

BACTERIAL SOFT ROT DISEASE CAUSED BY
NEW *DICKEYA* SPECIES AND ITS
BACTERIOPHAGE BIOCONTROL
POSSIBILITIES

Špela Alič

Doctoral Dissertation
Jožef Stefan International Postgraduate School
Ljubljana, Slovenia

Supervisor: Prof. Maja Ravnikar, PhD, National Institute of Biology, Ljubljana, Slovenia, and Jožef Stefan International Postgraduate School, Ljubljana, Slovenia
Co-Supervisor: Tanja Dreö, PhD, National Institute of Biology, Ljubljana, Slovenia

Evaluation Board:
Prof. Marina Dermastia, PhD, Chair, National Institute of Biology, Ljubljana, Slovenia, and Jožef Stefan International Postgraduate School, Ljubljana, Slovenia
Frédérique Van Gijsegem, PhD, Member, Institute for Ecology and Environmental Sciences – Paris, Paris, France
Prof. David Stopar, PhD, Member, Biotechnical Faculty, University of Ljubljana, Slovenia

MEDNARODNA PODIPLOMSKA ŠOLA JOŽEFA STEFANA
JOŽEF STEFAN INTERNATIONAL POSTGRADUATE SCHOOL



Špela Alič

BACTERIAL SOFT ROT DISEASE CAUSED BY NEW
DICKEYA SPECIES AND ITS BACTERIOPHAGE
BIOCONTROL POSSIBILITIES

Doctoral Dissertation

BAKTERIJSKA BOLEZEN MEHKIH GNILOB, KI JO
POVZROČA NOVA VRSTA RODU *DICKEYA*, IN MOŽNOST
BIOLOŠKEGA NADZORA BOLEZNI Z BAKTERIOFAGI

Doktorska disertacija

Supervisor: Prof. Maja Ravnikar, PhD

Co-Supervisor: Tanja Dreo, PhD

Ljubljana, Slovenia, February 2018

Acknowledgments

'Impossible' is not a scientific term.

(Vanna Bonta)

This work would never exist without the help and contributions of many incredible people that I was privileged to work with and the support of my friends and family.

First, I would like to thank my supervisor Maja Ravnikar and co-supervisor Tanja Dreo for their fostering me in the world of science. I am grateful for all your help, support and guidance, and for showing me that 'impossible' is truly not a scientific word. Thank you for opportunities and experiences that helped me grow as a scientist and as a person.

Big thanks to everyone that helped to complete my study and research. Tina Naglič, thank you for the introduction to the experimental world of bacteria and bacteriophages. Magda Tušek Žnidarič, thank you for all your help with transmission electron microscopy and endless positive energy (even when bacteria and bacteriophages did not cooperate). Lidiya Matičič, thank you for being my saving grace in the laboratory and offering irreplaceable help with my phylogenetic study. I also would like to extend my gratitude to Nejc Rački for helping with CIM chromatography and to Denis Kutnjak for lighting the way through the dark tunnel of NGS data analysis. Nataša Toplak and Simon Koren (Omega), thank you for your help with NGS sequencing and showing me how much fun lab work can be.

I am very grateful that I had an opportunity to spend a part of my PhD study at iEES-Paris under the supervision of Frédérique Van Gijsegem and Jacques Pédrón. There, I was able to learn much about *Dickeya* spp., comparative genomics and data analysis. I am appreciative for the experience because it did more than just widen my perspective of science. Frédérique and Jacques, thank you, I loved working with you.

I would like to express my appreciation to all the members of the evaluation board – Prof. Marina Dermastja, PhD; Frédérique Van Gijsegem, PhD; and Prof. David Stopar – for their insightful comments and thorough reviews.

It was never hard to work in our FITO department; it was always a very friendly, positive and encouraging environment. Therefore, I would like to wish a big thanks to all current and past members of the FITO group. You are and were great colleagues, and I am so happy that I had an opportunity to work with you. I would especially like to thank the bacteriology group (Manca, Tina, Špela, Lidiya, Tanja and Aleš and Vesna) for making me laugh, forming my psychological support system and being excellent lab companions. To my extended ex-DŠIWSO group, Alex, Živa, Tjaša and Dejan: I would like to sincerely express my gratitude for every moment we spent together. You are much more than colleagues: you became dear friends. Thank you!

I would like to acknowledge the support of the organisations that funded this research. The research presented in this thesis was supported by the Centre of Excellence for Biosensors, Instrumentation and Process Control, an operation financed by the European Union, European Regional Development Fund and the Republic of Slovenia's Ministry of Higher Education, Science and Technology, Slovenian Research Agency (contracts P4-0165, L7-5534 and 1000-15-0105) and the Euphresco Phytosanitary ERA-NET project on *Dickeya* and *Pectobacterium* spp. The Public Scholarship, Development, Disability and Maintenance Fund of the Republic of Slovenia (Ad Futura scholarship) and COST Action FA1208 financially supported my visit to iEES-Paris.

I am so grateful to all my friends for showing me the importance of the non-scientific part of life. You kept my life balanced.

Last, I want to convey my deepest appreciation to my family. Mum and Dad, big thanks for your support, love and the chance to fulfil my ambitions. Without you, I would not be the person I am now. Neža and Vida, you are the best sisters in the world. I am endlessly grateful for your support and shoulders to cry on (you know what I mean). Thank you!

Abstract

Bacterial soft rot is a destructive disease affecting various ornamental plants and crops worldwide. Causative bacteria are frequently identified as members of the genus *Dickeya*. Soft rot disease limits orchid production and causes severe economic losses in orchid nurseries globally. At the moment no effective chemical-based approach is available to control or treat *Dickeya* infections. Therefore, the bacteriophage biocontrol approach represents a promising management strategy for preventing and controlling *Dickeya* soft rots. Within this doctoral thesis, we aimed to comprehensively characterise and taxonomically classify causative pathogenic bacteria from symptomatic *Phalaenopsis* orchids from a commercial production site, as well as to isolate and characterise bacteriophages against bacterial isolates and assess their biocontrol potential.

In previous experiments, the most commonly isolated bacteria from diseased *Phalaenopsis* tissue were identified as *Dickeya* spp. Genetic diversity divided the isolates into two groups, with the greatest similarity to previously reported undefined *Dickeya* lineages from orchids (UDL-3 and UDL-4). Two model isolates, *Dickeya* sp. B16 (UDL-3) and S1 (UDL-4), representing the observed diversity, were comprehensively characterised. Virulence and aggressiveness comparison tests *in vivo* on *Phalaenopsis* orchids, potato plants and witloof chicory leaves indicated the high virulence and extreme maceration potential of these novel *Dickeya* isolates, compared to a reference panel of *Dickeya* spp.

In the last decade, the genus *Dickeya* has undergone multiple changes as multiple new taxa have been described, and just recently, a new putative *Dickeya* species was reported, namely, *Dickeya fangzhongdai*. However, *Dickeya* sp. B16 and S1 genetically and genomically corresponded to a, at the time, unclassified group of *Dickeya* strains isolated from monocotyledonous plants and water. Average nucleotide identity analysis and multilocus sequence analysis enabled us to classify these isolates as the new *D. fangzhongdai* species. However, variations among the isolates and *D. fangzhongdai* type strain could be observed on phenotypic level.

Bacteriophages active against causative *Dickeya* isolates were isolated from sewage water demonstrating the applicability of the convective interaction media monolith chromatography for bacteriophage isolation from environmental sources. Bacteriophages isolated from sewagewater and previously isolated bacteriophages from rotted *Phalaenopsis* tissue were classified into 3 different families, *Podoviridae*, *Myoviridae* and *Siphoviridae*, expressing slight variations in their host profiles. Bacteriophage BF25/12 from the *Podoviridae* family was comprehensively characterised (pH, UV and temperature stability, resistance development and whole-genome analysis), and molecular tools for tracking and quantification were developed and initial bacteriophage-host interactions were examined. Therefore, we have successfully established a bacteriophage-host system for studying

its dynamics that is essential for effective bacteriophage applicability.

The results of the research work presented in this thesis represent the first comprehensive characterisation of *Dickeya* isolates corresponding to the *D. fangzhongdai* species. Furthermore, we have established a well-defined system for a bacteriophage-host dynamics study, a first for the genus *Dickeya*.

Povzetek

Bakterijska bolezen mehkih gnilob je uničujoča rastlinska bolezen, ki prizadene številne vrste okrasnih rastlin in poljščin. Povzročitelji bolezni se zelo pogosto uvrščajo v rod *Dickeya*. Bolezen predstavlja hudo omejitev pri tržni vzgoji orhidej in posledično povzroča hudo ekonomsko škodo številnim pridelovalcem po celem svetu. Ker trenutno ni na voljo učinkovitega kemičnega sistema za zatiranje in preprečevanje okužb z *Dickeya* spp., bakteriofagni sistem biološkega nadzora omenjene bolezni predstavlja obetaven način nadzorovanja mehkih gnilob. V okviru predstavljene doktorske disertacije smo si prizadevali celostno opisati in taksonomsko razvrstiti povzročitelje mehkih gnilob na orhidejah *Phalaenopsis* iz rastlinjaka za tržno vzgojo orhidej. Obenem pa smo se usmerili v izolacijo bakteriofagov z ustreznimi lastnostmi za biološki nadzor bolezni.

Najpogostejše izolirani bakterijske povzročitelje bolezni iz okuženega tkiva orhidej *Phalaenopsis* so bile v predhodnih raziskavah uvrščene med predstavnike rodu *Dickeya*. Opažena genska raznolikost izolatov, ki je ustrezala predhodno opisani raznolikosti neuvrščenih linij *Dickeya* (UDL-3 in UDL-4) iz orhidej. Glede na zaznano raznolikost smo izbrali 2 reprezentativna izolata, *Dickeya* sp. B16 (UDL-3) in S1 (UDL-4), ki smo ju obsežno okarakterizirali. Bakterijska izolata sta izkazovala visoko stopnjo virulence na orhidejah in zelo visoko stopnjo agresivnosti na različnih vrstah rastlinskega tkiva (belgijski radič, gomolji krompirja). Stopnja in hitrost razgradnje rastlinskega tkiva je bila občutno višja od primerjalno testiranih sevov ostalih vrst rodu *Dickeya*.

Rod *Dickeya* je v preteklem desetletju doživel številne spremembe, vključno z dodatkom več novih vrst. Kot zadnja je bila nedavno opisana vrsta *Dickeya fangzhongdai*. Glede na genomsko in gensko podobnost sta *Dickeya* sp. B16 in S1 pripadali neuvrščeni skupini sevov *Dickeya* izoliranih iz rastlin enokaličnic in vode. S pomočjo izračuna povprečne identitete nukleotidov med genomi *Dickeya* (ang. average nucleotide identity) in tipizacijo na osnovi multilokusnih zaporedij pa smo uvrstili izolata v nedavno opisano vrsto *D. fangzhongdai*. Kljub visoki genomski podobnosti sevov *D. fangzhongdai* pa smo opazili raznolikosti na nivoju fenotipa.

Bakteriofage učinkovite proti izolatom *Dickeya* smo izolirali odpadne komunalne vode, na kateri smo demonstrirali uporabnost CIM monolitne kromatografije za izolacijo bakteriofagov iz okoljskih vzorcev. Izolirani bakteriofagi, skupaj s predhodnimi bakteriofagnimi izolati iz gnilega tkiva orhidej *Phalaenopsis*, so se glede na morfologijo uvrščali v 3 različne družine, in sicer *Podoviridae*, *Myoviridae*, in *Siphoviridae*. Razlike med njimi pa so bile opazne tudi na nivoju profila gostiteljskih bakterij. Preko obsežne karakterizacije bakteriofaga BF25/12, razvoja orodij za njegovo sledenje in kvantifikacijo ter analizo interakcij med gostiteljem in bakteriofagom, smo uspešno vzpostavili raziskovalni sistem bakteriofag – gostitelj, ki bo omogočal študij dinamike, ki je ključna za uspešno uporabo bakteriofagov za namene biološkega nadzora patogenih bakterij.

Rezultati predstavljenega raziskovalnega dela vključujejo prvi celosten opis izolatov vrste *D. fangzhongdai* in opišejo prvi sistem bakteriofag – gostitelj za raziskave dinamike za bakterije rodu *Dickeya*.

Contents

List of Figures	xix
List of Tables	xxi
Abbreviations	xxiii
1 Introduction	1
1.1 Bacterial Soft Rot Diseases	1
1.2 Soft Rots: An Important Limiting Factor in Orchid Production	2
1.3 <i>Dickeya</i> as a Major Soft Rot Pathogen	3
1.3.1 Genus <i>Dickeya</i> biology	3
1.3.2 Diversity of the genus <i>Dickeya</i>	4
1.4 Biocontrol Possibilities of Soft Rot Disease	4
1.4.1 Disease management strategies for bacterial plant diseases	4
1.4.2 Bacteriophages as biocontrol agents	6
1.4.3 <i>Dickeya</i> soft rot disease management using bacteriophages	7
1.5 Scientific Problems and Aims of This Thesis	8
1.5.1 Characterisation of bacteria associated with soft rots of <i>Phalaenopsis</i> orchids	8
1.5.2 Taxonomic positioning of unclassified <i>Dickeya</i> isolates	8
1.5.3 Isolation and characterisation of bacteriophages	9
1.6 Research Hypotheses	9
1.7 Previous Work and Contributions	10
2 Materials and Methods	11
2.1 Isolation and Characterisation of Bacterial Isolates	11
2.1.1 Plant material and bacterial isolation	11
2.1.2 DNA extraction and purification	12
2.1.3 Identification and molecular characterisation of bacteria	12
2.1.4 Phylogenetic analysis	13
2.1.5 Real-time PCR to detect <i>Dickeya</i> spp. and <i>Dickeya</i> spp. <i>fliC</i> lineages	13
2.1.6 Transmission electron microscopy	13
2.1.7 Maceration potential and aggressiveness of <i>Dickeya</i> strains	14
2.1.7.1 Maceration test	14
2.1.7.2 Orchid pathogenicity test	14
2.1.7.3 Potato inoculation	15
2.2 Genome Sequencing and Analysis of <i>Dickeya</i> Isolates	15
2.2.1 Bacteria whole genome sequencing	15
2.2.2 Average nucleotide identity and DNA-DNA hybridisation analysis	16
2.2.3 In-silico multilocus sequence analysis	18
2.3 Phenotypic Positioning of <i>Dickeya</i> Isolates from the <i>Phalaenopsis</i> Orchid to the Genus <i>Dickeya</i>	18
2.3.1 Colony morphology	18

2.3.2	Carbon utilisation assay	19
2.4	Isolation and Classification of Bacteriophages Active against <i>Dickeya</i>	19
2.4.1	Bacterial strains and media	19
2.4.2	Isolation of bacteriophages from wastewater	19
2.4.3	Host range and specificity of bacteriophages	20
2.4.4	Transmission electron microscopy	20
2.4.5	Bacteriophage genome characterisation	21
2.5	Genome Sequencing and Characterisation of Model Bacteriophage BF25/12.....	21
2.5.1	Bacteriophage whole-genome sequencing	21
2.5.2	UV radiation stability	22
2.5.3	pH stability	22
2.5.4	Temperature stability	22
2.5.5	Development of bacterial resistance	22
2.5.6	qPCR and ddPCR for bacteriophage detection	22
2.5.7	IZON qNANO bacteriophage particle analysis	24
3	Results	25
3.1	Genetic and Phenotypic Characterisation of Bacteria Causing Soft Rot Disease in the <i>Phalaenopsis</i> Orchid.....	25
3.1.1	Soft rot symptoms on orchids	25
3.1.2	<i>Dickeya</i> spp. isolated from orchid tissue with soft rot symptoms	25
3.1.3	Characterisation of the representative UDL-3 and UDL-4 bacteria: B16 and S1	28
3.1.4	Electron-dense ultrastructures	28
3.1.5	Pathogenicity and aggressiveness of <i>Dickeya</i> isolates	29
3.1.5.1	Maceration assay on witloof and potato tubers	29
3.1.5.2	Plant inoculation	31
3.1.5.3	Potato inoculation	33
3.2	Taxonomic Position of <i>Dickeya</i> Isolates Causing Soft Rots of Orchids and Their Relationships with Newly Described <i>Dickeya</i> Species.....	34
3.2.1	<i>Dickeya</i> sp. B16 and S1 genome sequencing	34
3.2.2	DNA sequence analysis	34
3.2.3	Colony morphology	36
3.2.4	Phenotypic identification and carbon utilisation tests	37
3.3	Isolation and Characterisation of Bacteriophages Active against <i>Dickeya</i> Isolates Causing Soft Rot on Orchids.....	40
3.3.1	Sourcing and their characterisation	40
3.3.2	Specificity of bacteriophages isolated against <i>Dickeya</i> spp.	45
3.3.3	Transmission electron microscopy of bacteria-bacteriophage interactions	48
3.4	Characterisation of the Model Bacteriophage BF25/12.....	49
3.4.1	Genome of the bacteriophage BF25/12	49
3.4.2	Development of qPCR and ddPCR to detect and quantify BF25/12	50
3.4.3	IZON qNANO bacteriophage particle analysis	50
3.4.4	UV radiation stability	51
3.4.5	pH stability	51
3.4.6	Temperature stability	52
3.4.7	Bacterial resistance	54
4	Discussion	55
4.1	Phenotypic and Genetic Characterisation of <i>Dickeya</i> Isolates from Diseased <i>Phalaenopsis</i> Orchids.....	56
4.2	Phylogenetic and Taxonomic Positioning of Unclassified <i>Dickeya</i> spp. Isolates into the <i>D. fangzhongdai</i> Species..	58

4.3 Bacteriophages Active against Causative <i>Dickeya</i> from Three Different <i>Caudovirales</i> Families	61
5 Conclusions	65
Appendix A Phylogenetic Study of <i>fliC</i> and <i>dnaX</i> Genes	67
A.1 Accession Numbers of Sequences of Strains Used in <i>fliC</i> and <i>dnaX</i> Phylogenetic Studies.	67
A.2 Phylogenetic Tree of Strains Used in <i>dnaX</i> Phylogenetic Study.	69
Appendix B Molecular Characterisation of <i>Dickeya</i> spp.	70
B.1 Performance of Real-Time PCR Assays UDL-3, UDL-4	70
B.1.1 Analytical specificity	71
B.1.2 General performance and analytical sensitivity	71
Appendix C Genetic and Genomic Comparison of <i>Dickeya</i> Species and Strains	73
C.1 MLSA Analysis of the Genus <i>Dickeya</i> , Including Japanese Isolates and DUC Strains	73
C.2 ANI Analysis of the Genus <i>Dickeya</i>	75
C.3 Bacterial Strains Used in the MLSA Study of Japanese <i>Dickeya</i> Isolates	76
Appendix D MALDI - TOF Analysis of the <i>D. fangzhongdai</i> Strains	79
References	82
Bibliography	91
Biography	93
List of Figures	xvii
List of Tables	xix
Abbreviations	xxi
1 Introduction	1
1.1 Bacterial Soft Rot Diseases	1
1.2 Soft Rots: An Important Limiting Factor in Orchid Production	2
1.3 <i>Dickeya</i> as a Major Soft Rot Pathogen	3
1.3.1 Genus <i>Dickeya</i> biology	3
1.3.2 Diversity of the genus <i>Dickeya</i>	3
1.4 Biocontrol Possibilities of Soft Rot Disease	4
1.4.1 Disease management strategies for bacterial plant diseases	4
1.4.2 Bacteriophages as biocontrol agents	5
1.4.3 <i>Dickeya</i> soft rot disease management using bacteriophages	6
1.5 Scientific Problems and Aims of This Thesis	7
1.5.1 Characterisation of bacteria associated with soft rots of <i>Phalaenopsis</i> orchids	7
1.5.2 Taxonomic positioning of unclassified <i>Dickeya</i> isolates	8
1.5.3 Isolation and characterisation of bacteriophages ...	8
1.6 Research Hypotheses	9
1.7 Previous Work and Contributions	9
2 Materials and Methods	10
2.1 Isolation and Characterisation of Bacterial Isolates ...	10
2.1.1 Plant material and bacterial isolation	10

2.1.2	DNA extraction and purification	11
2.1.3	Identification and molecular characterisation of bacteria	11
2.1.4	Phylogenetic analysis	11
2.1.5	Real-time PCR to detect <i>Dickeya</i> spp. and <i>Dickeya</i> spp. <i>fliC</i> lineages	12
2.1.6	Transmission electron microscopy	12
2.1.7	Maceration potential and aggressiveness of <i>Dickeya</i> strains	12
2.1.7.1	 Maceration test	12
2.1.7.2	 Orchid pathogenicity test	13
2.1.7.3	 Potato inoculation	14
2.2	Genome Sequencing and Analysis of <i>Dickeya</i> Isolates.....	14
2.2.1	Bacteria whole genome sequencing	14
2.2.2	Average nucleotide identity and DNA-DNA hybridisation analysis	15
2.2.3	In-silico multilocus sequence analysis	17
2.3	Phenotypic Positioning of <i>Dickeya</i> Isolates from the <i>Phalaenopsis</i> Orchid to the Genus <i>Dickeya</i>	17
2.3.1	Colony morphology	17
2.3.2	Carbon utilisation assay	17
2.4	Isolation and Classification of Bacteriophages Active against <i>Dickeya</i>	18
2.4.1	Bacterial strains and media	18
2.4.2	Isolation of bacteriophages from wastewater	18
2.4.3	Host range and specificity of bacteriophages	19
2.4.4	Transmission electron microscopy	19
2.4.5	Bacteriophage genome characterisation	20
2.5	Genome Sequencing and Characterisation of Model Bacteriophage BF25/12.....	20
2.5.1	Bacteriophage whole-genome sequencing	20
2.5.2	UV radiation stability	20
2.5.3	pH stability	20
2.5.4	Temperature stability	21
2.5.5	Development of bacterial resistance	21
2.5.6	qPCR and ddPCR for bacteriophage detection	21
2.5.7	IZON qNANO bacteriophage particle analysis	22
3	Results	23
3.1	Genetic and Phenotypic Characterisation of Bacteria Causing Soft Rot Disease in the <i>Phalaenopsis</i> Orchid.....	23
3.1.1	Soft rot symptoms on orchids	23
3.1.2	<i>Dickeya</i> spp. isolated from orchid tissue with soft rot symptoms	23
3.1.3	Characterisation of the representative UDL-3 and UDL-4 bacteria: B16 and S1	26
3.1.4	Electron-dense ultrastructures	26
3.1.5	Pathogenicity and aggressiveness of <i>Dickeya</i> isolates	27
3.1.5.1	 Maceration assay on witloof and potato tubers	27
3.1.5.2	 Plant inoculation	29
3.1.5.3	 Potato inoculation	31
3.2	Taxonomic Position of <i>Dickeya</i> Isolates Causing Soft Rots of Orchids and Their Relationships with Newly Described <i>Dickeya</i> Species.....	31
3.2.1	<i>Dickeya</i> sp. B16 and S1 genome sequencing	31
3.2.2	DNA sequence analysis	32

3.2.3 Colony morphology	34
3.2.4 Phenotypic identification and carbon utilisation tests	34
3.3 Isolation and Characterisation of Bacteriophages Active against <i>Dickeya</i> Isolates Causing Soft Rot on Orchids ...	37
3.3.1 Sourcing and their characterisation	37
3.3.2 Specificity of bacteriophages isolated against <i>Dickeya</i> spp.	41
3.3.3 Transmission electron microscopy of bacteria-bacteriophage interactions	44
3.4 Characterisation of the Model Bacteriophage BF25/12	45
3.4.1 Genome of the bacteriophage BF25/12	45
3.4.2 Development of qPCR and ddPCR to detect and quantify BF25/12	46
3.4.3 IZON qNANO bacteriophage particle analysis	46
3.4.4 UV radiation stability	47
3.4.5 pH stability	47
3.4.6 Temperature stability	48
3.4.7 Bacterial resistance	50
4 Discussion	51
4.1 Phenotypic and Genetic Characterisation of <i>Dickeya</i> Isolates from Diseased <i>Phalaenopsis</i> Orchids	52
4.2 Phylogenetic and Taxonomic Positioning of Unclassified <i>Dickeya</i> spp. Isolates into the <i>D. fangzhongdai</i> Species ..	53
4.3 Bacteriophages Active against Causative <i>Dickeya</i> from Three Different <i>Caudovirales</i> Families	56
5 Conclusions	61
Appendix A Phylogenetic Study of <i>fliC</i> and <i>dnaX</i> Genes	63
A.1 Accession Numbers of Sequences of Strains Used in <i>fliC</i> and <i>dnaX</i> Phylogenetic Studies.	63
A.2 Phylogenetic Tree of Strains Used in <i>dnaX</i> Phylogenetic Study.	65
Appendix B Molecular Characterisation of <i>Dickeya</i> spp.	66
B.1 Performance of Real-Time PCR Assays UDL-3, UDL-4	66
B.1.1 Analytical specificity	67
B.1.2 General performance and analytical sensitivity	67
Appendix C Genetic and Genomic Comparison of <i>Dickeya</i> Species and Strains	69
C.1 MLSA Analysis of the Genus <i>Dickeya</i> , Including Japanese Isolates and DUC Strains.	69
C.2 ANI Analysis of the Genus <i>Dickeya</i>	71
C.3 Bacterial Strains Used in the MLSA Study of Japanese <i>Dickeya</i> Isolates	72
Appendix D MALDI – TOF Analysis of the <i>D. fangzhongdai</i> Strains	73
References	76
Bibliography	85
Biography	87

List of Figures

Figure 3.1: Phylogenetic tree for the <i>fliC</i> sequence	2725
Figure 3.2: Colony morphology of the <i>Dickeya</i> spp. B16 and S1	2826
Figure 3.3: Ultrastructure of bacterial cells	2927
Figure 3.4: Maceration assay for witloof chicory leaves	3028
Figure 3.5: Maceration assay for potato tubers.	3129
Figure 3.6: Symptoms developed on <i>Phalaenopsis</i> leaves inoculated with <i>Dickeya</i> . 3230	
Figure 3.7: Consensus tree of the <i>purA</i> , <i>dnaN</i> , <i>gapA</i> , <i>gyrA</i> , <i>atpD</i> and <i>rpoS</i> 3532	
Figure 3.8: Colony morphology of <i>Dickeya</i> isolates	3734
Figure 3.9: Dendrogram of the relationships between the GENIII 3734	
Figure 3.10: <i>Dickeya</i> carbon utilisation heatmap	3835
Figure 3.11: Dendrogram of the relationships between the carbon utilisation profiles	3936
Figure 3.12: Representative bacteriophage plaque morphologies and RFLP profiles. 4239	
Figure 3.13: Representative transmission electron microscopy micrographs 4340	
Figure 3.14: Representative ultrastructure of bacteria-bacteriophage interactions.	4844
Figure 3.15: Annotated BF25/12 bacteriophage genome	4945
Figure 3.16: Size histogram of bacteriophage BF 25/12 particles 5147	
Figure 3.17: The effect of pH on the stability of bacteriophage BF25/12 5248	
Figure 3.18: Temperature stability with the time of bacteriophage BF25/12. 5349	
Figure 3.19: Emergence of <i>Dickeya</i> spp. B16 resistant to bacteriophage BF 25/12	5450
Figure A.1: Phylogenetic tree for <i>dnaX</i> sequence	6965
Figure B.2: Selected diagnostic parameters of real-time PCR assays. 7268	
Figure C.1 MLSA tree of the <i>recA</i> sequence	7470
Figure D.1: Peptide mass fingerprint product ion spectra of <i>D. fangzhongdai</i> . 8074	
Figure D.2: Clustering of <i>Dickeya</i> strains as a function of their MSP. 8175	

List of Tables

Table 2.1 Bacterial isolates used in genetic and phenotypic characterisation o	1110
Table 2.2 Bacterial strains used for the MLSA and ANI analysis	1716
Table 2.3: <i>Dickeya</i> spp. isolates used in the bacteriophage enrichment	1918
Table 2.4: Primers and probes	2321
Table 3.1: Diversity of the <i>Dickeya</i> isolates from <i>Phalaenopsis</i> orchids	2624
Table 3.2: Efficiency and aggressiveness for <i>Dickeya</i> isolates.	3230
Table 3.3: Bacteria detection in potato plants.	3331
Table 3.4: Pairwise ANI values	3633
Table 3.5: Similarity matrix of the <i>Dickeya</i> carbon utilization profiles	3936
Table 3.6: Carbon utilization pattern	4037
Table 3.7: Sampling timeline of the plant material and wastewater.	4138
Table 3.8: Bacteriophages isolated from infected <i>Phalaenopsis</i> tissue and wastewater	4340
Table 3.1 Host range of bacteriophages isolated against UDL-3 and UDL-4 isolates	4541
Table A.1: Accession numbers of sequences of strains used in <i>fliC</i> phylogenetic study.	6763
Table A.2: Accession numbers of sequences of strains used in <i>dnaX</i> phylogenetic study.	6764
Table B.1: Primers used for bacterial characterisations	7066
Table C.1 Pairwise ANI values	7571
Table C.3: GenBank Accession Numbers of the Japanese isolates and DUC isolates.	7672

Abbreviations

ABI	... Applied Biosystems
ANI	... Average Nucleotide Identity
BF	... Bacteriophage
BLAST	... Basic Local Alignment Search Tool
BUG	... Biolog Universal Growth Medium
CCD	... Charge Coupled Device
CFBP	... <i>Collection Française des Bactéries Phytopathogènes</i>
CFU	... Colony Forming Unit
CIM	... Convective Interaction Media
CISA	... Contig Integrator for Sequence Assembly
CPG	... Casamino Acid-Peptone-Glucose
Cq	... Quantification Cycle
ddPCR	... Digital Droplet Polymerase Chain Reaction
DNA	... Deoxyribonucleic Acid
dsDNA	... Double-Stranded Deoxyribonucleic Acid
DUC	... Dickeya Unassigned Clade
GBBC	... Culture Collection of ILVO Diagnostic Centre for Plants
IPO	... Plant Research International
LB	... Lysogeny Broth
LMG	... Laboratory of Microbiology, Ghent University
MALDI-TOF	... Matrix-assisted Laser Desorption Ionisation-time of Flight
MLSA	... Multilocus Sequence Analysis
MSP	... Main spectrum profile
NA	... Not Available
NCBI	... National Center for Biotechnology Information
NCPPB	... National Collection of Plant Pathogenic Bacteria
NGM	... Nematode Growth Medium
NIB Z	... National Institute of Biology Collection
OD ₆₀₀	... Optical Density at 600 nm
PB	... Phosphate-buffer
PBS	... Phosphate-buffered Saline
PCR	... Polymerase Chain Reaction
PFU	... Plaque Forming Units
pH	... Potential of Hydrogen
QA	... Quaternary Amine
qPCR	... Real-Time Polymerase Chain Reaction
rDNA	... Ribosomal Deoxyribonucleic Acid
RAST	... Rapid Annotations Using Subsystems Technology
RFLP	... Restriction Fragment Length Polymorphism
SD	... Standard deviation
SMG	... Saline-magnesium with Gelatin
SRE	... Soft Rot <i>Enterobacteriaceae</i>
UDL	... Unassigned <i>Dickeya</i> Lineage
UK	... United Kingdom
USA	... United States of America

UV . . . Ultraviolet

Chapter 1

Introduction

1.1 Bacterial Soft Rot Diseases

Bacterial soft rot diseases significantly affect the production of crops and ornamental plants in temperate and tropical regions worldwide. The diseases are mainly caused by bacteria of the family *Enterobacteriaceae*. The soft rot *Enterobacteriaceae* (SRE) belongs to gram-negative, rod-shaped, non-spore-forming motile facultative anaerobes (Charkowski et al., 2012). They are considered as hemibiotrophs, able to transit from a biotrophic lifestyle, at the initial host invasion, to a necrotrophic lifestyle. In the necrotrophic stage of the infection, bacteria kill the host tissue and derive energy and nutrients from dead host cells. Therefore, they were traditionally described as necrotrophs (Kraepiel & Barny, 2016). Distinctive soft rot symptoms are caused by the pathogen-producing and -secreting high level of plant cell wall-degrading enzymes (PCWDEs), like pectinases, cellulases, hemicellulases and proteinases. These enzymes cause general plant tissue maceration and are secreted mainly through the type II secretion system (Charkowski et al., 2012; Davidsson, Kariola, Niemi, & Palva, 2013; Toth, Bell, & Holeva, 2003).

A common characteristic of SRE is the low specificity of host-pathogen interactions, making them a broad host range of pathogens able to infect a wide range of plant species. The disease affects several important crop and ornamental species across the world, including orchids and potato (Davidsson et al., 2013; Toth et al., 2003; Toth et al., 2011).

A small number of SRE can be present on plant surfaces and in soil, entering the plant through wounds or natural openings. Inside the plant, they remain in the vascular system until conditions become favourable for disease development (Czajkowski, Boer, Velvis, & Wolf, 2009; Perombelon & Kelman, 1980). Favourable conditions include free water availability, suitably moderate temperatures and oxygen-limiting conditions that are critical for optimal disease development. The SRE, when not causing the disease, can live as endophytes, epiphytes and saprophytes in plants, soil, fresh surface water, ground water and insects in agricultural regions worldwide (Perombelon & Kelman, 1980; Toth et al., 2003). Commonly, SRE are spread mostly by contaminated plants. However, they can be present also in irrigation water, soil and insects. Furthermore, SREs can colonise plants latently without any symptoms; therefore, it is very difficult to control them and to keep the plants free of SRE (Davidsson et al., 2013; Perombelon & Kelman, 1980; Toth et al., 2003). Plant tissue maceration and the release of plant nutrients during infection attract other opportunistic microorganisms. Hence, other bacteria

can be isolated from the diseased plant tissue (Toth et al., 2003).

For a very long time, all the SRE were placed in the genus *Erwinia*, though the included species were not monophyletic. However, their taxonomical classification has been revised several times in recent years. Twenty years ago, the reclassification of the species *Erwinia carotovora* into the genus *Pectobacterium* was accepted (Gardan, Gouy, Christen, & Samson, 2003; Hauben et al., 1998). Later, another SRE species, *Erwinia chrysanthemi*, was placed into the new genus named *Dickeya* (Samson et al., 2005). Both genera, *Dickeya* and *Pectobacterium*, have been listed among the top 10 most important bacterial plant pathogens, based on their economic impact (Mansfield et al., 2012).

1.2 Soft Rots: An Important Limiting Factor in Orchid Production

The family *Orchidaceae* is one of the largest families of flowering plants and one of the best-selling plants in the horticultural trade with high added value. They are primarily grown as ornamental plants and represent a major export crop for various countries (Fu & Huang, 2011; Hinsley, Verissimo, & Roberts, 2015). Orchids grow naturally in numerous habitats worldwide. However, only a limited number of genera are cultivated as commercial ornamental crops (e.g. *Cymbidium*, *Dendrobium*, *Oncidium* and *Phalaenopsis*). Among all the orchid genera, *Phalaenopsis* is the prevalent genus in commercial production and comprises 85-90% of the potted orchid sales in the US (Blanchard & Runkle, 2006).

The productivity of the orchid industry has always been limited by phytopathogens. One of the most common and important limiting factors for the cultivation of *Orchidaceae* is soft rot disease. The disease is present in many nurseries all over the world, and outbreaks normally exceed economically damaging levels (Fu & Huang, 2011; Keith, Sewake, & Zee, 2005). Soft rot in orchids is primarily caused by the genera *Pectobacterium* and *Dickeya*. However, the soft rot disease caused by the genus *Dickeya* is one of the most devastating diseases that faces orchid production (Fu & Huang, 2011). In *Phalaenopsis* orchids, the disease spreads so rapidly through the plant that it can be completely rotten in 2-3 days (Joko, Subandi, Kusumandari, Wibowo, & Priyatmojo, 2014). Furthermore, optimal orchid cultivation and transport conditions, especially relatively high humidity and temperature, favour soft rot outbreak (Fu & Huang, 2011).

Dickeya spp. have been reported multiple times as a causative pathogen of orchid soft rot disease in the last decade. Cating et al. (2008) noted that *D. chrysanthemi* caused soft rot symptoms on *Vanda* orchids (Cating, Hong, Palmateer, Stiles, & Dickstein, 2008). Additionally, they described *Dickeya* infections of *Tolumnia* and *Oncidium* orchids (Cating & Palmateer, 2011; Cating, Palmateer, McMillan, & Dickstein, 2009; Li, Qiu, Fang, & Xie, 2009). Multiple *Dickeya* spp. isolates from *Oncidium* orchids were able to macerate, beside *Oncidium* orchids, as well as potato and radish tissue (Lin, Lee, Shie, Chern, & Chao, 2015). Zhou et al. (2012) isolated bacteria from symptomatic *Phalaenopsis* orchids and identified them as *D. dieffenbachiae*. A study surveying different bacterial species which cause soft rot on *Phalaenopsis* orchids summarised that the most severe symptoms in the pathogenicity assay were recorded for the isolates from the genus *Pectobacterium* (Joko et al., 2014). The study was conducted on isolates related to the genera *Pectobacterium*, *Klebsiella*, *Serratia*, *Enterobacter*, *Citrobacter*, *Providencia* and *Pseudomonas* isolated from various

orchid plants with soft rot symptoms. However, *Dickeya* isolates were not included in their study.

1.3 *Dickeya* as a Major Soft Rot Pathogen

1.3.1 Genus *Dickeya* biology

Bacteria from the genus *Dickeya* are very aggressive necrotrophic pathogens that have both a wide geographic distribution and host range, typical for soft rot *Enterobacteriaceae* (Hugouvieux-Cotte-Pattat, 2016). Originally, they were found mainly in warm tropical regions. However, in recent years, numerous *Dickeya* isolates and outbreaks have been reported in Europe, causing the substantial loss of potato crops and tubers. *Dickeya* isolates are normally spread over long distances by infected plant material. Nevertheless, the dissemination of the pathogen by infected water or vectors has been proposed. They can survive diverse environments not only as pathogens, but also as epiphytes or saprophytes in the soil and ground water. Therefore, *Dickeya* have been detected in a variety of water sources in different countries (Potrykus et al., 2016), and irrigation water was reported to be the probable source of the potato infection in Australia (Toth et al., 2011). The interaction between the pathogen and host plant is complex. The *Dickeya* infection process can be divided into three main steps, including (i) adhesion to the plant surface and penetration into plant tissue, exploiting wounds and natural openings, (ii) invasion of the plant cells' apoplast and (iii) degradation of plant tissue (Reverchon & Nasser, 2013; Toth et al., 2011).

An essential aspect of *Dickeya* pathogenesis is its metabolic functions. *Dickeya* spp. can catabolise a wide variety of plant oligosaccharides and glycosides. The *Dickeya* virulence strategy is fundamentally based on its ability to degrade the plant cell wall, resulting in visible soft rot symptoms. For this, bacterial cells secrete a range of polysaccharide-degrading enzymes mainly represented by pectate lyases that cleave the pectin polymer in the plant cell wall. The pectate lyases secreted by *Dickeya* have a double function as a virulence factor and as a nutrient provider. The specific or global regulators involved in the control of sugar assimilation enable *Dickeya* to associate nutritional status to virulence, optimising different phases of the host infection. However, an efficient colonisation of the host plant requires multiple additional elements that enable the adhesion of the bacterial cells and adaptation to the growth conditions encountered in the host. To facilitate this adaptation, *Dickeya* have developed complex regulatory networks that facilitate the optimal expression of virulence genes (Hugouvieux-Cotte-Pattat, 2016; Reverchon, Muskhelishvili, & Nasser, 2016).

Dickeya can latently infect host plants, residing in plants' intercellular spaces. Favourable conditions facilitate the expression of the *Dickeya* soft rot disease. There are currently no effective chemical management strategies to control *Dickeya* soft rot infections. Therefore, disease control is based only on preventive measures (Adriaenssens et al., 2012; Czajkowski, Ozymko, & Lojewska, 2013; Reverchon & Nasser, 2013).

1.3.2 Diversity of the genus *Dickeya*

The genus *Erwinia* was established in 1917 and included pectinolytic and non-pectinolytic plant pathogens. Members of the *Erwinia chrysanthemi* species caused the disease on a wide range of plant hosts and was therefore subdivided into six pathovars (Lelliott & Dickey, 1984). In 1998, the scientific community accepted the reclassification of the pectinolytic *Erwinia* to the genus *Pectobacterium* (Hauben et al., 1998; Waldee, 1945). Afterwards, it was shown that *Pectobacterium chrysanthemi* species form a clade distinct from other *Pectobacterium* spp. and was thus transferred to a new genus named *Dickeya* (Samson et al., 2005). The genus *Dickeya* currently comprises eight species – *Dickeya zeae*, *Dickeya dadantii*, *Dickeya chrysanthemi*, *Dickeya solani*, *Dickeya aquatica*, *Dickeya dianthicola*, *Dickeya paradisiaca* and *Dickeya fangzhongdai* (Parkinson, DeVos, Pirhonen, & Elphinstone, 2014; Samson et al., 2005; Tian et al., 2016; Toth et al., 2011). The genus *Dickeya* contains diverse isolates that cause soft rot disease on a wide variety of plant species, including economically important crops and ornamental plants, typically causing wilting, black leg and soft rot symptoms (Toth et al., 2011). However, until very recently, reports of soft rot disease caused by the genus *Dickeya* had been limited to herbaceous plants. The latest species that was recently reported, *Dickeya fangzhongdai*, includes isolates that have been described as causative agents of bleeding canker on pear trees in China (Tian et al., 2016). Tian et al. (2016) reported *D. fangzhongdai* as the first *Dickeya* species that infects trees. The symptoms do not look like a soft rot; rather, they resemble bleeding canker necrosis. However, no experimental evidence of the species' pathogenicity on pear trees or any other plant species was included in the report (Tian et al., 2016).

The use of molecular tools has revealed the further evolution of these species. Various authors have reported strains with characteristics different from the previously described species and described them as unassigned lineages (UDLs), complex unassigned clades (DUCs) or putative new species in the *Dickeya* phylogenetic backbone (Parkinson et al., 2009; Samson et al., 2005; Suharjo, Sawada, & Takikawa, 2014; van Vaerenbergh, Baeyen, de Vos, & Maes, 2012). Phylogenetic studies conducted on the partial sequencing of the *recA* gene grouped unassigned *Dickeya* isolates into clusters tentatively named DUC-1 to DUC-3 c. Likewise, van Vaerenbergh et al. (2012) described the division of unassigned isolates into UDLs, mostly for *Dickeya* isolates from ornamental plants, based on the partial sequencing of *fliC* genes. Suharjo et al. (2014) expanded DUC-2 and -3 clades, mostly comprising *Dickeya* isolates from monocotyledonous ornamental plants, with *Dickeya* isolates from Japan. All the Japanese strains were isolated in divergent locations from assorted monocotyledonous plants in the past 35 years, indicating that these strains are commonly present in the Asian environment. They suggested, based on genetic information, that these isolates may represent a new *Dickeya* species (Suharjo et al., 2014).

1.4 Biocontrol Possibilities of Soft Rot Disease

1.4.1 Disease management strategies for bacterial plant diseases

Bacterial plant diseases represent a major challenge for agricultural production. Disease control difficulties are the direct result of pathogen variability, the relatively high

probability of mutation or gene transfer in the pathogen when confronted with environmental constraints, a high pathogen multiplication rate during optimal conditions for disease development and a lack of adequate chemical-based control approaches. For efficient bacterial plant disease control, an integrated management strategy is crucial, including proper cultural practices, chemical and biological control strategies, and plant resistance (Obradovic et al., 2005; Obradovic, Jones, Momol, Balogh, & Olson, 2004).

The most important disease control strategy is to maintain a pathogen-free agricultural environment. The use of certified tubers in potato production helps to minimise the spread of contamination and infection. However, if a pathogen is introduced, the most likely source of infection is diseased plant material and the use of contaminated equipment. Therefore, the elimination of the infected plant material and the use of disinfected equipment reduce the risk of further spreading the disease.

The chemical control of bacterial diseases based mostly on copper-based bactericides and antibiotics has played a significant role in plant disease management though copper resistance as well as antibiotic resistance have been characterised in numerous plant pathogenic bacteria. Thus, considering the challenges in controlling bacterial diseases using conventional strategies, it is a good idea to replace chemical control methods with less toxic, biologically based strategies (Frampton, Pitman, & Fineran, 2012; Jones et al., 2012).

Biological control or biocontrol uses non-pathogenic microorganisms that are applied to plant tissue, resulting in the suppression of the disease. However, the implementation of these strategies has resulted in variable success, requiring additional improvements (Jeffrey B. Jones et al., 2012). In recent years, interest has been revived in bacteriophages as biocontrol agents of bacterial plant disease. Bacteriophages, viruses that specifically infect bacterial cells, were discovered at the beginning of the 20th century. The discovery raised high hopes of fighting human, animal and even plant diseases. The discovery of antibiotics decreased the interest in bacteriophages, as broad-spectrum antibiotics do not need accurately identified bacterial causal agents and are easier to standardise. However, the emergence of bacterial strains resistant to all known antibiotics renewed the interest in bacteriophage therapy. Advanced research methods and improved knowledge led to the successful application of bacteriophage therapy in humans and even implementation in the integrated management of plant pathogens (Frampton et al., 2012; J.B. Jones et al., 2007; Jones et al., 2012). The possibility of bacteriophage biocontrol management has been studied for various plant pathogenic bacteria (e.g. *E. amylovora*, *Agrobacterium tumefaciens*, *Ralstonia solanacearum*, *Streptomyces scabies*, *Pseudomonas* sp., *Xanthomonas* sp., *Pectobacterium* sp., *Xylella fastidiosa* and *Dickeya* sp.). For example, *E. amylovora* disease control, using bacteriophages in the bacterial delivery system, achieved a comparable efficiency level to the antibiotic streptomycin. Furthermore, purified bacteriophages active against *X. oryzae* significantly reduced leaf blight if applied prior to bacterial infection. A wide host range of bacteriophages was even used to disinfect *Streptomyces scabies*-infected seed potatoes (Buttimer et al., 2017; Jones et al., 2007). The first commercial bacteriophage product, AgriPhage, for agricultural use was registered in 2006 in the USA (AgriPhage from OmniLytics Inc., Salt Lake City, UT, EPA Registration # 67986-1) to control *Xanthomonas campestris* pv. *vesicatoria* and *Pseudomonas syringae* pv. *tomato* on tomato and pepper plants. It is now part of the

recommended integrated disease management approach for tomato growers in Florida.

However, *Dickeya* spp. soft rot disease control is still based on classic cultural practices, as there is no effective chemical control available. Nor is there sufficient data to implement specific biocontrol measurements against *Dickeya*. The prevention of the infection, removal of the infected plant material and prevention of pathogen transmission are currently the only *Dickeya* control possibilities (Frampton et al., 2012; Toth et al., 2011).

1.4.2 Bacteriophages as biocontrol agents

Bacteriophages are viruses that infect only bacterial cells, and like all viruses, they are obligatory parasites. The individual bacteriophage particle, known also as a *virion*, consists of a nucleic acid core surrounded by a protein coat named the *capsid*. Bacteriophages can be divided into four groups based on the nucleic acid-containing genomic information – single-stranded (ss) DNA, double-stranded (ds) DNA and ssRNA or dsRNA bacteriophages – though nucleotide molecules can be in linear or circular form (Bradley, 1965). The largest and most prevalent group of bacterial viruses comprises tailed bacteriophages, classified as the *Caudovirales* order. All members of *Caudovirales* consist the linear dsDNA molecule embedded in a protein shell. The heads of bacteriophages are icosahedral and connected to the tails that possess terminal adsorption structures, such as base plates, spikes or fibres. The genomes of tailed bacteriophages are normally large, complex and organised modularly. The bacteriophages' lifestyles divide them into lytic and temperate. However, temperate bacteriophages likely represent more than half of *Caudovirales*. As mentioned above, tailed bacteriophages are the most diverse, numerous and widespread. Based on their tail structure, they are additionally divided into three families: *Myoviridae*, *Siphoviridae* and *Podoviridae* (Kutter & Sulakvelidze, 2005).

The use of bacteriophages as control and therapy agents has been widely considered because of several advantageous characteristics. Bacteriophages are one of the most abundant organisms on Earth. Their presence in nature corresponds to the living habitats of their hosts. Therefore, they can be found in and isolated from soil, sewage, humans, animals, plants, ground and surface water, etc., where they can persist through host-dependent self-replication over long periods. However, in the absence of a host, they are eliminated. Bacteriophages can show high specificity for the bacterial hosts they infect, leaving members of native microflora unharmed and providing targeted disease management. They have been shown to be safe to use for human, animal and plant health purposes because they cannot infect eukaryotic cells. While only lytic bacteriophages can be used for bacteriophage therapy and biocontrol, their relatively easy and inexpensive production means that they are of commercial interest (Czajkowski et al., 2014; Jones et al., 2007; Kutter & Sulakvelidze, 2005).

Phage therapy proved to be successful in the field of human medicine, and a wide range of bacteriophage-based medical products were developed mostly in Poland and the former Soviet republics. Furthermore, bacteriophages are commercially used to ensure the food safety of packaged food (Intralix and Micros Food Safety) and for biocontrol in agriculture (Omnylites) (Frampton et al., 2012; Jones et al., 2007). Despite many advantages, only a few bacteriophages have demonstrated repeated, successful applications in plant disease management. Apart from the obvious

challenges, such as resistance development in bacteria (Katznelson, 1937; Levin & Bull, 2004), bacteriophage therapies must take into account the complex dynamics among bacteriophages, bacteria and their environment, which remain largely unexplored (Levin & Bull, 2004). Bacteriophages are applied to a very harsh environment. Environmental factors, especially UV radiation, pH and temperature, can inactivate or even destroy bacteriophage particles, resulting in the critical reduction of initial bacteriophage concentrations. Consequently, the suitable protective formulation and delivery of bacteriophages to affected plants must be optimised to ensure their efficient application and survival during this application (Frampton et al., 2012; Jones et al., 2007).

1.4.3 *Dickeya* soft rot disease management using bacteriophages

The idea of using bacteriophages as controlling agents has been proposed and tested for multiple plant pathogens, including *Dickeya* spp. Nevertheless, only limited attempts have been made to isolate lytic bacteriophages active against *Dickeya* spp. (Czajkowski, 2016). Bacteriophages active against the *Dickeya* species described in the literature were all classified as tailed bacteriophages. Furthermore, nearly 90% of them belong to the *Myoviridae* family. The vast majority of the bacteriophages exhibited a narrow host range, limiting the bacteriophages' efficiency to a few species within the genus.

Adriaenssens et al. (2012) described the T4-related bacteriophages LIMEstonel and LIMEstone2 that specifically infect *D. solani*. In their study, both bacteria and bacteriophages were isolated from soil taken from a potato field. The first reported an experimental field trial for the bacteriophage treatment of potato tubers infected with *D. solani*. It resulted in improved yields, which led to the proposal of the bacteriophages LIMEstonel and LIMEstone2 as effective therapeutics in an agricultural setting (Adriaenssens et al., 2012). Czajkowski et al. (2014) isolated and identified 9 additional bacteriophages that showed wider host ranges and that were all active against *D. solani*. All these bacteriophages prevented the growth of *D. solani* in vitro and protected potato tuber tissue from the maceration caused by *D. solani*. However, further studies are required to determine the long-term effectiveness of bacteriophages and to perform field trials. Some bacteriophages with broader host ranges have been reported to infect some *Pectobacterium* strains as well as *D. solani* (Czajkowski et al., 2015). Recently, bacteriophages that are active against *D. dadantii* isolates were isolated from potato plants. These bacteriophage isolates are members of the *Myoviridae* and *Siphoviridae* families and were all isolated from the water of the Caspian Sea. Their effectiveness was tested *in vitro*, and they showed promising biocontrol potential. However, no field trials have been conducted to date (Soleimani-Delfan, Etemadifar, Emtiazi, & Bouzari, 2015). Bacteriophages ΦD3 and ΦD5 exhibited a relatively wide host range, including *D. solani*, *D. dadantii*, *D. dianthicola*, *D. chrysanthemi* and *D. zeae* (Czajkowski et al., 2013), though preliminary in-vitro studies of phage effectiveness tested only the *D. solani* species. Numerous bacteriophages, active against *D. solani*, were isolated from sewage and river water in Scotland. However, only a few were applicable as a biocontrol agent, as most could affect horizontal gene transfer and were capable of transferring plasmids between *Dickeya* spp. (Day, Ahn, Fang, & Salmond, 2017). Bacteriophages able to transfer host genes to another host, like temperate bacteriophages or bacteriophages

containing transposon elements, cannot be used therapeutically because they may transfer bacterial virulence genes or drug resistant plasmids into unintended hosts, with unknown consequences.

1.5 Scientific Problems and Aims of This Thesis

1.5.1 Characterisation of bacteria associated with soft rots of *Phalaenopsis* orchids

Dickeya spp. are well-known soft rot pathogens of numerous crops, including orchids. The soft rot outbreaks in orchid nurseries cause significant losses in orchid production (Keith et al., 2005). Reports of soft rot disease of the *Phalaenopsis* orchid, a widely grown orchid species commercially, described *D. dieffenbachiae* as a causative pathogen. However, in wider studies on *Dickeya* spp. scientists have described many *Dickeya* isolates from *Phalaenopsis* that did not correspond to any currently described *Dickeya* species (Parkinson et al., 2009; Suharjo et al., 2014; van Vaerenbergh et al., 2012). Furthermore, neither of those isolates were comprehensively characterised on the genomic or phenotypic level, although some genetic similarities were indicated (Suharjo et al., 2014). Insufficient data on which bacteria are involved and what triggers lead to the soft rot of *Phalaenopsis* limit the possibilities for the control of the pathogen, warranting additional research.

Aim 1: Our first aim was to genetically and phenotypically characterise bacteria causing the soft rot disease of the *Phalaenopsis* orchid from a commercial production site in Slovenia.

1.5.2 Taxonomic positioning of unclassified *Dickeya* isolates

Throughout history, the taxonomy of SRE has been difficult to determine. For example, the *Dickeya* spp. taxonomy has undergone multiple revisions, genus reassignments and introductions of several new species. Traditional polyphasic approaches for species classification can be influenced by the subjective choice of which techniques are used for diagnosis and the complex mapping between genotype and phenotype (Pritchard et al., 2015). The whole-genome sequencing approach revealed multiple misaligned and misnamed *Dickeya* strains. The scientific community has proposed many times that current taxonomic practices and rules be reconsidered (Baltrus, 2016; Konstantinidis, Ramette, & Tiedje, 2006; Pritchard, Glover, Humphris, Elphinstone, & Toth, 2016; Zhang, Fan, & Loria, 2016). The problem of reliance on phenotypic data for taxonomic purposes persists regardless of evidence that a phenotype can be tightly connected to the bacterial environment and that, for some bacterial isolates, phenotyping is not even possible (Baltrus, 2016; Konstantinidis et al., 2006). However, information obtained from phenotypic data cannot be replaced by whole-genome data. Hence, phenotyping cannot be simply excluded from bacterial taxonomy. The taxonomy of plant pathogens is essentially important for pathogen control legislation for growers and plant pathology scientists. The correct bacterial classification is very important for the prediction and prevention of pathogen spread and the defining of appropriate control measures (Pritchard et al., 2016).

Aim 2: Our second aim was to clarify the taxonomic positioning of isolates causing the soft rot of orchids and their relationship with the newly described *Dickeya* species.

1.5.3 Isolation and characterisation of bacteriophages

Bacteriophage biocontrol strategies have been studied for the plant pathogen *Dickeya*, and initial field trials were conducted. Nevertheless, the variously reported investigations and trials including *Dickeya* spp. have only addressed bacteriophage biocontrol strategies to improve potato production (Adriaenssens et al., 2012; Czajkowski et al., 2013; Soleimani-Delfan et al., 2015). Ornamental plants with high added value, like orchids, have significant economic importance to agricultural production. Ornamentals are extensively traded worldwide, though the importation of ornamental plants into Europe is poorly regulated. If infected, plants can facilitate the spread and possible expansion of pathogenic bacteria beyond their original host plants. The pathogen transmission route from ornamental plants to potato crops has already been proposed for two *Dickeya* spp., namely *D. dianthicola* and *D. solani*. Accordingly, ornamental hosts can be regarded as a potential route for the future introduction of additional *Dickeya* spp. to agriculturally important crops throughout the world (Parkinson, Pritchard, Bryant, Toth, & Elphinstone, 2014). Effective *Dickeya* control strategies and the strictly controlled importation of ornamental plants would reduce the risk of pathogen spread and transmission.

Currently, no effective control agents or treatments are available for the management of soft rot in orchid production. Bacteriophages have many advantageous characteristics, giving them the great potential for the biocontrol of diseases in orchid nurseries, especially as part of preventive measures. Bacteriophages can be added to the plant production system to reduce the concentration of *Dickeya* spp. in the environment and to prevent outbreaks of latently infected plants. Furthermore, greenhouses represent a closed system with fewer environmental factors damaging bacteriophages and multiple possibilities for their application.

Aim 3: Our third aim was to isolate bacteriophages effective against the bacteria isolated from orchids, as well as to characterise and assess the biocontrol possibilities of bacteriophages effective against the causative bacteria of soft rot in *Phalaenopsis* orchids.

1.6 Research Hypotheses

i) *Dickeya* isolates from *Phalaenopsis* orchids genetically and phenotypically differ from any currently described *Dickeya* spp. and represent a new species within the genus *Dickeya*.

ii) Different bacteriophages active against *Dickeya* isolates from *Phalaenopsis* orchids are present in varying environments and are genetically and phenotypically diverse.

1.7 Previous Work and Contributions

The presented work is part of an integrated study performed at the National Institute of Biology, Ljubljana, Slovenia as part of a joint project with the Centre of Excellence for Biosensors, Instrumentation and Process Control, Ajdovšična, Slovenia. I joined the project in 2013 as part of my PhD studies. However, the work presented in this study is highly intertwined and includes multiple expert contributions. Accordingly, the whole study is presented to enable a comprehensive understanding of its problem.

Dr. Tanja Dreó and Tina Naglič isolated bacteria causing the soft rot disease of *Phalaenopsis* orchids in previous experiments and characterised them as members of the genus *Dickeya*. They placed the isolates into UDL-3 and UDL-4 lineages based on *fliC* diversity. Furthermore, dr. Tanja Dreó developed real-time PCR for the detection of *Dickeya* spp. *fliC* lineages, that enabled the specific detection of *Dickeya* sp. UDL-3 and UDL-4 isolates. Bacteriophages from rotted *Phalaenopsis* tissue that were active against UDL-3 bacteria were isolated by Tina Naglič and dr. Tanja Dreó (Alič et al., 2017b). The isolation of bacteriophages from wastewater was conducted on CIM concentrates that were provided by dr. Nejc Rački, and their preparation is described in Steyer et al. (2015). Transmission electron microscopy characterisation of bacteria, bacteriophages and their interactions was performed by dr. Magda Tušek Žnidarič (Alič et al., 2017a; Alič et al., 2017b).

All the other experiments presented in this study, including comprehensive phenotypic, genomic and phylogenetic characterisation of selected *Dickeya* isolates, isolation of bacteriophages from wastewater concentrates and characterisation of all bacteriophage isolates included in the study, were performed by the author of the thesis.

Chapter 2

Materials and Methods

2.1 Isolation and Characterisation of Bacterial Isolates

2.1.1 Plant material and bacterial isolation

Orchids were obtained from a commercial orchid production site at different times from the years 2010 to 2012. The symptoms observed varied concerning their soft rot spreading patterns: from the leaf base up, the leaf tip down and the middle of the leaves. Bacteria were isolated from *Phalaenopsis* orchids with soft rot symptoms by commuting or macerating the plant material (ca. halves of *Phalaenopsis* leaves) in 3 mL 10 mM phosphate-buffered (PB) (1.07 g Na₂HPO₄, 0.4 g NaH₂PO₄ × 2H₂O, per litre of water, pH 7.2). The suspensions were vortexed and left to stand at room temperature for 15 min. The samples were then streaked on CPG medium (1 g casamino acids, 10 g peptone, 10 g glucose, 18 g agar, per litre of water, pH 7.2–7.4). After 2 to 6 days' incubation at 25 °C to 28 °C, colonies were selected for further testing based on their prevalence and/or morphology. Bacteria were purified by sub-culturing and were stored in a MicroBank system (for long-term storage at temperatures below -76 °C; Pro-Lab Diagnostics) and grown on CPG or LB Luria (0.5 g/L NaCl) agar at 25 °C to 28 °C for testing. Reference bacterial strains of the *Dickeya* species were obtained from international culture collections ([Table 2.1](#)~~Table 2.1~~). The isolation of causative bacterial species was performed by dr. Tanja Dreo and Tina Naglič.

Table 2.1 Bacterial isolates used in the genetic and phenotypic characterisation of soft rot bacteria from *Phalaenopsis* orchids. Reprinted from "Putative new species of the genus *Dickeya* as major soft rot pathogens in *Phalaenopsis* orchid production" by Š. Alič et al., 2017, *Plant Pathology*, 66. Copyright 2018 by the John Wiley and Sons.

Collection number	Strain	Bacteria species	Year of isolation	Origin	Host plant
NIB Z 2098	COB B16	<i>Dickeya</i> sp.	2010	Slovenia	<i>Phalaenopsis</i> sp.
NIB Z 2099	COB S1	<i>Dickeya</i> sp.	2012	Slovenia	<i>Phalaenopsis</i> sp.
NIB Z 2238	COB S2	<i>Dickeya</i> sp.	2012	Slovenia	<i>Phalaenopsis</i> sp.
NIB Z 2243	COB S3-1	<i>Dickeya</i> sp.	2012	Slovenia	<i>Phalaenopsis</i> sp.
NIB Z 2244	COB S3-8	<i>Dickeya</i> sp.	2012	Slovenia	<i>Phalaenopsis</i> sp.
NIB Z 2245	COB S4-1	<i>Dickeya</i> sp.	2012	Slovenia	<i>Phalaenopsis</i> sp.
NIB Z 2246	COB S4-2	<i>Dickeya</i> sp.	2012	Slovenia	<i>Phalaenopsis</i> sp.

Collection number	Strain	Bacteria species	Year of isolation	Origin	Host plant
NIB Z 2247	COB S4-3	<i>Dickeya</i> sp.	2012	Slovenia	<i>Phalaenopsis</i> sp.
NIB Z 2248	COB S4-4	<i>Dickeya</i> sp.	2012	Slovenia	<i>Phalaenopsis</i> sp.
NIB Z 2249	COB S4-5	<i>Dickeya</i> sp.	2012	Slovenia	<i>Phalaenopsis</i> sp.
NIB Z 2250	COB S4-6	<i>Dickeya</i> sp.	2012	Slovenia	<i>Phalaenopsis</i> sp.
NIB Z 2251	COB S5-1	<i>Dickeya</i> sp.	2012	Slovenia	<i>Phalaenopsis</i> sp.
Continued on next page					
NIB Z 2252	COB S5-2	<i>Dickeya</i> sp.	2012	Slovenia	<i>Phalaenopsis</i> sp.
NIB Z 2253	COB S5-3	<i>Dickeya</i> sp.	2012	Slovenia	<i>Phalaenopsis</i> sp.
GBBC 500	GBBC 500	<i>D. solani</i>	2005	Belgium	<i>Solanum tuberosum</i>
GBBC 1021	GBBC 1021	<i>D. solani</i>	2008	France	<i>Solanum tuberosum</i>
GBBC 2040	GBBC 2040	<i>D. solani</i>	2007	Belgium	<i>Solanum tuberosum</i>
LMG 2485	LMG 2485 ^T	<i>D. dianthicola</i>	1956	UK	<i>Dianthus caryophyllus</i>
LMG 25991	LMG 25991 ^T	<i>D. dadantii</i> subsp. <i>dadantii</i>	1960	Comoro Is.	<i>Pelargonium capitatum</i>
LMG 25992	LMG 25992 ^T	<i>D. dadantii</i> subsp. <i>dieffenbachiae</i>	1957	USA	<i>Dieffenbachia</i> sp.
LMG 2497	LMG 2497	<i>D. zeae</i>	NA	USA	<i>Zea mays</i> var. <i>rugosa</i>

2.1.2 DNA extraction and purification

DNA was extracted from pure bacterial suspensions using Chelex DNA extraction, according to Bühlmann et al. (2014). The DNA was extracted from 100 µL of each plant extract using silica-coated magnetic beads based on QuickPick SML Plant DNA kits (BioNobile), according to Pirc et al. (2009), with the minor modification of 440 µL of lysate used for downstream purification.

2.1.3 Identification and molecular characterisation of bacteria

Bacterial identification was based on the morphology of media, oxidation and fermentation of glucose, indigoidine production (NGM medium), API20 and real-time PCR targeting DNA sequences from 16S rDNA amplified using the primers pA and pH (see paragraph 2.1.6; Edwards et al., 1989). The *Dickeya* isolates were further characterised through the partial sequencing of *dnaX* (Sławiak et al., 2009) and *fliC* (Van Vaerenbergh et al., 2012). The sequences were assembled, and their similarities to other known sequences were analysed using BLAST (Altschul, Gish, Miller, Myers, & Lipman, 1990) and CLC Main Workbench 6.8.2 (CLC Bio, Qiagen). Two isolates, *Dickeya* sp. B16 and S1, member of UDL-3 and UDL-4 lineage, respectively, were chosen as our model isolates, representing the observed *fliC* diversity.

2.1.4 Phylogenetic analysis

Nucleotide sequence alignment was carried out using the T-Coffee (Notredame, Higgins, & Heringa, 2000) alignment tool and edited using the JalView program (Waterhouse, Procter, Martin, Clamp, & Barton, 2009). The phylogenetic trees for both *fliC* and *dnaX* were constructed using the maximum likelihood method based on the Tamura three-parameter model. The initial trees for the heuristic search were obtained automatically by applying the Neighbour Joining and BioNJ algorithms. The bootstrap consensus tree inferred from 500 replicates was taken to represent the evolutionary history of the taxa analysed. Bootstrap percentages were calculated with 500 replicates. Branches corresponding to partitions reproduced in less than 50% of the bootstrap replicates were collapsed. Evolutionary analyses were conducted in MEGA7 (Kumar, Stecher, & Tamura, 2016).

2.1.5 Real-time PCR to detect *Dickeya* spp. and *Dickeya* spp. *fliC* lineages

DNA extracted from symptomatic plant tissue and infected plants was analysed using real-time PCR. The following assays were used for the molecular detection of all the samples (Appendix B, [Table B.1](#)): (i) UDL-3 and UDL-4 assay developed for the specific detection of *Dickeya* sp. UDL-3 and UDL-4 (Alič et al., 2017b; developed by Tanja Dreo, PhD, and Tina Naglič); (ii) ECH assays (Brierley et al., 2008) to detect all the known biovars and pathovars of *Dickeya* spp., except *D. paradisiaca* from the banana; and (iii) COX assays (Brierley et al., 2008; Nikolaeva, Kang, Olson, & Kim, 2012). Real-time PCR reactions were performed on a sequence detection system (ABI PRISM 7900 HT; Applied Biosystems and ViiA 7, Life Technologies) using universal cycling conditions: 2 min at 50 °C (AmpErase UNG activation), 10 min at 95 °C (AmpliTaQ Gold activation), followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C, with 9,600 emulation. The reaction volumes of 10 µL contained as final concentrations: 900 nM primers (IDT Technologies), 200 nM probe (Eurofins MWG Operon), 1× TaqMan Universal PCR Master Mix II (Applied Biosystems) and 1 µL to 2 µL of the sample (DNA). The SDS 2.4 software (Applied Biosystems) was used for the fluorescence acquisition and calculation of the threshold cycles (Cq). The baseline was set automatically, and the fluorescence threshold was set manually to intersect with the linear part of the amplification curves of all real-time PCR assays, which resulted in the final Cq value for each well.

2.1.6 Transmission electron microscopy

To examine the ultrastructural characteristics of the bacterial cells, suspensions of both isolated bacteria from genus *Dickeya* lineages 3 and 4, *Dickeya* sp. B16 and *Dickeya* sp. S1, respectively, (ca. 10⁸ cells per mL) were centrifuged at 5,000 × g for 10 min at room temperature. The supernatants were carefully removed, and the pellets were immediately resuspended in 3% (w/v) glutaraldehyde and 1% (w/v) paraformaldehyde in 0.1 M PB, pH 7.2 for 20 h at 4 °C. The samples were postfixed in 2% (v/v) OsO₄ in 0.1 M PB and embedded in Agar 100 resins (Agar Scientific). Ultrathin sections were stained with 2% (w/v) aqueous uranyl acetate and Reynolds lead citrate. The samples were examined with an electron microscope (Philips CM 100; FEI, the Netherlands) operating at an accelerating voltage of 80 kV. Images were recorded with a digital camera (Orius SC 200; Gatan) using Digital

Micrograph Software (Gatan Inc., USA). Transmission electron microscopy experiments were performed by Magda Tušek Žnidarič, PhD.

2.1.7 Maceration potential and aggressiveness of *Dickeya* strains

The aggressiveness of *Dickeya* model isolates B16 and S1 was tested in witloof chicory (Belgian endive) leaves, orchid plants and potato tubers, and compared to the virulence of other *Dickeya* spp.

2.1.7.1 Maceration test

The maceration test on witloof chicory was performed and compared for model isolates *Dickeya* B16 and S1, as well as *D. solani* GBBC 500, 1021 and 2040, *D. dianthicola* LMG 2485, *D. dadantii* subsp. *dieffenbachiae* LMG 25992 and *D. zeae* LMG 1497.

The inocula for leaf inoculation were prepared by resuspending 24-h cultures grown on a KB medium in 10 mM phosphate buffer to the final cell suspension of about 10^8 CFU/mL. The chicory leaves were inoculated by wounding the midrib with a sterile toothpick and infiltrating them with 50 μ L bacteria suspension using a 1-mL syringe. The aggressiveness was estimated by the measurement of the macerated area after an 18-h incubation in a dark chamber at 28 °C and 80% humidity. After incubation, each leaf was photographed and the macerated tissue area was estimated from obtained photography using ImageJ program.

Potato tuber inoculation was performed according to Lautier and Nasser (2007) with the following modifications: (i) bacterial inocula were prepared from a 16 h culture incubated at 28 °C on LB Luria plates, with the final cell concentration of about 10^6 CFU/mL 50 mM phosphate buffer; (ii) potato tubers (cv. Agata) were washed in tap water, surface-sterilised with 1% hypochlorite for 20 min and air-dried in a laminar flow chamber; and (iii) inoculated tubers were incubated at 28 °C for 48h with a high humidity rate. The potato tubers were obtained from the commercial production for the human consumption. The tubers used in the assay were about the same size and had no visible signs of a mechanical or biological damage. After 48h incubation, quantity of the macerated potato tuber tissue was evaluated. Each tuber was weighed. Then the macerated tissue was gently removed from each tuber and weighed separately.

2.1.7.2 Orchid pathogenicity test

The pathogenicity on *Phalaenopsis* orchids was tested for all the *Dickeya* isolates included in the chicory maceration test. The *Phalaenopsis* leaf surfaces were sterilised using 70% ethanol. Each leaf was inoculated using a sterile toothpick dipped in the bacteria culture grown on CPG plates for 24 h at 28 °C. The inoculation spot was in the middle of a leaf half. Each leaf was considered to be a replicate, and 3 or 4 leaves were inoculated per plant. However, the youngest and the oldest leaves were not used.

The inoculated plants were incubated and monitored for the development of soft rot symptoms over 18 days at 25 °C, with a 16-h photoperiod and 80% humidity in a controlled environment chamber. The lengths and widths of the rotting areas were measured, and the surface areas were calculated. The soft rot areas were shaped like an ellipse. Consequently, the ellipse formula was used for the surface evaluation. The plants inoculated

with *Dickeya* spp. B16 and S1, along with the negative control, were sampled (1 leaf per plant) on the fourth day of incubation. The sampling was performed next to the inoculation spot on the rotten tissue margin and the asymptomatic tissue at the tip of the leaf. Non-inoculated leaves on all the plants were sampled (1 leaf per plant) at the end of the incubation. The sampled tissues were macerated in 500 µL PB buffer using a micro-pestle. The plant extracts were centrifuged at 10,000 × g for 10 min. The supernatant was incubated at 95 °C for 5 min. The bacteria present and their levels were tested using qPCR.

The pathogenicity of the B16 and S1 was tested in *Phalaenopsis* orchids, and the pathogenicity of strains obtained as *Pantoea* from culture collections was tested in orchids of the genera *Phalaenopsis*, *Paphiopedium* and *Cattleyae*. The inoculated plants were incubated for up to 1 week at temperatures above 25 °C and under high humidity (>70%). The pathogenicity tests were scored as positive if soft rot developed around the point of inoculation.

2.1.7.3 Potato inoculation

The ability to spread and infect potato plants was tested for the model isolates B16 and S1, along with *D. solani* GBBC 2040. Potato (*Solanum tuberosum* L. cv. 'Désirée') was propagated in a tissue culture. After 6 weeks of growth in soil, the potato plants were inoculated. Pure bacteria cultures grown on CPG plates for 48 h at 28 °C were used as the inoculum. For the inoculation, the main stems of the plants were cut vertically with a razor blade that had been dipped in the bacteria inoculum. The cut was made 2 cm above the soil level, 1 cm long and a third of the stem thickness deep. The control plants were cut in the same manner with a clean razor blade. The inoculated plants were incubated in a greenhouse chamber, with a daily temperature of 26 °C and a nightly temperature of 22 °C. The plants were visually inspected weekly for the development of symptoms.

After 41 days of incubation, 6 plant stems of each group were sampled. The samples were 1-cm stem sections taken 3 cm and 9 cm from the inoculation wound. The plant extracts were prepared using the sampled stem segments. The samples were cut into smaller fragments and incubated in 200 µL 0.01 M PB for 10 min to 15 min, with an initial 30 s of vortexing. The DNA was extracted from 100 µL of the plant extract.

Potato tubers were grown on the inoculated plants for 72 days. The tubers were stored for a month in the dark at room temperature, and they were regularly visually inspected for soft rot symptoms. Additionally, the potato vascular system was collected from the tubers (EC Directive), and DNA was extracted from 100 µL of the prepared extract.

Specific qPCR assays were used to detect the inoculated bacteria: UDL-3, UDL-4 and ECH assays to detect *Dickeya* sp. B16, *Dickeya* sp. S1 and *D. solani* GBBC 2040, respectively.

2.2 Genome Sequencing and Analysis of *Dickeya* Isolates

2.2.1 Bacteria whole genome sequencing

The genomic DNA of representative *Dickeya* spp. isolates, namely B16 and S1, was extracted with an UltraClean Microbial DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA) and

concentrated to app. 500 ng/μL (GeneVac EZ-2Plus, Genevac, Ipswich, UK) as determined with the Qubit dsDNA HR Assay (Thermo Fischer Scientific, Life Technologies, Carlsbad, CA). Two single-read libraries were constructed with the Ion Xpress™ Plus Fragment Library Kit (Life Technologies) following the manufacturer's protocol (MA N0009847 Rev. C.0, 04/2014) with modifications: (i) 15 min DNA fragmentation time, (ii) six cycles in the library amplification and (iii) library purification with 340 μl of Agencourt® AMPure® XP Reagent (Beckman Coulter, Inc., Brea, USA). Fragments of app. 150–200 bp were selected (E-Gel® Size Select 2%, Life Technologies). An emulsion PCR was conducted with the Ion PGM™ Template OT2 200 Kit using the Ion OneTouch™ 2 System (both Life Technologies) according to the manufacturer's protocol (MA N0007220 Rev. 5.0, 10/2013), with a final library concentration of 13 pM. The quality of libraries was checked with Caliper LabChip® GX using the DNA High Sensitivity Assay (Perkin Elmer, Waltham, MA). An Ion 316 Chip v2 was used to sequence each library on the Ion Torrent Personal Genome Machine™ (PGM™) sequencer with Ion PGM™ Sequencing 200 Kit v2 (all Life Technologies).

FASTQ format sequences were quality checked, and adapters were trimmed using CLC Genomics Workbench v7.5 (Aarhus, Denmark), with the following modifications: (i) the sequence quality score was set at 0.1, (ii) eight nucleotides were trimmed from every read at the 5' terminal end and (iii) reads < 15 nucleotides were discarded. Trimmed reads were independently assembled with CLC Genomics Workbench v7.5 and the GS de novo assembler (454 Life Sciences, Roche Company, Branford, USA). Both assemblies were processed using standard program settings with minor modifications to the GS de novo assembler: (i) all contig thresholds were set to 200 bp, and (ii) long contig thresholds were changed to 1,000 bp. Assembled contigs were integrated using CISA from the Orione (Galaxy/CRS4) portal (<http://orione.crs4.it/>). Contigs with an average coverage < 10, contigs < 1,000 bp and all contaminants identified with BLASTn were discarded.

2.2.2 Average nucleotide identity and DNA–DNA hybridisation analysis

The average nucleotide identity (ANI) analysis used herein was based on whole-genome data using the method proposed by Goris et al. (2007). All calculations were performed using the blast option (ANiB), as found in the JSpecies software tool (Richter et al., 2009). The GenBank/European Molecular Biology Laboratory/DNA DataBank accession numbers for the genomes used in ANI analysis are listed in [Table 2.2](#)~~Table 2.2~~. Comparisons with the *D. fangzhongdai*-type strain were based on DNA–DNA hybridisation, as the genome of this strain is not yet publicly available. The DNA–DNA hybridisation itself was conducted by DSMZ (Leibniz Institute DSMZ–German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany), following standard spectroscopic DNA–DNA hybridisation protocols. *D. fangzhongdai* JS5^T was hybridised into isolates S1, B16 and NCPPB 3274.

Table 2.2 Bacterial strains used for the MLSA and ANI analysis, with corresponding GenBank accession numbers.

Species	Strain	Strain designation	GenBank accession number	Host	Year of isolation	Country of isolation
<i>Dickeya</i> sp.	B16	NIB Z 2098 ^T	JXBN000000000.1	<i>Phalaenopsis</i> sp.	2010	Slovenia
	S1	NIB Z 2099	JXBO000000000.1	<i>Phalaenopsis</i> sp.	2012	Slovenia
	MK7		AOOO000000000.1	River water	NA	Scotland
	NCPFB 3274	NCPFB 3274	AOOH000000000.1	<i>Aglaonema</i> sp.	1871	St. Lucia
<i>Dickeya solani</i>	IPO 2222 ^T	LMG 25993	AONU000000000.1	<i>Solanum tuberosum</i>	2007	The Netherlands
	GBBC 2040	LMG 25865	AONX000000000.1	<i>Solanum tuberosum</i>	2007	Belgium
	D s0432-1		AMWE000000000.1	<i>Solanum tuberosum</i>	NA	Finland
	RNS 08.23.3.1.A		AMYI000000000.1	<i>Solanum tuberosum</i>	2008	France
	IFB 0099	LMG 27554	JXRS000000000.1	<i>Solanum tuberosum</i>	2005	Poland
	RNS 05.1.2A		JWMJ000000000.1	<i>Solanum tuberosum</i>	2006	France
	PPO 9134		JWLT000000000.1	Hyacinth	2008	The Netherlands
	MK10		AOP000000000.1	<i>Solanum tuberosum</i>	NA	Israel
	MK16		AOOQ000000000.1	River water	NA	UK
	RNS 07.7.3B		JWLR010000000.1	<i>Solanum tuberosum</i>	2007	France
	M005		JSXD000000000.1	Waterfall	2013	Malaysia
	ND14b		CP009460.1	Waterfall	2013	Malaysia
<i>Dickeya dadantii</i>	NCPFB 898 ^T	NCPFB 898 ^T	AOOE000000000.1	<i>Pelargonium capitatum</i>	1960	Comoro Is.
	NCPFB 3537	NCPFB 3537	AOOL000000000.1	<i>Solanum tuberosum</i>	NA	Peru
	3937		CP002038.1	<i>Saintpaulia ionantha</i>	1972	France
<i>Dickeya dadantii</i> subsp. <i>dieffenbachiae</i>	NCPFB 2976 ^T	NCPFB 2976 ^T	AOOG000000000.1	<i>Dieffenbachia</i> sp.	1957	USA
<i>Dickeya dianthicola</i>	NCPFB 453 ^T	NCPFB 453 ^T	AOOB000000000.1	<i>Dianthus caryophyllus</i>	1956	UK
	RNS04.9		APVF000000000.1	<i>Solanum tuberosum</i>	2004	France
	GBBC 2039	LMG 25864	AOOM000000000.1	<i>Solanum tuberosum</i>	2004	Belgium
	IPO 980		AOS000000000.1	<i>Solanum tuberosum</i>	NA	The Netherlands
	NCPFB 3534	NCPFB 3534	AOOK000000000.1	<i>Solanum tuberosum</i>	NA	The Netherlands
<i>Dickeya chrysantemi</i>	NCPFB 402 ^T	NCPFB 402 ^T	AOOA000000000.1	<i>Chrysanthemum morifolium</i>	1956	USA
	L11		JSYH000000000.1	Lake water	2016	Malaysia
	NCPFB 3533	NCPFB 3533	AOOJ000000000.1	<i>Solanum tuberosum</i>	NA	USA
	NCPFB 516	NCPFB 516	AOOC000000000.1	<i>Parthenium argentatum</i>	1956	Denmark
	M074		JRWY000000000.1	Waterfall	2013	Malaysia
<i>Dickeya zeae</i>	NCPFB 2538 ^T	NCPFB 2538 ^T	AOOF000000000.1	<i>Zea mays</i>	1960	USA
	EC1		CP006929.1	NA	NA	
	CSL RW192		AONY000000000.1	River water	NA	UK
	DZ2Q		APMV000000000.1	NA	NA	
	ZJU1202		APWM000000000.1	Rice	2012	China

	MK19		AOR000000000.1	River water	NA	Scotland
	Ech586		CP001836.1	NA	NA	
	NCPPB 3532		AONW000000000.1	<i>Solanum tuberosum</i>	NA	Australia
<i>Dickeya zea</i>	NCPPB 3531		AOOI000000000.1	<i>Solanum tuberosum</i>	NA	Australia
	MS1		APWM000000000.1	Banana	2009	China
<i>Dickeya paradisiaca</i>	NCPPB 2511 ^T	NCPPB 2511 ^T	AONV000000000.1	<i>Musa paradisiaca</i> var. <i>dominico</i>	1973	Colombia
<i>Dickeya</i> sp.	DW0440		AON000000000.1	River water	NA	Finland
	NCPPB 569	NCPPB 569	AOD000000000.1	<i>Saccharum officinarum</i>	1958	Australia
	2B12		JSYG000000000.1	Lake water	2014	Malaysia
<i>Pectobacterium atrosepticum</i>	21A		GCA_000740965.1	<i>Solanum tuberosum</i>	1978	Belarus

2.2.3 In-silico multilocus sequence analysis

In-silico multilocus sequence analysis (MLSA) was performed on *purA*, *dnaN*, *gapA*, *gyrA*, *atpD* and *rpoS* gene sequences, based on previously reported phylogenetic analyses of the genus *Dickeya* (Brown et al., 2000; Brady et al., 2012; Marrero et al., 2013; Van der Wolf et al., 2014). Gene sequences were extracted from the available draft genomes based on gene prediction using the myRAST toolkit (Aziz et al., 2008; Overbeek et al., 2014). Furthermore, a tree-based MLSA of the genus *Dickeya*, including a set of *Dickeya* isolates from Japan (Suharjo et al., 2014) and DUC isolates described by Parkinson et al. (2009), was conducted on a partial *recA* gene sequence. SeaView, a multiplatform program for molecular phylogeny, was used to implement specified tools and options (Gouy, Guindon, & Gascuel, 2010). Sequences of housekeeping genes were aligned using the integrated MUSCLE multiple sequence alignment algorithm. Phylogenetic trees were first generated for each gene separately and then for the concatenated sequence data, using the maximum-likelihood approach and the PhyML tool. Alignment gaps were excluded using Gblocks. Bootstrap percentages were calculated with 1,000 replicates. The iTOL tool was used to visualise phylogenetic trees (Letunic & Bork, 2016). The GenBank/European Molecular Biology Laboratory/DNA DataBank accession numbers of the genomes used in multilocus sequence analysis are listed in [Table 2.2](#).

2.3 Phenotypic Positioning of *Dickeya* Isolates from the *Phalaenopsis* Orchid to the Genus *Dickeya*

2.3.1 Colony morphology

The colony's morphology was examined for *Dickeya* sp. B16, S1, MK7 and NCPPB 3274 and *D. fangzhongdai* JS5^T isolates on 4 different media. Bacteria were routinely grown on CPG, that is, 1 g casamino acids, 10 g peptone, 10 g glucose and 18 g agar per litre of ultrapure water (Mili-Q system, Merck, Darmstadt, Germany) with the water having a pH of 7.2–7.4, Luria Bertani (LB, using 0.5 g L⁻¹ NaCl), BUG (Biolog Inc., Darmstadt, CA, USA) and KingB with 20 g proteose peptone, 1.5 g dipotassium hydrogen phosphate, 1.5 g magnesium sulphate heptahydrate, 15 mL glycerol and 15g agar per litre of ultrapure water (Mili-Q system, Merck, Darmstadt, Germany) having a pH of 7.2. Plates were kept at 28°C for 40 h.

2.3.2 Carbon utilisation assay

Identification plates (GEN III Microplate™; Biolog Inc., Hayward, CA, USA) and phenotypic arrays (PM1, PM2A; MicroPlate, Biolog Inc., Hayward, CA, USA) were used according to the manufacturer's recommendations and incubated at 30 °C to identify the metabolic activities specific to the tested *Dickeya* spp. isolates. Utilization of the carbon sources, indicated by the dye changing colour to purple, were assessed colourimetrically. Biochemical reactions were carried out for *Dickeya*-type strains and isolates of interest - namely, *D. solani* GBBC 2040, *D. dadantii* subsp. *dadantii* NCPPB 898^T, *D. dianthicola* NCPPB 453^T, *D. chrysanthemi* NCPPB 402^T, *D. zeae* NCPPB 2538^T, *D. aquatica* NCPPB 4580^T, *D. paradisiaca* NCPPB 2511^T, *D. fangzhongdai* JS5^T, and *Dickeya* sp. S1, B16, MK7, and NCPPB 3274. Correlations among the carbon utilisation profile variables were calculated using the Pearson correlation coefficient. The unweighted pair group with arithmetic mean method was implemented to cluster the strains (Garcia-Vallve et al., 1999) with iTOL to visualise the carbon utilisation similarity dendrogram.

2.4 Isolation and Classification of Bacteriophages Active against *Dickeya*

2.4.1 Bacterial strains and media

To isolate the bacteriophages in [Table 2.1](#), a selection of *Dickeya* strains was used that was isolated from *Phalaenopsis* orchid leaves with soft rot symptoms and classified as members of UDL-3 and UDL-4. These bacteria were routinely grown in Luria Bertani (LB) with 1.5% agar and 0.05% (w/v) NaCl at 28 °C to 30 °C. For liquid preparations, the bacteria cultures were grown in LB broth at 28 °C, with agitation at 200 rpm, unless otherwise stated. LB with 0.4% agar was used for overlays. For long-term storage, the bacteria cultures and isolates were kept in a bacterial and fungal storage system (Microbank; bioTRADING, the Netherlands) at ≤-76 °C.

2.4.2 Isolation of bacteriophages from wastewater

Bacteriophages against *Dickeya* UDL-3 and UDL-4 strains were isolated from wastewater concentrates. Effluent water was collected from a wastewater treatment plant and concentrated using a convective interaction media (CIM) quaternary amine monolithic column. The CIM concentrates were provided by Nejc Rački, PhD. The preparation and characterisation of water samples was described in Steyer et al. (2015). The wastewater bacteriophage concentrates were diluted in double-distilled water (1:2; v/v) to a final 1% NaCl.

Table 2.3: *Dickeya* spp. isolates used in bacteriophage enrichment. Wastewater bacteriophage enrichment was conducted separately for the UDL-3 and UDL-4 bacteria mixtures. Adapted from "Newly Isolated Bacteriophages from the *Podoviridae*, *Siphoviridae*, and *Myoviridae* Families Have Variable Effects on Putative Novel *Dickeya* spp." by Š. Alič et al., 2017, *Frontiers in Microbiology*, 8, 3. Copyright 2018 by the Frontiers Media SA.

Sample	Bacteria mixture	<i>Dickeya</i> spp. isolates used
--------	------------------	-----------------------------------

Wastewater	UDL-3	COB10/12, COB11/12, COB16/12
	UDL-4	COB9/12, COB12/12, COB14/12

For the enrichment, 3 mL double-strength LB with 120 μL 1 M MgSO_4 and 24 μL 1 M CaCl_2 was mixed with 3 mL diluted bacteriophage concentrate. To that, 30 μL of bacteria in the log phase (12–14 h incubation at 28 °C, with shaking at 200 rpm) was added at an equal ratio (Van Twest & Kropinski, 2009). The enrichment of UDL-3 and UDL-4 bacteria ([Table 2.3](#)) was performed separately. Enrichments were incubated for approximately 24 h at 28 °C, with shaking at 50 rpm and sterile filtering through 0.2- μm membranes. The bacteriophage suspension (7 μL) was used in double agar overlay plaque assays on LB plates (Kropinski, Mazzocco, Waddell, Lingohr, & Johnson, 2009). The host bacterium for the plaque assays was prepared as follows: 1 μL inoculation loop of overnight bacteria grown on solid LB was transferred to 5 mL liquid LB with 50 μL 1 M MgSO_4 and 10 μL 1 M CaCl_2 , and incubated for 12 h to 14 h at 28 °C, with shaking at 100 rpm. The overnight culture was then diluted tenfold to obtain optical density at 600 nm (OD_{600}) of 0.5, which corresponded to approximately 10^8 cells per mL. Single plaques were selected and transferred to SMG for further purification using disposable pipette tips. The bacteriophages were purified by streaking 1 μL bacteriophage suspension on solid LB and the indicator bacterial lawn was poured over it. Three successive single plaque isolations were performed as described above. To amplify bacteriophages, double agar overlay plaque assays on LB were carried out (enumeration of bacteriophages by double agar overlay plaque assay) in the manner of Kropinski et al. (2009), with several modifications: (i) 2 mL of the top agar was dispensed into sterile tubes; (ii) tubes containing the top agar were incubated at 52 °C; and (iii) 63 μL bacteria suspension and bacteriophage dilution were mixed and incubated for 20 min at room temperature prior to their transfer to a warm overlay medium, with agitation at 150 rpm. Confluent plates (plaques evenly distributed over the plates as single plaques not touching others) were then poured with 3 mL SMG. The plates were sealed with Parafilm and incubated for approximately 20 h at room temperature (25 °C), with gentle shaking at 100 rpm. The bacteriophages were sterile filtered over 0.2- μm membranes and stored at +4 °C or at -80 °C for longer periods.

2.4.3 Host range and specificity of bacteriophages

For the host range determination, 56 bacterial strains and isolates ([Table 3.1](#)) were tested, including 14 *Dickeya* isolates from diseased *Phalaenopsis*, 8 *Dickeya* spp. reference strains from a culture collection (*D. solani*, *D. diffenbachiae*, *D. dianthicola*, *D. zeae*, *D. dadanti*, *D. aquatica*, 2 *Dickeya* spp.), 7 *Pectobacterium cypripedii* from a culture collection, 4 non-*Dickeya* spp. isolates from diseased *Phalaenopsis*, 10 isolates from healthy *Phalaenopsis* leaves and 14 different bacterial strains from the *Enterobacteriaceae* family. The host range was tested by spotting 10 μL of a bacteriophage suspension onto an LB bacterial lawn, which was performed with three biological repeats. All positive results were confirmed by double agar overlay plaque assays as described above to eliminate false positives.

2.4.4 Transmission electron microscopy

The presence and morphology of isolated bacteriophages was determined using transmission electron microscopy with a negative-

staining method. Here, 20- μ L bacteriophage suspensions were deposited on Formvar-coated and carbon-stabilised copper grids and stained with 1% (w/v) aqueous solution of uranyl acetate.

To accompany the bacteria-bacteriophage interactions, bacterial suspensions (i.e. UDL-3,UDL-4; separately) were mixed with the purified selected bacteriophage BF25/12, which was shown to be active against bacteria, and kept for 15 min at room temperature. The suspensions were centrifuged at $3,000 \times g$ for 10 min. The supernatants were carefully removed, and the pellets were resuspended in 3% glutaraldehyde and 1% paraformaldehyde in 0.1 M Sörensen phosphate buffer (Merck, USA), pH 7.2, for 20 h at 4 °C. For UDL-3, pieces of soft agar that contained borders of plaques from the plaque assay and soft agar with bacteria from the control plate were fixed in the same fixative. All the samples were post-fixed in 2% OsO_4 in 0.1 M Sörensen phosphate buffer (Merck) with a pH of 7.2 and embedded in Agar 100 resin (Agar Scientific, UK). Ultrathin sections were stained with 2% (w/v) aqueous uranyl acetate and Reynolds lead citrate (Agar Scientific).

The grids were observed using transmission electron microscopy (CM 100; Philips, the Netherlands), operated at 80 kV and equipped with a CCD camera (Orius SC 200) and Digital Micrograph Software (Gatan Inc., USA). Transmission electron microscopy experiments were performed by Magda Tušek Žnidarič, PhD.

2.4.5 Bacteriophage genome characterisation

Bacteriophage DNA was isolated according to Pickard (2009) using the Phase Lock Gel system (5 PRIME, Germany). To determine the diversity of the isolated bacteriophages, RFLP analysis was performed according to Adriaenssens et al. (2012) using the HindII (Thermo Fisher Scientific, USA) restriction enzyme. Here, 0.5 μ g bacteriophage DNA was used per reaction mixture. The restriction was performed at 37 °C for 16 h. The fragments were analysed on 0.8% agarose gels for 100 min to 120 min at 90 V. The bacteriophage BF25/12 was selected from among *Podoviridae* isolates for further characterisation.

2.5 Genome Sequencing and Characterisation of Model Bacteriophage BF25/12

2.5.1 Bacteriophage whole-genome sequencing

The whole genome of bacteriophage BF25/12 was sequenced using 454 sequencing technology. The paired-end library was sequenced in Microsynth, Switzerland, following the manufacturer's instructions to 36-fold coverage. Whole-genome assembly was performed with the GS De Novo Assembler using the default parameters (Microsynth, Balgach, Switzerland). The fully assembled genome was annotated with Rapid Annotations using Subsystems Technology (RAST) (Aziz et al., 2008; Brettin et al., 2015; Overbeek et al., 2014). Its default settings were selected. Additionally, genome annotation was verified and curated by BLAST analysis (Altschul et al., 1990). The assembled genome sequence was compared to other characterised bacteriophages using the PASC web tool (Bao, Chetvernin, & Tatusova, 2014) and BLAST analysis (Altschul et al., 1990).

2.5.2 UV radiation stability

Bacteriophage stability was tested for bacteriophage BF25/12. A bacteriophage suspension was prepared in SMG buffer, and the bacteriophage concentration was adjusted to the concentration of 10^8 pfu/mL. Testing was performed on 5 mL of prepared bacteriophage suspension in open Petri dishes (55 mm diameter, Golas) that were illuminated using double UV-C light (Philips TUV G30T8, UV dose approximately 100 mJ/cm^2 30 cm from the light source) for 1 and 2 minutes. Petri dishes were kept on ice covered with cloth to minimise the influence of heat produced by the UV light. The bacteriophage's stability was tested by the double agar overlay plaque assay described above in two biological repeats as two technical replicates.

2.5.3 pH stability

The pH stability of the bacteriophage BF25/12 was tested for a selected range of pH values, from 3 to 11. Testing was performed in SMG buffer and sterile demineralised water, and adjusted with HCl or NaOH to the pH values of 3, 5, 7, 9 and 11. Phage suspensions were mixed with buffer or water (1 ml total volume) giving final concentrations of 10^5 and 10^3 pfu/mL and incubated at room temperature with shaking (100 rpm) for 24 hours. The concentration of active bacteriophages were determined with the double agar overlay plaque assay described above in two biological repeats and two technical replicates.

2.5.4 Temperature stability

The stability of the bacteriophage BF25/12 was analysed by incubating a bacteriophage suspension (100 μL) in SMG at different temperatures: +4, +28, -20 and -80 °C. The stability was tested once per month for one year using double agar overlay plaque assays (described above) as three technical replicates.

2.5.5 Development of bacterial resistance

The emergence of bacterial resistance was measured for the combination of the selected bacteriophage BF25/12 and the bacteria *Dickeya* sp. B16. A double agar overlay assay was used to enumerate the emergence of resistant bacterial colonies at a bacterial concentration of 10^8 cfu/mL and 4 different bacteriophage concentrations: 10^3 , 10^4 , 10^5 and 10^6 pfu/mL. Resistant bacterial colonies were counted on plates, with double agar overlay plaque assays carried out after 27 h incubation at 28 °C. The test was performed in triplicate per bacteriophage concentration, with two technical repeats each.

2.5.6 qPCR and ddPCR for bacteriophage detection

To detect and quantify the model bacteriophage BF25/12, three qPCR and digital droplet PCR (ddPCR) assays were designed. Based on the automatic annotation of the phage genome, the genes encoding structural proteins (capside, tailspike, tail tubular protein) were selected to construct primers and probes. Primers and hydrolysis probes ([Table 2.4](#)) were designed using Primer Express 2.0 software (Applied Biosystems). Moreover, the quality of the designed oligonucleotides was investigated *in silico* with OligoAnalyzer 3.1 (<https://eu.idtdna.com/calc/analyzer>). The assay validation was performed through assessing (i) analytical sensitivity by analysing target DNA dilution series in water, (ii) analytical specificity by testing a range of non-target bacterial,

bacteriophage and plant DNA, (iii) repeatability and (iv) robustness by analysing performance on isolated DNA and purified phage particles. The probes were FAM labelled and contained Black Hole Quencher.

Table 2.4: Primers and probes designed for the detection and quantification of bacteriophage BF25/12 using qPCR and ddPCR assays.

Assay	Name	Sequence (5'-3')	Amplicon length
Capsid protein gene	BF25/12cp_F	CACATCGTCAGGGTATTGAGGAA	69 bp
	BF25/12cp_R	CGGTGATGAACTCGGTCAGA	
	BF25/12cp_P	FAM-TGGTTCGCCGTGACCTGGGC-BHQ	
Tailspike protein gene	BF25/12ts_F	GCGAATGCAGGGCGTAATAC	68 bp
	BF25/12ts_R	GCTTGCACCGCGAACTG	
	BF25/12ts_P	FAM-TTCGTAGGTGGGCAGTACTCATTG TGGAC-BHQ	
Tail tubular protein B	BF25/12ttB_F	TGCGTTATGAACTGACCCTGAA	71 bp
	BF25/12ttB_R	AAGCGTGCGTGAGCTGTCT	
	BF25/12ttB_P	FAM-CTGGTGAATTCCACGCGGTCATCA-BHQ	

The reaction (10 μ l) consisted in a final concentration of 1 $\times$ TaqMan® Universal PCR Master Mix II (Applied Biosystems), 900 nM of forward and reverse primers (Eurofins MWG Operon), 100 nM of the probe (Eurofins MWG Operon) and 2 μ l of the sample (DNA). Real-time PCR reactions were performed on an ABI ViiA® 7 (Applied Biosystems) using universal cycling conditions, i.e. 2 min at 50 °C (AmpErase® UNG activation) and 5 min at 95 °C (AmpliTaQ Gold activation), followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C, with 9,600 emulsion. Results were analysed using Applied Biosystems ViiA 7 software v1.1.

For the absolute quantification of target copy numbers, droplet digital PCR was performed using the QX100™ Droplet Digital™ PCR system (Bio-Rad, Pleasanton, CA, USA). ddPCR Supermix for probes (16 μ L, Bio-Rad) and a 4 μ L sample of DNA were used, with the primer and probe concentrations set as they were for the qPCR. After the droplet generation, 40 μ L of the generated droplet emulsion was transferred to a new 96-well PCR plate (Eppendorf) and amplified in a T100™ Thermal Cycler (Bio-Rad). The amplification conditions were a 10 min DNA polymerase activation at 95 °C, followed by 40-45 cycles of a two-step thermal profile of 30 s at 94 °C for denaturation and 60 s at 60 °C for annealing and extension, followed by a final hold of 10 min at 98 °C for droplet stabilisation and cooling to 4 °C. The temperature ramp rate was set to 2.5 °C/s, and the lid was heated to 105 °C, according to Bio-Rad recommendations. After thermal cycling, the plates were transferred to a droplet reader (Bio-Rad). The software package provided with the ddPCR system (QuantaSoft 1.3.2.0, Bio-Rad) was used for data acquisition and analysis.

2.5.7 IZON qNANO bacteriophage particle analysis

The bacteriophage BF25/12 suspension was filtered through a 0.2 μ m filter and diluted 100 times with 1X PBS buffer (1.7 mM KH₂PO₄, 5mM NaHPO₄, 50mM NaCl, pH 7,4). The calibration was performed with cpc70bis; diameter: 70nm; raw concentration: 1,4 $\times$ 10¹⁴; and dilution: 100. The measurement settings used were nanopore ID: Np100-; stretch: 43.98mm; pressure: 7; and voltage: 0.72V.

Chapter 3

Results

3.1 Genetic and Phenotypic Characterisation of Bacteria Causing Soft Rot Disease in the *Phalaenopsis* Orchid

3.1.1 Soft rot symptoms on orchids

The first and most commonly observed infection symptoms for *Phalaenopsis* orchids from commercial production sites were circular, water-soaked spots on leaves. Under favourable conditions, the symptoms rapidly progressed. In a few days, the whole inside of the leaf became macerated, while still contained by the waxy leaf cuticle. Less frequently, soft rot started at the base of the leaf and advanced toward the leaf's tip. Water-soaked spots were occasionally observed also on roots, but the flower parts were not affected. In general, the disease incidence was increased with higher outside temperatures (i.e. during summer).

3.1.2 *Dickeya* spp. isolated from orchid tissue with soft rot symptoms

Plant extracts were prepared from rotted *Phalaenopsis* tissues by macerating the surface-sterilised leaves in the buffer. On the media, colonies that resembled fried eggs were isolated repeatedly and were the dominant colony type. From 37 plants sampled from April to September, 14 isolates were purified ([Table 3.1](#)~~Table 3.1~~, next page) and provisionally identified as *Dickeya* spp. based on morphology on media, oxidation/fermentation of glucose and real-time PCR (Brierley et al., 2008). When inoculated into *Phalaenopsis* leaves, these all caused soft rot symptoms. Some variability among the *Dickeya* sp. strains was observed in indigoidine production on the NGM medium ([Table 3.1](#)~~Table 3.1~~, next page), as previously observed (Czajkowski et al., 2015).

Table 3.1: Diversity of *Dickeya* isolates from *Phalaenopsis* orchids for the partial sequencing of *fliC* and *dnaX* genes, as well as their pathogenicity on orchids and indigoidine production. Reprinted from "Putative new species of the genus *Dickeya* as major soft rot pathogens in *Phalaenopsis* orchid production" by Š. Alič et al., 2017, *Plant Pathology*, 66, 1360. Copyright 2018 by the John Wiley and Sons.

<i>Dickeya</i> isolate	<i>fliC</i> sequencing identificati on	GenBank accession number		Indigoidin e production	Pathogenicity on <i>Phalaenopsis</i>
		<i>fliC</i>	<i>dnaX</i>		
S1	UDL-4	KM979763	KM588693	No	Yes
COB S4-1	UDL-4	KM979767	KM588697	Yes	Yes
COB S4-4	UDL-4	KM979770	KM588700	Yes	Yes
COB S4-6	UDL-4	KM979772	KM588702	No	Yes
COB S4-2	UDL-3	KM979768	KM588698	Yes	Yes
COB S4-5	UDL-3	KM979771	KM588701	No	Yes
COB S5-2	UDL-3	KM979774	KM588704	Yes	Yes
B16	UDL-3	KM979762	KM588692	Yes	Yes
COB S2	UDL-3	KM979764	KM588694	Yes	Yes
COB S3-1	UDL-3	KM979765	KM588695	No	No
COB S3-8	UDL-3	KM979766	KM588696	No	Yes
COB S4-3	UDL-3	KM979769	KM588699	No	Yes
COB S5-1	UDL-3	KM979773	KM588703	Yes	Yes
COB S5-3	UDL-3	NA	KM588705	Yes	Yes

Based on the partial sequencing *fliC* (Edwards et al., 1989; Coenye et al., 1999; Van Vaerenbergh et al., 2012), all 14 isolates were confirmed to belong to the genus *Dickeya*. Again, some variability was observed in the partial *fliC* sequence, with most isolates corresponding to sequevars 13 (10/14 isolates) and 15 (4/14 isolates). The isolates were grouped within two previously detected, undefined lineages, UDL-3 and UDL-4, together with isolates PD 3786 and PD 814, isolated from *Oncidium* and *Phalaenopsis* orchids, respectively, and other similar isolates of *Dickeya* spp. from ornamental plants (Figure 3.1Figure 3.1, next page). The phylogenetic tree built on the *dnaX* sequences (Appendix A) confirmed the separate grouping of the isolates from the *Phalaenopsis* orchids, together with isolates from many monocots in Asia identified as a putative new species in the genus *Dickeya* (Suharjo et al., 2014) corresponding to the DUC-2 and DUC-3 groups described by Parkinson et al. (2009). Overall, the data indicated that the group of isolates are indeed a distinct genetic lineage within the genus *Dickeya* and might be more widespread than previously thought (Suharjo et al., 2014; Van Vaerenbergh et al., 2012).

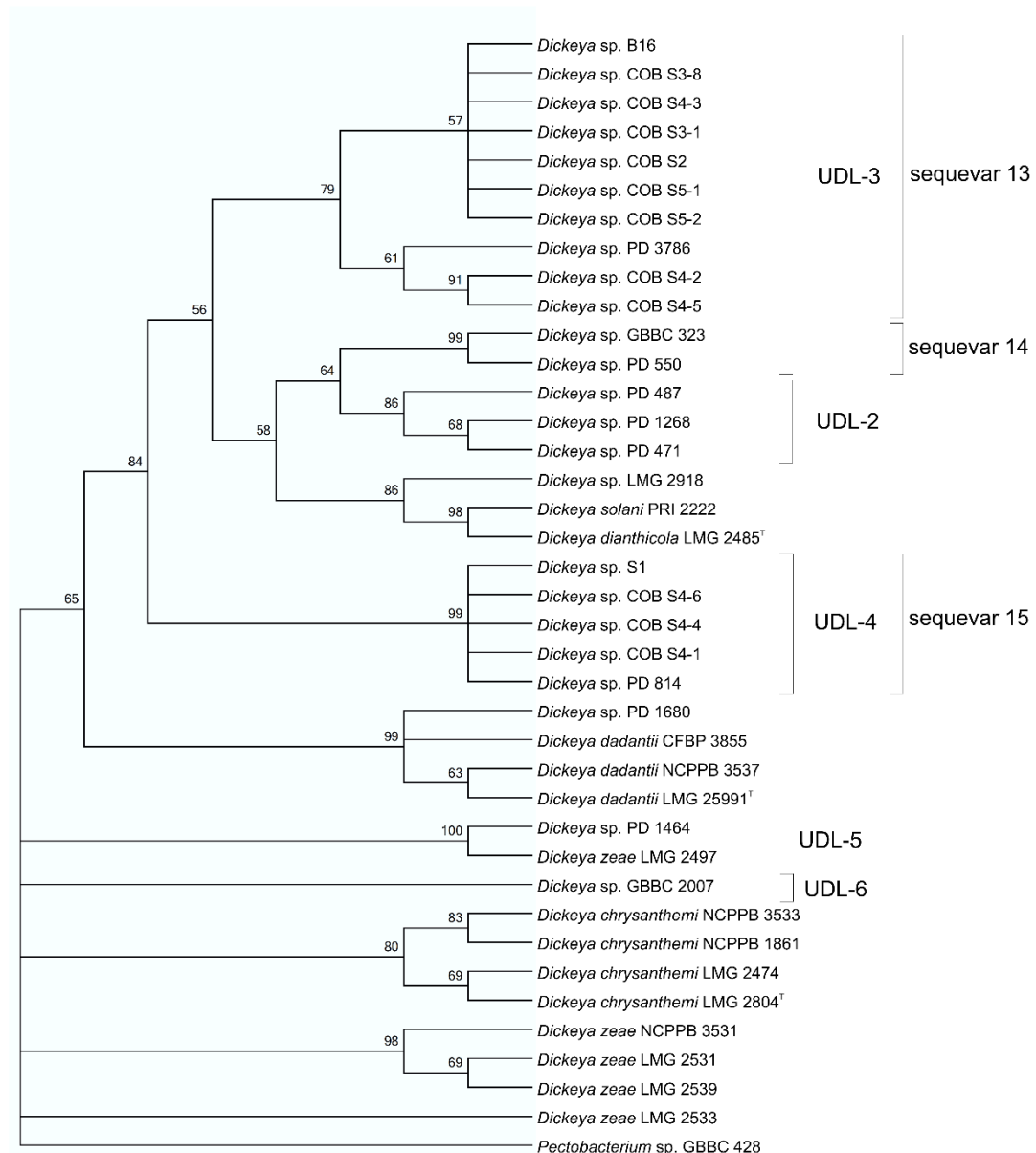


Figure 3.1: Phylogenetic tree for the *fliC* sequence of 39 members (Appendix A, [Table A.1](#)) of the genus *Dickeya*. The maximum likelihood method based on the Tamura three-parameter model was used for constructing the tree. The starting tree was created by neighbour joining and BioNJ algorithms. Bootstrap percentages were calculated with 500 replicates. Reprinted from "Putative new species of the genus *Dickeya* as major soft rot pathogens in *Phalaenopsis* orchid production" by Š. Alič et al., 2017, *Plant Pathology*, 66, p. 1361. Copyright 2018 by the John Wiley and Sons.

3.1.3 Characterisation of the representative UDL-3 and UDL-4 bacteria: B16 and S1

Two isolates that represent the most common UDL-3 and UDL-4 group strains, which are representative of the *dnaX* and *fliC* sequevars, *Dickeya* sp. B16 and S1, were further characterised. The colony's morphology differed between these two strains at the time of isolation, with B16 showing the typical fried-egg colonies and S1 producing colonies of a more uniform texture and colour ([Figure 3.2](#)). Upon repeated cultivation, the B16 colonies became more similar to the S1 colonies, despite the stability of the sequevars (based on qPCR) during storage and cultivation.

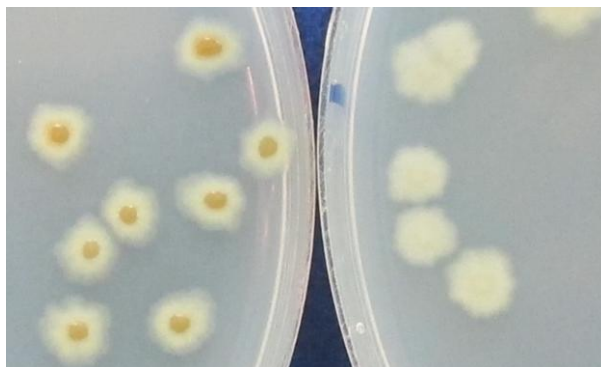


Figure 3.2: Colony morphology of the *Dickeya* spp. B16 (left) and S1 (right) at the time of isolation. Reprinted from "Putative new species of the genus *Dickeya* as major soft rot pathogens in *Phalaenopsis* orchid production" by Š. Alič et al., 2017, *Plant Pathology*, 66, p. 1363. Copyright 2018 by the John Wiley and Sons.

The two strains had identical Api 20NE (BioMérieux) profiles after 24 h of incubation (numerical profile 3477541) producing indole, hydrolysing esculin, gelatin and 4-nitrophenyl-D-galactopyranoside (enzyme β -galactosidase), but they were negative for arginine dihydrolase. The two strains did not assimilate D-maltose or phenylacetic acid.

3.1.4 Electron-dense ultrastructures

The bacterial ultrastructure was studied using thin sections of fixed and embedded bacterial suspensions ([Figure 3.3](#), next page). As Gram-negative bacteria, the cells contained cytoplasmic and outer membrane delimiting the periplasmic space, with the peptidoglycan layer located between them. Although *Dickeya* spp. B16 and S1 were both cultivated under the same conditions, only the cells of *Dickeya* sp. S1 showed the appearance of electron-lucent globular granules, with diameters <100 nm.

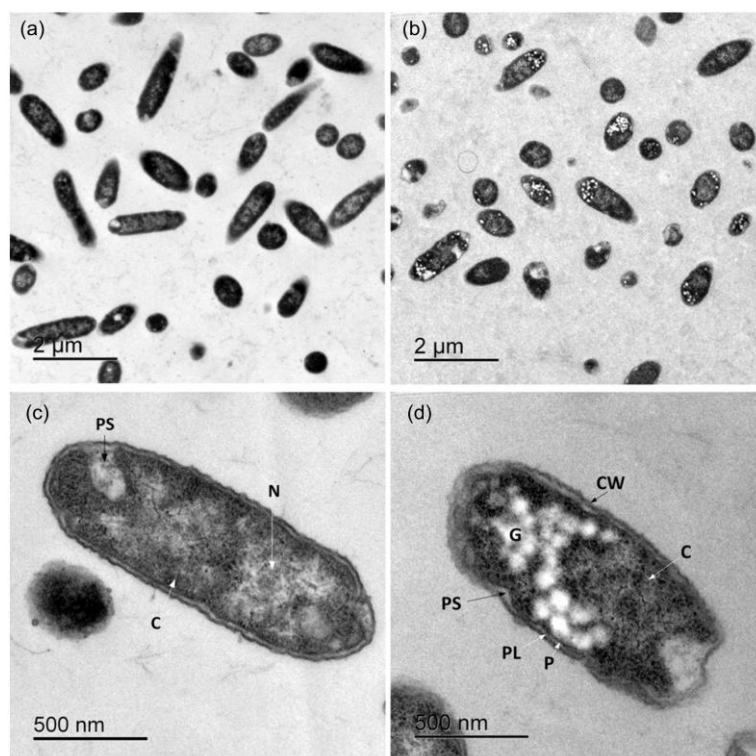


Figure 3.3: Ultrastructure of bacterial cells. (a) B16 (UDL-3) cells in suspension. (b) S1 (UDL-4) cells with electron-lucent granules. (c) B16 cell at higher magnification. (d) S1 cell at higher magnification. PS, periplasmic space; C, cytoplasm; N, nucleoid; CW, cell wall; P, plasmalemma; PL, peptidoglycan layer; G, electron-lucent granules. Reprinted from "Putative new species of the genus *Dickeya* as major soft rot pathogens in *Phalaenopsis* orchid production" by Š. Alič et al., 2017, *Plant Pathology*, 66, p. 1364. Copyright 2018 by the John Wiley and Sons.

3.1.5 Pathogenicity and aggressiveness of *Dickeya* isolates

3.1.5.1 Maceration assay on witloof and potato tubers

Two representative isolates of UDL-3 and UDL-4, *Dickeya* sp. B16 and *Dickeya* sp. S1, respectively, were tested for their aggressiveness in the maceration of witloof chicory, together with isolates of the other *Dickeya* spp. All the *Dickeya* strains tested showed maceration of the chicory tissue. However, the aggressiveness of the strains differed substantially. These data indicated significant variations in the maceration by B16 and S1 compared to the other *Dickeya* species included in the assay (Figure 3.4Figure 3.4, next page). The median of the maceration percentages for isolates B16 (41%) and S1 (39%) were similar. However, the medians of the macerated areas produced by the other tested *Dickeya* strains were significantly lower ($P < 0.05$; students' t-test). The third most aggressive strain in the testing was *D. solani* GBBC 500, with a median of 21%, about 20% lower than B16 and S1. The lowest level of maceration was observed for *D. zeae* IMG 2497. Conversely, the measured datasets of the *Dickeya* spp. B16 and S1 maceration diverged more substantially compared to the others. Indeed, the accuracy of the area measurements decreased with the section size. Hence, the leaf maceration precision was higher for the smaller areas.

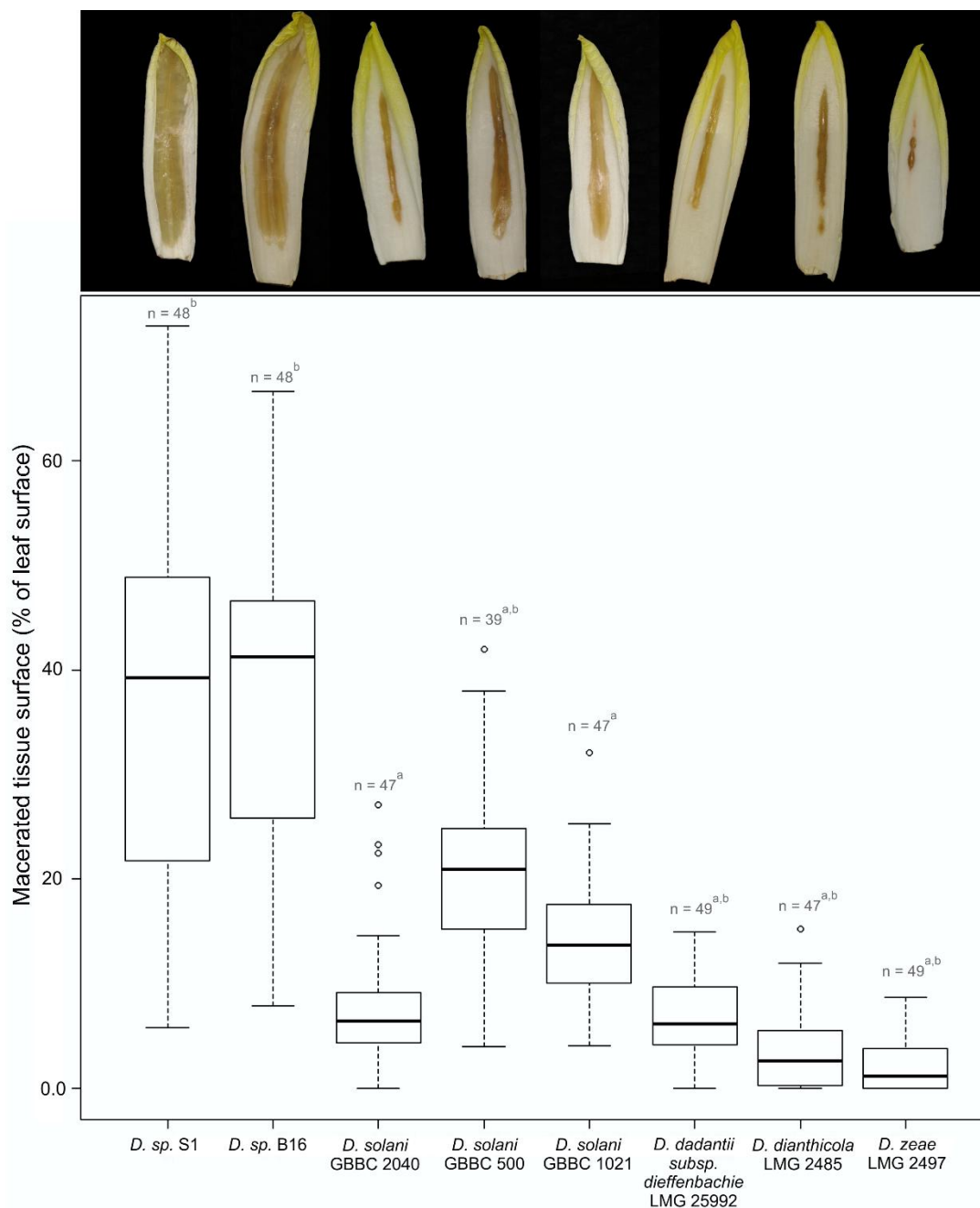


Figure 3.4: Maceration assay for witloof chicory leaves. Box plot for the macerated area produced by the *Dickeya* strains in the inoculation test. The number of inoculated leaves per strain is given at the top of each box plot. Top: Images of witloof chicory leaf inoculated with the corresponding *Dickeya* strain (below) after 18 h incubation in a dark, moist chamber at 28 °C and 80% humidity. The brown area represents macerated tissue. **Dickeya* sp. B16 and S1 maceration levels are significantly higher in comparison to other strains ($P < 0.05$; student's t-test). Adapted from "Putative new species of the genus *Dickeya* as major soft rot pathogens in *Phalaenopsis* orchid production" by Š. Alič et al., 2017, *Plant Pathology*, 66, p. 1365. Copyright 2018 by the John Wiley and Sons.

Furthermore, differences in aggressiveness were observed on potato tubers. *Dickeya* sp. B16, *Dickeya* sp. S1 and potato isolate *D. solani* GBBC 2040 all showed the ability to macerate potato tuber tissue. The median of the macerated potato tissue mass for

B16 (10.8 g) and S1 (11.6 g) were similar and higher than that observed in *D. solani* GBBC 2040 (1.0 g). However, the differences were not statistically significant because of the high variability among measurements (Figure 3.5).

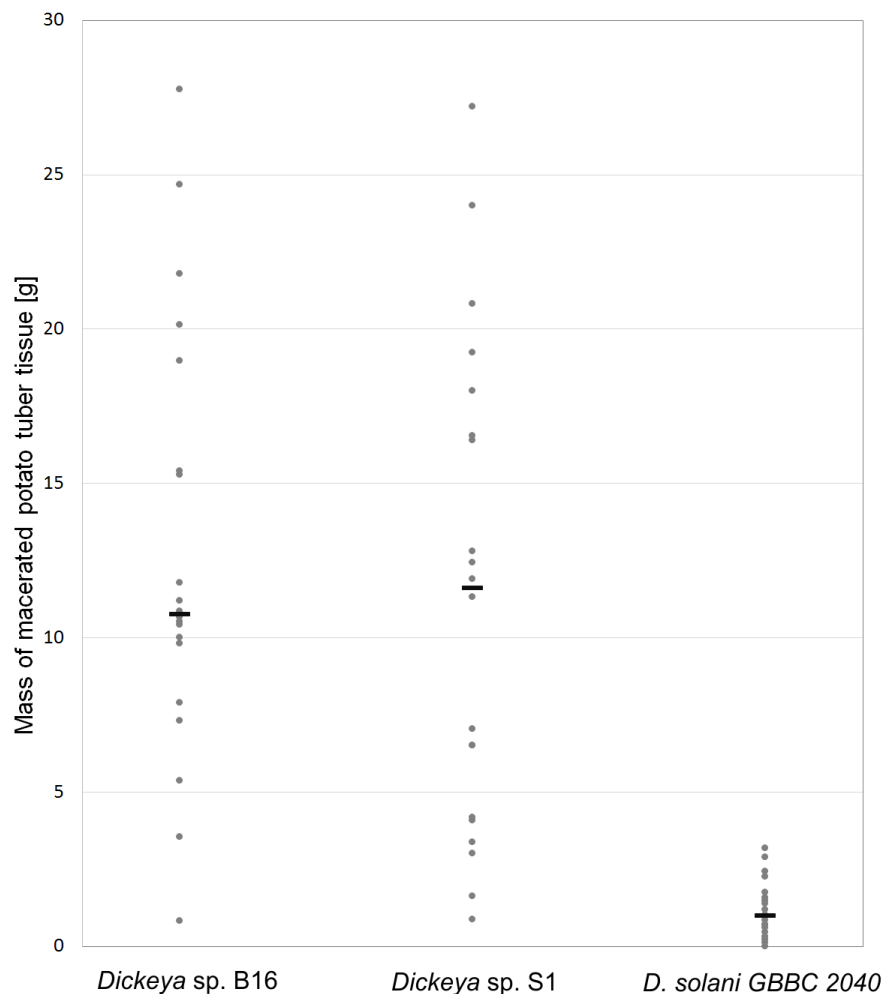


Figure 3.5: Maceration assay for potato tubers (cv. Agata). Twenty potato tubers were inoculated per strain. Grey dots represent individual measurements; the black line represents the median value of macerated tissue mass measurements. Reprinted from "Putative new species of the genus *Dickeya* as major soft rot pathogens in *Phalaenopsis* orchid production" by Š. Alič et al., 2017, *Plant Pathology*, 66, p. 1366. Copyright 2018 by the John Wiley and Sons.

3.1.5.2 Plant inoculation

Pathogenicity tests were performed on *Phalaenopsis* plants. The *Dickeya* isolates B16 and S1 infected orchid plants and caused soft rot symptoms, showing high infection efficiency. Most of the leaves inoculated with *Dickeya* spp. B16 and S1 showed typical soft rot symptoms (Figure 3.6). The other *Dickeya* isolates that displayed virulence on orchid plants were *D. solani* isolates GBBC 500 and GBBC 2040, *D. dianthicola* LMG 2485 and *D. zeae* LMG 2497. Furthermore, *D. dianthicola* and *D. zeae* showed a notably lower efficacy of infection. The brownish coloration of the rotted areas that was darker than the other *Dickeya* spp. *D. solani* and *D. dianthicola* caused variable reactions in the inoculated leaves, rotting symptoms or limited necrosis at the

inoculation points. The progression of symptoms in the early stages of infection was significantly faster in plants inoculated with *Dickeya* spp. B16 and S1 compared to other *Dickeya* spp., although the correlation between the efficacy of the infection and the aggressiveness of the *Dickeya* isolates was observed for all the tested species (Table 3.2). On the other hand, neither of these bacteria could disperse throughout the plant into uninoculated leaves.

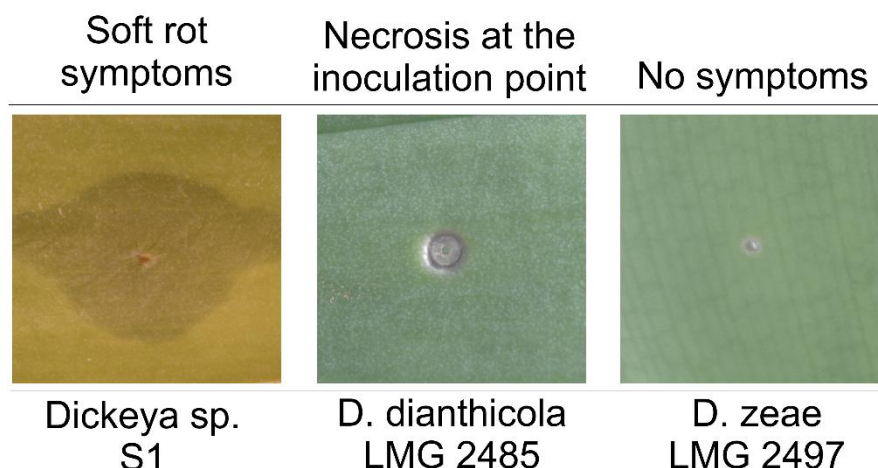


Figure 3.6: Symptoms developed on *Phalaenopsis* leaves inoculated with *Dickeya* after 24 h (soft rot symptoms) and 6 days (necrosis and no symptoms) post-inoculation. Typical soft rot symptoms were observed on plants inoculated with *Dickeya* sp. B16, *Dickeya* sp. S1, *D. solani* GBBC 500 and *D. solani* GBBC 2040. Reprinted from "Putative new species of the genus *Dickeya* as major soft rot pathogens in *Phalaenopsis* orchid production" by Š. Alič et al., 2017, *Plant Pathology*, 66. Copyright 2018 by the John Wiley and Sons.

Table 3.2: Efficiency and aggressiveness for *Dickeya* isolates according to the number of *Phalaenopsis* leaves with soft rot symptoms and average rotting area. Adapted from "Putative new species of the genus *Dickeya* as major soft rot pathogens in *Phalaenopsis* orchid production" by Š. Alič et al., 2017, *Plant Pathology*, 66, p. 1366. Copyright 2018 by the John Wiley and Sons.

Isolate	No. of leaves with soft rot symptoms	No. of inoculated leaves	Mean rotting area (cm ²)					
			24 h		48 h		3 days	
			Area	SD	Area	SD	Area	SD
<i>Dickeya</i> sp. B16	10	11	2.5	1.2	13.1	4.1	21.9	6.9
<i>Dickeya</i> sp. S1	11	11	3.5	4.9	14.2	8.5	24.5	12.2
<i>Dickeya solani</i> GBBC 500	6	11	0.4	1.0	7.5	4.3	15.6	7.6
<i>Dickeya solani</i> GBBC 2040	7	9	0.5	0.1	6.3	3.7	11.3	1.4
<i>Dickeya dianthicola</i> LMG 2485	3 ^{a,b}	11	0.2	0.1	1.2	1.0	4.0	2.4
<i>Dickeya dadantii</i> subsp. <i>diffenbachiae</i> LMG 25992	0	9	0.0	NA	0.0	NA	0.0	NA
<i>Dickeya zeae</i> LMG 2497	1 ^a	10	0.1	NA	1.8	NA	7.1	NA
Negative control	0	6	0.0	NA	0.0	NA	0.0	NA

^aRotting area darker coloured in comparison with others; ^bonly 2 leaves rotting at 24 h.

The bacteria concentrations next to the point of the inoculation (mean Cq, 20.7 for S1, 20.4 for B16) and at the edge of the rotting area (mean Cq, 18.2 for S1, 18.0 for B16) were very similar between these isolates. The bacteria were slightly more concentrated at the edges of symptomatic areas, which corresponded to the spread of the infection. *Dickeya* sp. B16 spread through the asymptomatic tissue to the ends of the leaves (mean Cq, 35.2). However, *Dickeya* sp. S1 was not detected in asymptomatic parts of the inoculated leaves.

3.1.5.3 Potato inoculation

The potential for the new *Dickeya* isolates to infect potato plants was tested. Potato plants were infected using the model *Dickeya* spp. isolates B16 and S1, along with *D. solani* GBBC 2040 as the positive control. No soft rot symptoms were observed on the inoculated potato plants over the 41-day incubation period. However, an analysis on molecular level confirmed the presence of the inoculated bacteria in the plants. Presence of *Dickeya* spp. B16 and *D. solani* GBBC 2040 were detected in all six of the tested potato plants and for five plants for *Dickeya* sp. S1. The bacteria concentrations were measured 3 cm and 9 cm from the point of infection, which resulted in nearly the same to slightly lower concentrations for the position further from the infection spot ([Table 3.3](#)~~Table 3.3~~). These data imply the spread of the bacteria along the plant stem, which indicates that *Dickeya* sp. B16 and S1 can undergo systemic spreading throughout the potato plant. Furthermore, the measured concentrations of bacteria in the plants infected with *Dickeya* spp. B16 and S1 corresponded to the *D. solani* concentration rates included in the testing.

Table 3.3: Bacteria detection in potato plants at 3 cm and 9 cm from the point of inoculation, 41 days post-inoculation, measured using real-time PCR. Reprinted from "Putative new species of the genus *Dickeya* as major soft rot pathogens in *Phalaenopsis* orchid production" by Š. Alič et al., 2017, *Plant Pathology*, 66, p. 1366. Copyright 2018 by the John Wiley and Sons.

<i>Dickeya</i> strain	Bacteria detected according to distance from inoculation					
	3 cm			9 cm		
	Positive (n)	Tested (n)	Cq range	Positive (n)	Tested (n)	Cq range
<i>Dickeya</i> sp. B16	6	6	23.1–24.6	6	6	22.4–31.5
<i>Dickeya</i> sp. S1	5	6	21.7–27.6	4	6	28.0–32.3
<i>Dickeya solani</i> GBBC 2040	5	5	19.5–31.4	5	5	22.4–34.7
Negative control	0	5	/	0	5	/

Because potato tubers produced by infected plants did not develop any soft rot symptoms over the 1-month period tested, they were tested for the presence of the inoculated bacteria. Bacteria were not detected in the potato tubers produced by the infected potato plants, including the plants infected with *D. solani*. Consequently, no conclusions can be made regarding the propagation of the *Dickeya* spp. B16 and S1 into potato tubers.

3.2 Taxonomic Position of *Dickeya* Isolates Causing Soft Rots of Orchids and Their Relationships with Newly Described *Dickeya* Species

3.2.1 *Dickeya* sp. B16 and S1 genome sequencing

Whole-genome sequencing was conducted on the model isolates *Dickeya* sp. B16 and S1 using Ion Torrent sequencing technology. The draft genomes comprise 61 and 59 contigs, with a total size of 4,885,657 bp and 4,892,969 bp and an N50 of 167,592 bp and 160,162 bp for strains B16 and S1, respectively.

3.2.2 DNA sequence analysis

In a phylogenetic analysis based on 6 housekeeping genes, the isolates B16, S1, MK7 and NCPPB 3274 were grouped with *D. fangzhongdai* JS5^T, forming a uniform clade within the *D. solani*/*D. dadantii*/*D. dianthicola* subgroup, which has relatively short phylogenetic distances ([Figure 3.7](#)~~Figure 3.7~~). The results indicated that the strains *Dickeya* sp. S1, B16, MK7 and NCPPB 3274, as well as 3 previously misidentified strains (M005, M074 and ND14b), belong to the species *D. fangzhongdai*. The close relation of these strains with a group of 16 strains isolated from monocotyledonous plants in Asia (Suharjo et al., 2014) and *Dickeya* isolates classified in the DUC-2 and DUC-3 group (Parkinson et al., 2009) further indicate that these, too, likely belong to the same species ([*Japanese strains; DUC strains have their clade classification written in brackets. Figure C.1](#)~~Figure C.1~~).

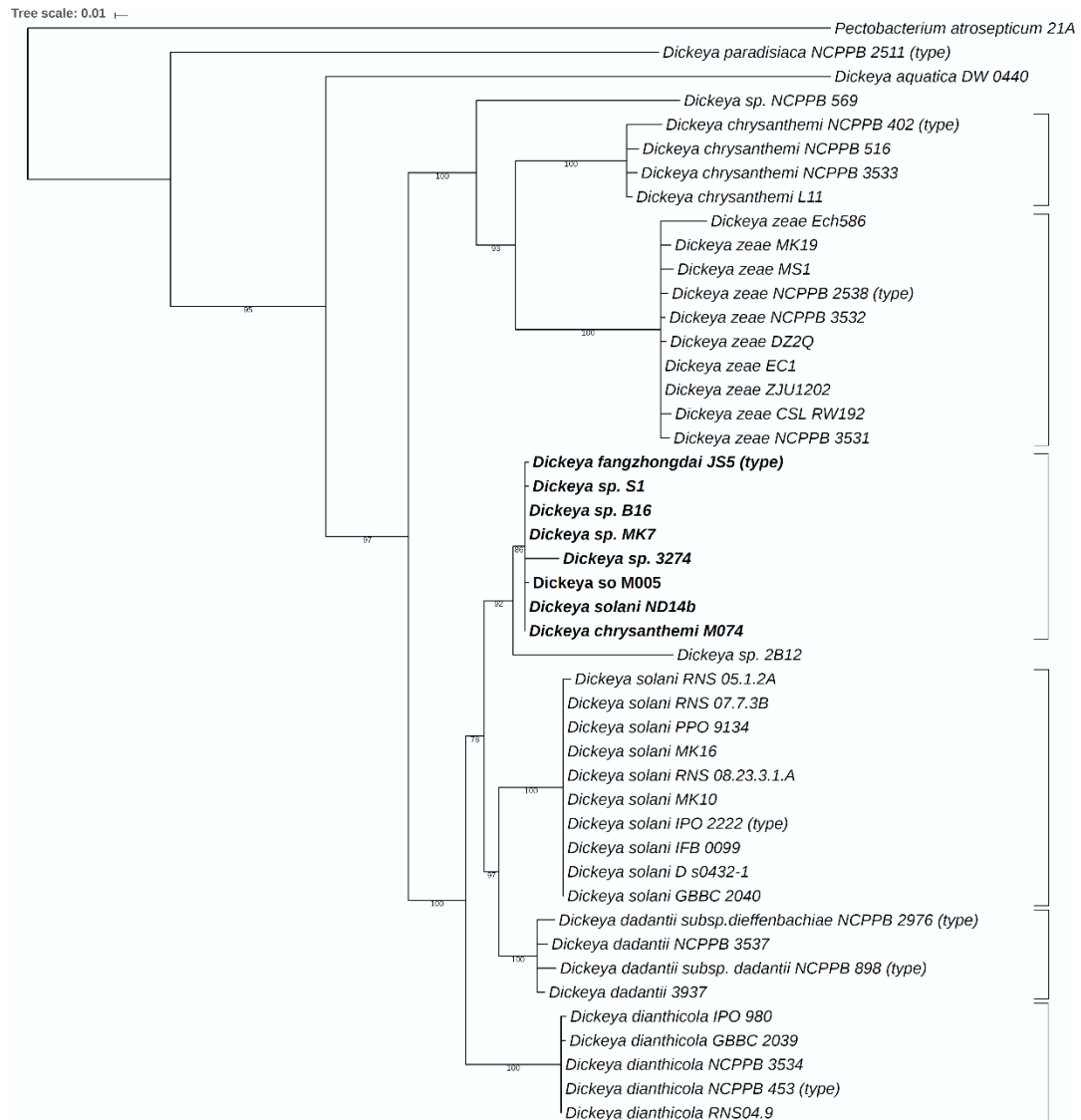
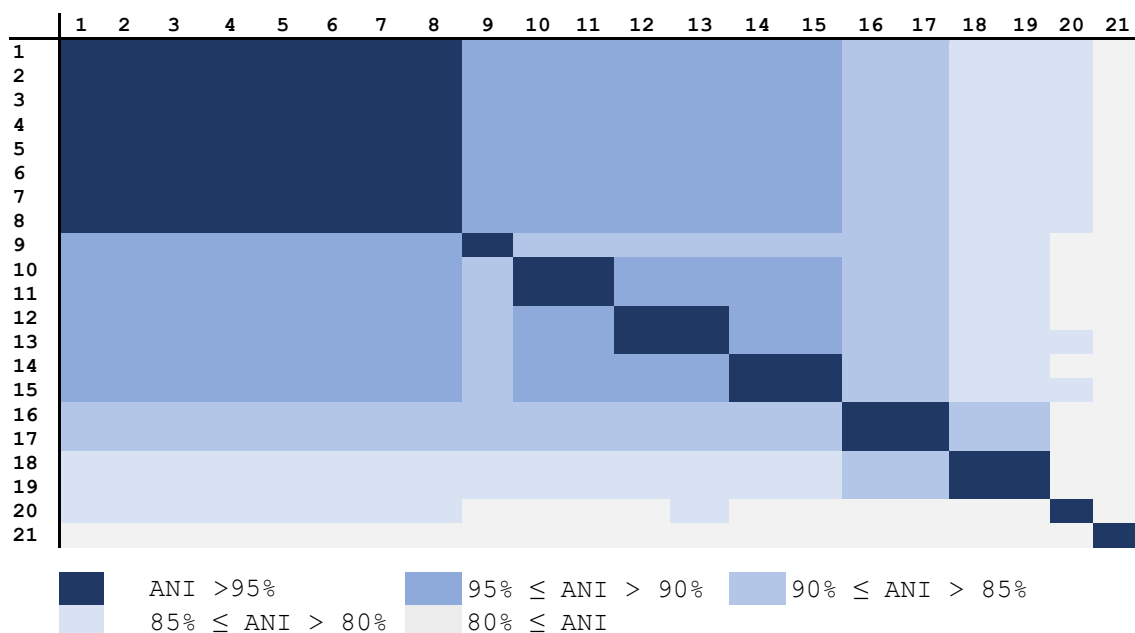


Figure 3.7: Consensus tree of the concatenated sequences (total 8274 bp) of *purA*, *dnaN*, *gapA*, *gyrA*, *atpD* and *rpoS* of 44 members of the genus *Dickeya* that belong to different species, including *D. fangzhongdai*. Brackets denote groups of strains with an ANI exceeding 95%. The maximum-likelihood method and generalised time-reversible model were used to construct the tree. The starting tree was created with the BioNJ algorithm. Bootstrap percentages were calculated with 1,000 replicates. *Pectobacterium atrosepticum* 21A was used as an outgroup to root the phylogenetic tree. Strains written in bold phylogenetically correspond to the *D. fangzhongdai* species.

The draft genomes of the isolates from diseased orchids, B16 and S1, were compared to other genomes of *Dickeya* spp. available in the National Center for Biotechnology Information (NCBI) database by determining the ANI values (Table C.1Table C.1). A group of strains (B16, S1, MK7, NCPPB 3274, M005, M074 and ND14b) showed ANI values exceeding 95%. Thus, we grouped them into the same species (Table 3.4Table 3.4, Table C.1Table C.1). The calculated ANI values in comparisons of these strains with other *Dickeya* spp. were all lower than 93% (i.e. below the threshold for assigning an isolate to a species) (Goris et al., 2007) and confirmed groupings identified during MLSA.

Table 3.4: Pairwise ANI values for genomes of the genus *Dickeya* representative strains.

Strains: 1, *Dickeya fangzhongdai* JS5^T; 2, *Dickeya* sp. B16; 3, *Dickeya* sp. MK7; 4, *Dickeya* sp. S1; 5, *Dickeya solani* M005; 6, *Dickeya solani* ND14b; 7, *Dickeya chrysanthemi* M074; 8, *Dickeya* sp. NCPPB 3274; 9, *Dickeya* sp. 2B12; 10, *Dickeya solani* IPO 2222^T; 11, *Dickeya solani* RNS 08.23.3.1.A; 12, *Dickeya dadantii* subsp. *dadantii* NCPPB 898^T; 13, *Dickeya dadantii* 3937; 14, *Dickeya dianthicola* NCPPB 453^T; 15, *Dickeya dianthicola* NCPPB 3534; 16, *Dickeya chrysanthemi* NCPPB 402^T; 17, *Dickeya chrysanthemi* NCPPB 516; 18, *Dickeya zeae* NCPPB 2538^T; 19, *Dickeya zeae* MS1; 20, *Dickeya aquatica* DW 440; and 21, *Dickeya paradisiaca* NCPPB 2511^T. Dark blue represents ANI values >95%, above the threshold for assigning strains to separate species. ANI values around 95% correspond to a similarity of 70% for DNA-DNA hybridisation.

To further confirm isolates' affiliation and examine their relationship to *D. fangzhongdai*, DNA-DNA hybridisation was performed. The hybridisation of *D. fangzhongdai* JS5 with *Dickeya* sp. S1, B16 and NCPPB 3274 exceeded 70% similarity for all tested combinations, placing these strains within *D. fangzhongdai* with an average of 92.5%, 86.3% and 75.3% similarity, respectively (means of two technical replicates).

3.2.3 Colony morphology

The tested *Dickeya* strains exhibited different colony morphologies on CPG, LB, BUG and KingB media ([Figure 3.8](#)). Strains S1, B16, MK7 and NCPPB 374 formed circular umbonate and nonmucoid colonies, which is common for *Dickeya* spp. However, *D. fangzhongdai* JS5^T colonies differed substantially from all other taxonomically related *Dickeya* spp. On all tested media, *D. fangzhongdai* JS5^T formed circular, mucoid colonies of more watery consistency than other strains.

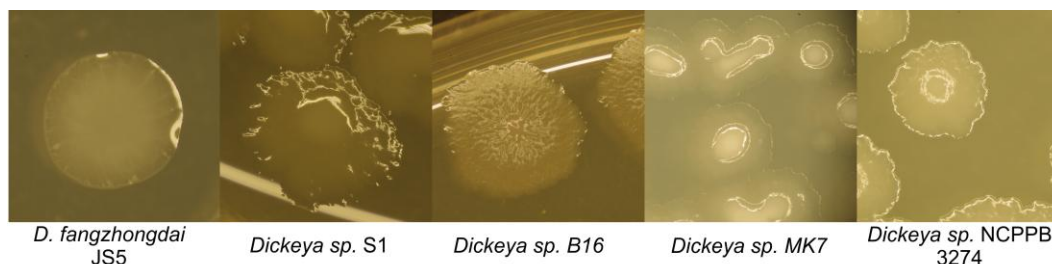


Figure 3.8: Colony morphology of *Dickeya* isolates taxonomically corresponding to the *D. fangzhongdai* species.

3.2.4 Phenotypic identification and carbon utilisation tests

Identification GEN III microplates and carbon utilisation assays were used to phenotypically identify and characterise strains. *Dickeya* sp. S1, B16, MK7, NCPPB 3274 and *D. fangzhongdai* JS5^T were identified by GENIII as members of the *Dickeya* genus. Three groups could be identified: MK7 and NCPPB 3274, clustering separately from the B16 and S1 isolates, and JS5^T, clustered separately from all the other strains (Figure 3.9). Both B16 and S1, as well as *D. fangzhongdai* JS5^T, were resistant to lincomycin and lithium chloride and were metabolically active in the presence of α -D-lactose and inosine.

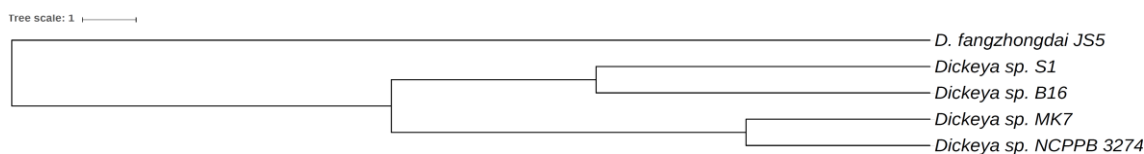
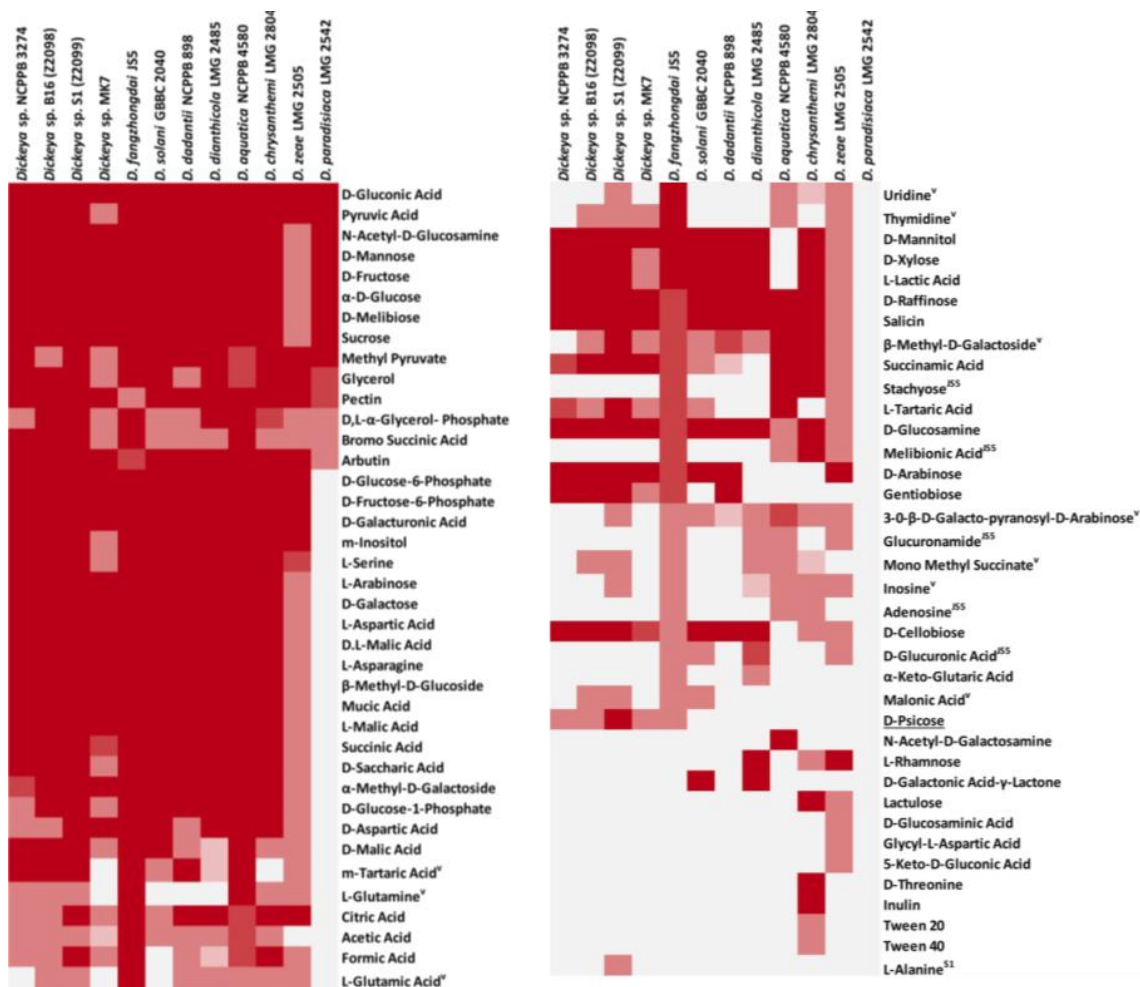


Figure 3.9: Dendrogram of the relationships between the GENIII plate phenotypic similarities of *D. fangzhongdai* strains. The dendrogram was constructed from the matrix of correlation distances (calculated using the Pearson correlation coefficient) using the UPMGA method.

The carbon utilisation assay results are reported as an average of two biological repeats (Figure 3.10, next page). Carbon sources showing low technical repeatability were omitted from the analysis (28 carbon sources); therefore, 166 carbon sources were included in the analysis. Ninety carbon sources could not be utilised by any tested strain, resulting in 76 differentially used carbon sources (Figure 3.10, next page). *D. paradisiaca* poorly utilised most of the tested carbon sources. Indeed, 37 carbon sources were commonly utilised by the tested *Dickeya* spp., and *D. paradisiaca* utilised only 14 of them.



^vThe variable utilisation of the carbon source among putative *D. fangzhongdai* isolates. ^{JS5}The carbon source was utilised only by *D. fangzhongdai* JS5^T among putative *D. fangzhongdai* isolates. ^{S1}The carbon source was utilised only by *Dickeya* sp. S1.

D. fangzhongdai strains (JS5^T, S1, B16, MK7 and NCPPB 3274) utilised 48 carbon sources (30%) and altogether showed 90% identity in their carbon utilisation profile ([Table 3.5](#)[Table 3.5](#), next page;

[Figure 3.11](#)[Figure 3.11](#), next page). The *Dickeya* sp. S1, B16, MK7 and NCPPB 3274 strains could not utilise 6 carbon sources that were utilised by JS5^T, corresponding to 93% identity in their carbon utilisation profile. Furthermore, all *D. fangzhongdai* strains were resistant to troleandomycin, rifamycin SV, Niaproof 4, vancomycin, tetrazolium violet and tetrazolium blue.

Table 3.5: Similarity matrix of *Dickeya* carbon utilisation profiles computed with the Pearson correlation coefficient.

	<i>Dickeya</i> sp. B16	<i>Dickeya</i> sp. S1	<i>Dickeya</i> sp. MK7	<i>Dickeya</i> sp. NCPPB 3274	<i>D. dadantii</i> NCPPB 898 ^T	<i>D. solani</i> GBBC 2040	<i>D. chrysanthemi</i> LMG 2804 ^T	<i>D. zeae</i> LMG 2505 ^T	<i>D. dianthicola</i> LMG 2485 ^T	<i>D. paradisiaca</i> LMG 2542 ^T	<i>D. aquatica</i> NCPPB 4580 ^T	<i>D. fangzhongdai</i> JS5 ^T
<i>Dickeya</i> sp. B16	1	0.96	0.92	0.97	0.94	0.91	0.80	0.76	0.84	0.45	0.81	0.90
<i>Dickeya</i> sp. S1		1	0.90	0.94	0.92	0.89	0.81	0.79	0.83	0.44	0.83	0.92
<i>Dickeya</i> sp. MK7			1	0.92	0.90	0.90	0.81	0.73	0.83	0.46	0.78	0.82
<i>Dickeya</i> sp. NCPPB 3274				1	0.94	0.92	0.79	0.76	0.83	0.49	0.78	0.86
<i>D. dadantii</i> NCPPB 898 ^T					1	0.92	0.82	0.77	0.89	0.43	0.77	0.85
<i>D. solani</i> GBBC 2040						1	0.81	0.77	0.92	0.47	0.77	0.83
<i>D. chrysanthemi</i> LMG 2804 ^T							1	0.77	0.83	0.44	0.81	0.83
<i>D. zeae</i> LMG 2505 ^T								1	0.78	0.44	0.76	0.81
<i>D. dianthicola</i> LMG 2485 ^T									1	0.48	0.75	0.81
<i>D. paradisiaca</i> LMG 2542 ^T										1	0.45	0.42
<i>D. aquatica</i> NCPPB 4580 ^T											1	0.87
<i>D. fangzhongdai</i> JS5 ^T												1

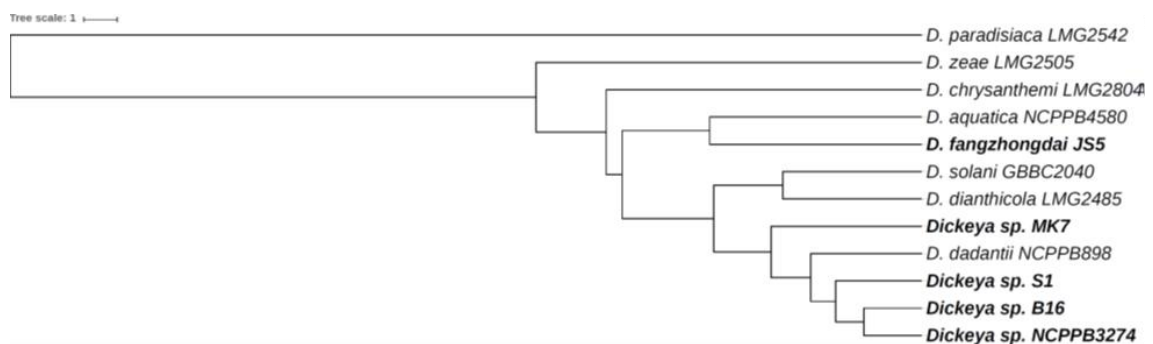


Figure 3.11: Dendrogram of the relationships between the carbon utilisation profiles of *Dickeya* spp. The dendrogram was constructed from the matrix of correlation distances (calculated using the Pearson correlation coefficient) with the UPMGA method. Names written in bold represent *D. fangzhongdai* strains.

The *D. fangzhongdai* strains JS5^T, S1, B16, MK7 and NCPPB 3274 differed from other *Dickeya* species in their ability to utilise D-psicose (D-ribo-2-hexulose or D-allulose), a C-3 epimer of D-fructose. D-Psicose is considered a rare, low-energy sugar that exists in nature in extremely small quantities (Chung, Oh, & Lee, 2012). Based on our results, only the isolates taxonomically

corresponding to the *D. fangzhongdai* species could metabolise D-psicose (Figure 3.10Figure 3.10). This finding runs counter to the observation of Parkinson et al. (2014), who report the metabolism of D-psicose by *D. aquatica* strains, namely, *D. aquatica* NCPPB 4580^T, 181 and Dw054 on Biolog identification plates (GN plates). Furthermore, *D. fangzhongdai* strains were phenotypically distinguished from other *Dickeya* species by their ability to metabolise L-tartaric acid, thymidine, D-arabinose and gentiobiose, as well as their inability to metabolise L-Rhamnose and D-Galactonic Acid- γ -Lactone (Table 3.6Table 3.6).

Table 3.6: Carbon utilisation pattern differentiating *D. fangzhongdai* from other *Dickeya* species.

Characteristic	1	2	3	4	5	6	7	8
D-Psicose	+	-	-	-	-	-	-	-
L-Tartaric Acid	+	+	-	-	-	+	-	+
Thymidine	v (80)	-	-	-	-	+	-	+
Gentiobiose	+	-	+	-	-	-	-	-
L-Rhamnose	-	-	-	+	+	+	-	-
D-Galactonic Acid- γ -Lactone	-	+	-	+	-	-	-	-

Species included in this study: 1. *D. fangzhongdai* (B16, S1, MK7, NCPPB 3274, JS5^T); 2. *D. solani* (GBBC 2040); 3. *D. dadantii* subsp. *dadantii* (NCPPB 898^T); 4. *D. dianthicola* (NCPPB 453^T); 5. *D. chrysanthemi* (LMG 402^T); 6. *D. zeae* (NCPPB 2538^T); 7. *D. paradisiaca* (NCPPB 2511^T); and 8. *D. aquatica* (NCPPB 4580^T). +, positive; -, negative; v, variable (percentage of positive strains).

Despite their high level of genetic relatedness, significant phenotypic differences were observed among *D. fangzhongdai* strains. Only some of the *D. fangzhongdai* were metabolically active on malonic acid, L-glutamic acid, mono-Methyl succinate, 3-O- β -D-galacti-pyranosyl-D-arabinose, uridine, inosine and thymidine. Compared to the other *D. fangzhongdai* strains, JS5^T exhibited stronger metabolic activity in the presence of, for example, 4% NaCl, D-maltose, N-acetyl neuraminic acid, stachyose, N-acetyl-D-galactosamine, L-rhamnose, D-arabitol, L-histidine, L-pyroglutamic acid or glucuronamide on GENIII plates. Significant differences in the carbon utilisation of *D. fangzhongdai* JS5^T on PM1 and PM2A plates were also observed. Unlike other *D. fangzhongdai* strains, JS5^T utilised D-glucuronic acid, α -keto-glutaric acid, glucuronamide, adenosine, stachyose and melibiononic acid (Figure 3.10Figure 3.10). However, discrepancies between GENIII and PM plates were noted in the utilisation of D-glucuronic acid and α -keto-glutaric acid.

3.3 Isolation and Characterisation of Bacteriophages Active against *Dickeya* Isolates Causing Soft Rot on Orchids

3.3.1 Sourcing and their characterisation

The target *Dickeya* spp. comprised a representative panel of *Dickeya* UDL-3 and UDL-4 isolates from diseased *Phalaenopsis* orchids. Together with a set of reference bacteria (Table 2.3Table 2.3), these were used as a mixture to enrich bacteriophages from

extracts of rotting orchid leaves and wastewater. Altogether, 18 bacteriophages against *Dickeya* spp. were isolated, 5 from diseased *Phalaenopsis* leaves and 13 from wastewater. Bacteriophages active against UDL-3 bacteria were successfully isolated from plant tissue, but bacteriophages with strong activity against UDL-4 were only isolated from municipal wastewater, which was distant from the orchid site of production. Bacteriophages were isolated from wastewater using a concentration of bacteriophage particles with CIM monolith chromatography. Together with the subsequent plaque assays, this demonstrates the suitability of this approach for the isolation of infective bacteriophages from wastewater samples.

The bacteriophage recovery from municipal wastewater varied with the season of the monthly sampling and isolation attempts. Bacteriophages active against *Dickeya* spp. were predominantly isolated in the warmer months (Table 3.7).

Table 3.7: Sampling timeline of the plant material and wastewater. Reprinted from "Newly Isolated Bacteriophages from the *Podoviridae*, *Siphoviridae*, and *Myoviridae* Families Have Variable Effects on Putative Novel *Dickeya* spp." by Š. Alič et al., 2017, *Frontiers in Microbiology*, 8, p. 6. Copyright 2018 by the Frontiers Media SA.

Month	Mean monthly temperature ^a (°C)	Diseased orchid tissue		Wastewater	
		Sampled	Bacteriophage isolated	Sampled	Bacteriophage isolated
January	1.6	–	–	+	–
February	–0.8	–	–	+	–
March	10.1	+	–	+	–
April	11.4	+	–	+	+
May	16.1	+	–	+	+
June	21.3	–	–	+	+
July	22.7	+	+	+	–
August	23.3	–	–	+	+
September	17.0	+	+	+	–
October	11.7	–	–	+	+
November	8.8	–	–	+	–
December	0.8	+	–	+	–

^aData derived from the Republic of Slovenia Statistical Office RS SI-Stat Data Portal (<http://pxweb.stat.si/pxweb/dialog/statfile1.asp>).

After overnight incubation, all the bacteriophages active against *Dickeya* spp. from diseased *Phalaenopsis* isolates produced large, clear plaques approximately 6 mm in diameter, with a halo effect, which suggested the presence of lysins (Figure 3.12A, next page). Mixed bacteriophage populations were ruled out by purifying the bacteriophages separately from the clear plaque zones and the halo rings, which resulted in identical morphology with a halo effect for all of them. Furthermore, the restriction of bacteriophage DNA with HindII (Figure 3.12E, next page) showed no differences in the patterns between the bacteriophages isolated from these diseased *Phalaenopsis* orchids. The bacteriophages active against *Dickeya* spp. that were isolated from wastewater showed two different plaque morphologies. The bacteriophages from the June samples produced large, clear

plaques approximately 5 mm in diameter with a halo effect ([Figure 3.12](#)~~Figure 3.12~~ B, next page) Those isolated in April, May, August and October produced medium to small plaques approximately 3 mm in diameter, with a clear zone encircled by a white ring and a halo effect ([Figure 3.12](#)~~Figure 3.12~~ C, next page). Restriction of the bacteriophage DNA derived from wastewater showed two RFLP patterns ([Figure 3.12](#)~~Figure 3.12~~ D, next page). The bacteriophages BF-CIM1/14 isolated in June had a uniform RFLP pattern that differed from all the other bacteriophages isolated from wastewater, which instead showed a common RFLP pattern.

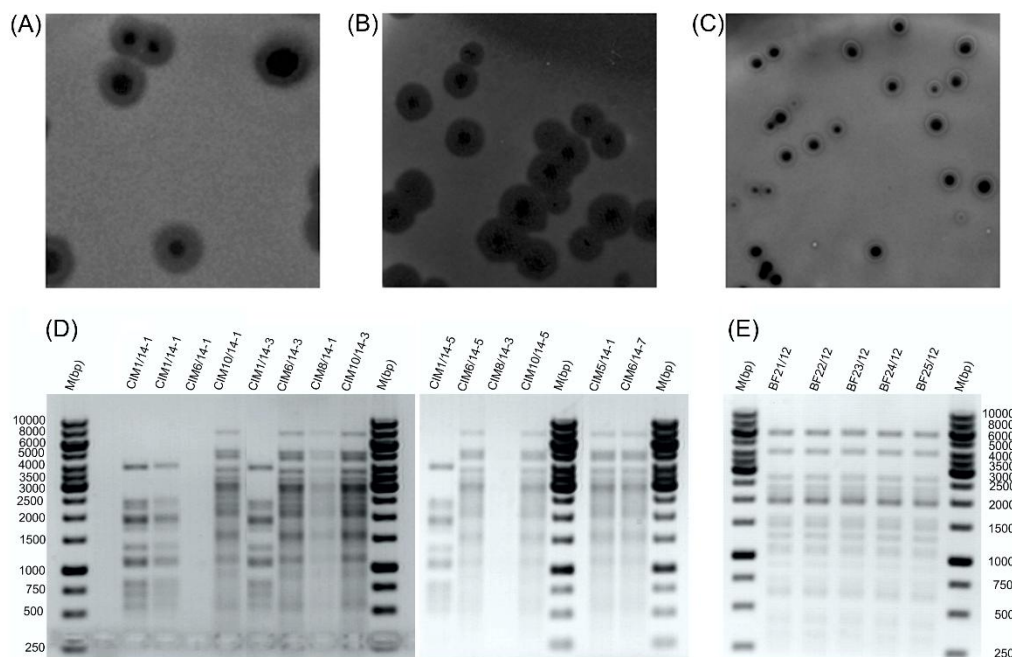


Figure 3.12: Representative bacteriophage plaque morphologies and restriction fragment length polymorphism profiles. Plaque morphologies for bacteriophages isolated from plant material (A) and wastewater (B,C). Bacteriophages against *Dickeya* spp. isolated from wastewater (D) and from diseased *Phalaenopsis* (E) digested with Hind II restriction enzyme. M, Gene Rule 1 kb DNA ladder. Reprinted from "Newly Isolated Bacteriophages from the *Podoviridae*, *Siphoviridae*, and *Myoviridae* Families Have Variable Effects on Putative Novel *Dickeya* spp." by Š. Alič et al., 2017, *Frontiers in Microbiology*, 8, p. 6. Copyright 2018 by the Frontiers Media SA.

Based on the morphologies of the bacteriophage particles examined with transmission electron microscopy (using negative staining), 2 of 5 isolated bacteriophages from diseased *Phalaenopsis* were placed in the *Podoviridae* family. They showed a head size of 55.8 ± 2.3 nm x 52.6 ± 3.1 nm ($n = 20$) ([Figure 3.13](#)~~Figure 3.13~~ A, next page). The bacteriophage tail dimensions were difficult to determine accurately because whole tails were rarely seen and the size highly depended on the bacteriophage's position on the grid. However, the tail size was estimated to be about 12.1 ± 2.4 nm x 16.3 ± 1.7 nm ($n = 13$) ([Figure 3.13](#)~~Figure 3.13~~ A, next page). Bacteriophages from wastewater were classified as *Siphoviridae* ([Figure 3.13](#)~~Figure 3.13~~ B, next page) and *Myoviridae* ([Figure 3.13](#)~~Figure 3.13~~ C, next page), both in the order *Caudovirales*. Bacteriophages from the *Siphoviridae* family ([Figure 3.13](#)~~Figure 3.13~~ B, next page) had an icosahedral head

(54.6 ± 2.6 nm x 53.9 ± 2.2 nm, $n = 24$) and slightly flexible tail (131.7 ± 8.4 nm x 10.6 ± 0.6 nm, $n = 23$) which continued into thin terminal fibres. Bacteriophages characterised as members of the *Myoviridae* family (Figure 3.13C) had a larger icosahedral head (138.2 ± 3.7 nm x 136.6 ± 4.1 nm, $n = 47$) and rigid contractile tail (228.1 ± 7.0 nm x 24.8 ± 1.4 nm, $n = 47$). The base plate was rather large (approximate length of 30 nm). Attached spikes were short and could only be observed as a net. Therefore, they were impossible to count.

In summary, all these bacteriophages that were active against *Dickeya* spp. belong to 1 of 3 groups with common RFLP profiles and plaque and bacteriophage morphologies (Table 3.8, next page).

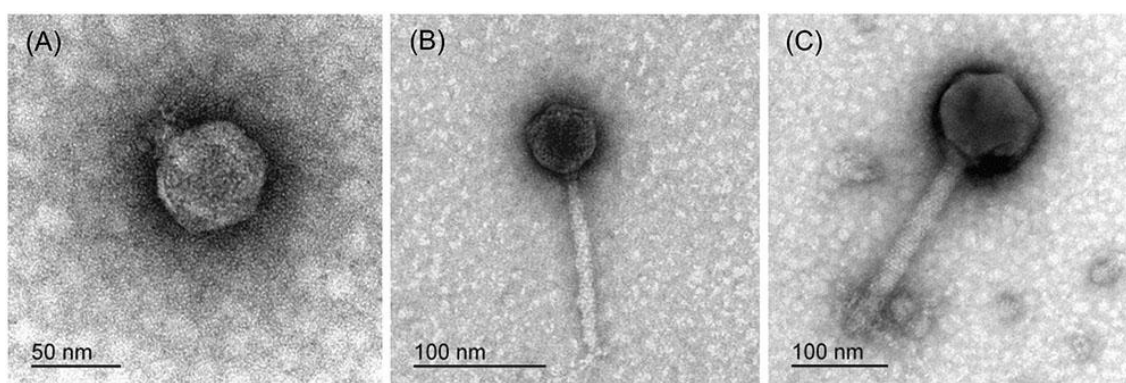


Figure 3.13: Representative transmission electron microscopy micrographs of bacteriophages isolated from diseased *Phalaenopsis* leaves (A) and sewage water (B, C), all of which were only active against *Dickeya* spp. Reprinted from "Newly Isolated Bacteriophages from the *Podoviridae*, *Siphoviridae*, and *Myoviridae* Families Have Variable Effects on Putative Novel *Dickeya* spp." by Š. Alič et al., 2017, *Frontiers in Microbiology*, 8, p. 7. Copyright 2018 by the Frontiers Media SA.

Table 3.8: Bacteriophages isolated from infected *Phalaenopsis* tissue and wastewater concentrates using CIM monolith chromatography. Reprinted from "Newly Isolated Bacteriophages from the *Podoviridae*, *Siphoviridae*, and *Myoviridae* Families Have Variable Effects on Putative Novel *Dickeya* spp." by Š. Alič et al., 2017, *Frontiers in Microbiology*, 8, p. 7. Copyright 2018 by the Frontiers Media SA.

Sample origin	Bacteriophage code	Plaque description	Bacteriophage family
<i>Phalaenopsis</i> tissue	BF 21/12	Large, clear plaques with halo effect	<i>Podoviridae</i>
	BF 22/12		
	BF 23/12		
	BF 24/12		
	BF 25/12		
Wastewater	CIM 1/14-1	Large, clear plaques with halo effect	<i>Siphoviridae</i>
	CIM 1/14-3		
	CIM 1/14-5		
	CIM 5/14-1	Medium to small plaques with a clear cone encircled by a white ring and halo effect	<i>Myoviridae</i>
	CIM 6/14-1		
	CIM 6/14-3		
	CIM 6/14-5		
	CIM 6/14-7		
	CIM 8/14-1		
	CIM 8/14-3		
	CIM 10/14-1		

CIM 10/14-3
CIM 10/14-5

3.3.2 Specificity of bacteriophages isolated against *Dickeya* spp.

The specificities of the interactions between the bacteria and bacteriophages were examined using spot tests (i.e. the isolation of bacteriophages via the induction of lysogenesis) and confirmed with plaque assays. The specificities of the bacteriophages included in the testing were limited to *Dickeya* spp., excluding *D. aquatica* and *D. zeae*. All the bacteriophages from orchid tissue showed identical host-range profiles; that is, they were active against *Dickeya* sp. UDL-3 isolates, *Dickeya* sp. MK7 and *D. dadantii* NCPPB 898. On the other hand, the bacteriophages isolated from wastewater showed variability in their host specificities. The host ranges of the bacteriophages BF-CIM5/14, BF-CIM6/14, BF-CIM8/14 and BF-CIM10/14 were very similar, and their specificities were close for both *Dickeya* spp. belonging to UDL-3 and UDL-4 lineage. However, substantial differences were observed in the specificities against *Dickeya* spp. from the bacteria collection tested here. The bacteriophages BF-CIM8/14 and BF-CIM10/14 are able to infect all the tested *Dickeya* spp., but *D. aquatica* and *D. zeae*. Conversely, infection by the bacteriophages BF-CIM5/14 and BF-CIM6/14 was limited to *Dickeya* sp. MK7 and weakly to *D. solani* GBBC 500 and *D. solani* GBBC 2040. Finally, all 3 bacteriophage isolates from the BF-CIM1/14 group showed a uniform infection profile; they were active only against *Dickeya* sp. UDL-4 isolates, *D. solani* GBBC 500 and *D. dianthicola* LMG 2458 ([Table 3.1](#)). However, none of the bacteriophages was able to infect other tested enterobacteria.

Table 3.1 Host range of bacteriophages isolated against UDL-3 and UDL-4 isolates. The host range for a representative bacteriophage is given from each isolated group. Reprinted from "Newly Isolated Bacteriophages from the *Podoviridae*, *Siphoviridae*, and *Myoviridae* Families Have Variable Effects on Putative Novel *Dickeya* spp." by Š. Alič et al., 2017, *Frontiers in Microbiology*, 8, p. 8-9. Copyright 2018 by the Frontiers Media SA.

[illegible]

Bacterial species and strain	Origin		Bacteriophage cone of lysis			
	Source country	Year	Wastewater ^a			Orchid ^b BF25/12
			BF-CIM1/14	BF-CIM5/14, BF-CIM6/14	BF-CIM8/14, BF-CIM10/14	
S1	Slovenia	2012	Clear	Clear	Clear	No
COB9/12	Slovenia	2012	Clear	Clear	Clear	No
COB12/12	Slovenia	2012	Clear	Clear	Clear	No
COB14/12	Slovenia	2013	Clear	Clear	Clear	No

Continued on next page

<i>Dickeya</i> spp. from bacteria collection						
<i>D. solani</i> NIB Z 1821 (GBBC 500)	NA	NA	Clear	Weak	Clear	No
<i>D. solani</i> NIB Z 1823 (GBBC 2040)	Belgium	2007	No	Weak	Clear	No
<i>D. dianthicola</i> NIB Z 1824 (LMG 2485 ^T)	UK	1956	Clear	No	Clear	No
<i>D. dieffenbachiae</i> NIB Z 1826 (LMG 25992)	USA	1957	No	No	Clear	No
<i>D. zeae</i> NIB Z 1827 (LMG 2497)	USA	1966	No	No	No	No
<i>D. dadantii</i> NIB Z 2131 (NCPBP 898 ^T)	Comro Is.	1961	No	No	Clear	Clear
<i>Dickeya</i> spp. NIB Z 2132 (NCPBP 3274)	St. Lucia	1983	No	No	Clear	No
<i>D. aquatica</i> NIB Z 2133 (NCPBP 4580 ^T)	UK	2008	No	No	No	No
<i>Dickeya</i> spp. MK7, NIB Z 2211	Scotland	NA	No	Clear	Clear	Clear
Other bacteria isolated from infected orchid tissue						
COB3/12	Slovenia	2012	No	No	No	No
COB4/12	Slovenia	2012	No	No	No	No
COB 5/12	Slovenia	2012	No	No	No	No
COB 6/12	Slovenia	2012	No	No	No	No
Bacteria isolates from asymptomatic orchids						
COB 1/13	Slovenia	2013	No	No	No	No
COB 2/13	Slovenia	2013	No	No	No	No
COB 3/13	Slovenia	2013	No	No	No	No
COB 4/13	Slovenia	2013	No	No	No	No
COB 5/13	Slovenia	2013	No	No	No	No
COB 6/13	Slovenia	2013	No	No	No	No
COB 7/13	Slovenia	2013	No	No	No	No
COB 8/13	Slovenia	2013	No	No	No	No
COB 9/13	Slovenia	2013	No	No	No	No
COB 10/13	Slovenia	2013	No	No	No	No

Bacterial species and strain	Origin		Bacteriophage cone of lysis			
	Source country	Year	Wastewater ^a			Orchid ^b
			BF-CIM1/14	BF-CIM5/14, BF-CIM6/14	BF-CIM8/14, BF-CIM10/14	BF25/12
<i>Pectobacterium cypripedii</i>						
NIB Z 1559 (NCPPB 750)	USA	1950	No	No	No	No
NIB Z 1560 (NCPPB 751)	USA	1950	No	No	No	No
NIB Z 1562 (NCPPB 3129)	USA	NA	No	No	No	No
NIB Z 1563 (NCPPB 3889)	UK	1994	No	No	No	No
NIB Z 1564 (NCPPB 4059)	UK	1999	No	No	No	No
NIB Z 1529 (NCPPB 3004)	USA	NA	No	No	No	No
<i>Enterobacteriaceae</i> from culture collection						
<i>Pantoea agglomerans</i> NIB Z 25	Slovenia	2002	No	No	No	No
<i>Proteus vulgaris</i> NIB Z 856	NA	NA	No	No	No	No
<i>Proteus mirabilis</i> NIB Z 857	NA	NA	No	No	No	No
<i>Deftia acidovorans</i> NIB Z 858	NA	NA	No	No	No	No
<i>Erwinia billingiae</i> NIB Z 1142 (DSM 17872)	UK	1999	No	No	No	No
Continued on next page						
<i>Erwinia tasmaniensis</i> NIB Z 1143 (DSM 17950)	Australia	2006	No	No	No	No
<i>Enterobacter</i> sp. NIB Z 1701 (NCCPB 4168)	NA	NA	No	No	No	No
<i>E. coli</i> NIB Z 2036	NA	NA	No	No	No	No
<i>E. coli</i> NIB Z 2037 (DSM 423)	NA	NA	No	No	No	No
<i>E. coli</i> NIB Z 2038 (DSM 13127)	NA	NA	No	No	No	No
<i>E. coli</i> NIB Z 2039 (APEC_FUW1)	NA	NA	No	No	No	No
<i>E. coli</i> NIB Z 2040 (DSMZ 613)	NA	NA	No	No	No	No

^aBacteriophages isolated from wastewater; ^bmodel bacteriophage isolated from diseased orchids; all other bacteriophages isolated from plant material were characteristically the same as the model bacteriophage.

Altogether, according to plaque morphology, RFLP profiles, host ranges and transmission electron microscopy analysis, 3 different types of bacteriophages were isolated. Two were derived from wastewater and were members of the families *Myoviridae* and *Siphoviridae*. The third bacteriophage belonged to the family *Podoviridae* and was obtained from soft rot orchid tissue. Based on the characteristics of these bacteriophages, bacteriophage BF25/12 was chosen for further analysis.

3.3.3 Transmission electron microscopy of bacteria-bacteriophage interactions

The interactions of the selected bacteriophage BF25/12 with the host bacteria were examined at the ultrastructure level. The interactions of BF25/12 and the UDL-4 bacteria activated granule degradation in the bacteria cells without bacterial lysis. Most of these UDL-4 bacteria contained electron-lucent intracellular granules (diameter, 20-70 nm), which were similar to intracellular inclusion bodies (Figure 3.14A). When bacteria UDL-4 and bacteriophages BF25/12 were incubated together for 15 min, these granules almost completely disappeared, and bacteriophages attached to the bacterial surface were observed (Figure 3.14B). Mixtures of *Dickeya* sp. UDL-3 (Figure 3.14C) and BF25/12 also showed attachment of the bacteriophages to the bacterial surface (Figure 3.14D). From plaque assays, plaques' borders were analysed. Most of the UDL-3 cells here were lysed, and the plasmalemma of the resting cells were detached (Figure 3.14E). New bacteriophages could also be seen in the cytoplasm of some bacteria (Figure 3.14F).

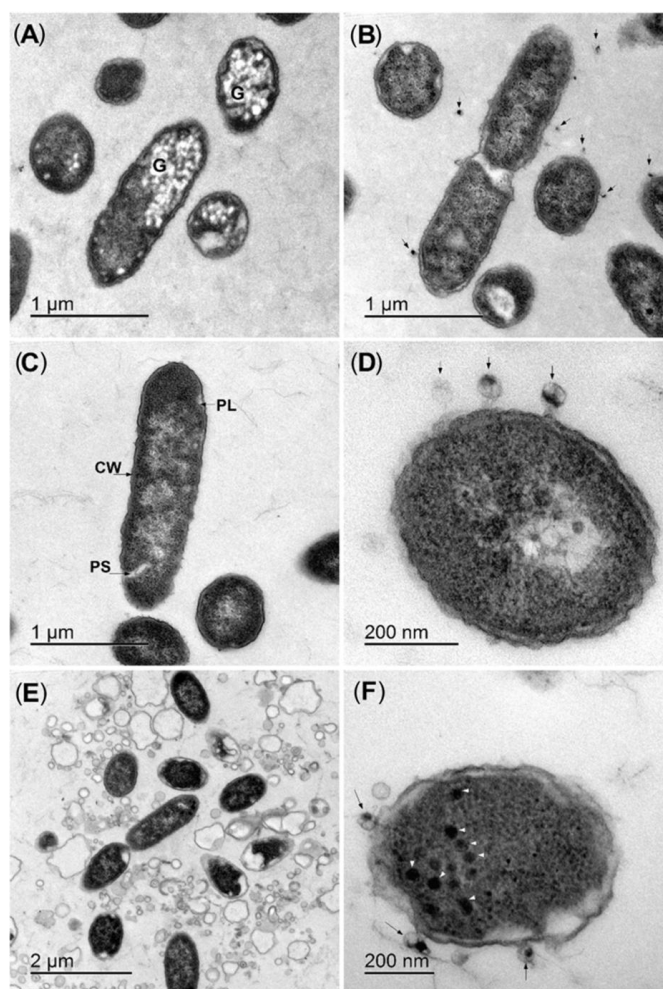


Figure 3.14: Representative ultrastructure analyses of bacteria-bacteriophage interactions. (A, B) Strain UDL-4 before (A) and after (B) mixing with bacteriophage BF25/12 (arrows). (C, D) Strain UDL-3 before (C) and after (D) mixing with bacteriophage BF25/12 (arrows). (E, F) Lysed UDL-3 bacteria from the border of a plaque (E) and new bacteriophages in the cytoplasm (arrowheads) (F). G, intracellular granules; CW, cell wall; PL, plasmalemma; PS, periplasmic space. Reprinted from "Newly Isolated

Bacteriophages from the *Podoviridae*, *Siphoviridae*, and *Myoviridae* Families Have Variable Effects on Putative Novel *Dickeya* spp." by Š. Alič et al., 2017, *Frontiers in Microbiology*, 8, p. 10. Copyright 2018 by the Frontiers Media SA.

3.4 Characterisation of the Model Bacteriophage BF25/12

3.4.1 Genome of the bacteriophage BF25/12

The genome of bacteriophage BF25/12 was additionally characterised using genome sequencing and automatic annotation. BF25/12 contains linear double-stranded (ds) DNA that is 43,872 bp long with 52.2% GC, which substantially supports its classification into the *Podoviridae* family. The genome of bacteriophage BF25/12 contains 52 open reading frames that were determined with RAST (Aziz et al., 2008; Brettin et al., 2015; Overbeek et al., 2014). They were mainly coded for hypothetical proteins (27 genes), along with structural proteins and proteins involved in bacteriophage replication. Furthermore, a single putative endolysine gene was identified, corresponding to the halo effect observed in plaque morphology. However, no genes that encode antibiotic resistance or toxins were detected (Figure 3.15). The annotated BF25/12 genome sequence is available in GenBank under Accession No. KT240186.

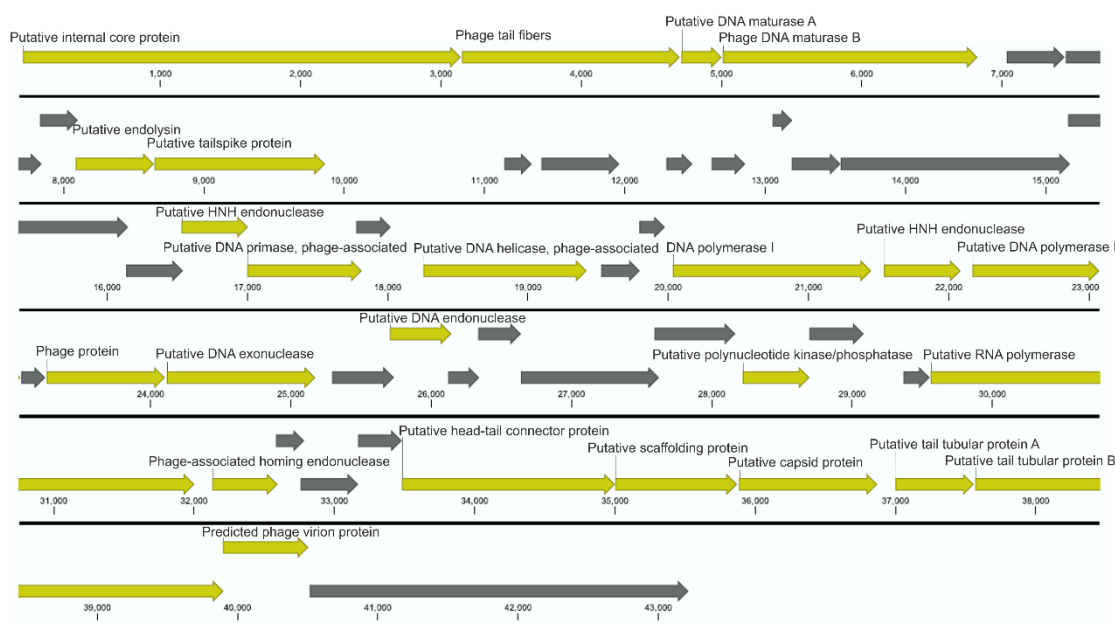


Figure 3.15: Annotated BF25/12 bacteriophage genome. According to the annotation, the genome does not contain any genes encoding antibiotic resistance or toxins. The open reading frames coding for structural proteins, proteins involved in bacteriophage replication and other conserved proteins are shown in yellow, and those coding for hypothetical proteins are shown in grey. The unit of the presented genome annotation is bp. Adapted from "Newly Isolated Bacteriophages from the *Podoviridae*, *Siphoviridae*, and *Myoviridae* Families Have Variable Effects on Putative Novel *Dickeya* spp." by Š. Alič et al., 2017, *Frontiers in Microbiology*, 8, p. 11. Copyright 2018 by the Frontiers Media SA.

The genomic sequence of the bacteriophage BF25/12 was compared to the characterised genera from the *Podoviridae* family using the

PASC tool (Bao et al., 2014). BF25/12 showed no homology (identity < 3%) to any member of the *Picovirinae* or *Sequivirinae* subfamily. However, it shared a higher identity to the *Autographivirinae* subfamily, especially *Klebsiella* virus F19 from the genus Kp34virus (33% identity). Based on the conducted pairwise identity analysis, the BF25/12 genome was 57-62% identical to 3 unclassified *Podoviridae* bacteriophages. All 3 genomically similar bacteriophages were *Pectobacterium* bacteriophages, namely, *Pectobacterium* phage PP16, PP90 and Peat1, that could not be assigned to any *Podoviridae* subfamily. The results correspond to nBLAST analysis. The assembled BF25/12 genome showed substantial similarity (74-79% identity and 21-56% query coverage) to 5 bacteriophage genomes in the BLAST database. All 5 genomes belonged to *Pectobacterium* bacteriophages, 4 of which were assigned to the family *Podoviridae*, like BF25/12. The last was unclassified. The closest hit represented the *Pectobacterium* bacteriophage PP16 (79% identity over 56% query coverage).

3.4.2 Development of qPCR and ddPCR to detect and quantify BF25/12

The development of 3 qPCR and ddPCR assays for the tracking and quantification of our model bacteriophage was based on the BF25/12 annotated genome sequence. Each test detects a different gene encoding phage structural protein, namely, capsid protein, tail tubular protein B and tail spike protein. Analytical sensitivity varied between 416.7-4167.0 copies of DNA per ml sampled. All 3 assays proved to be specific, as none cross-reacted with any tested bacterial DNA (54 samples), DNA extracted from plant material (18 samples) and DNA of other bacteriophages (15 samples), proving sufficient analytical specificity. All three tests could detect phage DNA in samples isolated with phenol-chloroform extraction and Bionobile kit as well as in isolated phage particles in SMG buffer with a negligible effect on assays' efficiency, indicating the possibility of tracking and quantifying our model phage without prior DNA extraction. ddPCR was used for reference material quantification. Determined concentrations of DNA with all three tests were alike, approximately 4.2×10^{10} copies of DNA per mL.

3.4.3 IZON qNANO bacteriophage particle analysis

Phage BF25/12 particle size was measured using TEM and IZON qNANO (Izon Science Ltd., Oxford, UK). The measured mean particle dimensions were 55.8 ± 2.3 nm x 52.6 ± 3.1 nm (n = 20) and 72 nm (n = 787; Figure 3.16, next page), respectively. Phage particles were not completely spherical. Therefore, the size determination with qNANO proved rather difficult and imprecise. Particle diameter measured with qNANO varied from 44.9 to 120.1 nm (n = 787; Figure 3.16, next page). Observed broad size span might result from the broken and damaged phage particles that cannot be eliminated from the measurement.

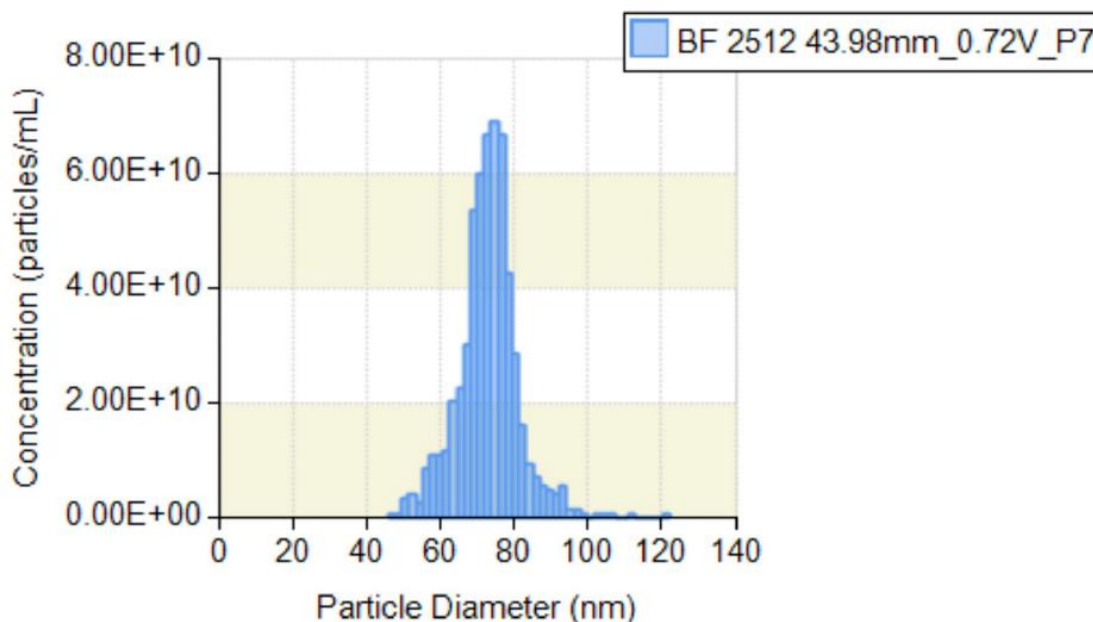


Figure 3.16: Size histogram of bacteriophage BF 25/12 particles measured with IZON qNANO. The histogram is based on measurements of 787 BF 25/12 particles.

The concentration of infective phage particles BF25/12 was determined using a plaque assay, and all particle concentrations were evaluated by IZON qNANO to 4.8×10^9 pfu/ml and 5.8×10^{10} particles/ml, respectively. The concentration of infective virus particles corresponded to 8.3% of all virus particles regarding the measured differences in the tested phage suspension. Additionally, the concentration of DNA isolated from the phage suspension was determined using ddPCR assays and calculated to the initial phage concentration. The determined concentration of 4.6×10^{10} phage particles/ml corresponds with the qNANO concentration measurement. Slight differences can be explained by losses during the DNA extraction process.

3.4.4 UV radiation stability

Bacteriophage BF25/12 could not survive the 2 min exposure to UV-C light. The reduction of active bacteriophages was 10^5 , corresponding to almost 100% in the first minute of exposure.

3.4.5 pH stability

The bacteriophage pH stability analysis in SMG buffer and sterile demineralised water at two bacteriophage concentrations (10^5 and 10^3 pfu/mL) showed that bacteriophage BF25/12 pH stability is affected by both its concentration and environment (Figure 3.17/ Figure 3.17, next page). Bacteriophage BF25/12 lost its infectivity completely at pH 3, regardless of the incubation media (SMG buffer or sterile demineralised water). It showed maximum stability at pH 7 and 9, which indicated bacteriophage BF25/12's favourable pH is neutral to mildly alkaline. Furthermore, no significant difference in the drop of the bacteriophage titre could be detected between tested incubation media or bacteriophage concentrations after 24 hours' incubation at pHs 7 and 9. At higher concentrations in SMG buffer, mildly acidic pH (pH 5) did not cause significant phage de-activation. A bacteriophage incubated in SMG buffer completely lost its

infectivity at pH 11 in 24 hours' incubation, but its infectivity was not affected in sterile demineralised water in the same conditions. The exposure of highly concentrated bacteriophage lysate (10^8 pfu/mL) to an acidic (pH 3) and alkaline (pH 11) environment for 24 hours had almost no detectable effect on bacteriophages' survival and infectivity (data not shown).

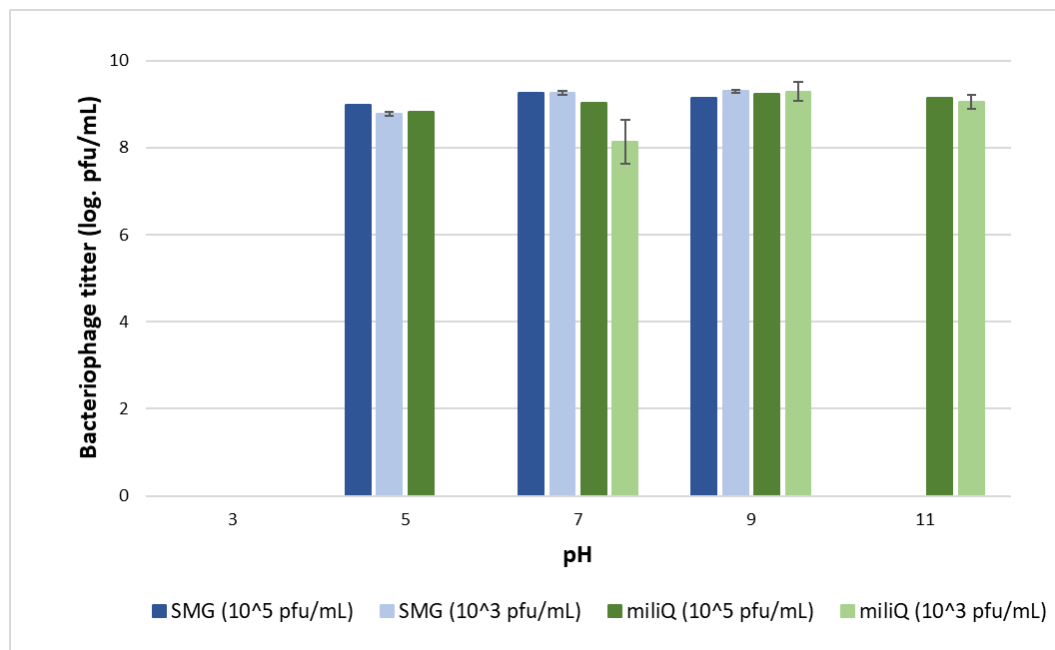


Figure 3.17: The effect of pH on the stability of bacteriophage BF25/12. Stability was tested in SMG buffer and sterile demineralised water at 2 different bacteriophage concentrations, 10^5 and 10^3 pfu/mL. Adapted from "Newly Isolated Bacteriophages from the *Podoviridae*, *Siphoviridae*, and *Myoviridae* Families Have Variable Effects on Putative Novel *Dickeya* spp." by Š. Alič et al., 2017, *Frontiers in Microbiology*, 8, p. 11. Copyright 2018 by the Frontiers Media SA.

3.4.6 Temperature stability

The stability of the bacteriophage BF25/12, which is active against *Dickeya* spp., was monitored at different temperatures over a year. The bacteriophage was stable at 4 °C, as the changes in the bacteriophage titres were negligible, which indicated this to be a favourable temperature for long-term bacteriophage storage (Figure 3.18Figure 3.18, next page). The bacteriophage's stability at -80 °C appeared similar to its stability at 4 °C. The least favourable temperature tested was 28 °C.

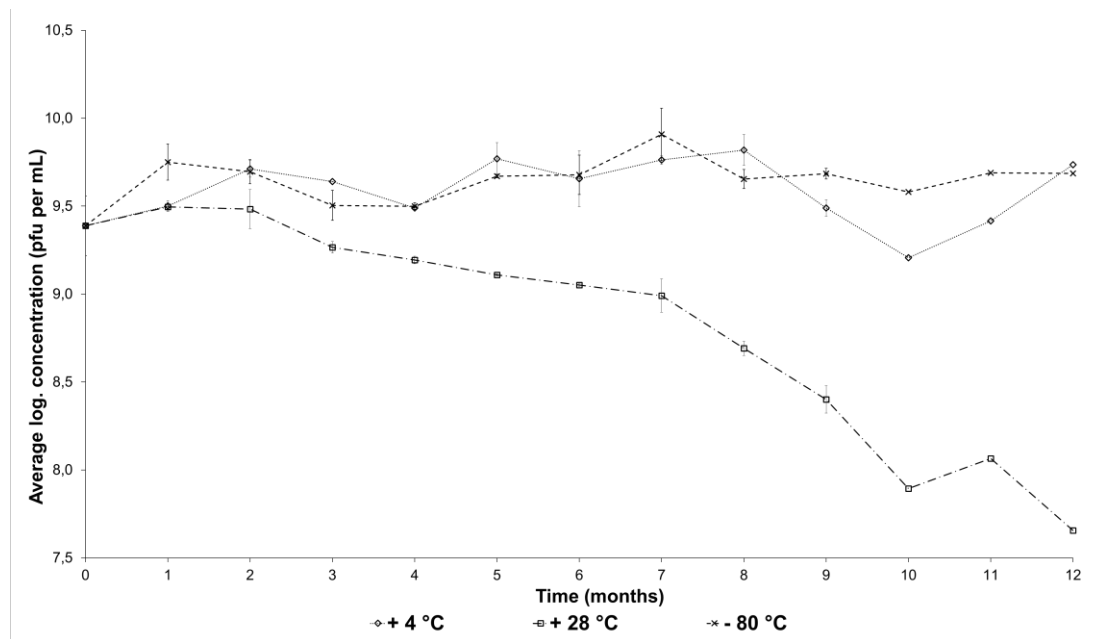


Figure 3.18: Temperature stability with the time of *Dickeya* spp. bacteriophage BF25/12 incubated at temperatures of +4, +28 and -80 °C (as indicated). Adapted from "Newly Isolated Bacteriophages from the *Podoviridae*, *Siphoviridae*, and *Myoviridae* Families Have Variable Effects on Putative Novel *Dickeya* spp." by Š. Alič et al., 2017, *Frontiers in Microbiology*, 8, p. 11. Copyright 2018 by the Frontiers Media SA.

3.4.7 Bacterial resistance

The emergence of resistance against bacteriophage BF25/12 was determined for host bacterium *Dickeya* sp. B16. The number of growth-resistant bacterial colonies depended greatly on the bacteriophage concentration in the medium ([Figure 3.19](#)). The highest rate of bacterial resistance development against bacteriophage BF25/12 was at the final bacteriophage concentration of between 10^4 pfu/mL and 10^5 pfu/mL, at the initial bacterial concentration of 10^8 cfu/mL. Bacteriophage concentrations $<\log 4$ and $>\log 5$ provided lower selection pressure, which resulted in the decreased occurrence of resistant bacterial colonies.

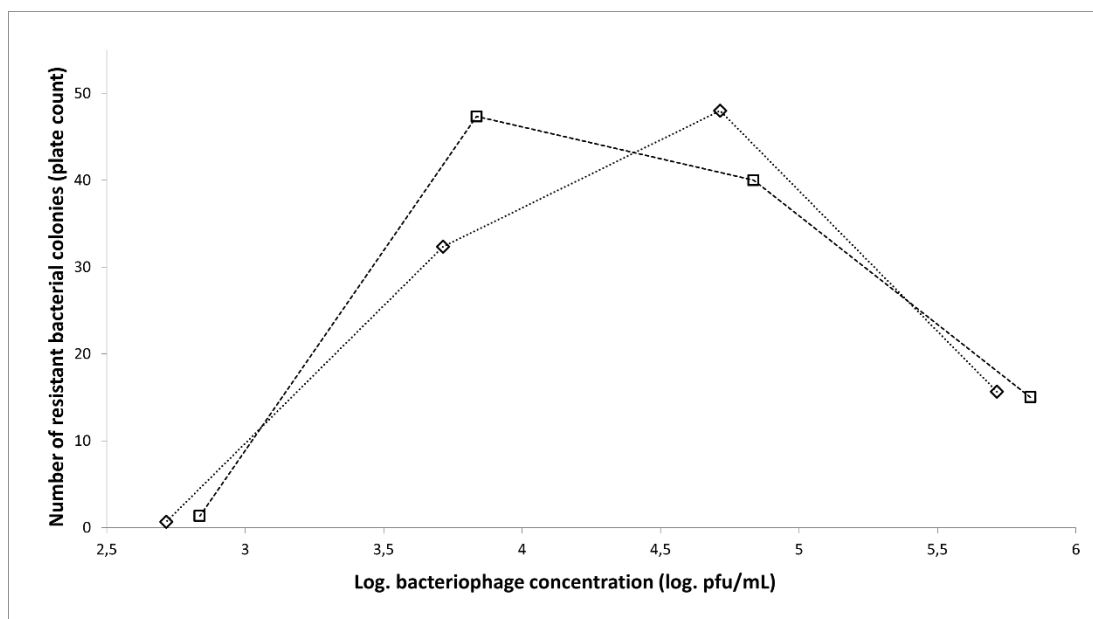


Figure 3.19: Emergence of *Dickeya* spp. B16 resistant to bacteriophage BF 25/12. The experiment was performed as 2 technical repeats, shown on the graph as squares and diamonds. Initial bacterial concentration prior bacteriophage addition was 10^8 cfu/mL. Adapted from "Newly Isolated Bacteriophages from the *Podoviridae*, *Siphoviridae*, and *Myoviridae* Families Have Variable Effects on Putative Novel *Dickeya* spp." by Š. Alič et al., 2017, *Frontiers in Microbiology*, 8, p. 12. Copyright 2018 by the Frontiers Media SA.

Chapter 4

Discussion

Bacterial soft rot disease represents a significant economic liability for agriculture, especially for the production of ornamental plants such as orchids, as optimal cultivation conditions are favourable for the development of the disease. Multiple papers report *Dickeya* isolates from orchids that could not be assigned to a specific *Dickeya* species, indicating the presence of a new species within the genus (Parkinson et al., 2009; Suharjo et al., 2014; Van Vaerenbergh et al., 2012). However, no comprehensive characterisation and description of those isolates has been published. A characterisation of the pathogen is essential for discriminating between the pathogenic bacterial species and setting efficient diagnostic controls. In the scope of our study, we have characterised new *Dickeya* isolates from *Phalaenopsis* orchids from Slovene commercial production sites that are genetically similar to previously described isolates from orchid and monocot plants. However, comprehensively characterised isolates did not correspond to any *Dickeya* species at the time. Therefore, classification into a putative new *Dickeya* species was proposed (as described in Alič et al., 2017b). A combination of genomic, genetic and suitable phenotype analyses were conducted to taxonomically place the isolates as independent species within the genus *Dickeya*. However, in the middle of our investigation in 2016, a new *Dickeya* species was described named *D. fangzhongdai*. *D. fangzhongdai* was reported as a tree pathogen associated with bleeding canker disease. *Dickeya* isolates included in our study that were grouped as the putative new species passed the taxonomy requirements to be classified in the *D. fangzhongdai* species.

The control of the soft rot disease caused by *Dickeya* spp. is still based on the prevention of the disease and elimination of the infected plants and plant tissue (Czajkowski et al., 2015; Toth et al., 2011). Classic chemical control agents are not efficient for either of the *Dickeya* spp. Pathogens. Therefore, biocontrol management strategies give great expectations for the management of this destructive disease (Czajkowski, 2016). Studies exploring bacteriophage biocontrol strategies for *Dickeya* spp. were limited mostly to potato pathogens *D. solani* and *D. dadantii*, though *Dickeya* spp. present a major economic liability to the production of ornamentals like orchids (Adriaenssens et al., 2012; R. Czajkowski et al., 2013; Czajkowski et al., 2015; Day et al., 2017; Soleimani-Delfan et al., 2015).

This thesis focuses on the *Dickeya* isolates from *Phalaenopsis* orchids with soft rot symptoms from commercial production sites and the potential of bacteriophages as a relevant research tool and biocontrol agents. The two main aims of the thesis were to (i) genetically and phenotypically characterise novel *Dickeya* isolates and (ii) resolve their taxonomic positions within the genus *Dickeya*. The third aim was to isolate bacteriophages that are active against new *Dickeya* sp. isolates and explore their

potential as a biocontrol agent. A well-characterised host-bacteriophage model is a valuable research tool, providing the needed knowledge of bacteria-bacteriophage dynamics in a given host environment for the successful establishment of the bacteriophage biocontrol system.

The results of our study were summarised in three scientific publications (Alič et al., 2015; Alič et al., 2017a; Alič et al., 2017b; Alič et al., In press).

4.1 Phenotypic and Genetic Characterisation of *Dickeya* Isolates from Diseased *Phalaenopsis* Orchids

Causative bacteria of the soft rot affecting *Phalaenopsis* orchids were identified as members of the genus *Dickeya* on all the tested plants. Other soft rot *Enterobacteriaceae*, including *Pantoea* and *Pectobacterium*, were not detected. Based on the variability in the *fliC* nucleotide sequence, these isolates were divided into two groups, namely UDL-3 and UDL-4 (Van Vaerenbergh et al., 2012), which clustered with similar *Dickeya* isolates from ornamental plants. The UDL-3 lineage was more prevalent among these isolates. A phylogenetic analysis based on the partial sequencing of the *dnaX* gene placed these isolates from *Phalaenopsis* in a cluster with *Dickeya* isolates from various monocot plants in Asia corresponding to the DUC-2 group (Suharjo et al., 2014). All of our *Dickeya* isolates were clustered together with the previously described UDL-3 and UDL-4 isolates and *Dickeya* isolates described by Suharjo et al. (2014). The isolates formed a separate group, causing soft rot infections on monocot plants, which indicated the prevalence of the group in monocots. However, the pathogenicity tests on potato plants also indicated a dicotyledonous plant infection, which supports the broad host range of the pathogens belonging to the genus *Dickeya* (Ma et al., 2007).

The phenotypic characteristics of the model isolates *Dickeya* sp. B16 (UDL-3) and *Dickeya* sp. S1 (UDL-4) were tested in *in vivo* plant models (orchid, Belgian chicory, potato) and compared to other *Dickeya* spp. Belgian chicory (witloof) is known as a model plant for testing the aggressiveness of *Dickeya* strains. However, our study represents the first report that describes aggressiveness comparisons of multiple *Dickeya* spp. tested on the witloof plant. These data confirm that the bacterial aggressiveness is not solely species specific; rather, it is strain characteristic (Potrykus, Golanowska, Hugouvieux-Cotte-Pattat, & Lojkowska, 2014). The model *Dickeya* isolates from diseased orchids showed significantly higher rates of leaf-tissue maceration in comparison with the other *Dickeya* strains included in the testing, which indicates the increased aggressiveness of the novel isolates.

Cross-infections between ornamental hosts and potato have been suggested for *D. dianthicola* and *D. solani*. Ornamental plants can present potential routes for the introduction of additional *Dickeya* species and strains with a broad host range, which represents a new threat to potato production (Parkinson et al., 2009). Therefore, the pathogenicity of the new *Dickeya* isolates for the potato host was also tested. These data indicate the potential virulence of the *Dickeya* spp. B16 and S1 isolates for potato plants. The model bacteria spread through these plants and showed high maceration potential in the witloof model. The measured bacteria concentrations in the plant tissues corresponded to the bacteria rates detected in the positive control using the

D. solani GBBC 2040 strain. *D. solani* can spread through potato plants via the vascular tissue, with the systemic colonisation of potato plants and the progeny tubers as already reported (Czajkowski et al., 2009). However, in the present study, *D. solani* GBBC 2040 was not detected in the tubers produced by the inoculated potato plants. For unknown reason the bacteria did not spread from the potato plant to the tuber tissue. Therefore, no conclusions can be drawn regarding the possible systemic spread of *Dickeya* spp. B16 and S1 into the tuber progeny. Nevertheless, both *Dickeya* spp. B16 and S1 showed high maceration potential on potato tuber tissue.

There are no reports in the literature regarding the *Dickeya* cell ultrastructure that enables the storage of energy and compounds. Electron microscopy of these *Dickeya* isolates of the UDL-4 group revealed the presence of small electron-lucent granules. However, these structures were only observed in the cells of the UDL-4 strain, even though these two strains (i.e. UDL-3 and UDL-4) were grown under identical conditions. In these micrographs, these spherical granules were similar to the polyhydroxyalkanoate granules that are found as cytoplasmic inclusions in many microorganisms and are probably most frequently carbon storage compounds and energy reserves (Jendrossek & Pfeiffer, 2014). Polyhydroxyalkanoate granules are usually 200 nm to 500 nm in diameter (Jendrossek & Pfeiffer, 2014). In UDL 4, the granule diameter was <100 nm, they were located mostly at the cell poles, and they rarely formed groups in the centres of the cells. Due to these inconclusive transmission electron microscopy data, these granules were additionally stained with Sudan black B and Nile blue A dye (data not shown), which are dyes for the specific staining of polyhydroxyalkanoate and polyhydroxybutyrate structures. However, no staining of these granules was observed. Glycogen is another inclusion that functions as a metabolic reserve in bacteria, and it can be accumulated as granules or inclusions that range from 20 nm to 100 nm in size (Shively et al., 2011). Glycogen forms a reddish-brown complex with iodine, which provides the staining of glycogen-producing cells (Cheng, Hironaka, Roberts, & Costerton, 1973). Both the UDL-3 and UDL-4 bacteria cultures stained reddish-brown with iodine solution, even though these cell structures were not seen for UDL-3. However, the iodine staining intensity was lower in the UDL-3 cultures. Therefore, no conclusions can be made at this point regarding these granule structures and their function, although they appear most likely to represent metabolic reserves.

These novel *Dickeya* isolates expressed high aggressiveness and maceration potential on tested plant models. They do not represent a risk only for the production of ornamental plants, their original host, but also for crop production, especially for the potato, as these data also indicate the possibility of cross-infections. The impact of the production of intracellular granules on the virulence of these *Dickeya* isolates is not known. However, only minor roles of glycogen in virulence and colonisation were shown for *Salmonella enterica*, although it had a significant contribution to the survival of bacteria, giving these cells the potential for longer persistence in the environment (McMeechan et al., 2005).

4.2 Phylogenetic and Taxonomic Positioning of Unclassified *Dickeya* spp. Isolates into the *D. fangzhongdai* Species

Further genomic and phenotypic methods were used to decipher the relationships among our model isolates *Dickeya* sp. B16 and S1, commonly involved in the soft rot of greenhouse-grown *Phalaenopsis* orchids, other unclassified *Dickeya* isolates and all currently described *Dickeya* spp., including the just-added *D. fangzhongdai*.

Based on the whole genome similarity - including the ANI analysis of draft genomes and DNA-DNA hybridisation - the strains *Dickeya* sp. S1 (*Phalaenopsis* sp.), *Dickeya* sp. B16 (*Phalaenopsis* sp.), *Dickeya* sp. MK7 (river water), *Dickeya* sp. NCPPB 3274 (*Aglaonema* sp.), *D. solani* ND14b (previously *Cedecea neteri*; waterfall water), *D. solani* M005 (waterfall water) and *D. chrysanthemi* M074 (waterfall water) were all found to belong to the *D. fangzhongdai* species, with ANI values exceeding 96%. The classification of these isolates into the *D. fangzhongdai* species was confirmed by *in silico* MLSA using 6 housekeeping gene sequences. The isolates of *D. fangzhongdai* are placed as a distinct clade within the genus *Dickeya*, which is genetically closest to the *D. dadantii*/*D. solani*/*D. dianthicola* subgroup. The tree-based MLSA constructed on partial sequence of the *recA* gene available for all the sets of previously described isolates indicated that, additionally, strains of *Dickeya* sp. isolated from many monocotyledon plants in Asia (Suharjo et al., 2014) belong to the *D. fangzhongdai* sp., as well as strains previously placed in DUC-2 and DUC-3 groups (Parkinson et al., 2009). Genomically, *Dickeya* sp. NCPPB 3274 was placed on the border of species designation by ANI and DNA-DNA hybridisation analysis. However, the strain shares most of the phenotypic characteristics of *D. fangzhongdai*, confirming its position within the species.

The strains *D. fangzhongdai* JS5^T, S1, B16, MK7 and NCPPB 3274 could be phenotypically distinguished from other *Dickeya* species by their ability to assimilate D-psicose, L-tartaric acid, thymidine, D-arabinose and gentiobiose. Furthermore, proteomic fingerprinting using MALDI-TOF mass spectrometry showed higher similarity among *D. fangzhongdai* strains compared to other *Dickeya* spp. in the Brunker database ([Error! Reference source not found. Appendix—D](#)). Based on our results, the only diagnostic phenotypic feature of *D. fangzhongdai* isolates - which differentiates them from all other *Dickeya* spp. - is the utilisation of D-psicose. Parkinson et al. (2014) reported the ability of *D. aquatica* to utilise D-psicose on GN plates. However, no utilisation of D-psicose by *D. aquatica* could be observed in our experiment. D-psicose, also known as D-allulose, is a rare, low-energy hexose. Some bacterial isolates can convert D-fructose to D-psicose using D-psicose-3-epimerase (He, Mu, Jiang, Yan, & Zhang, 2016). Little is known of the microbial utilisation of D-psicose, and no particular function of D-psicose in bacteria has been identified. However, it has been shown that D-psicose is involved in plant signalling and has been proposed to be an inducer of systemic resistance in agricultural systems (see e.g. U.S. Patent No. 8,017,828, 2011). The bacterial ability to metabolise it may therefore be important regarding plant pathogenic bacteria (Cho & Yoo, 2011; Kano et al., 2011).

Overall, phenotypic variability was noted among *D. fangzhongdai* strains and the entire genus *Dickeya*. The phenotypic differences among the strains of *D. fangzhongdai* did not reflect their genetic and genomic relationships determined by MLSA and ANI, respectively. *D. fangzhongdai* JS5^T differed substantially from all

the other tested putative *D. fangzhongdai* isolates, as only JS5^T could utilise 6 additional carbon sources (i.e. D-glucuronic acid, α -keto-glutaric acid, glucuronamide, adenosine, stachyose and melibiononic acid).

While most of the isolates corresponding to the *D. fangzhongdai* species originate from Asia and were isolated from herbaceous monocotyledonous plants or water (Alič et al., 2017b; Pritchard et al., 2013), the species was originally described on 3 strains (including the reference-type strain of the species, JS5^T), isolated from pear trees exhibiting bleeding canker disease. The pathogenicity of the *D. fangzhongdai* isolates on the pear trees have not yet been scientifically proven. Therefore, *D. fangzhongdai* cannot be proclaimed as a species pathogenic also for trees. However, observed differences in the phenotypic profiles among the isolates could originate from ecological differences in the environments in which the various *D. fangzhongdai* isolates are found. It was shown that, for *Pectobacterium carotovorum*, species diversity strongly correlates with plant evolution and that the specialisation of strains within the species towards monocotyledonous hosts can be observed for *Pectobacterium carotovorum*. In the genus *Pectobacterium*, the bacterial isolates favouring monocotyledonous or dicotyledonous hosts were distinctly different at both the genetic and phylogenetic levels (Yishay et al., 2008). Similarly, differentiation between *D. fangzhongdai* isolates from monocotyledonous and dicotyledonous plants could be observed, but only at the phenotypic level. Konstantinidis et al. (2006) explained the effect of those ecological constraints that can modulate phenotypic and ecological potential, as well as how they contribute to the emergence of novel species – even among strains that show higher than 98–99% ANI values based on current species designation.

A bacterial species is a group of strains characterised by at least one diagnostic phenotypic characteristic showing 70% DNA–DNA hybridisation (Wayne et al., 1987). However, multiple researchers have reported in the last decade that the current taxonomy and binomial naming of bacterial species is clearly insufficient – particularly for *Enterobacteriaceae*, since there is no established classification system to describe diversity within bacterial species (Konstantinidis et al., 2006; Pritchard et al., 2016; Vinatzer et al., 2017). This problem is particularly relevant to plant pathogenic bacterial species, and it was also observed in the present study. The observed contradiction between *D. fangzhongdai* genomic and phenotypic data is most likely due to strong host and environmental constraints that differ significantly among the strains. The relationship between taxonomy/genotype and phenotype is complex; therefore, the assumption that taxonomic classification implies bacterial phenotype can lead to misclassifications (Pritchard et al., 2016; Vinatzer et al., 2017). The current species designation does not consider ecological potential; however, ecological niches have a crucial influence on the phenotypic characteristics of a strain (Konstantinidis et al., 2006). While next-generation sequencing experiments could enable a more comprehensible taxonomy and species designation, even for highly diverse bacterial species (Pritchard et al., 2016; Vinatzer et al., 2017), the phenotype must remain an integral part of research on plant pathogenic bacteria. Analyses of the *D. fangzhongdai* pear isolates accessory genome could shed more light on this apparent discrepancy between genetic and phenotypic relatedness.

Dickeya strains S1, B16, MK7, NCPPB 3274, ND14b, M005 and M074 showed very high genomic similarity to the *D. fangzhongdai* species. Regardless of the significant diversity observed at the

phenotypic level, based on current taxonomic classification practices, the strains share phenotypic traits to a sufficient degree to be classified into a uniform *D. fangzhongdai* species. Therefore, we propose the classification of *Dickeya* sp. S1, *Dickeya* sp. B16, *Dickeya* sp. MK7, *Dickeya* sp. NCPPB 3274, *Dickeya* solani ND14b, *D. solani* M005 and *D. chrysanthemi* M074 into *D. fangzhongdai* S1, *D. fangzhongdai* B16, *D. fangzhongdai* MK7, *D. fangzhongdai* NCPPB 3274, *D. fangzhongdai* ND14b, *D. fangzhongdai* M005 and *D. fangzhongdai* M074, respectively.

To date, only a few instances of isolates corresponding to *D. fangzhongdai* have been reported in Europe. It can be safely assumed that the bacteria have been introduced to Europe several times through the propagation of ornamental plants as early as 1985 (*Dickeya* sp. PD 550, PD 813 and GBBC 323, isolated in 1985, 1987 and 1998, respectively) (Van Vaerenbergh et al., 2012). The species is not known to be associated with any significant plant disease in Europe's open environment. However, the orchid pathogens *Dickeya* sp. S1 and B16 also were able to persist in potato plants. Coupled with the extended pathogenic potential of some *D. fangzhongdai* strains, the high adaptability of the genus *Dickeya* in general and the phenotypic fluidity of the strains warrant caution against *D. fangzhongdai* in the production of many economically important plants.

4.3 Bacteriophages Active against Causative *Dickeya* from Three Different Caudovirales Families

Bacteriophages active against *Dickeya* spp. isolates that cause the soft rot of orchids at *Phalaenopsis* production sites were isolated from two different sources: diseased *Phalaenopsis* tissue and wastewater. For the last, CIM monolith chromatography was used for the bacteriophage particle concentration. CIM monolith chromatography has been reported as an effective technique for virus concentration and for the purification of virus particles (Steyer et al., 2015). However, we believe that we are the first group that used CIM monolith chromatography as a technique for the isolation of infectious bacteriophages from environmental samples. This method proved to be very efficient for the concentration of bacteriophages from environmental samples, which allowed more efficient bacteriophage enrichment on the host bacterium and, therefore, better yields in the bacteriophage isolation. Seventy-two percent of obtained bacteriophages active against *Dickeya* spp. were isolated from wastewater using this CIM monolith chromatography, which indicates the presence of viable bacteria of *Dickeya* spp. in wastewater or in plants that are in direct contact with wastewater. Similarly, Day et al. (2017) successfully isolated 67 bacteriophages active against *D. solani* from sewage and river water. An abundance of active bacteriophages in water sources, specifically against *Dickeya* spp., supports reports of water being a possible source of plant infection (Toth et al., 2011). Furthermore, different sources of bacteriophage isolation resulted in the identification of different bacteriophages from different families with different RFLP patterns, although they showed similar host-specificity profiles. The bacteriophages were isolated from orchid samples in summer (i.e. July, September), when the highest concentrations of the bacteria are expected. The isolates from wastewater were obtained from late spring to early autumn, excluding July and September. The attempts to isolate bacteriophages from soil were not successful, regardless of the month when the sampling was conducted (data not shown). Thus, these differences cannot be explained in terms of seasonal changes, which indicates the specificity of each bacteriophage source.

On the other hand, the bacteriophages active against *Dickeya* spp. that showed great specificities provide a great opportunity to serve as biocontrol agents, as the treatment of a diseased orchid with these bacteriophages can specifically eliminate pathogenic bacteria while leaving the normal microbiota unharmed. Adriaenssens et al. (2012) and Czajkowski et al. (2013, 2014, 2015) reported the isolation of bacteriophages specific against the aggressive potato pathogen *D. solani*, bacteriophages with multiple hosts across *Dickeya* spp. and also broad host-range bacteriophages against *Pectobacterium* spp. and *Dickeya* spp. All the bacteriophages active against *Dickeya* spp. described in the literature belong to the order Caudovirales as members of the family Myoviridae (Adriaenssens et al., 2012; Czajkowski et al., 2013; Czajkowski et al., 2015; Soleimani-Delfan et al., 2015), and the first report of a bacteriophage from the Siphoviridae family was recently made (Soleimani-Delfan et al., 2015). Podoviridae active against *Dickeya* spp. are also rarely isolated (Day et al., 2017), and none of the members of Podoviridae has been characterised before. However, in our study, we were able to isolate bacteriophages from all three Caudovirales families. The bacteriophages from different families show some small variations

in their specificities. All the bacteriophages from the families *Podoviridae* (BF21/12-BF25/12) and *Myoviridae* (BF-CIM5/14-BF-CIM10/14) infected all the *Dickeya* isolates here, as well as the aggressive potato pathogen *D. solani* and other reference *Dickeya* strains, excluding *D. paradisiaca* and *D. aquatica*. However, bacteriophages BF-CIM1/14-BF-CIM5/14, as members of the family *Siphoviridae*, showed divergent host ranges. The activity against the *Dickeya* sp. UDL-3 isolates varied and was relatively low. Conversely, all the *Dickeya* sp. UDL-4 and *D. solani* strains were highly susceptible, which supports a relationship between those 2 *Dickeya* spp.

The selected bacteriophage from the *Podoviridae* family, BF25/12, was further evaluated and characterised at the genome level. It showed no similarities to any previously described bacteriophages that are active against *Dickeya* spp. However, closely related bacteriophages deposited in GenBank were identified using BLAST and PASC analysis. The BF25/12 genome showed the highest similarity to bacteriophages active against *Pectobacterium* species characterised as members of the *Podoviridae* family, like BF25/12. *Pectobacterium* bacteriophages did not share any similarity to BF25/12 throughout the whole genome. Rather, the homology was limited to sections of the genome. This is the first member of the family *Podoviridae* among bacteriophages active against *Dickeya* spp., therefore the lack of genetically similar bacteriophages was expected. Bacteriophage BF25/12 did not show significant similarities to any currently described and characterised genus *Podoviridae* at the genome level. Based on the genomic and morphologic characteristics, it is closest to genera in the *Autographivirinae* subfamily, which includes the T7 virus genus and the *Escherichia coli* model bacteriophage T7. The bacteriophage T7 virion consists of a head with a diameter of 55 nm and a 23 nm-long tail (Stevens & Trus, 1986). The size of the T7 virus particle corresponds to measurements of the BF25/12 virion (head approx. 56 x 52 nm and tail 16 x 12 nm). The BF25/12 genome size of 43.8 kbp as well as the 52.2% GC content matches the small genome of the T7 bacteriophage (around 40 kbp and 48.4% GC content) (Dunn, Studier, & Gottesman, 1983). Furthermore, T7 and BF25/12 have almost an identical number of coding sequences in the genome: 50 and 52 genes, respectively (Dunn et al., 1983). The genome analysis showed the potential of BF25/12 for field-application studies due to its lack of gene coding for the bacteriophage lysogenic cycle and of genes encoding toxins or antibiotic resistance.

Methods slightly varied regarding the determined quantity (titre) and size of BF25/12 virus particles. IZON qNANO measurements differed from the titre determined with a plaque assay and the size measured with TEM. The deviation in measured concentrations occurred because a plaque assay detects only infective virus particles, unlike qNANO, which measures whole particle concentrations in tested phage suspensions. However, some difficulties in the measurement of the qNANO size were noticed. The determined particle diameter ranged from 44.9 to 120.1 nm and differed from the size determined with TEM (55.8 ± 2.3 nm x 52.6 ± 3.1 nm). The observed discrepancies could be explained by the particle shape and possible contribution of the solvation to the measured size. The calibration process only involves spherical beads, with no possibility for testing the instrument in differently shaped calibration particles, more adequate for bacteriophage measurements. Based on the observed results, the qNANO instrument can closely quantify the number of *Podoviridae* particles in a suspension, yet size measurements are imprecise due to the geometry of virus particles.

Bacteriophage BF25/12 showed differences in activity against the UDL-3 and UDL-4 bacteria, which are genetically very similar bacterial isolates. Variations in the infections of these two isolates were observed under transmission electron microscopy, which resulted in phenomena that have not been reported previously. The interactions of the bacteriophage with the UDL-4 bacteria-activated granule degradation in the bacterial cells were similar to intracellular storage granules, with no bacterial lysis. The structure and function of these observed inclusions remain unknown, although a type of intracellular metabolic reserve is most likely. Some metabolic inclusions that function as a metabolic reserve are directly connected to persistence of bacteria. A glycogen metabolism has been linked to bacterial survival in the environment, colonisation and virulence (reviewed in Wilson et al., 2010). Moreover, in *Pseudomonas oleovorans*, it was shown that the metabolism of intracellular polyhydroxyalkanoates granules contributes to bacterial cells' survival. Wild-type strain *Pseudomonas oleovorans* that was able to degrade intracellular polyhydroxyalkanoates showed enhanced resistance to stress agents compared to the corresponding mutant strain incapable of polyhydroxyalkanoates' depolymerisation (Ruiz, López, Fernández, & Méndez, 2001). This observed granule degradation in UDL-4 cells might represent a bacterial stress response to the bacteriophage infection process, whereby metabolic inclusions might enhance the environmental survival of the bacteria and therefore limit the development of the bacteriophage's infection. However, the correlation between a bacterial resistance to bacteriophage lysis and intercellular storage granule degradation has not been described.

One of the main challenges in bacteriophage applications are environmental factors, including temperature, pH and UV radiation. Temperature can affect the ability of bacteriophages to lyse bacteria as well as their survival on a plant surface (Jones et al., 2007). Monitoring the bacteriophage's stability here revealed decreased bacteriophage titres at 28 °C. The observed outcome corresponds to a study of Jepson and March (2004), who reported a decrease in liquid bacteriophage λ stock at 37 °C over 120 days to less than the limit of detection. However, the decline in the viability of bacteriophage BF25/12 was only 27.34% in 1 year, which indicates its applicability due to this appreciable stability at the implementation temperature. As expected, the bacteriophage titre at 4 °C remained stable over the observed period. Bacteriophage BF25/12 belongs to the tailed bacteriophages, and these are known to be extremely viable at 4 °C, with a report that some can retain their viability for 10–12 years (Jepson & March, 2004; Jończyk, Kłak, Międzybrodzki, & Górski, 2011). The storage of bacteriophages at -20 °C is not recommended, as the formation of ice crystals can damage and destroy bacteriophage particles (Jończyk et al., 2011). Furthermore, a suboptimal pH can inactivate bacteriophages. Therefore, stability and an optimal pH for bacteriophages is very important in plant disease control applications (Jones et al., 2007). Bacteriophage BF25/12 was sensitive to acid pH and showed maximum stability at neutral and alkaline conditions. Only bacteriophages incubated in sterile demineralised water were stable at pH 11, indicating the influence of the incubation medium to bacteriophage stability at a certain pH. It was reported that a buffer and salts concentration can affect a bacteriophage's stability and activity (Burnett & Stanley, 1960; Chow, Lin, & Kuo, 1971; Jończyk et al., 2011). Chow et al. (1971) reported the higher stability of bacteriophage Xp12 in Tris buffer at a higher pH. The effect is more prominent at higher ionic strengths. However, the effect was studied only for the pH range 7 to 9. The

most damaging environmental factor for bacteriophage stability is UV radiation (Jones et al., 2007) and, as reported for other bacteriophages, BF25/12 was highly susceptible to UV radiation. However, our aim was to study bacteriophages as biocontrol agents of ornamental plants grown in greenhouses. Sunlight's UV radiation cannot penetrate the glass of a greenhouse; therefore, UV radiation does not represent a crucial limiting factor for bacteriophage survival (Jones et al., 2007).

The occurrence of resistant bacterial mutants represents a major concern when using bacteriophages as biocontrol agents (Jones et al., 2007). Here, the dependence between bacteriophage concentration and the development of bacterial resistance was optimal from 10^4 to 10^5 pfu/mL. These data indicate the importance of using suitable bacteriophage concentrations for bacteriophage treatments and the danger of using a single uniform type of bacteriophage isolate in any active treatment approaches. The active bacteriophage used in this treatment undergoes extensive reproduction above the threshold needed to kill the bacteria through a secondary infection (Payne & Jansen, 2000). The extensive enrichment of bacteriophage-resistant bacteria might occur if the initial bacteriophage concentration used in any active approach is below the observed optimum for resistance development. Accordingly, for *Dickeya* spp. B16 and BF25/12, this was below 10^4 pfu/mL. However, the emergence of resistant bacteria during bacteriophage biocontrol can be reduced with the use of bacteriophage cocktails instead of individual bacteriophages (Jones et al., 2007).

For the applicability of the bacteriophages defined in our study, the infection parameters (e.g. adsorption rate, one-step growth assay, burst size) should be determined to define the potential effectiveness of these bacteriophages as biocontrol agents. The combination of these isolated bacteriophages could in the future form an intriguing bacteriophage cocktail due to the combination of the 3 bacteriophage families with similar specificities. However, the biocontrol properties of the bacteriophages should be evaluated by pot or field experiment. Bacteriophage biocontrol is a dynamic process, which requires extensive knowledge of a specific bacteria-bacteriophage system and understanding pharmacokinetic principles of the system. The variable success of bacteriophage therapy and biocontrol is largely due to insufficient knowledge of bacteria-bacteriophage dynamics in a given host environment (Payne & Jansen, 2000). Therefore, we have developed 3 specific qPCR and ddPCR assays, each enabling quantitative phage detection without a DNA extraction step for tracking bacteriophage BF25/12 in *in-vivo* systems. Furthermore, bacteriophages can be used as well in combined biocontrol management strategies, as they enable the decrease of bacteria inoculum and therefore allow the higher efficiency of other substances used in bacterial disease control and treatment. The newly isolated bacteriophages and the described bacteriophage-host system represent a good research platform to study phage-bacterium interactions that could be used to further evaluate their applicability in *Dickeya* spp. biocontrol.

Chapter 5

Conclusions

This thesis presents a comprehensive characterisation of the new *Dickeya* isolates from the *Phalaenopsis* orchid with soft rot symptoms from a commercial production site. Results of the thesis provides initial insight into the specific *Dickeya* bacteriophages and bacteria - bacteriophage interactions.

Dickeya spp. was confirmed as a causative agent of the *Phalaenopsis* soft rot disease at commercial production sites. In the range of virulence and aggressiveness testing performed, new isolates indicated high virulence and maceration potential, compared to a reference set of *Dickeya* spp. Representative bacterial isolates also could persist and systematically colonise potato plants, which are not their original host. These results support indications of the possible introduction and spread of the *Dickeya* spp. from ornamental plants to crop plant species. Electron-dense ultrastructures resembling energy reserve granules and firstly described in SRE were observed in new *Dickeya* isolates. This phenomenon is opening a range of unexplored possibilities regarding *Dickeya* persistence and survival in the environment.

Whole genome information about the selected isolates, *Dickeya* sp. B16 and S1, enables the genetic end genomic positioning of the new isolates onto the *Dickeya* phylogenetic tree. Both isolates correspond to a group of *Dickeya* isolates from monocotyledonous plants and water. A combination of genetic, genomic and phenotype comparisons to the currently described *Dickeya* spp. placed these isolates in the newly described *D. fangzhongdai* species. Therefore, the classification of the *Dickeya* sp. S1, *Dickeya* sp. B16, *Dickeya* sp. MK7, *Dickeya* sp. NCPPB 3274, *Dickeya* solani ND14b, *D. solani* M005 and *D. chrysanthemi* M074 into the *D. fangzhongdai* species has been proposed. However, a phenotypic characterisation reveals variations among the tested isolates and a *D. fangzhongdai*-type strain. The correct bacterial classification is essential for the prediction and prevention of pathogen spread and the establishment of appropriate control measures, yet a variable species phenotype makes it difficult to classify all species members accurately. Current taxonomic practice should be revised in light of novel methods, techniques and knowledge to gain its full potential and usefulness.

Finally, CIM chromatography was successfully utilised to isolate viable bacteriophages from wastewater, demonstrating the applicability of this approach for routine bacteriophage isolation from water sources. Bacteriophages were isolated from different natural sources sampled multiple times through the year. Isolated bacteriophages were classified to 3 different *Caudovirales* families, including previously rarely isolated *Podoviridae* and *Syphoviridae*. The characterisation of a *Podoviridae* bacteriophage on the level of bacteria - bacteriophage interactions revealed the importance of understanding a given bacteria-bacteriophage

system for successful bacteriophage therapy or biocontrol applications. Therefore, specific molecular tools for bacteriophage tracking and quantification were developed that are useful for studies of the dynamics of a bacteriophage-host system.

The work of this doctoral thesis represents the first comprehensive description and characterisation of *Dickeya* isolates corresponding to the *D. fangzhongdai* species. It is also the first characterisation of a bacteriophage-host system for the genus *Dickeya*, the latter as a research tool providing adequate knowledge for successful bacteriophage biocontrol applications in the future.

Appendix A

Phylogenetic Study of *fliC* and *dnaX* Genes

A.1 Accession Numbers of Sequences of Strains Used in *fliC* and *dnaX* Phylogenetic Studies.

Table A.1: Accession numbers of sequences of strains used in *fliC* phylogenetic study. Reprinted from "Putative new species of the genus *Dickeya* as major soft rot pathogens in *Phalaenopsis* orchid production" by Š. Alič et al., 2017, *Plant Pathology*, 66. Copyright 2018 by the John Wiley and Sons.

Species	Strain	Accession no.	Species	Strain	Accession no.
<i>D. chrysanthemi</i>	LMG 2474	JN617655	<i>Dickeya</i> sp.	GBBC 2007	JN617651
	LMG 2804	JN617659		PD 1464	JN617649
	NCPFB 1861	JN617652		PD 1680	JN617639
<i>D. dadantii</i>	LMG 25991	JN617642		PD 814	JN617638
	NCPFB 3537	JN617641		PD 3786	JN617637
	CFBP 3855	JN617640		PD 471	JN617636
<i>D. zeae</i>	LMG 2497	JN617650		PD 1268	JN617635
	LMG 2539	JN617647		PD 487	JN617634
	LMG 2531	JN617646		GBBC 323	JN617633
	NCPFB 3531	JN617645		PD 550	JN617632
	LMG 2533	JN617643		LMG 2918	JN617631
<i>D. solani</i>	PRI 2222	JN617630	<i>Dickeya</i> sp. (novel isolates)	S1	KM979763
<i>D. dianthicola</i>	LMG 2485	JN617628		COB S4-1	KM979767
<i>Pectobacterium</i> sp.	GBBC 428	JN617653		COB S4-4	KM979770
				COB S4-6	KM979772
				COB S4-2	KM979768
				COB S4-5	KM979771
				COB S5-2	KM979774
				B16	KM979762
				COB S2	KM979764
				COB S3-1	KM979765
				COB S3-8	KM979766
				COB S4-3	KM979769
				COB S5-1	KM979773

Table A.2: Accession numbers of sequences of strains used in *dnaX* phylogenetic study. Reprinted from "Putative new species of the genus *Dickeya* as major soft rot pathogens in *Phalaenopsis* orchid production" by Š. Alič et al., 2017, *Plant Pathology*, 66. Copyright 2018 by the John Wiley and Sons.

Species	Strain	Accession no.	Species	Strain	Accession no.
<i>Dickeya</i> spp. (putative new species, previous studies)	SUPP2451	AB713594	<i>D. solani</i>	IPO 3336	KC844510
	SUPP40	AB713587		LMG 25990	KC844485
	SUPP1034	AB713589		IPO 2187	KC844499
	SUPP2736	AB713610		NCPPB 4575	KC844500
	SUPP2737	AB713611		IPO 2276	KC844501
	SUPP2735	AB713609		IPO 3239	KC844502
	SUPP2586	AB713599		IPO 3294	KC844503
	MAFF 311171	AB713590		IPO 3295	KC844504
	MAFF 311172	AB713583	<i>D. dadantii</i>	MAFF 301767	AB713584
	SUPP420	AB713602		MAFF 106634	AB713580
	SUPP1152	AB713604		NCPPB 2976	KC844493
	SUPP2738	AB713612		SUPP877	AB713603
	SUPP1164	AB713606		SUPP2162	AB713614
	SUPP1352	AB713608		SUPP2200	AB713601
	SUPP1399	AB713607		CFBP 1360	KC844492
	SUPP2739	AB713613		NCPPB 4424	KC844491
	SUPP1539	AB713595		NCPPB 898	KC844490
<i>Dickeya</i> spp. (this study)	S1	KM588693		IPO 3332	KC844509
	COB S4-1	KM588697	<i>D. dianthicola</i>	MAFF 302984	AB713576
	COB S4-4	KM588700		SUPP2525	AB713600
	COB S4-6	KM588702		MAFF 311149	AB713582
	COB S4-2	KM588698		SUPP215	AB713598
	COB S4-5	KM588701		SUPP2565	AB713615
	COB S5-2	KM588704		MAFF 311041	AB713577
	B16	KM588692		MAFF 311042	AB713591
	COB S2	KM588694		NCPPB 453	KC844486
	COB S3-1	KM588695	<i>D. zeae</i>	NCPPB 3345	KC844487
	COB S3-8	KM588696		IPO 3327	KC844505
	COB S4-3	KM588699		NCPPB 3534	KC844508
	COB S5-1	KM588703		IPO 980	KC844511
	COB S5-3	KM588705		NCPPB 2538	KC844496
<i>Dickeya</i> spp.	MAFF 301677	AB713575		NCPPB 2339	KC844497
	SR149	AB713618		CFBP 6466	KC844498
	SUPP1844	AB713585		NCPPB 3531	KC844506
<i>D. chrysanthemi</i>	SUPP20	AB713588		MAFF 311098	AB713581
	MAFF 311043	AB713579		SR 120 ^{s2}	AB713617
	MAFF 311151	AB713578		SUPP1158	AB713605
	MAFF 302132	AB713592		SR 171	AB713619
	NCPPB 402	KC844489		SR 261	AB713620
	NCPPB 1861	KC844488		SR 90 ^{s1}	AB713616
<i>E. tasmaniensis</i>	NCPPB 3533	KC844507		MAFF 106502	AB713593
	ET1/99	CU468135		SUPP 410	AB713596
<i>E. carotovora</i> subsp. <i>atroseptica</i>	SCRI1043	BX950851		SUPP739	AB713597

A.2 Phylogenetic Tree of Strains Used in *dnaX* Phylogenetic Study.

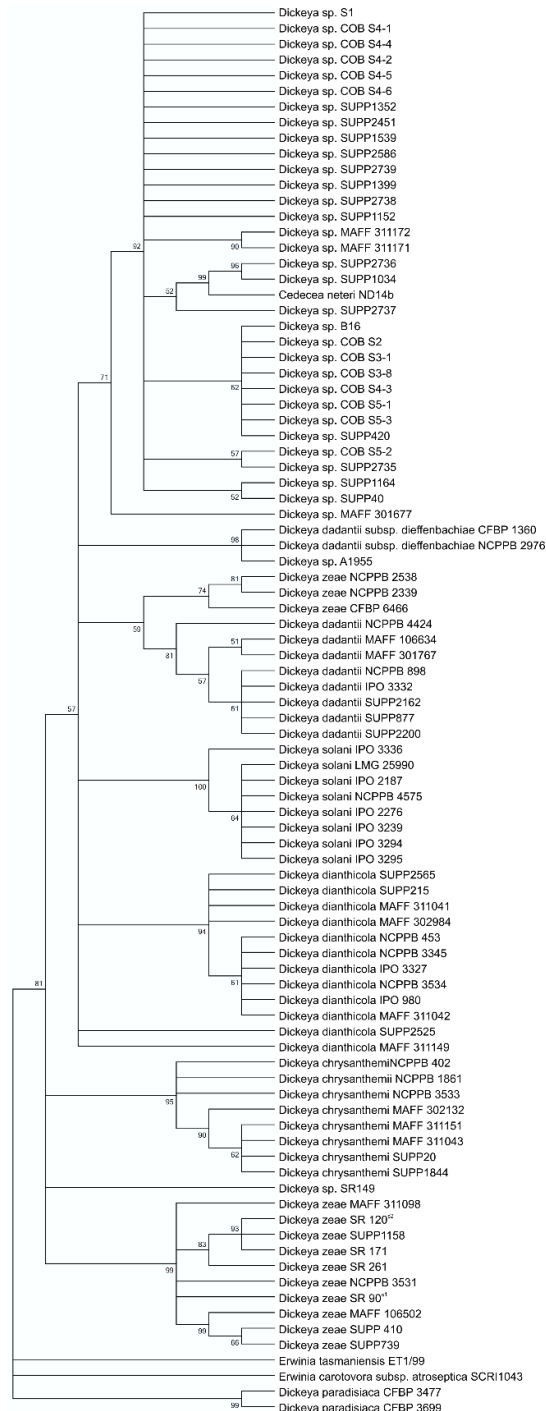


Figure A.1: Phylogenetic tree for *dnaX* sequence of 90 members of the genus *Dickeya* (Table A.2). The maximum likelihood method based on the Tamura three-parameter model was used for constructing the tree. The starting tree was created by Neighbour Joining and BioNJ algorithms. Bootstrap percentages were calculated with 500 replicates. Reprinted from "Putative new species of the genus *Dickeya* as major soft rot pathogens in *Phalaenopsis* orchid production" by Š. Alič et al., 2017, *Plant Pathology*, 66. Copyright 2018 by the John Wiley and Sons.

Appendix B

Molecular Characterisation of *Dickeya* spp.

B.1 Performance of Real-Time PCR Assays UDL-3, UDL-4

The real-time PCR assays developed for the specific detection of *Dickeya* sp. UDL-3 and UDL-4 were evaluated. The matrices tested include pure bacterial cultures (identification of isolates), plant extracts prepared from orchid leaves and orchid roots, and potato tubers. The diagnostic parameters defined hereafter are as described in the standard PM 7/98(2) of the European and Mediterranean Plant Protection Organization (EPPO; available from www.eppo.org). All tests were performed on 7900HT Fast Real-Time PCR, Applied Biosystems and ViiA 7, Life Technologies instruments. Primers and probes of the assays used and/or designed in this study are listed in [Table B.1](#) ~~Table B.1~~.

Table B.1: The primers used for bacterial characterisation through partial gene sequencing and primers and probes used in real-time PCR assays designed and evaluated in this study. Reprinted from "Putative new species of the genus *Dickeya* as major soft rot pathogens in *Phalaenopsis* orchid production" by Š. Alič et al., 2017, *Plant Pathology*, 66. Copyright 2018 by the John Wiley and Sons.

Assay	Target sequence	Name	Sequence (5'-3')	Published
<i>dnaX</i>	DNA polymerase III, tau and gamma subunit	<i>dnaXf</i>	TATCAGGTYCTTGCCCGTAAGTGG	Slawiak et al., 2009
		<i>dnaXr</i>	TCGACATCCARC GCYTTGAGATG	
<i>fliC</i>	flagellin gene	<i>fliC-1</i>	TATCAACAGCGCCAAAGACAACGC	Van Vaerenbergh, 2012
		<i>fliC-2</i>	ACGGCTCATGTTGGATACTTCGTT	
Ech		Ech-F	GAGTCAAAAGCGTCTTGCGAA	Brierley et al.; 2008
		Ech-R	CCCTGTTACCGCCGTGAA	
		Ech-P	FAM/CTGACAAGTGATGTCCCCTTCGTCTAGAGG/TAMRA	
COX	Plant cytochrome oxidase gene	COX-F	CGTCGCATTCCAGATTATCCA	Nikoleva et al.; 2012
		COX-RW	CAACTACGGATATATAAGRRCCRAACTG	
		COX-P	FAM/TGCTTACGCTGGATGGAATGCCCT/TAMRA	Brierley et al.; 2008

Continued on next page

Assay	Target sequence	Name	Sequence (5'-3')	Published
UDL-3	flagellin gene <i>fliC</i>	1-UDL3Taq-F	TGCCAGAACCAACCTGAATG	This study, evaluated
		1-UDL3Taq-R	CGAACTTCAACGGCAAAGC	
		1-UDL3Taq-P	FAM/AAGCCAGTAGCAGTAACAGTGCCGT CCA/BHQ1	
UDL-4	flagellin gene <i>fliC</i>	37-UDL4Taq-F	TGCTGCCAGCGCAAACCT	This study, evaluated
		37-UDL4Taq-R	GGCGCCAACTTGGATGTT	
		37-UDL4Taq-P	FAM/CAACGGCAAAGCGCTGCTGGA/BHQ1	

B.1.1 Analytical specificity

The analytical specificity of the assays was determined *in silico* and experimentally by analysing several different isolates of *Dickeya* and other selected species, as well as plant extracts.

For the experimental evaluation of the assays, bacteria were suspended in water (app. concentration of 10^6 cells/mL determined turbidimetrically), and the DNA were isolated by Chelex. Briefly, 1 mL of bacterial suspension was concentrated through centrifugation (5 min, 9.900 g) and mixed with 300 μ L of Chelex (6% w/V of aqueous suspension). The mixtures were incubated for 20 min at 56 °C, vortexed, incubated for 8 minutes at 99 °C, vortexed for 10 seconds, immediately cooled on ice and centrifuged for 7 minutes at 14.000 g. Supernatant containing DNA was analysed in real-time PCR. DNA was isolated from plant material using magnetic-beads-based DNA extraction (QuickPick™ SML Plant DNA, Bio-Nobile, Turku, Finland).

All *Dickeya* sp. isolates identified as *Dickeya* sp. and classified into UDL-3 or UDL-4 lineages based on *fliC* sequences were correctly identified. With UDL-3 and UDL-4 assays, no amplification was observed (no false positives) when amplifying other bacteria isolated from orchids with soft rot symptoms (8 isolates), isolates from asymptomatic leaves and roots of orchids (10 isolates), plant extracts from asymptomatic orchid leaves (10 extracts), selected other *Dickeya*, *Pectobacterium* and *Pantoea* spp. (9 isolates), isolates from potato tubers with soft rot symptoms (10 isolates) and healthy orchids (10 isolates), isolates from potato tubers with soft rot symptoms (10 isolates) and plant extracts of potato tubers with soft rot (3 extracts). Overall, UDL-3 and UDL-4 assays had 100% analytical specificity ([Figure B.2](#)[Figure B.1](#)).

B.1.2 General performance and analytical sensitivity

The UDL-3 and UDL-4 assays were able to detect down to 10^4 CFU/mL when analysing DNA extracted from bacterial suspensions using magnetic-beads-based DNA extraction (QuickPick™ SML Plant DNA, Bio-nobile, Turku, Finland).

UDL-3		sanitary status		
		pos	neg	total
Result of technique	pos	right 11	false positive 0	11
	neg	false negative 0	right 49	49
	total	11	49	60

prevalence	0,18
sensitivity	1,00
specificity	1,00
false positive rate	0,00
false negative rate	0,00
positive predictive value	1,00
negative predictive value	1,00
positive likelihood ratio	NA
negative likelihood ratio	0,00

UDL-4		sanitary status		
		pos	neg	total
Result of technique	pos	right 4	false positive 0	4
	neg	false negative 0	right 56	56
	total	4	56	60

prevalence	0,07
sensitivity	1,00
specificity	1,00
false positive rate	0,00
false negative rate	0,00
positive predictive value	1,00
negative predictive value	1,00
positive likelihood ratio	NA
negative likelihood ratio	0,00

Figure B.2: Selected diagnostic parameters of real-time PCR assays designed to specifically detect *Dickeya* sp. UDL-3 and UDL-4. Adapted from "Putative new species of the genus *Dickeya* as major soft rot pathogens in *Phalaenopsis* orchid production" by Š. Alič et al., 2017, *Plant Pathology*, 66. Copyright 2018 by the John Wiley and Sons.

Figure C.1 MLSA tree of the *recA* sequence (367 bp) comprising 86 members of the genus *Dickeya*, including Japanese isolates (Suharjo et al. 2014) and *Dickeya* strains classified in DUC clades (Parkinson et al. 2009). Strains written in bold phylogenetically correspond to the *D. fangzhongdhai* species. The maximum-likelihood method and generalised time-reversible model were used to construct the tree. The starting tree was created with the BioNJ algorithm. Bootstrap percentages were calculated with 1,000 replicates. *Pectobacterium atrosepticum* 21A was used as an outgroup to root the phylogenetic tree.

C.2 ANI Analysis of the Genus *Dickeya*

Table C.1: Pairwise ANI values for the genomes of the genus *Dickeya* available in the NCBI database. Bold type indicates ANI values >95%, above the threshold for assigning strains to separate species.

	Dickeya fangzhongdai J55 [†]	Dickeya sp. B16	Dickeya sp. MK7	Dickeya sp. S1	Dickeya solani ND14b	Dickeya chrysanthemii IM074	Dickeya solani MO05	Dickeya chrysanthemii NCPPB 3274	Dickeya solani IBF 0099	Dickeya solani MK10	Dickeya solani GBBC 2040	Dickeya solani D-0432-1	Dickeya solani RNS 08.23.3.1.A	Dickeya solani MK16	Dickeya solani IPO 2222 [‡]	Dickeya solani RNS 07.7.3.B	Dickeya solani IPO 9134	Dickeya solani strain RNS 05.05.1.2A	Dickeya dadantii subsp. diffeinbachiae NCPPB 3976 [†]	Dickeya dadantii 3937	Dickeya dadantii NCPPB 3537	Dickeya dadantii subsp. dadantii NCPPB 898 [†]	Dickeya dianthicola GBBC 2039	Dickeya dianthicola RNS 04.9	Dickeya dianthicola NCPPB 453 [†]	Dickeya dianthicola NCPPB 3534	Dickeya dianthicola IPO 980	Dickeya sp. 2812	Dickeya chrysanthemii NCPPB 402 [†]	Dickeya chrysanthemii NCPPB 3533	Dickeya chrysanthemii L11	Dickeya chrysanthemii NCPPB 516	Dickeya sp. NCPPB 569	Dickeya zeae DZQ	Dickeya zeae EC1	Dickeya zeae ZJ102	Dickeya zeae CSL RW192	Dickeya zeae NCPPB 3531	Dickeya zeae EcH 586	Dickeya zeae MS1	Dickeya zeae NCPPB 3532	Dickeya zeae NCPPB 2538 [‡]	Dickeya zeae MK19	Dickeya paradisiaca NCPPB 2511 [†]	Dickeya aquatica DW 0440	Dickeya sp. CSL RW240	
Dickeya fangzhongdai J55 [†]	---	99.08	99.07	99.11	97.04	97.11	97.12	96.30	92.69	92.66	92.67	92.65	92.65	92.64	92.64	92.63	92.61	92.59	92.62	92.53	92.50	92.42	92.26	92.21	92.15	92.14	92.14	91.78	87.77	87.72	87.65	87.67	87.32	86.41	86.45	86.36	86.35	86.34	86.47	86.38	86.41	86.37	86.33	85.01	84.65	84.63	
Dickeya sp. B16	99.06	---	99.88	99.13	97.14	97.16	97.11	96.28	92.66	92.66	92.66	92.64	92.64	92.64	92.64	92.64	92.64	92.59	92.59	92.52	92.48	92.46	92.18	92.16	92.14	92.11	92.10	91.76	87.77	87.72	87.66	87.63	87.30	86.38	86.41	86.34	86.31	86.30	86.30	86.43	86.37	86.34	86.30	85.04	84.70	84.68	
Dickeya sp. MK7	99.07	99.91	---	99.14	97.15	97.18	97.12	96.29	92.68	92.67	92.68	92.65	92.65	92.66	92.66	92.66	92.66	92.61	92.61	92.50	92.49	92.46	92.19	92.16	92.15	92.13	92.12	91.76	87.78	87.73	87.66	87.65	87.30	86.34	86.35	86.33	86.30	86.28	86.39	86.35	86.33	86.31	86.28	85.03	84.69	84.67	
Dickeya sp. S1	99.08	99.12	99.11	---	97.08	97.10	97.04	96.29	92.69	92.68	92.69	92.67	92.68	92.67	92.67	92.67	92.66	92.60	92.61	92.54	92.50	92.47	92.19	92.17	92.15	92.14	92.14	91.78	87.82	87.74	87.76	87.71	87.28	86.47	86.51	86.40	86.37	86.33	86.46	86.42	86.33	86.31	86.31	84.94	84.71	84.68	
Dickeya solani ND14b	97.06	97.15	97.15	97.11	---	99.96	98.81	96.57	92.74	92.71	92.73	92.71	92.72	92.70	92.70	92.70	92.70	92.70	92.64	92.63	92.61	92.51	92.47	92.27	92.27	92.22	92.22	92.18	92.05	87.74	87.66	87.69	87.66	87.29	86.48	86.55	86.40	86.36	86.28	86.49	86.36	86.38	86.32	86.33	84.84	84.68	84.66
Dickeya chrysanthemii M074	97.07	97.14	97.14	97.09	99.92	---	98.81	96.57	92.74	92.72	92.73	92.70	92.70	92.70	92.71	92.71	92.71	92.71	92.65	92.64	92.56	92.53	92.48	92.27	92.23	92.22	92.22	92.19	92.02	87.78	87.68	87.70	87.67	87.31	86.43	86.41	86.38	86.40	86.32	86.45	86.39	86.38	86.34	86.35	84.87	84.70	84.68
Dickeya solani MO05	97.07	97.10	97.09	97.04	98.77	98.81	---	96.52	92.74	92.72	92.74	92.71	92.70	92.71	92.71	92.71	92.71	92.71	92.65	92.61	92.53	92.53	92.48	92.27	92.19	92.18	92.19	92.15	92.00	87.80	87.71	87.71	87.69	87.31	86.37	86.41	86.37	86.39	86.31	86.45	86.40	86.38	86.37	86.35	84.85	84.77	84.75
Dickeya chrysanthemii NCPPB 3274	96.30	96.29	96.29	96.30	96.57	96.59	96.54	---	92.53	92.52	92.53	92.51	92.50	92.51	92.51	92.51	92.51	92.48	92.56	92.49	92.49	92.42	92.14	92.06	92.05	92.06	92.01	91.66	88.02	87.74	87.88	87.72	87.42	86.46	86.52	86.49	86.48	86.42	86.59	86.49	86.36	86.31	86.37	84.86	84.77	84.81	
Dickeya solani IBF 0099	92.77	92.71	92.68	92.74	92.95	92.81	92.79	92.51	---	99.92	99.93	99.92	99.91	99.93	99.93	99.88	99.85	98.72	94.11	94.29	94.12	94.02	92.33	92.37	92.21	92.15	92.16	89.69	87.67	87.70	87.79	87.57	87.19	86.69	86.84	86.39	86.21	86.23	86.64	86.51	86.42	86.44	86.28	84.75	84.88	84.89	
Dickeya solani MK10	92.65	92.66	92.67	92.68	92.71	92.74	92.74	92.52	99.95	---	99.96	99.96	99.96	99.96	99.96	99.94	99.90	98.76	94.12	94.10	94.12	94.02	92.27	92.24	92.22	92.22	92.18	92.17	89.59	87.70	87.70	87.66	87.53	87.15	86.31	86.33	86.28	86.19	86.17	86.35	86.34	86.24	86.27	86.21	84.70	84.53	84.51
Dickeya solani GBBC 2040	92.66	92.66	92.68	92.70	92.72	92.75	92.75	92.53	99.95	99.96	---	99.96	99.96	99.96	99.96	99.97	99.95	99.91	98.76	94.15	94.12	94.14	94.04	92.27	92.24	92.24	92.20	92.21	89.61	87.73	87.72	87.69	87.57	87.16	86.33	86.36	86.32	86.23	86.20	86.37	86.36	86.25	86.29	86.23	84.73	84.55	84.54
Dickeya solani D-0432-1	92.65	92.64	92.65	92.68	92.71	92.72	92.72	92.51	99.95	99.96	99.96	---	99.94	99.97	99.97	99.94	99.91	98.73	94.11	94.12	94.12	94.02	92.26	92.25	92.21	92.17	92.17	89.59	87.70	87.70	87.67	87.55	87.16	86.33	86.39	86.29	86.20	86.18	86.38	86.34	86.24	86.27	86.22	84.74	84.58	84.53	
Dickeya solani RNS 08.23.3.1.A	92.65	92.64	92.65	92.68	92.72	92.72	92.72	92.51	99.93	99.96	99.96	99.95	---	99.96	99.96	99.94	99.91	98.74	94.12	94.12	94.12	94.03	92.26	92.25	92.21	92.17	92.17	89.59	87.71	87.70	87.68	87.56	87.15	86.32	86.38	86.29	86.20	86.18	86.39	86.35	86.23	86.27	86.22	84.73	84.55	84.54	
Dickeya solani MK16	92.64	92.64	92.65	92.67	92.70	92.73	92.72	92.51	99.95	99.96	99.96	99.97	99.96	---	99.97	99.95	99.91	98.77	94.12	94.11	94.12	94.03	92.25	92.21	92.20	92.16	92.16	89.58	87.70	87.70	87.66	87.54	87.15	86.32	86.34	86.29	86.20	86.17	86.35	86.35	86.23	86.27	86.22	84.74	84.54	84.52	
Dickeya solani IPO 2222 [‡]	92.63	92.64	92.66	92.68	92.70	92.73	92.73	92.51	99.96	99.96	99.97	99.97	99.96	99.97	---	99.95	99.91	98.76	94.12	94.10	94.12	94.03	92.26	92.21	92.21	92.17	92.18	89.58	87.72	87.71	87.67	87.55	87.15	86.31	86.34	86.30	86.21	86.18	86.35	86.34	86.23	86.27	86.21	84.72	84.53	84.51	
Dickeya solani RNS 07.7.3.B	92.61	92.63	92.64	92.66	92.68	92.71	92.71	92.49	99.88	99.91	99.92	99.91	99.91	99.92	99.93	---	99.85	98.76	94.10	94.08	94.11	94.02	92.24	92.20	92.20	92.15	92.15	89.57	87.71	87.70	87.67	87.55	87.15	86.31	86.34	86.28	86.20	86.17	86.36	86.35	86.23	86.27	86.22	84.73	84.53	84.52	
Dickeya solani IPO 9134	92.61	92.62	92.63	92.66	92.67	92.71	92.70	92.49	99.85	99.88	99.88	99.88	99.89	99.89	99.85	---	98.67	94.08	94.05	94.09	93.99	92.29	92.28	92.27	92.24	92.24	92.24	89.57	87.70	87.70	87.66	87.55	87.16	86.31	86.35	86.29	86.21	86.17	86.36	86.35	86.24	86.27	86.22	84.73	84.52	84.51	
Dickeya solani strain RNS 05.1.2A	92.57	92.58	92.60	92.59	92.63	92.66	92.66	92.47	98.72	98.75	98.75	98.72	98.73	98.75	98.74	98.77	---	98.67	94.08	94.11	94.02	92.27	92.23	92.22	92.22	92.19	92.19	89.53	87.66	87.69	87.61	87.55	87.14	86.28	86.32	86.27	86.17	86.15	86.37	86.33	86.25	86.27	86.22	84.77	84.57	84.57	
Dickeya dadantii subsp. diffeinbachiae NCPPB 2976 [†]	92.61	92.59	92.61	92.61	92.63	92.65	92.62	92.56	94.14	94.12	94.15	94.11	94.11	94.12	94.11	94.09	94.13	---	96.75	96.81	96.66	92.19	92.16	92.16	92.12	92.12	92.19	89.59	88.04	88.02	88.04	88.03	87.93	87.31	86.38	86.48	86.46	86.44	86.35	85.99	86.60	86.38	86.40	86.50	84.89	84.88	84.92
Dickeya dadantii 3937	92.52	92.51	92.49	92.54	92.61	92.57	92.54	92.48	94.13	94.10	94.11	94.13	94.12	94.11	94.10	94.09	94.06	94.08	96.75	---	98.46	98.30	92.20	92.19	92.12	92.11	92.07	89.65	88.19	88.19	88.23	88.04	87.28	86.48	86.47	86.36	86.39	86.33	86.78	86.61	86.53	86.53	86.45	84.82	84.84	84.78	
Dickeya dadantii NCPPB 3537	92.49	92.49	92.48	92.51	92.50	92.53	92.54	92.48	94.13	94.11	94.14	94.12	94.12	94.11	94.12	94.09	94.12	94.12	96.82	98.46	---	98.47	92.19	92.13	92.12	92.14	92.09	89.58	87.96	88.01	87.85	87.87	87.23	86.34	86.36	86.33	86.33	86.31	86.77	86.49	86.39	86.36	86.43	84.73	84.63	84.61	
Dickeya dadantii subsp. dadantii NCPPB 898 [†]	92.41	92.45	92.45	92.47	92.46	92.49	92.52	92.41	94.04	94.02	94.04	94.02	94.03	94.03	94.03	94.03	94.03	94.03	96.66	98.33	98.47	---	92.17	92.11	92.11	92.12	92.10	89.64	88.03	88.00	87.89	87.87	87.20	86.24	86.31	86.28	86.38	86.25	86.43	86.50	86.35	86.43	86.40	86.40	84.74	84.66	84.64
Dickeya dianthicola GBBC 2039	92.25	92.17	92.17	92.24	92.26	92.22	92.13	92.26	92.27	92.26	92.25	92.25	92.25	92.25	92.25	92.29	92.27	92.19	92.20	92.19	92.17	---	99.39	99.38	99.38	99.35	99.25	89.27	88.10	88.11	87.97	87.98	87.28	86.59	86.58	86.55	86.56	86.48	86.59	86.49	86.48	84.99	84.85	84.96	84.94		
Dickeya dianthicola RNS 04.9	92.20	92.16	92.14	92.16	92.25	92.22	92.18	92.05	92.25	92.22	92.24	92.25	92.24	92.21	92.20	92.28	92.23	92.16	92.18	92.13	92.11	99.39	---	99.96	99.41	99.51	89.22	88.08	88.05	87.89	87.87	87.96	87.28	86.59	86.73	86.61	86.56	86.48	86.62	86.51	86.46	86.45	86.43	84.88	84.85	84.84	
Dickeya dianthicola NCPPB 453 [†]	92.13	92.14	92.14	92.14	92.19	92.21	92.17	9																																							

C.3 Bacterial Strains Used in the MLSA Study of Japanese *Dickeya* Isolates

Table C.2: GenBank accession numbers of the Japanese isolates (Suharjo et al., 2014) and DUC isolates (Parkinson et al., 2009) used for MLSA analysis.

Strain	GenBank Accession Number	Host	Year of isolation	Country of isolation
<i>recA</i>				
SUPP2451	AB713677	Welsh onion	2004	Japan
SUPP40	AB713675	Welsh onion	1982	Japan
SUPP1034	AB713673	<i>Phalaenopsis</i> sp.	1988	Japan
SUPP2736	AB713685	<i>Phalaenopsis</i> sp.	1988	Japan
SUPP2737	AB713686	<i>Phalaenopsis</i> sp.	1989	Japan
SUPP2735	AB713684	<i>Phalaenopsis</i> sp.	1988	Japan
SUPP2586	AB713678	Taro	2004	Japan
MAFF 311171	AB713706	Taro	1998	Japan
MAFF 311172	AB713668	Taro	1998	Japan
SUPP420	AB713707	Bush lily	1985	Japan
SUPP1152	AB713697	<i>Oncidium</i> sp.	1989	Japan
SUPP2738	AB713687	<i>Oncidium</i> sp.	1990	Japan
SUPP1164	AB713698	<i>Vanda</i> sp.	1989	Japan
SUPP1352	AB713682	<i>Dracaena</i> sp.	1990	Japan
SUPP1399	AB713695	<i>Cattleya</i> sp.	1990	Japan
SUPP1539	AB713696	Iris	1992	Japan
MAFF 301677	AB713663	<i>Solanum</i> <i>tuberosum</i>	1977	Japan
PD 813	FJ217022	<i>Phalaenopsis</i> sp.	1987	Netherlands
PD 1750	FJ217067	<i>Yuca</i> sp.	1990	Netherlands
20714261	FJ216975	<i>Phalaenopsis</i> sp.	2007	Taiwan
NCPPB 2915	FJ217098	<i>Musa</i> sp.	1977	Panama
20711883	FJ216974	<i>Solanum</i> <i>tuberosum</i>	2007	Israel
G-121	FJ216976	<i>Solanum</i> <i>tuberosum</i>	2007	Israel
20710970	FJ217004	<i>Solanum</i> <i>tuberosum</i>	2007	Netherlands
G-115	FJ217007	<i>Solanum</i> <i>tuberosum</i>	2007	Israel
G-118	FJ217008	<i>Solanum</i> <i>tuberosum</i>	2007	Israel
G-120	FJ217009	<i>Solanum</i> <i>tuberosum</i>	2007	Israel
G-122	FJ217010	<i>Solanum</i> <i>tuberosum</i>	2007	Israel
G-169	FJ217011	<i>Solanum</i> <i>tuberosum</i>	2007	Israel

G-87	FJ217012	<i>Solanum tuberosum</i>	2007	Israel
Imp1	FJ217013	<i>Solanum tuberosum</i>	2007	Israel
Imp23.1	FJ217014	<i>Solanum tuberosum</i>	2007	Israel
Imp24	FJ217015	<i>Solanum tuberosum</i>	2007	Israel
Pc26.4	FJ217073	<i>Solanum tuberosum</i>	2007	Israel
Pc3	FJ217074	<i>Solanum tuberosum</i>	2007	Israel
Pc36.1	FJ217075	<i>Solanum tuberosum</i>	2007	Israel
Pc41.1	FJ217076	<i>Solanum tuberosum</i>	2007	Israel
NCPPB 3306	FJ217153	<i>Polyscias filicifolia</i>	1984	UK

Appendix D

MALDI – TOF Analysis of the *D. fangzhongdai* Strains

Peptide mass fingerprint product ion spectra were generated using a Microflex LT Biotyper operating system (Bruker Daltonics, Bremen, Germany). The data were analyzed in the automatic mode using the Bruker Biotyper 3.0 software and the spectra comparison libraries provided by Bruker. *D. fangzhongdai* isolates were compared against each other and *Dickeya* strains included in the spectra library.

Generated main spectrum profile (MSP, [Error! Reference source not found.Figure D.1](#)) were further used to perform a hierarchical clustering of *Dickeya* strains using the MSP dendrogram tool of the MALDI Biotyper software. The resulting score-oriented dendrogram showed clustering of the *D. fangzhongdai* strains separately from other *Dickeya* spp. ([Error! Reference source not found.Figure D.2](#)). However, some unassigned *Dickeya* isolates obtained in National Institute Bacteria Collection, that were previously added to the MSP library, were also placed to *D. fangzhongdai* cluster.

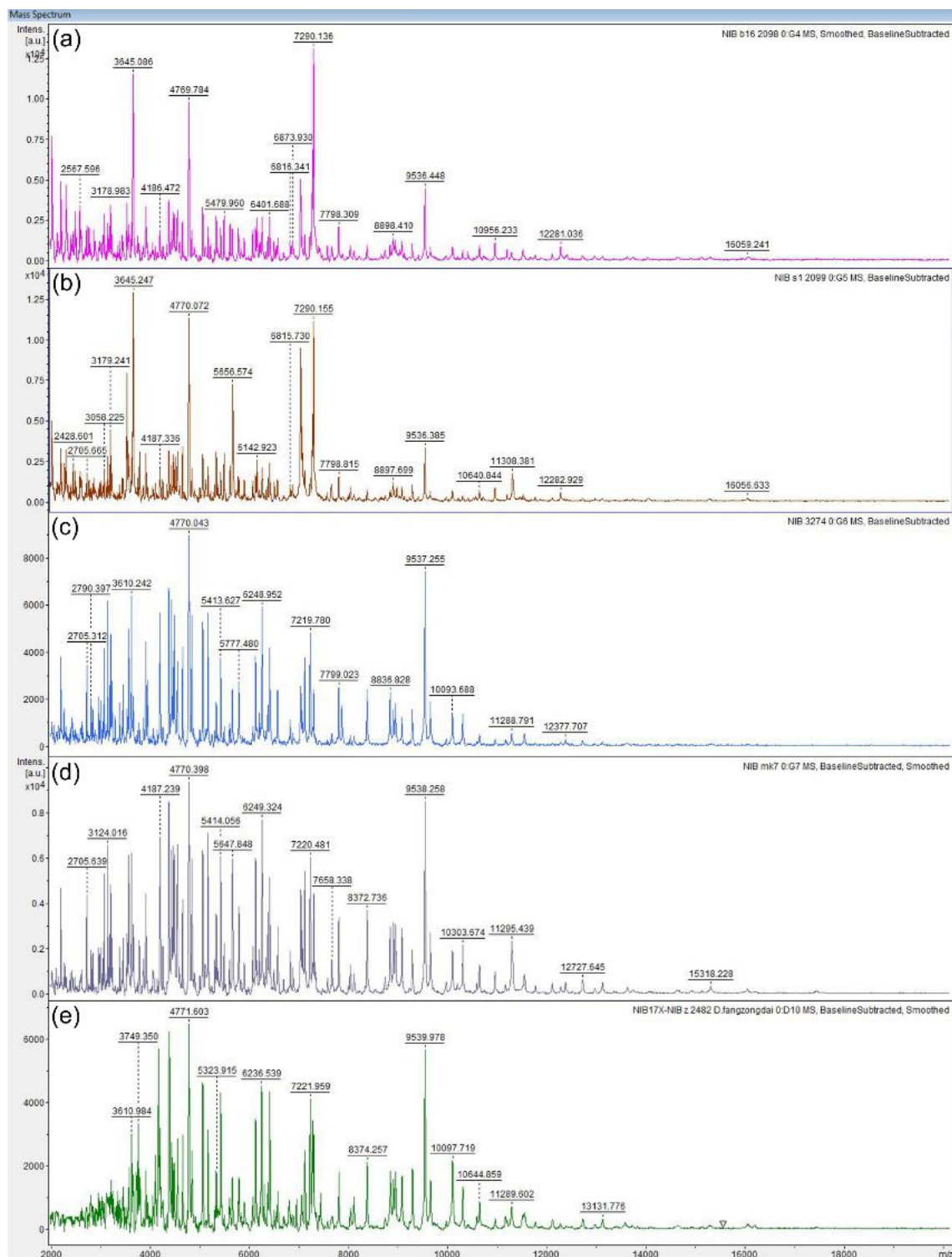


Figure D.1: Peptide mass fingerprint product ion spectra of *D. fangzhongdai* B16 (a), S1 (b), NCPB 3274 (c), MK7 (d) and JS5^T (e). MSP profiles were automatically calculated from mass spectra by Biotyper 3.0 software.

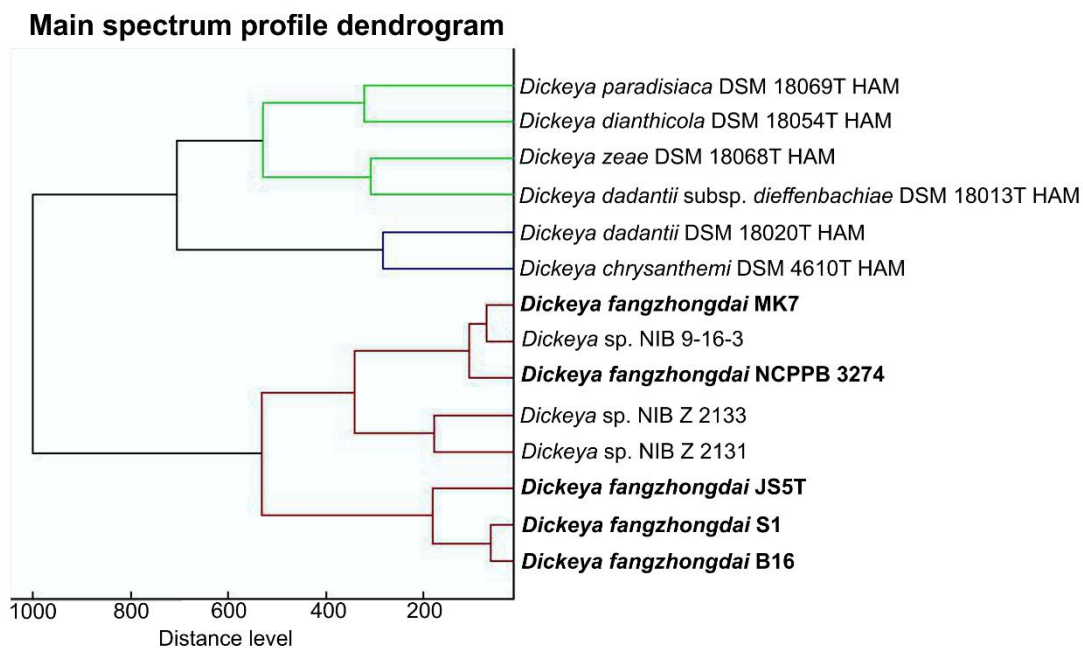


Figure D.2: Clustering of *Dickeya* strains as a function of their MSP. *D.fangzhongdai* strains are shown in bold in the MSP dendrogram. The MSP dendrogram shows clustering of *D. fangzhongdai* isolates separately from other *Dickeya* spp. However, some unclassified *Dickeya* strains were similar to *D. fangzhongdai* on the peptide mass fingerprint level.

References

- Adriaenssens, E. M., Van Vaerenbergh, J., Vandenheuvel, D., Dunon, V., Ceyssens, P. J., de Proft, M., ... Lavigne, R. (2012). T4-related bacteriophage LIMEstone isolates for the control of soft rot on potato caused by "*Dickeya solani*." *PLoS ONE*, 7(3). <https://doi.org/10.1371/journal.pone.0033227>
- Alič, Š., Naglič, T., Llop, P., Toplak, N., Koren, S., Ravnikar, M., & Dreo, T. (2015). Draft genome sequences of *Dickeya* sp. isolates B16 (NIB Z 2098) and S1 (NIB Z 2099) causing soft rot of *Phalaenopsis* orchids. *Genome Announcements*, 3(5). doi.org/10.1128/genomeA.00973-15
- Alič, Š., Naglič, T., Tušek-Žnidarič, M., Ravnikar, M., Rački, N., Peterka, M., & Dreo, T. (2017a). Newly isolated bacteriophages from the *Podoviridae*, *Siphoviridae*, and *Myoviridae* families have variable effects on putative novel *Dickeya* spp. *Frontiers in Microbiology*, 8, 1-14. doi.org/10.3389/fmicb.2017.01870
- Alič, Š., Naglič, T., Tušek-Žnidarič, M., Peterka, M., Ravnikar, M., & Dreo, T. (2017b). Putative new species of the genus *Dickeya* as major soft rot pathogens in *Phalaenopsis* orchid production. *Plant Pathology*, 66(8), 1357-1368. doi.org/10.1111/ppa.12677
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, 215(3), 403-410. [doi.org/10.1016/S0022-2836\(05\)80360-2](https://doi.org/10.1016/S0022-2836(05)80360-2)
- Aziz, R. K., Bartels, D., Best, A. A., DeJongh, M., Disz, T., Edwards, R. A., ... Zagnitko, O. (2008). The RAST server: Rapid annotations using subsystems technology. *BMC Genomics*, 9(1), 75. doi.org/10.1186/1471-2164-9-75
- Baltrus, D. A. (2016). Divorcing strain classification from species names. *Trends in Microbiology*, 24(6), 431-439. doi.org/10.1016/j.tim.2016.02.004
- Bao, Y., Chetvernin, V., & Tatusova, T. (2014). Improvements to pairwise sequence comparison (PASC): A genome-based web tool for virus classification. *Archives of Virology*, 159(12), 3293-304. doi.org/10.1007/s00705-014-2197-x
- Blanchard, M. G., & Runkle, E. S. (2006). Temperature during the day, but not during the night, controls flowering of *Phalaenopsis* orchids. *Journal of Experimental Botany*, 57(15), 4043-4049. doi.org/10.1093/jxb/erl176
- Bradley, D. E. (1965). The morphology and physiology of bacteriophages as revealed by the electron microscope. *Journal of Microscopy*, 84(3), 257-316. doi.org/10.1111/J.1365-2818.1965.TB02129.X
- Brady, C., Cleenwerck, I., Venter, S., Coutinho, T., & De Vos, P.

- (2013). Taxonomic evaluation of the genus *Enterobacter* based on multilocus sequence analysis (MLSA). *Systematic and Applied Microbiology*, 36(5), 309-319. <https://doi.org/10.1016/j.syapm.2013.03.005>
- Brady, C., Cleenwerck, I., Venter, S., Vancanneyt, M., Swings, J., & Coutinho, T. (2008). Phylogeny and identification of *Pantoea* species associated with plants, humans and the natural environment based on multilocus sequence analysis (MLSA). *Systematic and Applied Microbiology*, 31(6-8), 447-460. doi.org/10.1016/j.syapm.2008.09.004
- Brady, C. L., Cleenwerck, I., Venter, S. N., Engelbeen, K., De Vos, P., & Coutinho, T. A. (2010). Emended description of the genus *Pantoea*, description of four species from human clinical samples, *Pantoea septica* sp. nov., *Pantoea eucrina* sp. nov., *Pantoea brenneri* sp. nov. and *Pantoea conspicua* sp. nov., and transfer of *Pectobacterium cyprapedii* (Hori 1911) Brenner et al. 1973 emend. Hauben et al. 1998 to the genus as *Pantoea cyprapedii* comb. nov. *International Journal of Systematic and Evolutionary Microbiology*, 60(10), 2430-2440. <https://doi.org/10.1099/ijs.0.017301-0>
- Brettin, T., Davis, J. J., Disz, T., Edwards, R. A., Gerdes, S., Olsen, G. J., ... Xia, F. (2015). RASTtk: A modular and extensible implementation of the RAST algorithm for building custom annotation pipelines and annotating batches of genomes. *Scientific Reports*, 5, 8365. <https://doi.org/10.1038/srep08365>
- Brierley, J., Lees, A., Hilton, A., Wale, S., Peters, J., Elphinstone, J., & Boonham, N. (2008). *Improving decision making for the management of potato diseases using real-time diagnostics*.
- Burnett, F. M., & Stanley, W. M. (1960). *The viruses. Volume 1, General virology: Biochemical, biological, and biophysical properties*. Cambridge, MA: Academic Press.
- Buttimer, C., McAuliffe, O., Ross, R. P., Hill, C., O'Mahony, J., & Coffey, A. (2017). Bacteriophages and bacterial plant diseases. *Frontiers in Microbiology*, 8, 1-15. doi.org/10.3389/fmicb.2017.00034
- Cating, R. A., Hong, J. C., Palmateer, A. J., Stiles, C. M., & Dickstein, E. R. (2008). First report of bacterial soft rot on *Vanda* orchids caused by *Dickeya chrysanthemi* (*Erwinia chrysanthemi*) in the United States. *Plant Disease*, 92(6), 977-977. doi.org/10.1094/PDIS-92-6-0977A
- Cating, R. A., & Palmateer, A. J. (2011). Bacterial soft rot of *Oncidium* orchids caused by a *Dickeya* sp. (*Pectobacterium chrysanthemi*) in Florida. *Plant Disease*, 95(1), 74-74. doi.org/10.1094/PDIS-07-10-0523
- Cating, R. A., Palmateer, A. J., McMillan, R. T., & Dickstein, E. R. (2009). First report of a bacterial soft rot on *Tolumnia* orchids caused by a *Dickeya* sp. in the United States. *Plant Disease*, 93(12), 1354-1354. doi.org/10.1094/PDIS-93-12-1354B
- Charkowski, A., Blanco, C., Condemine, G., Expert, D., Franza, T., Hayes, C., ... Yedidia, I. (2012). The role of secretion systems and small molecules in soft-rot *Enterobacteriaceae* pathogenicity. *Annual Review of Phytopathology*, 50(1), 425-449. doi.org/10.1146/annurev-phyto-081211-173013

- Cheng, K. J., Hironaka, R., Roberts, D. W., & Costerton, J. W. (1973). Cytoplasmic glycogen inclusions in cells of anaerobic gram-negative rumen bacteria. *Canadian Journal of Microbiology*, 19(12), 1501-6. Retrieved from www.ncbi.nlm.nih.gov/pubmed/4131088
- Cho, Y. H., & Yoo, S. D. (2011). Signaling role of fructose mediated by FINS1/FBP in *Arabidopsis thaliana*. *PLoS Genetics*, 7(1), 1-10. doi.org/10.1371/journal.pgen.1001263
- Chow, T.-Y., Lin, Y.-T., & Kuo, T.-T. (1971). Stability of phage Xp12. *Botanical Bulletin of Academia Sinica*, 12, 57-65.
- Chung, M.-Y., Oh, D.-K., & Lee, K. W. (2012). Hypoglycemic health benefits of D-Psicose. *Journal of Agricultural and Food Chemistry*, 60(4), 863-869. doi.org/10.1021/jf204050w
- Coenye, T., Falsent, E., Hostef, B., Govant, J. R., Kersters, K., & Vandamme, P. (1999). Classification of *Alcaligenes faecalis*-like isolates from the environment and human clinical samples as *Ralstonia gilardii* sp. nov. *International Journal of Systematic Bacteriology*, 49, 405-41.
- Czajkowski, R. (2016). Bacteriophages of soft rot *Enterobacteriaceae*--A minireview. *FEMS Microbiology Letters*, 363(2), fnv230. doi.org/10.1093/femsle/fnv230
- Czajkowski, R., De Boer, W. J., Velvis, H., & Van Der Wolf, J. M. (2009). Systemic colonization of potato plants by a soilborne, green fluorescent protein-tagged strain of *Dickeya* sp. Biovar 3. *Phytopathology*, 100(2), 134-42. doi: 10.1094/PHYTO-100-2-0134
- Czajkowski, R., Ozymko, Z., De Jager, V., Siwinska, J., Smolarska, A., Ossowicki, A., ... Lojkowska, E. (2015). Genomic, proteomic and morphological characterization of two novel broad host lytic bacteriophages Φ PD10.3 and Φ PD23.1 infecting pectinolytic *Pectobacterium* spp. and *Dickeya* spp. *PLoS ONE*, 10(3). doi.org/10.1371/journal.pone.0119812
- Czajkowski, R., Ozymko, Z., & Lojkowska, E. (2013). Isolation and characterization of novel soilborne lytic bacteriophages infecting *Dickeya* spp. biovar 3 ('*D. solani*'). *Plant Pathology*, 63(4), 758-772. doi.org/10.1111/ppa.12157
- Czajkowski, R., Ozymko, Z., Zwirowski, S., & Lojkowska, E. (2014). Complete genome sequence of a broad-host-range lytic *Dickeya* spp. bacteriophage Φ D5. *Archives of Virology*, 159(11), 3153-3155. doi.org/10.1007/s00705-014-2170-8
- Czajkowski, R., Pérombelon, M. C. M., Jafra, S., Lojkowska, E., Potrykus, M., Van Der Wolf, J. M., & 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(1), 18-38. doi.org/10.1111/aab.12166
- Davidsson, P. R., Kariola, T., Niemi, O., & Palva, E. T. (2013). Pathogenicity of and plant immunity to soft rot pectobacteria. *Frontiers in Plant Science*, 4, 1-13. doi.org/10.3389/fpls.2013.00191
- Day, A., Ahn, J., Fang, X., & Salmond, G. P. C. (2017). Environmental bacteriophages of the emerging enterobacterial phytopathogen, *Dickeya solani*, show genomic conservation and capacity for horizontal gene transfer between their bacterial

- hosts. *Frontiers in Microbiology*, 8(AUG), 1-9. doi.org/10.3389/fmicb.2017.01654
- DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., ... Andersen, G. L. (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Applied and Environmental Microbiology*, 72(7), 5069-5072. doi.org/10.1128/AEM.03006-05
- Dunn, J. J., Studier, F. W., & Gottesman, M. (1983). Complete nucleotide sequence of bacteriophage T7 DNA and the locations of T7 genetic elements. *Journal of Molecular Biology*, 166(4), 477-535. doi.org/10.1016/S0022-2836(83)80282-4
- Edwards, U., Rogall, T., Blöcker, H., Emde, M., & Böttger, E. C. (1989). Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. *Nucleic Acids Research*, 17(19), 7843-53.
- Frampton, R. A., Pitman, A. R., & Fineran, P. C. (2012). Advances in bacteriophage-mediated control of plant pathogens. *International Journal of Microbiology*, 2012. doi.org/10.1155/2012/326452
- Fu, S., & Huang, H. (2011). Molecular characterization of the early response of orchid *Phalaenopsis amabilis* to *Erwinia chrysanthemi* infection, (1). doi.org/10.1142/9789814327930_0015
- Garcia-Vallve, S., Palau, J., & Romeu, A. (1999). Horizontal gene transfer in glycosyl hydrolases inferred from codon usage in *Escherichia coli* and *Bacillus subtilis*. *Molecular Biology and Evolution*, 16(9), 1125-1134. doi.org/10.1093/oxfordjournals.molbev.a026203
- Gardan, L., Gouy, C., Christen, R., & Samson, R. (2003). Elevation of three subspecies of *Pectobacterium carotovorum* to species level: *Pectobacterium atrosepticum* sp. nov., *Pectobacterium betavascularum* sp. nov. and *Pectobacterium wasabiae* sp. nov. *International Journal of Systematic and Evolutionary Microbiology*, 53(2), 381-391. doi.org/10.1099/ijs.0.02423-0
- Goris, J., Konstantinidis, T. K., Klappenbach, J. A., Coenye, T., Vandamme, P., & Tiedje, J. M. (2007). DNA-DNA hybridization values and their relationship to whole-genome sequence similarities. *International Journal of Systematic and Evolutionary Microbiology*, 57(1), 81-91. doi.org/10.1099/ijs.0.64483-0
- Gouy, M., Guindon, S., & Gascuel, O. (2010). SeaView Version 4: A multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Molecular Biology and Evolution*, 27(2), 221-224. doi.org/10.1093/molbev/msp259
- Hauben, L., Moore, E. R. B., Vauterin, L., Steenackers, M., Mergaert, J., Verdonck, L., & Swings, J. (1998). Phylogenetic position of phytopathogens within the *Enterobacteriaceae*. *Systematic and Applied Microbiology*, 21(3), 384-397. doi.org/10.1016/S0723-2020(98)80048-9
- He, W., Mu, W., Jiang, B., Yan, X., & Zhang, T. (2016). Construction of a food grade recombinant *Bacillus subtilis* based on replicative plasmids with an auxotrophic marker for biotransformation of D-fructose to D-allulose. *Journal of Agricultural and Food Chemistry*, 64(16), 3243-3250. doi.org/10.1021/acs.jafc.6b00278

- Hinsley, A., Verissimo, D., & Roberts, D. L. (2015). Heterogeneity in consumer preferences for orchids in international trade and the potential for the use of market research methods to study demand for wildlife. *Biological Conservation*, 190, 80–86. doi.org/10.1016/j.biocon.2015.05.010
- Hugouvieux-Cotte-Pattat, N. (2016). Metabolism and virulence strategies in *Dickeya*-host interactions. *Progress in Molecular Biology and Translational Science*, 142, 93–129. doi.org/10.1016/BS.PMBTS.2016.05.006
- Izumori, K., Akimitsu, K., Tajima, S., Agarie, M., Yanagi, T., & Mochioka, R. (2011). U.S. Patent No. 8,017,828. doi.org/10.1016/j.(73)
- Jendrossek, D., & Pfeiffer, D. (2014). New insights in the formation of polyhydroxyalkanoate granules (carbonosomes) and novel functions of poly(3-hydroxybutyrate). *Environmental Microbiology*, 16(8), 2357–2373. doi.org/10.1111/1462-2920.12356
- Jepson, C. D., & March, J. B. (2004). Bacteriophage lambda is a highly stable DNA vaccine delivery vehicle. *Vaccine*, 22(19), 2413–2419. doi.org/10.1016/j.vaccine.2003.11.065
- Joko, T., Subandi, A., Kusumandari, N., Wibowo, A., & Priyatmojo, A. (2014). Activities of plant cell wall-degrading enzymes by bacterial soft rot of orchid. *Archives of Phytopathology and Plant Protection*, 47(10), 1239–1250. doi.org/10.1080/03235408.2013.838374
- Jończyk, E., Kłak, M., Międzybrodzki, R., & Górski, A. (2011). The influence of external factors on bacteriophages—review. *Folia Microbiologica*, 56(3), 191–200. doi.org/10.1007/s12223-011-0039-8
- Jones, J. B., Jackson, L. E., Balogh, B., Obradovic, A., Iriarte, F. B., & Momol, M. T. (2007). Bacteriophages for plant disease control. *Annual Review of Phytopathology*, 45(1), 245–262. doi.org/10.1146/annurev.phyto.45.062806.094411
- Jones, J. B., Vallad, G. E., Iriarte, F. B., Obradović, A., Wernsing, M. H., Jackson, L. E., ... Momol, M. T. (2012). Considerations for using bacteriophages for plant disease control. *Bacteriophage*, 2(4), e23857. https://doi.org/10.4161/bact.23857
- Kano, A., Hosotani, K., Gomi, K., Yamasaki-Kokudo, Y., Shirakawa, C., Fukumoto, T., ... Akimitsu, K. (2011). D-Psicose induces upregulation of defense-related genes and resistance in rice against bacterial blight. *Journal of Plant Physiology*, 168(15), 1852–1857. doi.org/10.1016/j.jplph.2011.04.003
- Katznelson, H. (1937). Bacteriophage in relation to plant diseases. *The Botanical Review*, 3(10), 499–521. doi.org/10.1007/BF02870486
- Keith, L. M., Sewake, K. T., & Zee, F. T. (2005). Isolation and characterization of *Burkholderia gladioli* from orchids in Hawaii. *Plant Disease*, 89(12), 1273–1278. doi.org/10.1094/PD-89-1273
- Kim, O.-S., Cho, Y.-J., Lee, K., Yoon, S.-H., Kim, M., Na, H., ... Chun, J. (2012). Introducing EzTaxon-e: A prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. *International Journal of Systematic and Evolutionary Microbiology*, 62(Pt 3), 716–721.

- doi.org/10.1099/ijs.0.038075-0
- Konstantinidis, K. T., Ramette, A., & Tiedje, J. M. (2006). The bacterial species definition in the genomic era. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 361(1475), 1929-1940. doi.org/10.1098/rstb.2006.1920
- Kraepiel, Y., & Barny, M. A. (2016). Gram-negative phytopathogenic bacteria, all hemibiotrophs after all?. *Molecular plant pathology*, 17(3), 313-316. 10.1111/mpp.12345
- Kropinski, A. M., Mazzocco, A., Waddell, T. E., Lingohr, E., & Johnson, R. P. (2009). Enumeration of bacteriophages by double agar overlay plaque assay. In *Methods in Molecular Biology (Clifton, N.J.)* (Vol. 501, pp. 69-76). doi.org/10.1007/978-1-60327-164-6_7
- Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Molecular Biology and Evolution*, 33(7), 1870-1874. doi.org/10.1093/molbev/msw054
- Kutter, E., & Sulakvelidze, A. (2005). *Bacteriophages: Biology and applications*. CRC Press.
- Lautier, T., & Nasser, W. (2007). The DNA nucleoid-associated protein Fis co-ordinates the expression of the main virulence genes in the phytopathogenic bacterium *Erwinia chrysanthemi*. *Molecular Microbiology*, 66(6), 1474-90. doi.org/10.1111/j.1365-2958.2007.06012.x
- Lelliott, R. A., & Dickey, R. S. (1984). Genus VII. *Erwinia*. In N. R. Krieg & J. G. Holt (Eds.), *Bergey's Manual of Systematic Bacteriology*, vol. 1 (pp. 469-476). Baltimore: Williams & Wilkins.
- Letunic, I., & Bork, P. (2016). Interactive tree of life (iTOL) v3: An online tool for the display and annotation of phylogenetic and other trees. *Nucleic Acids Research*, 44, 242-245. doi.org/10.1093/nar/gkw290
- Levin, B. R., & Bull, J. J. (2004). Opinion: Population and evolutionary dynamics of phage therapy. *Nature Reviews Microbiology*, 2(2), 166-173. doi.org/10.1038/nrmicro822
- Li, B., Qiu, W., Fang, Y., & Xie, G. L. (2009). Bacterial stem rot of *Oncidium* orchid caused by a *Dickeya* sp. (ex *Pectobacterium chrysanthemi*) in Mainland China. *Plant Disease*, 93(5), 552-552. doi.org/10.1094/PDIS-93-5-0552B
- Lin, Y. H., Lee, P. J., Shie, W. T., Chern, L. L., & Chao, Y. C. (2015). *Pectobacterium chrysanthemi* as the dominant causal agent of bacterial soft rot in *Oncidium* "Grower Ramsey." *European Journal of Plant Pathology*, 142(2), 331-343. doi.org/10.1007/s10658-015-0618-7
- Ma, B., Hibbing, M. E., Kim, H.-S., Reedy, R. M., Yedidia, I., Breuer, J., ... Charkowski, A. O. (2007). Host range and molecular phylogenies of the soft rot enterobacterial genera *Pectobacterium* and *Dickeya*. *Phytopathology*, 97(9), 1150-1163. doi.org/10.1094/PHTO-97-9-1150
- Mansfield, J., Genin, S., Magori, S., Citovsky, V., Sriariyanum, M., Ronald, P., ... Foster, G. D. (2012). Top 10 plant pathogenic bacteria in molecular plant pathology. *Molecular Plant Pathology*, 13(6), 614-629. doi.org/10.1111/j.1364-3703.2012.00804.x
- McMeechan, A., Lovell, M. A., Cogan, T. A., Marston, K. L.,

- Humphrey, T. J., & Barrow, P. A. (2005). Glycogen production by different *Salmonella enterica* serotypes: Contribution of functional glgC to virulence, intestinal colonization and environmental survival. *Microbiology*, 151(12), 3969–3977. doi.org/10.1099/mic.0.28292-0
- Nikolaeva, E. V., Kang, S., Olson, T. N., & Kim, S. (2012). Real-time PCR detection of *Rhodococcus fascians* and discovery of new plants associated with *R. fascians* in Pennsylvania. *Plant Health Progress*. doi.org/10.1094/PHP-2012-0227-02-RS
- Notredame, C., Higgins, D. G., & Heringa, J. (2000). T-coffee: A novel method for fast and accurate multiple sequence alignment 1. Edited by J. Thornton. *Journal of Molecular Biology*, 302(1), 205–217. doi.org/10.1006/jmbi.2000.4042
- Obradovic, A., Jones, J. B., Momol, M. T., Balogh, B., & Olson, S. M. (2004). Management of tomato bacterial spot in the field by foliar applications of bacteriophages and SAR inducers. *Plant Disease*, 88(7), 736–740. doi.org/10.1094/PDIS.2004.88.7.736
- Obradovic, A., Jones, J. B., Momol, M. T., Olson, S. M., Jackson, L. E., Balogh, B., ... Iriarte, F. B. (2005). Integration of biological control agents and systemic acquired resistance inducers against bacterial spot on tomato. *Plant Disease*, 89(7), 712–716. doi.org/10.1094/PD-89-0712
- Overbeek, R., Olson, R., Pusch, G. D., Olsen, G. J., Davis, J. J., Disz, T., ... Stevens, R. (2014). The SEED and the rapid annotation of microbial genomes using subsystems technology (RAST). *Nucleic Acids Research*, 42(Database issue), D206–14. doi.org/10.1093/nar/gkt1226
- Parkinson, N., DeVos, P., Pirhonen, M., & Elphinstone, J. (2014). *Dickeya aquatica* sp. nov., isolated from waterways. *International Journal of Systematic and Evolutionary Microbiology*, 64(Pt 7), 2264–2266. doi.org/10.1099/ijs.0.058693-0
- Parkinson, N., Pritchard, L., Bryant, R., Toth, I., & Elphinstone, J. (2014). Epidemiology of *Dickeya dianthicola* and *Dickeya solani* in ornamental hosts and potato studied using variable number tandem repeat analysis. *European Journal of Plant Pathology*, 141(1), 63–70. doi.org/10.1007/s10658-014-0523-5
- Parkinson, N., Stead, D., Bew, J., Heeney, J., Tsrer, L., & Elphinstone, J. (2009). *Dickeya* species relatedness and clade structure determined by comparison of *recA* sequences. *International Journal of Systematic and Evolutionary Microbiology*, 59(10), 2388–2393. doi.org/10.1099/ijs.0.009258-0
- Payne, R. J. H., & Jansen, V. A. A. (2000). Phage therapy: The peculiar kinetics of self-replicating pharmaceuticals. *Clinical Pharmacology and Therapeutics*, 68(3), 225–230. doi.org/10.1067/mcp.2000.109520
- Peng, G., Zhang, W., Luo, H., Xie, H., Lai, W., & Tan, Z. (2009). *Enterobacter oryzae* sp. nov., a nitrogen-fixing bacterium isolated from the wild rice species *Oryza latifolia*. *International Journal of Systematic and Evolutionary Microbiology*, 59(7), 1650–1655. doi.org/10.1099/ijs.0.65484-0
- Perombelon, M. C. M., & Kelman, A. (1980). Ecology of the soft rot *Erwinias*. *Annual Review of Phytopathology*, 18(1), 361–387.

- doi.org/10.1146/annurev.py.18.090180.002045
- Pickard, D. J. J. (2009). Preparation of bacteriophage lysates and pure DNA. In *Methods in molecular biology* (Clifton, N.J.) (Vol. 502, pp. 3-9). doi.org/10.1007/978-1-60327-565-1_1
- Potrykus, M., Golanowska, M., Hugouvieux-Cotte-Pattat, N., & Lojkowska, E. (2014). Regulators involved in *Dickeya solani* virulence, genetic conservation, and functional variability. *Molecular Plant-Microbe Interactions*, 27(7), 700-711. doi.org/10.1094/MPMI-09-13-0270-R
- Potrykus, M., Golanowska, M., Sledz, W., Zoledowska, S., Motyka, A., Kolodziejska, A., ... Lojkowska, E. (2016). Biodiversity of *Dickeya* spp. isolated from potato plants and water sources in temperate climate. *Plant Disease*, 100(2), 408-417. doi.org/10.1094
- Pritchard, L., Glover, R. H., Humphris, S., Elphinstone, J. G., & Toth, I. K. (2016). Genomics and taxonomy in diagnostics for food security: Soft-rotting enterobacterial plant pathogens. *Anal. Methods*, 8(1), 12-24. doi.org/10.1039/C5AY02550H
- Pritchard, L., Humphris, S., Saddler, G. S., Elphinstone, J. G., Pirhonen, M., & Toth, I. K. (2013). Draft genome sequences of 17 isolates of the plant pathogenic bacterium *Dickeya*. *Genome Announcements*, 1(6). doi.org/10.1128/genomeA.00978-13
- Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., ... Glöckner, F. O. (2012). The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Research*, 41(D1), D590-D596. doi.org/10.1093/nar/gks1219
- Reverchon, S., Muskhelishvili, G., & Nasser, W. (2016). Virulence program of a bacterial plant pathogen: The *Dickeya* model. *Progress in Molecular Biology and Translational Science*, 142, 51-92. doi.org/10.1016/BS.PMBTS.2016.05.005
- Reverchon, S., & Nasser, W. (2013). *Dickeya* ecology, environment sensing and regulation of virulence programme. *Environmental Microbiology Reports*, 5(5), 622-636. doi.org/10.1111/1758-2229.12073
- Ruiz, J. A., López, N. I., Fernández, R. O., & Méndez, B. S. (2001). Polyhydroxyalkanoate degradation is associated with nucleotide accumulation and enhances stress resistance and survival of *Pseudomonas oleovorans* in natural water microcosms. *Applied and Environmental Microbiology*, 67(1), 225-30. doi.org/10.1128/AEM.67.1.225-230.2001
- Samson, R., Legendre, J. B., 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, Dick. *International Journal of Systematic and Evolutionary Microbiology*, 55(4), 1415-1427. doi.org/10.1099/ijs.0.02791-0
- Shively, J. M., Cannon, G. C., Heinhorst, S., Bryant, D. A., DasSarma, S., Bazylnski, D., ... Dahl, C. (2011). *Bacterial and archaeal inclusions*. doi.org/10.1002/9780470015902.A0000302.PUB3
- Sławiak, M., van Beckhoven, J. R. C. M., Speksnijder, A. G. C. L., Czajkowski, R., Grabe, G., & van der Wolf, J. M. (2009).

- Biochemical and genetical analysis reveal a new clade of biovar 3 *Dickeya* spp. strains isolated from potato in Europe. *European Journal of Plant Pathology*, 125(2), 245-261. doi.org/10.1007/s10658-009-9479-2
- Soleimani-Delfan, A., Etemadifar, Z., Emtiazi, G., & Bouzari, M. (2015). Isolation of *Dickeya dadantii* strains from potato disease and biocontrol by their bacteriophages. *Brazilian Journal of Microbiology*, 46(3), 791-797. doi.org/10.1590/S1517-838246320140498
- Stevens, A. C., & Trus, B. L. (1986). *The structure of bacteriophage T7*. (J. R. Harris & R. W. Horne, Eds.), *Electron Microscopy of Proteins, Viral Structure* (Vol. 5). London: Academic Press.
- Steyer, A., Gutiérrez-Aguirre, I., Rački, N., Beigot Glaser, S., Brajer Humar, B., Stražar, M., ... Rupnik, M. (2015). The detection rate of enteric viruses and *Clostridium difficile* in a waste water treatment plant effluent. *Food and Environmental Virology*, 7(2), 164-172. doi.org/10.1007/s12560-015-9183-7
- Suharjo, R., Sawada, H., & Takikawa, Y. (2014). Phylogenetic study of Japanese *Dickeya* spp. and development of new rapid identification methods using PCR-RFLP. *Journal of General Plant Pathology*, 80(3), 237-254. doi.org/10.1007/s10327-014-0511-9
- Tian, Y., Zhao, Y., Yuan, X., Yi, J., Fan, J., Xu, Z., ... Li, X. (2016). *Dickeya fangzhongdai* sp. nov., a plantpathogenic bacterium isolated from pear trees (*Pyrus pyrifolia*). *International Journal of Systematic and Evolutionary Microbiology*, 66(8), 2831-2835. doi.org/10.1099/ijsem.0.001060
- Toth, I., Bell, K., & Holeva, M. (2003). Soft rot erwinia: From genes to genomes. *Molecular Plant Pathology*, 4(1), 17-30.
- Toth, I. K., Van der Wolf, J. M., Saddler, G., Lojkowska, E., Hélias, V., Pirhonen, M., ... Elphinstone, J. G. (2011). *Dickeya* species: An emerging problem for potato production in Europe. *Plant Pathology*, 60(3), 385-399. doi.org/10.1111/j.1365-3059.2011.02427.x
- Van Twest, R., & Kropinski, A. M. (2009). Bacteriophage enrichment from water and soil. *Methods in molecular biology*, 501, 15-21. doi.org/10.1007/978-1-60327-164-6_2
- Van Vaerenbergh, J., Baeyen, S., de Vos, P., & Maes, M. (2012). Sequence diversity in the *Dickeya fliC* gene: Phylogeny of the *Dickeya* genus and taqman® PCR for "D. solani," new biovar 3 variant on potato in Europe. *PLoS ONE*, 7(5), 1-9. doi.org/10.1371/journal.pone.0035738
- Vinatzer, B. A., Weisberg, A. J., Monteil, C. L., Elmarakeby, H. A., Sheppard, S. K., & Heath, L. S. (2017). A proposal for a genome similarity-based taxonomy for plant-pathogenic bacteria that is sufficiently precise to reflect phylogeny, host range, and outbreak affiliation applied to *Pseudomonas syringae sensu lato* as a proof of concept. *Phytopathology*, 107(1), 18-28. doi.org/10.1094/PHYTO-07-16-0252-R
- Waldee, E. L. (1945). Comparative studies of some peritrichous phytopathogenic bacteria. *Iowa State College Journal of Science*, 19(4). Retrieved from

- <https://www.cabdirect.org/cabdirect/abstract/19481101232>
- Waterhouse, A. M., Procter, J. B., Martin, D. M. A., Clamp, M., & Barton, G. J. (2009). Jalview Version 2--a multiple sequence alignment editor and analysis workbench. *Bioinformatics* (Oxford, England), 25(9), 1189-91. doi.org/10.1093/bioinformatics/btp033
- Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., ... Truper, H. G. (1987). Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *International Journal of Systematic and Evolutionary Microbiology*, 463-464.
- Wilson, W. A., Roach, P. J., Montero, M., Baroja-Fernández, E., Muñoz, F. J., Eydallin, G., ... Pozueta-Romero, J. (2010). Regulation of glycogen metabolism in yeast and bacteria. *FEMS Microbiology Reviews*, 34(6), 952-985. doi.org/10.1111/j.1574-6976.2010.00220.x
- Yishay, M., Burdman, S., Valverde, A., Luzzatto, T., Ophir, R., & Yedidia, I. (2008). Differential pathogenicity and genetic diversity among *Pectobacterium carotovorum* ssp. *carotovorum* isolates from monocot and dicot hosts support early genomic divergence within this taxon. *Environmental Microbiology*, 10(10), 2746-2759. doi.org/10.1111/j.1462-2920.2008.01694.x
- Zhang, Y., Fan, Q., & Loria, R. (2016). A re-evaluation of the taxonomy of phytopathogenic genera *Dickeya* and *Pectobacterium* using whole-genome sequencing data. *Systematic and Applied Microbiology*, 39(4), 252-259. doi.org/10.1016/j.syapm.2016.04.001

Bibliography

Publications Related to the Thesis

Journal Articles

- Alič, Š., Naglič, T., Llop, P., Toplak, N., Koren, S., Ravnikar, M., & Dreó, T. (2015). Draft genome sequences of *Dickeya* sp. isolates B16 (NIB Z 2098) and S1 (NIB Z 2099) causing soft rot of *Phalaenopsis* orchids. *Genome Announcements*, 3(5). doi.org/10.1128/genomeA.00973-15
- Alič, Š., Naglič, T., Tušek-Žnidarič, M., Ravnikar, M., Rački, N., Peterka, M., & Dreó, T. (2017a). Newly isolated bacteriophages from the *Podoviridae*, *Siphoviridae*, and *Myoviridae* families have variable effects on putative novel *Dickeya* spp. *Frontiers in Microbiology*, 8, 1-14. doi.org/10.3389/fmicb.2017.01870
- Alič, Š., Naglič, T., Tušek-Žnidarič, M., Peterka, M., Ravnikar,

- M., & Dreo, T. (2017b). Putative new species of the genus *Dickeya* as major soft rot pathogens in *Phalaenopsis* orchid production. *Plant Pathology*, 66(8), 1357-1368. doi.org/10.1111/ppa.12677
- Alič, Š., Van Gijsegem, F., Pédrón, J., Ravnikar, M., & Dreo, T (In press). Diversity within the novel *Dickeya fangzhongdai* sp., isolated from infected orchids, water, and pears. *Plant Pathology*.

Biography

Špela Alič was born on March 13, 1989, in Kranj, Slovenia. She finished her primary education in Poljane and secondary education in Škofja Loka. In summer 2008, she attended the International Biology Olympiad held in India and received a bronze medal. She started her studies at the Faculty of Chemistry and Chemical Technology, University of Ljubljana, Slovenia, in 2008. In 2013, she was awarded a Diploma in Biochemistry for the thesis entitled '*DNA-binding properties of human stefin B dimer*' from the University of Ljubljana. She received the Prešern Award of the Faculty of Chemistry and Chemical Technology for her thesis.

In 2013, she was selected as a PhD candidate at the National Institute of Biology, Ljubljana, Slovenia, under the supervision of Prof. Maja Ravnikar, PhD, and co-supervision of Tanja Dreo, PhD, at the Department of Biotechnology and Systems Biology. At the same time, she started her postgraduate studies in nanoscience and nanotechnologies at the Jožef Stefan International Postgraduate School, Ljubljana, Slovenia, also under the supervision of Prof. Maja Ravnikar, PhD.

Her research focuses on the bacterial soft rot disease of the *Phalaenopsis* orchid and disease biocontrol options using bacteriophages. During her studies she visited the Institute of Ecology and Environmental Sciences-Paris, France, first in 2015 and second in 2017, for a total 5-month stay at, during which she was trained in bacterial genome assembly and comparative genomics under the supervision of Jacques Pedron, PhD, and Frédérique Van Gijsegem, PhD. She has attended and presented her work at several conferences and workshops on the topics of bacteriophages and biocontrol, next-generation sequencing, genome assembly and annotation, genomics and evolutionary genomics.

She is currently involved in Slovenian Research Agency projects and projects funded by companies.

In her spare time, she is an active volunteer, mostly as a member of the local firefighting unit, first responders and first aid team.