Chapter 5
Isolation, Detection and Characterization of *Pectobacterium* and *Dickeya* Species

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Abstract This chapter outlines isolation, detection and characterization methods for soft rot *Pectobacteriaceae* (SRP) and finishes with recommendations for diagnostics of SRP and perspectives for improved detection using metagenomic and pangenomic approaches. For dilution plating and isolation of SRP, crystal violet pectate is still the medium of preference, although it is poorly selective. To improve the diagnostic sensitivity of detection methods, enrichment methods are used in which selective growth of the pathogen is enhanced by incubation in a pectate broth under low oxygen conditions. For molecular characterization, various finger printing techniques are described, but today analysis based on phylogenetic markers are preferred, in particular multi-locus sequence typing of housekeeping genes and comparative genetics using whole-genome sequences. For phenotypic characterization, methods are used based on serological, biochemical and physiological features. Currently the most precise phenotyping method is protein mass fingerprinting using a MALDI-TOF Mass Spectrometry. For detection of the pathogen, DNA-based amplification

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methods are generally used, including conventional PCR, real time (TaqMan) PCR assays and LAMP assays. They can detect the pathogen at a low density and allow recognition of the pathogens at different taxonomic levels. An inventory has been included of recently developed primer and probe combinations.

5.1 Introduction

Methods for detection and diagnostics are crucial in the management of diseases caused by the soft rot *Pectobacteriaceae* (SRP) genera *Dickeya* and *Pectobacterium.* Detection methods are required to test symptomless plant material for the presence of SRP, but also for studies on the ecology and management of the pathogens. Isolation to pure cultures is needed for full characterization, which may also comprise assessment of the virulence and determination of virulence factors. For routine detection, high throughput methods with an adequate diagnostic sensitivity are indispensable. In the period between 1960 and 2000 antibody-based solutions were often the method of choice. However, as the serological diversity of disease-causing SRP was high and difficult to standardize, in recent decades DNA-based amplification methods have been the preferred option, since specificity can be adapted to allow all variants of the target organism to be detected. Nevertheless, for risk assessment studies, methods for phenotypic characterization of the bacteria are also needed. In this chapter the various methods for isolation, detection and characterization of SRP are briefly outlined.

5.2 Dilution Plating and Isolation

Understanding the ecology, taxonomy and epidemiological potential of SRP requires methods to selectively detect, isolate and quantify them in their different habitats. Many of SRP are widespread in nature and can be found in complex matrices (e.g. soil, plants) and aquatic ecosystems (e.g. rivers, lakes, aerosols, snow), which can contain large microbial populations. Artificial selective media are therefore essential for isolation and identification of *Pectobacterium* and *Dickeya* spp. from these environments.

5.2.1 Chrystal Violet Pectate (CVP): A Selective Medium Extensively Used to Isolate SRP

To recover SRP from the environment, semi-selective media are used (Meneley and Stanghellini 1976; Burr and Schroth 1977) of which crystal violet pectate (CVP) medium in various forms is the preferred option (Cuppels and Kelman 1974;

Pérombelon and Burnett 1991; Hyman et al. 2001; Helias et al. 2012). CVP can be used in either single or double layer forms. Its selectivity is based on the presence of pectin as the major carbon source and the addition of crystal violet, which inhibits the growth of Gram-positive bacteria. SRP metabolize pectin and form characteristic deep cup-like cavities (Fig. 5.1) that are easily distinguishable from shallower and wider cavities formed by pectinolytic pseudomonads. CVP-based media facilitate the isolation of SRP from diverse environments but the cavity forming colonies are frequently mixed with non-target bacteria, requiring additional steps to grow them to pure cultures. Genus-specific growth media exist, such as NGM medium (Lee and Yu 2006), which is useful for *Dickeya* spp. identification by targeting the ability of this group to produce indigoidine. However, their performance as selective isolation media is poor, especially for samples with a high microbial background. Therefore, CVP remains the medium of choice for isolation of pectinolytic bacteria, including *Dickeya* and *Pectobacterium* spp. from diseased or asymptomatic plants and tubers. Preparation of test material depends on the infection level: isolation can be made directly from symptomatic plant material but an enrichment step is advisable prior to isolation when latent infections are suspected.

The source of pectin is critical to CVP preparation as it needs to have a strong gelling capacity, allowing the medium to solidify and to produce deep cavities, making them clearly visible when metabolized by the target bacteria. In addition, it must also be nontoxic in order to allow high recovery rates of all SRP. Few pectin sources have been shown to combine these characteristics since the first pectin media were described. Today, two pectin sources are commercially available, supplied by Sigma-Aldrich (pectin from citrus peel P3850) and Agdia (DIPECTA, AG366). The

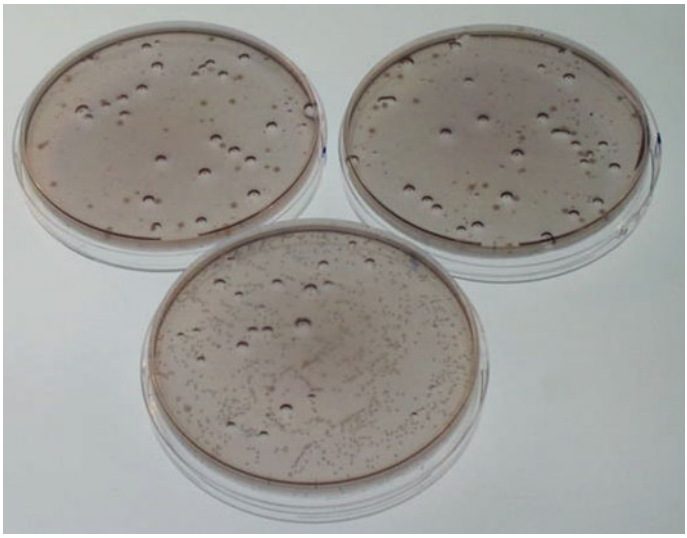

Fig. 5.1 Isolation of SRP on double layer CVP medium: *Pectobacterium* and *Dickeya* form characteristic cavities in the medium (image credit Valérie Hélias, FN3PT-RD3PT)

use of Sigma-Aldrich pectin in CVP failed to detect several pectinolytic strains and produced only small cavities from others, making isolation difficult (Helias et al. 2012). Because of its excellent properties, AG366 pectin is now used worldwide for research and diagnostic purposes on pectinolytic bacteria (Helias et al. 2012).

5.2.2 Liquid Enrichment Medium

As many studies require the recovery of SRP from latent infections or environmental samples, where bacteria are present at low levels (e.g. soil, water, latently contaminated plants), it is frequently necessary to selectively enrich the population of pectinolytic bacteria to enhance recovery. This preliminary step aims to promote the growth of pectinolytic bacteria and reduce competition from antagonistic or saprophytic microorganisms, which can be present in the environmental sample, prior to dilution and plating on CVP medium. For this purpose, a pectin enrichment broth (PEB) (Meneley and Stanghellini 1976) is used to enrich SRP populations prior to isolation on CVP media or direct detection with other techniques. The sample, tenfold diluted PEB, is incubated under anaerobic conditions (Pérombelon and van der Wolf 2002) in order to restrict the proliferation of competing (saprophytic) bacteria, whereas the growth of pectinolytic bacteria is enhanced. Enrichment for one day in PEB at room temperature is helpful to selectively increase the number of SRP prior to plating on the CVP agar (van der Wolf, Wageningen, the Netherlands, unpublished results).

5.2.3 Isolation from Plant Material or Environmental Samples

CVP remains the preferred diagnostic selective medium for the isolation of SRP from diseased plants and tubers (Laurila et al. 2008; Tsror et al. 2009; Potrykus et al. 2016). The selectivity of the medium is sufficient for the isolation of the pathogen from symptomatic stems, tubers or leaves. In practice, it is recommended that a small amount of tissue, about 1 cm of infected stem/tuber sections from the leading edge of the lesion, is sampled to minimize interference from contaminating saprophytes, including *P. carotovorum.* After incubation in an appropriate buffer the supernatant is used directly, or after further clarification and concentration, for dilution plating (Humphris et al. 2015).

In latent infections of potato plants, bacteria may be present in all tissues, stems, roots or leaves. They are more frequently found in the stolon end than in the rose end of the tuber (Czajkowski et al. 2009) but are also often present in lenticels and suberized wounds. Tuber extract consists of peel strips, including the stolon end or stolon end cores, as used in quarantine bacterial tests (see EU Commission Directive

2006/56/EC). After crushing or macerating the tuber tissue in buffer, the supernatant is centrifuged in order to concentrate the bacteria. The resulting pellet is then either enriched to enhance bacterial recovery or dilution plated directly onto selective CVP media (Humphris et al. 2015).

Densities of SRP in water are generally low and will not exceed 10^3 cfu per ml, therefore bacteria should be concentrated by filtration or differential centrifugation. Centrifugation is done twice, firstly at a low speed to clarify the supernatant and secondly at a high speed to form a concentrated bacterial pellet, before dilution plating onto CVP media. Alternatively, an enrichment step can be applied after the first centrifugation by mixing equal amounts of the clarified sample and PEB. The mixture is incubated for 24–48 h prior to the second centrifugation.

After processing, samples are serially diluted from 10^0 to 10^{-3} for latent infections and 10^0 to 10^{-6} for symptomatic samples to ensure that background saprophytes are diluted out and isolated SRP colonies can be recovered. One hundred μL of each dilution are spread onto duplicate CVP plates previously dried to remove excess surface moisture. The plates are then incubated upside down: one set of plates at 27 °C and one at 37 °C for 48–72 h. Depending on bacterial species, characteristic cavities formed by SRP appear after 24–48 h. Selected cavity forming colonies are purified on nutrient agar before being used for further molecular/phenotypic characterization.

5.3 Molecular Detection Methods

Molecular detection methods, based on the ability to target and amplify sections of bacterial genomic DNA, are the most commonly used methods to detect and differentiate SRP. These approaches include conventional PCR (De Boer and Ward 1995; Nassar et al. 1996; Kang et al. 2003), quantitative PCR (qPCR; Pritchard et al. 2013; Kim et al. 2011), multi-locus sequence typing (MLSA; Ma et al. 2007a), multiplex identification using padlock probes (Sławiak et al. 2013) and loop-mediated isothermal amplification (LAMP; Yasuhara-Bell et al. 2017). These PCR-based methods rely on amplification of target-specific DNA sequences and can be designed with specificity at any level of taxonomic organisation such as genus, species or subspecies. Although these assays can be cheaper and faster than standard plating techniques, and more specific than serological techniques, they do have some disadvantages. Target-specific primers cannot distinguish between viable or non-viable bacterial cells, while false-positive and false-negative reactions can still occur with off-target DNA or a failure to detect target region in the presence of the pathogen, respectively. Development of specific primers for diagnostics is made more difficult by the complicated taxonomic history of SRP, which has undergone several significant revisions of both genus and species-level classification and the introduction of new species (see Chap. 2). This has sometimes led to strains in culture collections and online sequence databases having the incorrect taxonomic assignments that, in turn, have led to problems when these strains are used for the design and validation of diagnostic primers (Pritchard et al. 2016).

PCR primer design to identify species and sub-species of SRP has commonly targeted genes associated with virulence (Nassar et al. 1996; Frechon et al. 1998); housekeeping (Park et al. 2006; van der Wolf et al. 2014) or phylogenetically-divergent regions such as 16S rDNA or intergenic transcribed region (Duarte et al. 2004). However, the increasing availability of whole genome sequences for SRP has recently made it possible to use a computational primer prediction pipeline to bulk search across complete genome sequences to identify PCR primers that target the species or sub-species of interest but not related non-target bacteria. The specificity of the predicted primers is first tested in silico before in vitro validation of promising diagnostic test candidates (Pritchard et al. 2016; Karim et al. 2019).

A comprehensive overview of detection methods, including molecular techniques, for differentiating between SRP can be found in Czajkowski et al. (2015), Humphris et al. (2015) and Motyka et al. (2017). A complete table of PCR primer sets used to detect SRP with conventional, multiplex and real-time assays can also be found in Czajkowski et al. (2015). PCR primer sets developed since publication of these papers are referenced in the relevant section below and summarized in Table 5.1.

5.3.1 Conventional and Multiplex PCR

PCR identification of SRP has been possible since the 1990s and many of those primer sets are still routinely used in microbiology laboratories. These include Eca1 and Eca2r primers (De Boer and Ward 1995) and Y45 and Y46 primers (Frechon et al. 1998), specific for *P. atrosepticum*; ADE1 and ADE2 primers, specific for *Dickeya* spp. (Nassar et al. 1996); BR1F and L1R primers, specific for *P. brasiliense* (Duarte et al. 2004) and EXPCCF and EXPCCR, specific for *P. carotovorum/P. wasabiae/P. parmentieri* (Kang et al. 2003). A few multiplex PCR assays have also been developed that simultaneously detect *Dickeya* spp. and *P. atrosepticum* (Peters et al. 2007; Diallo et al. 2009) and a more comprehensive assay based on existing primer sets has been developed to detect *Dickeya* spp., *P. atrosepticum* and, in a single reaction *P. carotovorum*, *P. wasabiae* and *P. parmentieri* (Potrykus et al. 2014).

For exact identification of *P. wasabiae* and *P. parmentieri,* application of species-specific PCR is necessary. Multiplex PCR reduces time and effort in the laboratory by allowing rapid detection of multiple targets in a single reaction. However, additional optimisation is often required and specificity can be reduced (Humphris et al. 2015).

5.3.2 Real-Time (Quantitative) PCR

Real-time PCR has the advantage over conventional PCR of allowing both detection and quantification of blackleg and soft rot pathogens. Real-time PCR assays can either be based on SYBR green (Laurila et al. 2010; Kim et al. 2012) or TaqMan chemistries (Pritchard et al. 2013; Humphris et al. 2015) and, while they do improve

Table 5.1 Primer sets developed between 2015 and 2020 to detect *Pectobacterium* and *Dickeya* spp.[a]

Target organism	Assay type	Primer name	Primers (5′ → 3′)	PCR product size (bp)	References
Dickeya spp., *P. atrosepticum*, *P. carotovorum*, *P. wasabiae/parmentieri*	Convention, multiplex	Df	AGAGTCAAAAGCGTCTTG	133	Potrykus et al. (2014)
		Dr	TTTCACCCACCGTCAGTC		
		Y45	TCACCGGACGCCGAACTGTGGCGT	439	
		Y46	TCGCCAACGTTCAGCAGAACAAGT		
		ExpccF	GAACTTCGCACCGCCGACCTTCTA	550–400	
		ExpccR	GCCGTAATTGCCTACCTGCTTAAG		
D. dianthicola	Conventional and qPCR	DDI-F1	CTGACTATGCCTGCGTGAAA	206	Karim et al. (2019)
		DDI-R1	GGAATCAGGCAGAACAGAT		
		DDI-F2	GTATTCAGCTCCGCCACTTC	201	
		DDI-R2	TTAACCTGACCAGCGGAGT		
D. fangzhongdai	qPCR TaqMan	DfR	ATCAGGGCGTGACCTTCGTT		Tian et al. (2020)
		DfP	TGCTGCAGACTCGATCAGGTTCTGA		
D. chrysanthemi, *D. dadantii*, *D. dianthicola*, *D. solani* and *D. zeae*	qPCR Taqman	Fw109	GCGCGCAGCACTHGAT*		Zijlstra et al. (2020)
		Rv109	CGACGGCACGCTCAGAAT		
		probe P109	AAGCCGCGGAAAT		
		Fw284	TGTGCGTTTTCGGGCTASTC*		
		Rv284	CCYTGTCTTCTGTTATCAATTCATTAAC*		
		probe P284	AACCAGAATAAGGCCC		
Pectobacterium and *Dickeya* spp.	qPCR Taqman	PEC-1F	GTG CAA GCG TTA ATC GGA ATG		Humphris et al. (2015)
		PEC-1R	CTC TAC AAG ACT CTA GCC TGT CAG TTT T		
		PEC-P	CTG GGC GTA AAG CGC ACG CA		
P. atrosepticum	qPCR Taqman	ECACSL-1F	CGGCATCATAAAAACACGCC		Humphris et al. (2015)
		ECA-CSL-89R	CCTGTGTAATATCCGAAAGGTGG		
		ECA-CSL-36 T-P	ACATTCAGGCTGATATTCCCCCTGCC		
Dickeya spp.	qPCR Taqman	ECH-1F	GAG TCA AAA GCG TCT TGC GAA		Humphris et al. (2015)

(continued)

Table 5.1 (continued)

Target organism	Assay type	Primer name	Primers (5′ → 3′)	PCR product size (bp)	References
		ECH-1R	CCC TGT TAC CGC CGT GAA		
		ECH-P	CTG ACA AGT GAT GTC CCC TTC GTC TAG AGG		
D. dianthicola	qPCR Taqman	DIA-A F	GGCCGCCTGAATACTACATT		Pritchard et al. (2013)
		DIA-A R	TGGTATCTCTACGCCCATCA		
		DIA-A P	ATTAACGGCGTCAACCCGGC		
D. solani	qPCR Taqman	SOLC-F	GCCTACACCATCAGGGCTAT		Pritchard et al. (2013)
		SOLC-R	ACACTACAGCGCGCATAAAC		
		SOLC-P	CCAGGCCGTGCTCGAAATCC		
D. solani	qPCR Taqman	*fusA* –F	GGTGTCGTTGACCTGGTGAAA		Kelly et al. (2012)
		fusA –R	ATAGGTGAAGGTCACACCCTCATC		
		fusA- P	TGAAAGCCATCAACTGGAATGATTC		
All *Dickeya* spp. *D. dianthicola*	Multiplex qPCR Taqman	DICg-F1 wf	**ATTATCTCT**GCATTGTCGAAACCAAGAACAC		Dobhal et al. (2020)
		DICg-R1 wf	AAATTATTTCTTGTCTTTCAGCCAGGTGAGC		
		DICg-P	ATGATGCAAGGGCTGTTACCATGAAAGC		
		Ddia-F1 wf	TCTACTATTTTGTGAGCTTGGCATCAAGGAA		
		Ddia-R1 wf	TTATAACATTCCGTCGTCCAACAAAATGCAG		
		Ddia-P	CAAGGCCGAACTGCTGGCGATGTAT		
P. carotovorum	LAMP	**Primer set 1**			Yasuhara-Bell et al. (2016)
		P4HA-F3	CATGAAACCCCGTTCCAGT		
		P4HA-B3	AAGGCGTCAGAGGTCAGC		
		P4HA-FIP	CGCCGTTACGTTCACGGTAGTTTATGGCGTAACAGCAGCATC		
		P4HA-BIP	TTCCTTTAGCCTCCGGCAAAGTTCGTTACACATTCCCAGCC		
		P4HA-Loop	GGAACTCATGGGCAAGCG		
		P4HA-Loop probe	/56FAM/ACGCTGAGGACCCGGATGCGAATGCGGATGCGGATGCCGAGGAACTCATGGGCAAGCG		

(continued)

Table 5.1 (continued)

Target organism	Assay type	Primer name	Primers (5′ → 3′)	PCR product size (bp)	References
		Quencher probe	TCGGCATCCGCATCCGCATTCGCATCCGGGTCCTCAGCGT/3BHQ_1/		
		Primer set 2			
		P4HA-F3	CATGAAACCCCGTTCCAGT		
		P4HA-B3	AAGGCGTCAGAGGTCAGC		
		P4HA-QFIP	/5IABkFQ/CGCCGTTACGTTCACGGTAGTTTATGGCGTAACAGCAGCATC		
		P4HA-Probe	AACTACCGTGAACGTAACGGCG/36-FAM/		
		P4HA-BIP	TTCCTTTAGCCTCCGGCAAAGTTCGTTACACATTCCCAGCC		
		P4HA-Loop	GGAACTCATGGGCAAGCG		
Dickeya spp.	LAMP	MglC-F3	TCGCTATCGGCGGTAACC		Yasuhara-Bell et al. (2017)
		MglC-B3	ACCACCGGCAAAAGACAC		
		MglC-FIP	GCCGGACAGCATATACACCCAAGGAAGCCGCCAAAGTGTC		
		MglC-BIP	CCTTCGGCGGTATGCTGGAAGCGATGGCGTCAAGTTCGTA		
		MglC-Loop	CGGTAGTGCCACTAACAACCTGG		
		MglC-Loop probe/56	FAM/ACGCTGAGGACCCGGATGCGAATGCGGATGCGGATGCCGATTTTCGGTAGTGCCACTAACAACCTGG		
		Quencher probe	TCGGCATCCGCATCCGCATTCGCATCCGGGTCCTCAGCGT/3BHQ_1		
D. dianthicola	LAMP	Dd-FIP	GGAATTCGGCAATCACGCGGATGTTTCCATCGGTGCTCACA		Ocenar et al. (2019)
		Dd-BIP	GCCGTTGCGAATGGCAAGGATGTTGAAGGCCATTCCAGC		
		Dd-F3	TGACTCACGCAATTGAAGCG		
		Dd-B3	GCGAATGCACATAGCCAAGA		
		Dd-LF	AACGCGGAGTGGTCTGTCAG		
		Dd-LB	TCAAGGCGCGCGAAATGATGG		
Pectobacterium and *Dickeya* spp.	Amplification + sequencing	GapA-7-F	ATC AAA GTA GGT ATC AAC GG	932	Cigna et al. (2017)
		GapA-938-R	TCR TAC CAR GAA ACC AGT T		

H = A or C or T; S = C or G; Y = C or T

[a]PCR based assays previously developed are summarized in Czajkowski et al. (2015)

specificity and can be carried out directly from plant extracts, the overall cost can increase due to the use of fluorescent dyes (SYBR green) or unique reporter probes (TaqMan). SYBR green based detection uses a dsDNA binding dye to detect PCR products as it accumulates during the PCR reaction, whereas TaqMan-based detection uses a fluorogenic probe specific to the target. TaqMan assays therefore have the advantage of being more specific, sensitive and reproducible than SYBR green but they do require more optimisation and the cost is higher. There are several real-time assays for detection of SRP at genus, species or subspecies level. These include TaqMan assays for the detection and quantification of all SRP, *Dickeya* spp. (Zijlstra et al. 2019), *P. brasiliense* (Brierley et al. 2008), *D. solani* (Vaerenbergh et al. 2012; Pritchard et al. 2013) and *D. dianthicola* (Pritchard et al. 2013; Karim et al. 2019), for six *Dickeya* spp. (van der Wolf et al. 2014) and SYBR green assays for *Dickeya* spp. (Laurila et al. 2010) and *P. wasabiae*/*P. parmentieri* (Kim et al. 2011).

There are currently only a few multiplex qPCR assays available for SRP, including multiplex TaqMan assays for (i) the simultaneous detection of blackleg causing SRP (De Haan and Van den Bovenkamp 2009); (ii) detection of the *Dickeya* genus and *D. dianthicola* (Dobhal et al. 2020) and (iii) the simultaneous amplification of *Pectobacterium* spp. and *P. parmentieri* (Arizala et al. 2019).

5.3.3 Loop-Mediated Isothermal Amplification (LAMP)

Loop-mediated isothermal amplification (LAMP) is gaining popularity as a sensitive and rapid amplification method that can be completed in a single step by incubating at a constant temperature. LAMP assays can be used in the field for rapid non-quantitative detection of pathogens or for quantitative detection using standard real-time fluorescence monitoring equipment in the lab (Yasuhara-Bell et al. 2017). There are numerous LAMP assays available for detection of SRP including for the *Dickeya* genus (Yasuhara-Bell et al. 2017); *D. solani* (Dobhal et al. 2020); *D. dianthicola* (Ocenar et al. 2019); *P. carotovorum* (Yasuhara-Bell et al. 2017) and *P. brasiliense* (Li et al. 2011). The advantage of LAMP assays over real-time PCR is that they do not require thermocyclers, thus making them suitable for incorporation into in-field detection devices. However, primer design for LAMP is far more complicated in that it requires four or six different primers specifically designed to recognize four or six distinct regions on the target gene (Lees et al. 2019).

5.4 Phenotyping for Detection and Identification

The most frequently used assay for SRP identification is based on pectinolytic activity and colony characteristics on CVP medium (see Sect. 5.2). An additional, easy-to-perform, test for characterization of SRP is a pathogenicity assay to determine the ability of the isolate to macerate potato tuber tissue (Lelliott and Dickey 1984;

Pérombelon and van der Wolf 2002; Potrykus et al. 2016; Zoledowska et al. 2018). The method is reliable but it should be taken into account that, on occasion, other bacteria present in the potato ecosystem can also macerate potato tissue, including *Pseudomonas* spp.

5.4.1 Biochemical Methods

Biochemical methods applied for the differentiation of SRP are based on a restricted number of characteristics that sometimes indicate the natural variability within bacteria from the same species. In general, SRP are catalase positive, oxidase negative, ferment glucose, reduce nitrate, produce β-galactosidase and H_2S, utilize L-arabinose, D-galactose, D-glucose, glycerol, D-mannose, D-ribose and sucrose but do not produce urease or acid from adonitol (De Boer and Kelman 2001). Most strains utilize L-rhamnose and D-mannitol but not dextrin (De Boer and Kelman 2001). To differentiate isolates belonging to the *Pectobacterium* genus from those of the *Dickeya* genus, and to discriminate between different species, various physiological and biochemical assays are used. For example, the most characteristic features of *P. atrosepticum* strains are the utilization of α-methyl-D-glucoside, production of reducing sugars from sucrose, growth on medium with 5% NaCl and lack of growth at 37 °C (De Boer and Kelman 2001; Gardan et al. 2003). *P. carotovorum* has similar features but is not able to utilize α-methyl-D-glucoside but grows at 37 °C (Gardan et al. 2003). *P. parmentieri* strains utilize maltose/maltodextrin, raffinose, lactose, β-galactose and α-melibiose as the sole source of carbon but not methyl α-glycopyranoside, maltose or malonic acid. These features differentiate *P. parmentieri* from closely related *P. wasabiae* strains (Goto and Matsumoto 1987; Khayi et al. 2016; Waleron et al. 2013b, 2018). About 50% of *P. parmentieri* strains are able to grow at 37 °C and are resistant to 5% NaCl (Moleleki et al. 2013; Waleron et al. 2013a).

Dickeya strains show phosphatase activity, the ability to produce indole and sensitivity to erythromycin. They can grow in a wide range of temperatures, from 21 to 41 °C with an optimal temperature of about 32 °C (Du Raan et al. 2016). Palacio-Bielsa et al. (2006) developed a modern version of a biochemical test for the differentiation of *D. chrysanthemi* biovars using a microtiter plate system. Slawiak and Lojkowska (2009) and Palacio-Bielsa et al. (2006) modified the microtiter assay and used it for the differentiation of different *Dickeya* spp. Additional features were used for differentiation such as: growth at 25 °C, 39 °C and 41 °C in nutrient broth, anaerobic hydrolysis of arginine, and polysaccharide inulin utilization in phenol red peptone water. In addition, eight carbon sources were implemented in the testing systems including acidification/alkalization on liquid Ayers, Rupp and Johnson medium (Ayers and Rupp 1919) with bromothymol blue mixed with different carbohydrates: (−)-D-arabinose, 5-keto-D-gluconate, mannitol, (+)-D-melibiose, (+)-D-raffinose and (−)-D-tartrate, β-gentiobiose and (+)-L-tartrate.

Strains of the same species show variation in their biochemical and physiological features. In addition, results of biochemical tests can be ambiguous, and procedures are sometimes difficult to standardize and are time consuming. As an alternative, commercial automated techniques such as the Biolog system (Biolog, Inc, Hayward, CA), for testing substrate utilization by SRP strains, are applied. The system is useful for rapid identification of some species and subspecies, e.g. for *P. atrosepticum*, *P. betavasculorum, P. carotovorum* and *Dickeya* spp. (De Boer and Kelman 2001; Waleron et al. 2018).

5.4.2 Serological Assays

Serological methods, including immunofluorescence staining and immunofluorescence colony staining, as well as fatty acid methyl ester analysis and volatile profiling, are also used for distinguishing SRP (Czajkowski et al. 2015). Serological or immunological methods are based on the application of antibodies against the specific antigenic properties of the target microorganisms. Serological techniques were commonly and routinely used for detection and characterization of a specific serogroup of *P. atrosepticum* (serogroup I), which was until recently the most prevalent blackleg causing organism (De Boer et al. 1987; Pérombelon and Kelman 1987; Pérombelon et al. 1998; Sledz et al. 2000). For the differentiation of SRP, polyclonal antibodies are mostly used against whole living cells or glutaraldehyde fixed cells (van der Wolf and Gussenhoven 1992; De Boer et al. 1987). Sporadically, polyclonal antibodies against specific bacterial antigens have been used. Yarkus and Schaad (1979) reported the effectiveness of polyclonal antibodies against the ribosome fraction of five *Dickeya* strains for identification of *Dickeya* spp. The obtained antibodies were specific to *Dickeya* spp. and did not react with the *P. carotovorum*, *P. atrosepticum*, *Escherichia coli* or *Pantoea agglomerans* cells. In contrast, polyclonal antibodies against whole cells cross-reacted with fluorescent pseudomonads (van der Wolf et al. 1993). The highest specificity can be obtained with monoclonal antibodies, which have been produced, for example, against the lipopolysaccharides of *P. atrosepticum* serogroup I (Vernon Shirley and Burns 1992; Gorris Maria et al. 1994). However, since their production costs are high, and the cost of reagents for molecular methods have decreased considerably, monoclonal antibodies are no longer used for routine analysis.

Immunofluorescence staining (IF) methods are based on the application of antibodies conjugated with a fluorophore (e.g. fluorescein or rhodamin) to recognize specific epitopes on the bacterial cell surface (e.g. proteins, exopolymeric substances, lipopolysaccharides). The specific interaction can be visualized under UV-light using a fluorescent microscope. IF based methods were successfully used for *P. atrosepticum* (Allan and Kelman 1977) and *D. dianthicola* (Janse and Ruissen 1988). For *P. carotovorum*, serological methods were found less useful due to their high serological variation (De Boer et al. 1987).

5.4.3 Fatty Acid Methyl Ester Analysis

Fatty Acid Methyl ester analysis (FAME) has been used to differentiate *P. carotovorum* and *P. atrosepticum* strains (De Boer and Sasser 1986). Species of *Pectobacterium* were reported to produce at least 10 different/characteristic fatty acids with chain lengths between 12 and 18 carbon atoms. A database was created that allowed differentiation of *Pectobacterium* spp. on the basis of the ratios between different fatty acids (Dawyndt et al. 2006; Waleron et al. 2018). Cother et al. (1992) used FAME to identify *Dickeya* spp. isolates in alpine water. All isolates contained cis-9-hexadecanoic, hexadecanoic, tetradecanoic acids, with most strains also containing dodecanoic acid. Nevertheless, FAME analyses allow differentiation of *Dickeya* spp. from *Pectobacterium* spp. but are not able to differentiate *Dickeya* isolates up to the species level (van der Wolf et al. 2013).

5.4.4 Electronic Sensor Systems

Volatile compounds produced by SRP or SRP-infected plant tissues have also been used for detection and characterization purposes. For example, in *Pectobacterium*-infected plant tissue, acetaldehyde, ethanol, 1-propanol, acetone 3-hydroxy-2-butanone, 2-butanone and ethanol are detected (Lui et al. 2005). However, not all these compounds can be attributed to *Pectobacterium,* as some are also detected in uninfected plant tissue. Only a few can be related solely to the presence of *Pectobacterium* spp.. Lui et al. (2005) found that acetic acid ethenyl ester (vinyl acetate) was uniquely associated with *P. atrosepticum* and cyclohexene, diazene and methoxy-(1, 1-dimethyl-2-hydroxyethyl)-amine were exclusively associated with *P. carotovorum* in potato tissue.

The first electronic sensor system (so-called ‘electronic nose’) was developed in 1999 and contained sensors able to detect compounds produced by *Pectobacterium* spp. during potato tuber infections in storage (de Lacy Costello et al. 1999). The authors expected that the “electronic nose” would be useful for application in commercial storage but to date there has been little uptake of the method.

5.4.5 MALDI-TOF Mass Spectrometry

Currently, the most precise phenotyping method is protein mass fingerprinting using a MALDI-TOF Mass Spectrometry. Together with dedicated software and a fingerprint databank, it allows quick and reliable analysis of SRP strains. MALDI TOF MS was able to generate specific spectra for the various *Dickeya* spp. (van der Wolf et al. 2013). Results indicated that *D. solani* strains clustered tightly but differed significantly from strains of *D. dadantii* subsp. *dadantii*, *D. dadantii* subsp. *dieffenbachia* and

D. dianthicola. MALDI-TOF MS analysis was also able to identify *P. brasiliense* strains from blackleg diseased plants (de Werra et al. 2015), and was used for the description of *P. peruviense* as a new species (Waleron et al. 2018).

A combination of capillary isoelectric focusing and MALDI-TOF MS allowed rapid and reliable identification of *Dickeya* and *Pectobacterium* spp. in plant tissue (Šalplachta et al. 2015). The obtained protein profiles, with several unique peaks, were species-specific and allowed *D. dianthicola*, *D. dadantii*, *D. dieffenbachiae*, *D. chrysanthemi*, *D. zeae*, *D. paradisiaca*, *D. solani*, *P. carotovorum*, and *P. atrosepticum* to be identified by hierarchical cluster analysis. It was found that the presence of plant tissue did not affect the results, making the proposed procedure very promising with respect to fast and reliable detection and identification of bacteria in plant tissues (Šalplachta et al. 2015; Horká et al. 2013).

5.5 Genotyping for Identification

Genotyping is a technology where genetically related genera, species, subspecies or individual strains are discriminated through analysis of small genetic variations, which can have major impacts on phenotype, metabolism, virulence and pathogenicity. Multiple strategies have been developed as genotyping tools for the identification and differentiation of economically important SRP at both genus and species levels. These include pulsed-field gel electrophoresis (PFGE) (Kim et al. 2009; Tsror et al. 2013; Potrykus et al. 2016), amplified fragment length polymorphism (AFLP) (Nabhan et al. 2012; Ngadze et al. 2012), PCR-restriction fragment length polymorphism (PCR–RFLP) (Darrasse et al. 1994b; Nassar et al. 1996; Toth et al. 2001; Waleron et al. 2002), DNA amplification using arbitrary primers (AP-PCR) (Parent Jean et al. 1996), repetitive sequence-based PCR (RS-PCR) (Potrykus et al. 2014; Degefu et al. 2013), phylogenetic analysis of rDNA gene sequences (Fessehaie et al. 2002; Duarte et al. 2004), multi-locus sequence typing (MLSA) of housekeeping genes (Ma et al. 2007a; De Boer et al. 2012; Waleron et al. 2013a) and whole-genome sequence analysis (Zhang et al. 2016). Such investigations of the genetic diversity have been used in the classification and taxonomy of SRP as well as in disease diagnosis and have been of fundamental importance in disease management and seed potato certification programs.

5.5.1 PCR-Restriction Fragment Length Polymorphism (PCR–RFLP)

The most common strategy for developing genotyping methods has been through the targeting of pathogenicity related genes, e.g. *hrpB* genes and *pel* genes (Darrasse et al. 1994a) including *pelADE* (Nassar et al. 1996), *pelY* (Frechon et al. 1998) and

pelI (Diallo et al. 2009). PCR–RFLP has been used successfully in classification of SRP (Darrasse et al. 1994b; Nassar et al. 1996), where the target PCR products are digested with several restriction endonucleases in independent reactions, and the banding patterns displayed by agarose gel electrophoresis for identification of signature PCR–RFLP patterns. Using this strategy, Darrasse et al. (1994b) classified *Pectobacterium* isolates into several groups, confirming the potato blackleg pathogen *P. atrosepticum* to be a homogenous group, whereas *P. carotovorum* was shown to be more genetically diverse. Nassar et al. (1996) digested a *pelADE* gene cluster from 78 *Dickeya* strains and formed 16 RFLP groups that were highly correlated with the biovar and pathovar groupings established previously based on host specificity and geographical distribution.

An alternative PCR–RFLP protocol based on the 16S-23S intergenic spacer of soft rot bacteria was used to differentiate among species and subspecies of SRP (Toth et al. 2001). Waleron et al. (2002) applied a PCR–RFLP assay to differentiate these pectinolytic species based on the housekeeping gene *recA*. Restriction analyses of the *recA* gene with four restriction endonucleases revealed the presence of 57 restriction groups with two distinct RFLP profiles for *P. atrosepticum*, 16 profiles for *P. carotovorum*, 14 profiles for *Dickeya* spp., and single profiles for each of *P. betavasculorum, P. odoriferum* and *P. wasabiae.* These analyses led to the establishment of *Pectobacterium* and *Dickeya* as separate genera (Gardan et al. 2003; Samson et al. 2005).

5.5.2 Amplified Fragment Length Polymorphism (AFLP)

Another widely used genotyping strategy involves randomly surveying for genomic polymorphisms among closely-related bacteria by means of amplified fragment length polymorphism (AFLP) (Avrova et al. 2002; Nabhan et al. 2012; Ngadze et al. 2012), DNA amplification using arbitrary primers (AP-PCR) (Parent Jean et al. 1996), and repetitive sequence-based PCR (RS-PCR) (Degefu et al. 2013). In AFLP protocols a set of short oligo adaptors are ligated to the sticky ends of genomic DNA fragments after restriction enzyme digestion. A subset of the restriction fragments is selectively amplified using primers complementary to the adaptor sequence, and separated and visualized by denaturing agarose gel electrophoresis, or on an automated capillary gel apparatus, followed by cluster analysis of similarity matrices derived from the AFLP patterns (Avrova et al. 2002). Using AFLP analysis, *P. carotovorum* and *Dickeya* spp. exhibited a high level of molecular diversity, whereas *P. odoriferum*, *P. betavasculorum*, *P. atrosepticum*, and *P. wasabiae* showed considerably less variation (Avrova et al. 2002; Nabhan et al. 2012; Ngadze et al. 2012). Yishay et al. (2008) adapted the AFLP analysis to separate *P. carotovorum* isolates of monocot from isolates of dicot plants in diverse geographical locations, which led to the establishment of *P. aroidearum* for isolates of *P. carotovorum* from monocot plants (Nabhan et al. 2013). Furthermore, AFLP analyses provided the earlier evidence to separate *P. carotovorum* from *P. brasiliense*, and group the latter

into 12 clusters, reflecting their geographical origins (Nabhan et al. 2012; Yishay et al. 2008; Ngadze et al. 2012).

5.5.3 Random Amplification of Polymorphic DNA (RAPD)

Random amplification of polymorphic DNA (RAPD), also known as DNA amplification, uses a set of decamer (8–12 nucleotides long) arbitrary primers for random amplification of genomic DNA, resulting in differentiable amplification patterns in agarose gel electrophoresis. The technique has proved to be useful in discriminating between *P. atrosepticum* and *P. carotovorum* (Parent Jean et al. 1996). However, its resolving power is much lower than targeted, species-specific DNA comparison methods, such as repetitive sequence-based PCR (RS-PCR) (Degefu et al. 2013). RS-PCR (ERIC-PCR or BOX-PCR) employs primers that specifically amplify target regions containing multiple noncoding repetitive sequences called enterobacterial repetitive intergenic consensus (ERIC) element or BOX elements, enabling the generation of unique DNA fingerprints with sufficient resolution to differentiate soft rot bacteria at species, and even strain level. RS-PCR analyses were used to classify *D. solani* in Finland (Degefu et al. 2013) and to differentiate potato isolates of *Pectobacterium* and *Dickeya* spp. in South Africa and Zimbabwe (Ngadze et al. 2012).

5.5.4 Multi-Locus Sequence Analysis (MLSA)

While *Pectobacterium* and *Dickeya* spp. can be taxonomically separated from other *Erwinia* and *Enterobacter* spp. on the basis of 16S rRNA sequence analysis (Kwon Soon et al. 1997), the interspecies and intraspecies relationships within SRP are illustrated using sequences of specific housekeeping genes or the multi-locus sequence analysis (MLSA) technique. *DnaX* sequences have been found useful for differentiation of *Dickeya* spp. (Slawiak et al. 2009), whereas *recA* (Parkinson et al. 2009) and *gapA* (Cigna et al. 2017) are used to differentiate all SRP. However, MLSA studies based on the sequences of multiple housekeeping genes will further improve the reliability of the characterization (Ma et al. 2007a; De Boer et al. 2012; Waleron et al. 2013b; van der Wolf et al. 2013). Housekeeping genes, including *acnA*, *gapA*, *icdA*, *mdh*, *mtlD*, *pgi* and *proA,* were chosen for MLSA analysis since they are present in all enterobacteria and have a wide scattering within the genome, as their products are involved in diverse aspects of bacterial metabolism, and none of them are clustered in the genome (Ma et al. 2007b; De Boer et al. 2012). Furthermore, *P. carotovorum* strains isolated by various researchers during 1970 to 1985 were re-characterized using MLSA based on housekeeping genes, e.g. strains from different regions worldwide were identified as *P. brasiliense*, while others from the United States were identified as *P. wasabiae*. For example, Duarte et al. (2004) previously classified the

potato blackleg and soft rot pathogens in Brazil as a new taxon, *P. brasiliense*, which later proved to be widely distributed and caused blackleg on potato and soft rot in many host plants. *P. brasiliense* has been the major causal agent of potato blackleg in South Africa (van der Merwe et al. 2010), Kenya (Onkendi and Moleleki 2014), Israel (Ali et al. 2013), the Netherlands (Nunes Leite et al. 2014) and China (Zhao et al. 2018). More recently, *P. brasiliense* was also found widely spread in potato growing regions of the United States and Canada (Ma et al. 2007a; De Boer et al. 2012; Waleron et al. 2013b), and has now become the dominant pathogen causing blackleg disease in the Netherlands (van der Wolf et al. 2017). With new advances in genome sequencing and bioinformatic toolkits, *P. brasiliense* was found to be relatively heterogeneous (Li, Charlottetown, Canada, unpublished data; Portier et al. 2019). Three indels (insertions and deletions) were readily identified and used for genotyping to demonstrate the relatively high intraspecies variation of *P. brasiliense* (Table 5.2, Li, Charlottetown, Canada, unpublished data). For instance, in tropical strains of *P. brasiliense* three unique indels were identified that also have putative pathogenicity islands or similar gene structures not present in strains from China and North America. The pathogenicity islands possibly originate from lateral gene transfer (Li and De Boer, Charlottetown, Canada, unpublished data).

Table 5.2 Signature features of three indels encoding putative pathogenicity islands for separating *Pectobacterium brasiliense* (tropical strains with high virulence) in genome comparison with temperate isolates from North America

PAI-associated features	Measurement methods	*P. brasiliense* V_{High} strain		
		Indel I	Indel II	Indel III
Sequence signature	G + C content, GC-skew, codon usage, etc	50.01	52.00	56.68
Virulence factors	Virulence factor database, VFDB	++	++	+++
Presence of mobile elements (integrases, transposes, etc.)	NCBI-nr/nt, UniprotKB, Pfam or COG database	–	–	++
Phage or prephage-related genes/gene cluster	NCBI-nr/nt, UniprotKB, Pfam or COG database	++	++	–
tRNA genes	Use tRNA gene search tool of tRNAscan-SE	?	?	?
High % of hypothetic protein genes	NCBI-nr/nt, UniprotKB, Pfam or COG database	++	++	+++
Presence of direct repeats	Use repeat finder software REPuter	+++	+++	+++
Presence of insertion sequences	Search through ISfinder database	?	?	+++

5.5.5 *Future Directions for Genotyping*

With the support and rapid progress of next generation sequencing (NGS), the growing number of sequenced bacterial genomes provides a rich source of information for new approaches to resolve complex diagnostic questions in plant pathology. Developments in real-time long-read sequencing, such as Oxford Nanopore technologies (MinION and etc.) have made significant progress in generating high quality genome sequences with decreased error rates (Votintseva et al. 2017). The genotyping of soft rot bacteria increasingly benefits from NGS and bioinformatics, such as full genome strategies for pairwise comparison by ANI and dDDH (Zhang et al. 2016), genome-wise identification of indels and SNPs (Khayi et al. 2015; Golanowska et al. 2018; Li and De Boer Charlottetown, Canada, unpublished data), pangenome description and characterization of within species diversity (Golanowska et al. 2018, Zoledowska et al. 2018) and supervised machine learning (Ma et al. 2014) (see also Chap. 2). Newly emerging techniques and applications should enable genomics-based strategies to become the first-line genotyping tools of the future.

5.6 Concluding Remarks

Isolation of SRP is a prerequisite for phenotypic and genetic characterization of strains. Currently, CVP-medium is still widely used but the selectivity is limited and there is certainly a need for improvements to increase isolation efficiency. For rapid and cost-effective characterization of strains the sequencing of one or multiple housekeeping genes is recommended. Biochemical methods that were used in the recent past are time-consuming and the reproducibility is limited, although these methods are still useful for full characterization of strains and for studies on biological features.

For detection of SRP in plant material PCR-based amplification methods are recommended, with testing of symptomless material preferably being preceded by an enrichment step. For high throughput testing, TaqMan assays, following automated DNA extraction, are the methods of choice. TaqMan assays have shown their robustness and, with the availability of a high number of whole genome sequences of various SRP and the development of automatic pipelines for selection of primers and TaqMan probes, the development of new methods with the desired specificity is relatively easy. In case of latent infections, enrichment is also required to detect low densities of SRP; TaqMan assays commonly cannot detect the pathogens below a density of 10^4 cells per ml. For testing of potato, the assays should be able to detect all pathogens that can cause blackleg, as there are few seed lots entirely free from soft rot causing pathogens. It can mean that additional assays may need to be developed.

Serological detection methods may still be used in epidemiological studies in which strains with a specific serogroup are introduced but the serological variation is too high for testing of plant material. However, whole genome sequence analysis

becomes increasingly the standard for characterization of SRP, which allows the determination of the exact taxonomic position based on MLSA, ANI, isDNA-DNA hybridization and similar methods (see Sect. 2.2.2). The information of many strains is now available in public databases. The increasing amount of information will make a pan-genomic approach to characterization an increasingly attractive proposition in which the entire gene set of all strains of a species can be analysed efficiently with advanced bioinformatic tools (Snipen et al. 2009).

Sequences continue to be used for the development of target specific DNA based amplification assays, including TaqMan and increasingly for LAMP assays, which are less prone to disturbances by sample compounds and more suitable for on-site detection. It is expected that the use of metagenomics, in which detection and diagnostics are combined, will be increasingly important in the future, allowing unbiased analysis of potential pathogens but also of the microbial background on or within a host plant.

Even within species, large differences in pathogenicity exist. Therefore, there is still a need for rapid methods to characterize strains phenotypically, in particular for methods to reliably determine the virulence of strains across plant genotypes. Currently, the capacity of strains to macerate tubers is often used as an indicator of pathogenicity and, while useful in determining its ability to cause soft rot, is not an accurate indicator of blackleg disease. For this, only (repeated) field experiments with inoculated planting material are reliable. The availability of new, high throughput methods to determine the potential to cause disease in the field, possibly based on the response of axenically grown plants on infections with SRP, will strongly support ongoing and future diagnostic work.

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