* sp. nov., a pectinolytic plant-pathogenic bacterium isolated from potato (*Solanum tuberosum*). International Journal of Systematic and Evolutionary Microbiology.
17. **Slawiak M, van Beckhoven JRCM, Speksnijder AGCL, Czajkowski RL, Grabe G, van der Wolf JM.** 2009. Biochemical and genetical analysis reveal a new clade of biovar 3 *Dickeya* spp. strains isolated from potato in Europe Eur J Plant Pathol **125**:245-261.
18. **Gardan L, Gouy C, Christen R, Samson R.** 2003. Elevation of three subspecies of *Pectobacterium carotovorum* to species level: *Pectobacterium atrosepticum* sp. nov., *Pectobacterium betavasculorum* sp. nov. and *Pectobacterium wasabiae* sp. nov. Int J Syst Evol Microbiol **53**:381-391.
19. **Duarte V, De Boer SH, Ward LJ, de Oliveira MC.** 2004. Characterization of atypical *Erwinia carotovora* strains causing blackleg of potato in Brazil. J Appl Microbiol **96**:535-545.
20. **Nabhan S, De Boer SH, Maiss E, Wydra K.** 2012. Taxonomic relatedness between *Pectobacterium carotovorum* subsp. *carotovorum*, *Pectobacterium carotovorum* subsp. *odoriferum* and *Pectobacterium carotovorum* subsp. *brasiliense* subsp. nov. Journal of Applied Microbiology **113**:904-913.
21. **Nabhan S, De Boer SH, Maiss E, Wydra K.** 2013. *Pectobacterium aroidearum* sp. nov., a soft rot pathogen with preference for monocotyledonous plants. Int J Syst Evol Microbiol **63**:2520-2525.
22. **Khayi S, Cigna J, Chong TM, Quetu-Laurent A, Chan KG, Helias V, Faure D.** 2016. Transfer of the potato plant isolates of *Pectobacterium wasabiae* to *Pectobacterium parmentieri* sp. nov. International Journal of Systematic and Evolutionary Microbiology **66**:5379-5383.
23. **Ma B, Hibbing ME, Kim H-S, Reedy RM, Yedidia I, Breuer J, Breuer J, Glasner JD, Perna NT, Kelman A, Charkowski AO.** 2007. The host range and molecular phylogenies of the soft rot enterobacterial genera *Pectobacterium* and *Dickeya*. Phytopathology **97**:1150-1163.
24. **Marrero G, Schneider KL, M. JD, M. AA.** 2013. Phylogeny and classification of *Dickeya* based on multilocus sequence analysis. International Journal of Systematic and Evolutionary Microbiology **63**:3524-3539.