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Ecological role of tailocins (phage tail-like particles) in Soft Rot Pectobacteriaceae

Ekologiczna rola tailocyn (cząstek przypominających ogonki fagowe) u bakterii pektynolitycznych z rodziny Pectobacteriaceae

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"*Go then, there are other worlds than these.*"

Stephen King, *The Dark Tower*

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Table of contents

Abstract

Bacteria colonising plant surfaces and tissues face intense competition for space and nutrients and therefore deploy a diverse arsenal of interference mechanisms to inhibit rivals. Among plant-associated bacteria, Soft Rot Pectobacteriaceae (SRP) are particularly exposed to such competitive pressure. These bacteria – *Pectobacterium*, *Dickeya*, and *Musicola* species that cause soft rot and other maceration-associated diseases in economically important crops – are found across habitats ranging from surface water and agricultural soil to infected plant tissue, where they encounter both closely related SRP and taxonomically distant competitors. Under competitive pressure, these bacteria have evolved several antagonistic mechanisms, including tailocins – phage tail–like bacteriocins encoded by domesticated prophage regions in their genomes. Tailocins are bactericidal particles that kill susceptible cells by puncturing their cell envelope. What makes tailocins unusual among bacterial weapons is that their production requires the death of the producing cell: particles are assembled in the cytoplasm and can only be released through lysis. Despite this cost, tailocin gene clusters are found across diverse bacterial taxa, which raises the question of what selective advantage sustains such expensive weapons. To date, among SRP, only one type of tailocin has been described in detail – carotovoricin Er from *Pectobacterium carotovorum* – and the selective advantage of tailocins in this group remains largely unexplored.

This thesis, which consists of four published studies, characterises tailocin-mediated interactions in SRP, from the molecular properties of individual particles to their potential ecological consequences in plant-associated habitats. To establish a model system, I characterised the first R-type tailocin from *Dickeya dadantii* strain 3937, designated dickeyocin P2D1. The gene cluster encoding P2D1 shares sequence homology with the genes encoding the tail of *Peduovirus* P2 – a lineage phylogenetically distinct from that of carotovoricin Er – indicating that the two SRP genera acquired their tailocins independently from different phage ancestors. Further, to identify the conditions and timing of tailocin production, the temporal dynamics of P2D1 release were resolved, showing that production follows a tightly coordinated sequence and can be triggered by structurally unrelated DNA-damaging agents, including mitomycin C, hydrogen peroxide, ciprofloxacin, and norfloxacin. The finding that H_2O_2 induces tailocin release is of particular ecological relevance, because SRP

bacteria encounter plant-derived reactive oxygen species during the oxidative burst that accompanies infection.

To determine how widespread tailocin production is among environmental SRP, I surveyed 27 strains isolated from the Durance River in France. This river connects ecosystems in which different SRP species co-occur, making it a source of strains that are likely to encounter one another in nature. Of the 27 strains tested, 24 produced tailocin particles. A broader genomic survey, including screening of 190 complete SRP genomes, confirmed that tailocin clusters are present in 83% of *Pectobacterium* and 69% of *Dickeya* genomes. All *Dickeya* clusters are homologous to the P2D1 locus and all *Pectobacterium* clusters to the carotovoricin Er locus, with no genome carrying both types, confirming independent acquisition after the divergence of the two genera. Systematic pairwise testing among these strains revealed that *Dickeya* tailocins kill more frequently and across broader taxonomic boundaries than *Pectobacterium* tailocins, including activity against *Pectobacterium* strains – an asymmetry not observed in the opposite direction. Tailocin particles were also detected in experimentally *Dickeya*-infected potato tissue, indicating that these particles are present during plant infection.

Tailocins are generally considered to target kin strains. In this work, to test whether the ecological reach of tailocins extends beyond SRP, I screened 480 soil and rhizosphere bacterial isolates for sensitivity to P2D1. Nine strains were found susceptible, all spanning different phylogenetic clades within genus *Pseudomonas*. Tests involving a P2D1-deficient mutant of *D. dadantii* confirmed that the activity is tailocin-mediated. Six of the nine susceptible *Pseudomonas* are non-pathogenic and can suppress *D. dadantii* soft rot on potato, whereas three cause soft rot themselves. P2D1 can therefore eliminate bacteria with contrasting ecological roles relative to its producer – both direct antagonists and organisms that exploit the same host.

Together, these findings indicate that tailocins are a widespread and functional component of the competitive arsenal of SRP. The convergent maintenance of tailocin clusters of independent phage origin in both genera, the detection of particles in infected plant tissue, and the demonstration that H_2O_2 triggers their production all point toward a selective advantage that has sustained tailocin carriage despite the lethal cost of deployment. While direct evidence for tailocin-mediated competition under natural conditions remains to be

obtained, this thesis establishes the molecular, genomic, and ecological foundations on which such studies can now be built.

Streszczenie

Bakterie zasiedlające powierzchnie i tkanki roślin stale rywalizują o przestrzeń i składniki odżywcze, wykorzystując w tym celu szeroki wachlarz mechanizmów konkurencji. Wśród bakterii związanych z roślinami, patogeny z grupy Soft Rot Pectobacteriaceae (SRP) są szczególnie narażone na presję konkurencyjną. Gatunki należące do rodzajów *Pectobacterium*, *Dickeya* i *Musicola* powodują mokrą zgniliznę i inne choroby roślin uprawnych o dużym znaczeniu gospodarczym, a jednocześnie występują w szerokim zakresie siedlisk, od wód powierzchniowych i gleb rolniczych po tkanki infekowanych roślin, gdzie napotykają zarówno blisko spokrewnione szczepy SRP, jak i odległe filogenetycznie mikroorganizmy. Genomy tych bakterii zawierają liczne elementy pochodzenia profagowego, a ewolucyjne „udomowienie" genów kodujących ogonki profagów jest uważane za źródło pochodzenia tailocyn – bakteriobójczych cząstek, które zabijają wrażliwe komórki przez mechaniczne przebicie ich ściany i błon komórkowych. Tym, co wyróżnia tailocyny spośród innych mechanizmów konkurencji międzybakteryjnej, jest fakt, że ich produkcja wymaga śmierci komórki produkującej: cząstki są składane w cytoplazmie i mogą zostać uwolnione wyłącznie w wyniku lizy. Pomimo tego kosztu klastry genów tailocyn występują u wielu różnych taksonów bakteryjnych, co wskazuje na istnienie przewagi selekcyjnej, która rekompensuje koszt ich produkcji. W SRP charakter tej przewagi pozostawał niezbadany, a jedyną szczegółowo opisaną tailocyną typu R była carotovorocyna Er z *Pectobacterium carotovorum*.

Niniejsza rozprawa doktorska, składająca się z czterech opublikowanych prac eksperymentalnych, charakteryzuje oddziaływania międzybakteryjne pośredniczone przez tailocyny w obrębie SRP – od właściwości molekularnych pojedynczych cząstek po ich potencjalną rolę ekologiczną w środowiskach związanych z roślinami. W celu opracowania systemu modelowego scharakteryzowałem pierwszą tailocynę typu R u *Dickeya dadantii* szczep 3937, nazwaną dickeyocyną P2D1. Jej klaster genowy wykazuje homologię z genami ogonka *Peduowirusa* P2 – linią filogenetycznie odrębną od carotovorocyny Er. Wskazuje to, że oba rodzaje SRP nabyły swoje tailocyny niezależnie od różnych dawców fagowych. W celu określenia warunków i dynamiki produkcji tailocyn zbadano kinetykę uwalniania P2D1, wykazując, że produkcja tych cząstek przebiega w sposób ściśle skoordynowany i może być indukowana przez strukturalnie różne czynniki uszkadzające DNA, w tym mitomycynę C, nadtlenek wodoru, ciprofloksacynę i norfloksacynę. Stwierdzenie, że H_2O_2 indukuje

uwalnianie tailocyn, ma szczególne znaczenie ekologiczne, ponieważ bakterie SRP narażone są na reaktywne formy tlenu produkowane przez roślinę podczas infekcji.

W celu określenia, jak powszechna jest produkcja tailocyn wśród środowiskowych szczepów SRP, przebadałem 27 szczepów wyizolowanych z rzeki Durance we Francji: 24 z nich produkowało tailocyny, a analiza genomowa 190 kompletnych genomów SRP potwierdziła obecność klastrów tailocyn u 83% szczepów *Pectobacterium* i 69% szczepów *Dickeya*. Wszystkie klastry u *Dickeya* są homologiczne do locus P2D1, a wszystkie klastry u *Pectobacterium* do locus carotovorocyny Er, przy czym żaden z przebadanych genomów nie zawierał obu typów klastrów jednocześnie, co potwierdza niezależne nabycie genów tailocyn już po dywersyfikacji rodzajowej. Niektóre tailocyny *Dickeya* były aktywne wobec szczepów *Pectobacterium*, natomiast żadne tailocyny *Pectobacterium* nie wykazywały aktywności wobec szczepów *Dickeya*. Cząstki tailocyn wykryto również w eksperymentalnie zainfekowanej tkance ziemniaka, co wskazuje na ich obecność podczas infekcji roślin.

W celu sprawdzenia, czy ekologiczny zasięg działania tailocyn wykracza poza SRP, przebadałem 480 izolatów glebowych i ryzosferowych pod kątem wrażliwości na P2D1: dziewięć szczepów *Pseudomonas* należących do różnych kladów filogenetycznych okazało się wrażliwych, a delecja genów kodujących strukturalne elementy tailocyny P2D1 u *D. dadantii* potwierdziła, że obserwowana aktywność zależy od cząstek tailocyn. Sześć z dziewięciu wrażliwych szczepów *Pseudomonas* nie wywoływało objawów chorobowych na ziemniaku i hamowało mokrą zgniliznę wywoływaną przez *D. dadantii*, podczas gdy trzy pozostałe same wywoływały objawy mokrej zgnilizny. Oznacza to, że P2D1 może eliminować bakterie o odmiennych rolach ekologicznych, w tym względem swojego producenta.

Uzyskane w tej pracy wyniki wskazują, że tailocyny są powszechnym i funkcjonalnym elementem arsenału konkurencyjnego SRP. Utrzymanie klastrów wywodzących się z odrębnych linii fagowych w obu rodzajach, wykrycie cząstek w zainfekowanej tkance roślinnej oraz wykazanie, że H_2O_2 indukuje ich produkcję, wskazują na przewagę selekcyjną, która utrzymała tailocyny pomimo kosztu związanego z lizą komórki produkującej. Chociaż bezpośredni dowód na udział tailocyn w rywalizacji międzybakteryjnej w warunkach naturalnych nie został dotychczas uzyskany, niniejsza rozprawa tworzy molekularne, genomowe i ekologiczne podstawy, na których takie badania mogą być prowadzone.

General Introduction

Competition in plant-associated bacterial communities

In the densely populated habitats that plants provide, bacteria are under persistent competition for limited resources. On leaf surfaces, bacterial populations commonly reach 10^6 to 10^7 cells per square centimetre, with cells concentrated in aggregates at epidermal cell junctions, stomata, and trichome bases where nutrient availability is highest (Lindow & Brandl, 2003; Monier & Lindow, 2004; Vorholt, 2012). The rhizosphere – the narrow zone of soil directly influenced by root exudation – supports even higher densities, with bacterial populations reaching 10^8 to 10^{12} cells per gram of soil and steep gradients in nutrient concentration, oxygen availability, and pH enriching for specific bacterial populations within micrometres of the root surface (Aufrecht et al., 2022; Kennedy & de Luna, 2005; Zhalnina et al., 2018). Because many of the species present in these habitats depend on the same pool of plant-derived carbon and nitrogen, as well as micronutrients, high cell density translates directly into competition for shared resources (Granato et al., 2019). Although bacteria in these communities also engage in cooperative behaviours such as cross-feeding during co-infection (Barny et al., 2024), the intensity of resource limitation means that antagonistic interactions are a constant selective force, favouring the evolution and maintenance of competitive traits (Ghoul & Mitri, 2016; Hibbing et al., 2010).

The strategies that bacteria deploy under such pressure can be broadly divided into two categories, although they are not mutually exclusive (Ghoul & Mitri, 2016). In **exploitative competition**, one organism reduces the availability of a shared resource to its neighbours, either by consuming it more efficiently or by modifying local conditions – such as acidifying the environment or sequestering iron through siderophore production – in ways that exclude competitors, without requiring direct contact between cells (Ghoul & Mitri, 2016; Hibbing et al., 2010). In **interference competition**, bacteria produce molecules or structures that directly harm or kill neighbouring cells. Because a dead competitor no longer consumes resources, interference also provides an indirect competitive benefit (Hibbing et al., 2010). Theoretical and experimental work has shown that interference mechanisms are most effective in spatially structured environments, where producers and targets remain in close proximity and the benefits of killing are not diluted across a large, well-mixed population (Cornforth & Foster,

2013). Plant-associated habitats meet this condition: bacteria on leaf surfaces and in the rhizosphere are organised into discrete aggregates rather than homogeneous lawns (Monier & Lindow, 2004; Remus-Emsermann & Schlechter, 2018). The range of interference mechanisms deployed in these habitats is broad, including low-molecular-weight antibiotics, nonribosomal peptide and polyketide compounds, contact-dependent inhibition systems, type VI secretion systems that inject toxic effectors into adjacent cells, volatile organic compounds, and proteinaceous bacteriocins of various sizes and mechanisms of action (Granato et al., 2019; Ossowicki et al., 2017; Stubbendieck & Straight, 2016). A single bacterial strain commonly carries genes for several of these systems simultaneously, which means that the competitive outcome between any two strains is likely shaped by more than one mechanism (Booth et al., 2023; Hibbing et al., 2010; Raaijmakers & Mazzola, 2012).

Despite this this broad arsenal of weapons, our understanding of how individual competitive mechanisms contribute to bacterial fitness in plant habitats remains uneven (Granato et al., 2019). Type VI secretion systems and antibiotic production have received considerable attention in rhizosphere and phyllosphere ecology (Bernal et al., 2018; Raaijmakers & Mazzola, 2012), but other components of the interference arsenal are less studied. Among these are tailocins – bactericidal particles resembling phage tails. Tailocin-encoding clusters are present in the genomes of many plant-associated bacteria, both pathogenic and beneficial (Patz et al., 2019; Vacheron et al., 2021). Direct evidence that they influence competitive outcomes in plant environments has, however, not been obtained. Whether this gap reflects an absence of ecological relevance or the technical difficulty of detecting a killing event that is short-lived, spatially confined, and lethal to the producing cell remains an open question (Backman, Latorre, et al., 2024; Fautt et al., 2025; Vacheron et al., 2021).

Addressing this gap requires a model system in which tailocin production is common, the producing species are ecologically well characterised, and the habitats they occupy are accessible to experimental study. Soft Rot Pectobacteriaceae (SRP) meet these criteria.

Soft Rot Pectobacteriaceae as a model for studying bacterial antagonism

SRP are Gram-negative plant pathogens belonging to three genera – *Pectobacterium*, *Dickeya*, and *Musicola* – formerly classified collectively as pectinolytic *Erwinia* species (Hugouvieux-Cotte-Pattat et al., 2021; Samson et al., 2005). These bacteria cause soft rot, the enzymatic maceration of plant parenchyma tissue, and blackleg, a vascular disease of potato stems, in a wide range of economically important crops including potato, onion, carrot, chicory, and ornamental species, and are responsible for substantial pre- and post-harvest losses worldwide (Toth et al., 2011; Van Gijsegem et al., 2021). Because soft rot symptoms can be caused by more than ten different SRP species on a single crop such as potato (Ge et al., 2021; Ma et al., 2024), and because several species are frequently isolated together from the same diseased plant (Barny et al., 2024; Ge et al., 2021), SRP function as a species complex in which closely related pathogens share the same host and the same infected tissue. Because these species also share the same enzymatic strategies and therefore compete for the same plant-derived nutritional resources, co-occurrence intensifies competition between them (Ge et al., 2021; Ghoul & Mitri, 2016).

SRP are not restricted to cultivated plants: they have also been isolated from wild hosts such as bittersweet nightshade (Fikowicz-Krosko & Czajkowski, 2018; Fikowicz-Krosko et al., 2016), demonstrating that these pathogens maintain populations outside agricultural settings. What makes SRP a particularly suitable model for studying interbacterial competition is the range of environments they occupy outside of their plant hosts. SRP have been isolated from rainwater, surface water, aerosols, irrigation systems, agricultural and natural soils, sewage, and the surfaces and guts of insects (Ben Moussa et al., 2022; Potrykus et al., 2016; Rossmann et al., 2018; Van Gijsegem et al., 2021). These varied sources yield taxonomically diverse populations that include both plant-pathogenic species and species originally described from freshwater environments, such as *Dickeya aquatica*, *D. lacustris*, and *D. undicola* (Hugouvieux-Cotte-Pattat et al., 2019; Hugouvieux-Cotte-Pattat et al., 2023; Oulghazi et al., 2019; Parkinson et al., 2014). However, the boundary between aquatic and plant-associated populations is not fixed, as *D. aquatica* has since been isolated from carrot (Zaczek-Moczydłowska et al., 2019). Because water connects agricultural fields to natural ecosystems, river-borne SRP strains may eventually colonise the same plant host, where they would encounter both related SRP and

the broader resident microbiota (Van Gijsegem et al., 2021). Consequently, SRP are under continuous pressure to compete not only against close relatives but also against taxonomically distant organisms that share the same habitats (Bellieny-Rabelo et al., 2019; Van Gijsegem et al., 2021). SRP are also frequent subjects of biological control research that explores the use of antagonistic bacteria or bacteriophages against these pathogens (Czajkowski, 2016; Czajkowski et al., 2017; Krzyzanowska et al., 2019; Krzyzanowska et al., 2012; Maciag et al., 2020), which means that the competitive interactions these pathogens experience are of interest in both ecological and applied contexts.

Genomic analyses indicate that SRP carry an extensive arsenal of competitive tools (Bellieny-Rabelo et al., 2019). In addition to type VI secretion systems, SRP genomes encode nonribosomal peptide and polyketide biosynthetic clusters with demonstrated antibacterial and antifungal activity (Brual et al., 2023; Shyntum et al., 2019). SRP genomes also harbour abundant prophage-like elements – between one and four regions per genome in sequenced strains (Czajkowski, 2019). Experimental work on lytic bacteriophages of SRP has shown that these phages retain killing activity in plant-associated environments and that phage resistance in SRP frequently reduces bacterial virulence and fitness *in planta* (Bartnik et al., 2021; Bartnik et al., 2022; Czajkowski et al., 2017; Sokolova et al., 2023).

Despite this body of work on antibiotics, secretion systems and lytic phages, tailocins – particles that share their structural ancestry with bacteriophage tails – had not been well characterised in SRP prior to the work presented in this thesis (Nguyen et al., 1999; Yamada et al., 2006).

Prophage domestication as a source of competitive tools

Prophages, the genomes of temperate bacteriophages integrated into the bacterial chromosome, are widespread across bacterial taxa. A typical sequenced genome carries approximately three prophage sequences, and in some lineages prophage-derived DNA constitutes up to 20% of the total genome content (Bondy-Denomy & Davidson, 2014; Canchaya et al., 2004; Casjens, 2003). Given this abundance, the cumulative effect of prophages on host biology extends well beyond the phage life cycle itself. Integrated prophages can carry accessory genes, termed cargo genes, that are not required for phage replication but that confer new traits on the bacterial host upon expression (Boyd & Brussow, 2002; Czajkowski, 2019). This phenomenon, known as lysogenic conversion, accounts for virulence acquisition in several bacterial pathogens, including Shiga toxin production in *Escherichia coli* (O'Brien et al., 1984), diphtheria toxin expression in *Corynebacterium diphtheriae* (Freeman, 1951), and cholera toxin production in *Vibrio cholerae* (Waldor & Mekalanos, 1996). Prophages can also protect their host against further phage infection: prophage-encoded membrane proteins block adsorption or DNA injection by incoming related virions, a process termed superinfection exclusion (Braun et al., 1994; Lu & Henning, 1994).

Beyond their effects on individual cell phenotypes, prophages can function as population-level competitive weapons (Bossi et al., 2003; Burns et al., 2015; Duerkop et al., 2012). In *Salmonella enterica* serovar Typhimurium, a minority lysogenic population that underwent spontaneous prophage induction outcompeted a non-lysogenic competitor in mixed culture, because the released phage particles lysed the non-immune strain (Bossi et al., 2003). A similar advantage has been demonstrated in *Enterococcus faecalis*, where two distinct prophage elements cooperate to produce a single composite phage particle that kills susceptible competing strains and enhances colonisation of the mouse intestine (Duerkop et al., 2012). In *Pseudomonas aeruginosa*, strains carrying multiple prophages outcompeted non-lysogenic rivals in an insect infection model, because competitors needed to acquire resistance to each prophage separately, which prolonged the period during which they remained susceptible to lysis (Burns et al., 2015). In all three systems, the competitive benefit arises because spontaneous prophage induction in a small fraction of cells produces virions that lyse non-immune neighbours while the immune majority of the lysogenic population remains unharmed.

Likewise, even prophages that have lost the capacity for autonomous replication can retain structural components with bactericidal activity. Over evolutionary time, many prophages undergo precisely this type of mutational decay: genes required for capsid assembly, DNA packaging, and viral replication are progressively deleted or rendered nonfunctional, while genes encoding structural components that benefit the host are selectively retained (Bobay et al., 2014; Brussow et al., 2004). This process, termed prophage domestication, is the most widely accepted explanation for the origin of tailocins (Bobay et al., 2014; Ghequire & De Mot, 2015). Two structural types of tailocins have been described: R-type, which derive from contractile myovirus tails, and F-type, which derive from non-contractile siphovirus tails and are less well characterised (Gu et al., 2025; Nakayama et al., 2000).

R-type tailocins belong to a broader group of structures collectively termed contractile phage tail-like particles (CPTPs; Patz et al., 2019), all of which share structural homology with the contractile tails of myoviruses – tailed bacteriophages whose tails shorten upon injection of DNA into the host cell. CPTPs lack the capsid head and DNA-associated genes of their phage relatives and can be divided into three functional categories on the basis of their site of action and whether they deliver a molecular cargo. Type VI secretion systems (T6SS) remain anchored in the membrane of the producing cell and inject effector proteins directly into adjacent cells upon contact; because they do not require lysis of the producer, T6SS can be deployed repeatedly and have been shown to contribute to competitive fitness in rhizosphere and other plant-associated bacteria (Bernal et al., 2018; Cascales & Cambillau, 2012). Extracellular contractile injection systems (eCIS) are released from the producing cell and deliver a protein cargo into their targets, as in the antifeeding prophage of *Serratia entomophila* and the *Photorhabdus* virulence cassettes (Hurst et al., 2004; Yang et al., 2006). R-type tailocins, the third category, are also released from the cell but carry no cargo: they kill by puncturing the cell envelope of susceptible bacteria (Nakayama et al., 2000; Patz et al., 2019). Both tailocins and eCIS are assembled in the cytoplasm and can only exit the cell through lysis of the producer, making every deployment event lethal for the producing cell – a cost that distinguishes these released CPTPs from other bacteriocins, which are secreted without cell death, and from antibiotics, which can be produced continuously (Backman, Burbano, et al., 2024; Nobrega et al., 2018).

Of these three categories, tailocins are unique in combining cargo-free killing with the death of the producing cell, properties that raise the question of how such a costly weapon can persist across diverse bacterial lineages (Backman, Burbano, et al., 2024).

Tailocins as agents of interbacterial competition

R-type tailocin particles retain the core architecture of a contractile phage tail: a rigid, rod-shaped structure of 80 to 180 nm in length, composed of a contractile sheath surrounding a hollow inner tube, with a baseplate at one end from which tail fibers extend outward (Ge et al., 2020; Patz et al., 2019). When the tail fibers of an R-type tailocin bind to a receptor on the surface of a susceptible cell, the sheath contracts and drives the rigid tube through the cell envelope, creating a channel that collapses the proton motive force and causes rapid cell death (Fraser et al., 2021; Ge et al., 2020; Uratani & Hoshino, 1984). Under laboratory conditions, a single particle is sufficient to kill one cell (Carim et al., 2021; Scholl & Martin, 2008), although in natural settings non-specific adsorption to cell debris or free polysaccharides may reduce the effective number of particles available (Yao et al., 2017). Because each particle can be used only once and its release is fatal to the producing cell, tailocin deployment carries an irreversible cost that is absent from most other competitive mechanisms (Backman, Burbano, et al., 2024; Nobrega et al., 2018).

The specificity of tailocin killing is determined by the tail fibers, which recognise primarily lipopolysaccharide (LPS) components on the target cell surface (Kohler et al., 2010; Meadow & Wells, 1978; Yao et al., 2017). Because LPS structure varies among bacterial strains, tailocins have traditionally been described as narrow-spectrum weapons that target close phylogenetic relatives, and they have consequently been framed as mediators of kin competition (Backman, Burbano, et al., 2024; Scholl, 2017). Killing activity that extends well beyond close neighbours has, however, been reported under laboratory conditions in several systems: tailocins from *Burkholderia cenocepacia* can kill *Pseudomonas aeruginosa* (Yao et al., 2017), those from *Pseudomonas fluorescens* suppress the plant pathogen *Xanthomonas vesicatoria* (Principe et al., 2018), and tailocins of *P. syringae* target not only other plant-associated bacteria but also the human pathogen *Salmonella enterica* (Weaver et al., 2022). None of these cross-genus interactions has been demonstrated in natural plant environments. Tailocins from *Pseudomonas fluorescens* SF4c have been evaluated as a biocontrol agent against plant pathogens under greenhouse conditions (Principe et al., 2018), but whether tailocins contribute to competitive outcomes in natural habitats remains an open question. What determines whether a given tailocin can target phylogenetically distant taxa is unclear,

but the repeated observation of cross-genus activity suggests that some LPS epitopes are conserved more broadly than the kin-killer framework assumes (Fautt et al., 2025).

The breadth of taxa that tailocins can target, despite the lethal cost of their production, suggests a strong selective advantage (Backman, Burbano, et al., 2024). Consistent with this, tailocin gene clusters are found across a broad range of bacterial taxa. Among Gram-negative bacteria, tailocin loci have been identified in *Pseudomonas*, *Escherichia*, *Pectobacterium*, *Burkholderia*, *Xenorhabdus*, and *Serratia* (Burk, 2024; Jabrane et al., 2002; Kageyama et al., 1964; Nguyen et al., 1999; Thaler et al., 1995; Yao et al., 2017). Tailocin production is not limited to Gram-negative species: R-type tailocins or their gene clusters have also been described in *Clostridioides* and *Listeria* (Gebhart et al., 2012; Sigal et al., 2024), in which the target receptors are not yet defined, as these bacteria lack LPS. Phylogenetic analyses suggest that tailocin loci in both Gram-negative and Gram-positive lineages arose through multiple independent domestication events rather than from a single ancestor (Backman, Burbano, et al., 2024; Ghequire & De Mot, 2015). This recurrence implies that the fitness advantage conferred by tailocins has repeatedly outweighed their cost across varied bacterial lineages (Backman, Burbano, et al., 2024).

Tailocin gene clusters are regulated by the SOS response, which is activated upon DNA damage through the RecA and LexA proteins (Blasco et al., 2023; Penterman et al., 2014). In the laboratory, tailocin production is most commonly triggered by mitomycin C or UV irradiation, both of which cause DNA lesions that activate the SOS cascade (Principe et al., 2018; Vacheron et al., 2021; Yao et al., 2017). Mitomycin C has no established role as an environmental stressor; UV irradiation is more plausibly relevant, particularly for bacteria colonising aerial plant surfaces (Vorholt, 2012), but the doses required for tailocin induction have not been compared with those encountered in the phyllosphere. What triggers tailocin production under natural conditions therefore remains unresolved. In plant-associated environments, a plausible candidate is the oxidative stress that bacteria encounter during infection of plant tissue: when plant cells detect microbial challenge, NADPH oxidases and cell wall peroxidases generate a rapid local accumulation of reactive oxygen species (ROS), including hydrogen peroxide, in a response known as the apoplastic oxidative burst (Bolwell et al., 2002; Torres et al., 2006; Van Gijsegem et al., 2017). Because H_2O_2 causes oxidative DNA damage in bacteria (Goerlich et al., 1989; Imlay & Linn, 1987), it could in principle activate the

SOS response and thereby trigger tailocin production in bacteria colonising infected plant tissue. If this occurs, plant innate immunity would link directly to interbacterial competition among colonising bacteria, and the high cell densities typical of plant surfaces and the rhizosphere would place producers and potential targets within the effective range of released particles. At the time this thesis work was initiated, however, this possibility had not been tested, and more broadly, the role of tailocins in Soft Rot Pectobacteriaceae – bacteria that routinely encounter such oxidative stress during plant infection – had received little experimental attention.

Aims and scope of this thesis

The overarching aim of this thesis was to establish the role of tailocins as a component of the competitive arsenal of Soft Rot Pectobacteriaceae and to determine the scope of their killing activity, from interactions among closely related SRP strains to cross-genus targeting of phylogenetically distant bacteria. Prior to this work, the only R-type tailocin described in detail from SRP was carotovoricin Er from *Pectobacterium carotovorum* (Nakayama et al., 2000; Yamada et al., 2006), and no tailocin had been characterised in any *Dickeya* species. To address this gap, the thesis is built around four questions:

1. **Can a functional tailocin be identified and characterised in *Dickeya* spp., and what are its structural, genetic, and physicochemical properties?**

2. **Which conditions trigger tailocin production, and are any of these conditions encountered by SRP in their natural habitats?**

3. **How widespread is tailocin production among environmental SRP strains, and what patterns of killing emerge when diverse strains interact?**

4. **Does tailocin killing extend beyond SRP to phylogenetically distant bacteria that share the same plant-associated environments?**

Chapter 1 (Borowicz et al., 2023) explores whether a functional tailocin can be identified in *Dickeya* and what its properties are. As a result of my study, I reported the first R-type tailocin from *Dickeya dadantii* strain 3937, designated dickeyocin P2D1. Further, the study identified a 22-kb genomic cluster with homology to the tail of *Peduovirus* P2, characterised the P2D1 particle by transmission electron microscopy, and determined its killing spectrum and environmental stability. P2D1 was shown to kill strains of several *Dickeya* species but not the tested *Pectobacterium* strains, and it retained activity across a wide range of pH and temperature conditions relevant to plant habitats, while being inactivated by freezing and strong acidity.

Chapter 2 (Sobolewska, Krzyżanowska, **Borowicz et al., 2025**) addresses the question of what triggers tailocin production and whether any of these triggers are ecologically relevant. I contributed to resolving the temporal dynamics of P2D1 production and to identifying new inducers. Expression of tailocin structural genes peaked within two hours of

induction, whereas particle accumulation in the culture supernatant peaked at six hours and remained stable for at least eighteen hours. Beyond mitomycin C, the study demonstrated that hydrogen peroxide, norfloxacin, and ciprofloxacin all trigger P2D1 production, consistent with SOS-dependent regulation. The finding that H_2O_2 induces tailocin production is of particular ecological relevance, because SRP bacteria encounter plant-derived H_2O_2 during the oxidative burst that accompanies infection.

Whether tailocin production is restricted to individual reference strains or is a common trait among environmental SRP populations was unknown. **In Chapter 3 (Borowicz et al., 2025)**, I addressed this question by surveying tailocin production across 27 SRP strains isolated from the Durance River in France, an aquatic habitat that connects ecosystems in which SRP are found. The study showed that 88% of tested strains produce tailocin particles and mapped 351 pairwise killing interactions among them. *Dickeya* strains showed a higher incidence of killing (57%) than *Pectobacterium* strains (21%). Moreover, *Dickeya* tailocins can target strains across genus boundaries within SRP, whereas *Pectobacterium* tailocins act only within their own genus. Genomic comparison revealed that *Pectobacterium* and *Dickeya* tailocin clusters differ in gene organisation and sequence, supporting independent origins from different phage donors. The study also provides the first detection of tailocin particles in experimentally infected potato tissue, indicating that these particles are produced during plant infection.

Chapter 4 (Borowicz et al., 2026) investigated whether the killing range of dickeyocin P2D1 extends beyond SRP to phylogenetically distant bacteria that share the same plant-associated environments. I screened 480 soil and rhizosphere isolates from plant-associated habitats in Poland and identified nine *Pseudomonas* strains susceptible to P2D1, spanning several phylogenetic clades. Deletion of the genes encoding the P2D1 sheath and tube in *D. dadantii* 3937 abolished killing, confirming that the observed activity is tailocin-mediated. Six of the nine susceptible *Pseudomonas* strains are non-pathogenic and can suppress *D. dadantii* soft rot on potato, whereas the remaining three cause soft rot themselves under permissive conditions, indicating that P2D1 can eliminate bacteria with contrasting ecological roles relative to each other and to the tailocin producer. These results demonstrate that the killing range of tailocins produced by SRP is not restricted to close relatives but can extend to ecologically co-occurring bacteria across genera.

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Chapter 1

Soft rot pathogen *Dickeya dadantii* 3937 produces tailocins resembling the tails of *Peduovirus* P2

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Soft rot pathogen *Dickeya dadantii* 3937 produces tailocins resembling the tails of *Peduovirus* P2

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Tailocins are nanomolecular machines with bactericidal activity. They are produced by bacteria to contribute to fitness in mixed communities, and hence, they play a critical role in their ecology in a variety of habitats. Here, we characterized the new tailocin produced by *Dickeya dadantii* strain 3937, a well-characterized member of plant pathogenic Soft Rot *Pectobacteriaceae* (SRP). Tailocins induced in *D. dadantii* were *ca.* 166 nm long tubes surrounded by contractive sheaths with baseplates having tail fibers at one end. A 22-kb genomic cluster involved in their synthesis and having high homology to the cluster coding for the tail of the Peduovirus P2 was identified. The *D. dadantii* tailocins, termed dickeyocins P2D1 (phage P2-like dickeyocin 1), were resistant to inactivation by pH (3.5–12), temperature (4–50°C), and elevated osmolarity (NaCl concentration: 0.01–1 M). P2D1 could kill a variety of different *Dickeya* spp. but not any strain of *Pectobacterium* spp. tested and were not toxic to *Caenorhabditis elegans*.

KEYWORDS

bacteriophage, phage tail, tailocin, *Erwinia chrysanthemi*, bacteriocin, bacteria-bacteria interactions, *Caenorhabditis elegans*

Introduction

Under natural conditions, bacterial species inhabit shared environments, developing spatial and temporal interspecies associations and communities having complex networks of interactions (Little et al., 2008; Gorter et al., 2020). In such communities, a particular member needs to continuously compete for limited resources (i.e., scarce nutrients and limited space) with most other members of the community to gain a competitive edge (Bauer et al., 2018; Wagner, 2022). Given such challenging conditions, bacteria have evolved diverse strategies to successfully coexist with both closely and distantly related microbes (Granato et al., 2019). Such strategies, although employing a broad range of mechanisms, can be distinguished as either (1) indirect, exploitative competition that occurs through the

consumption of resources and (2) direct, interference competition, where individual cells directly kill one another, limiting their lifespan (Ghoul and Mitri, 2016). Whereas exploitative competition depends primarily on the utilization of limited resources by a strain, thereby restricting it from the competitor, interference competition relies on producing various antimicrobial agents that aim to kill other cells (Hibbing et al., 2010). These antimicrobials include, but are not limited to, broad-spectrum antibiotics, toxins, contact-dependent inhibition, effectors transported via type VI secretion system (T6SS effectors), low molecular weight bacteriocins, and tailocins (Stubbendieck and Straight, 2016; Granato et al., 2019). A given bacterial cell may often use several such systems to gain fitness advantages in the environment (Hibbing et al., 2010). Among the systems bacteria exploit to fight competitive microbes, tailocins are now receiving increasing attention (Scholl, 2017; Patz et al., 2019).

Tailocins are syringe-like nanomolecular entities that are evolutionary and morphologically related to bacteriophage tails, type VI secretion systems, and extracellular contractile injection systems (Scholl, 2017). These particles, also known as high molecular weight bacteriocins or phage tail-like particles, are chromosomally encoded and ribosomally synthesized toxins that usually express a narrow killing range, interacting only with closely-related bacterial species that typically would occupy the same niche (Patz et al., 2019). These agents adsorp to the surface of susceptible cells, thereby puncturing the cell envelope, leading to depolymerization of the cell membrane and, ultimately, the death of the attacked cell (Ge et al., 2015).

Tailocins are classified into two distinct families: rigid and contractile (R-type) and noncontractile but flexible particles (F-type; Ghequire and De Mot, 2015). The R-type tailocins have features of tails of *Peduovirus* P2 or T-even bacteriophages infecting *Escherichia coli*. In contrast, the F-type tailocins resemble the flexible tails of bacteriophage lambda (λ). Although tailocins exhibit remarkable morphological similarity to bacteriophage tails of the viruses mentioned above, it is now believed that they have evolved independently from bacteriophages and should not be considered exclusively as domesticated prophages or phage remnants that bacteria harness for their advantage (Scholl, 2017).

The production of tailocins has been demonstrated both in Gram-negative and Gram-positive bacterial species. Producing strains include both human, animal, and plant pathogens and saprophytic bacteria residing in various environments (Morales-Soto et al., 2012; Liu et al., 2013; Ghequire and De Mot, 2014; Gebhart et al., 2015). Until recently, tailocins have been best characterized in *Pseudomonas* species (Michel-Briand and Baysse, 2002; Fischer et al., 2012). There are, however, reports of tailocins isolated from *Clostridioides* spp., *Serratia* spp., *Xenorhabdus* spp., *Burkholderia* spp., *Kosakonia* spp., *Budvicia* spp., *Pragia* spp., *Pectobacterium* spp. as well as from other bacteria (Smarda et al., 2005; Becker et al., 2022).

The omnipresence of tailocins in phylogenetically unrelated bacterial genera suggests that these particles are important for fitness in various habitats (Scholl, 2017). However, the ecological role of tailocins in the natural environment of the producing strains has received little attention, especially for plant-pathogenic bacteria residing in agricultural locations. This issue is important given the diversity of conditions such bacteria encounter in such natural settings. No comprehensive studies have addressed tailocins produced by Soft Rot *Pectobacteriaceae* (SRP) bacteria (Van Gijsegem et al., 2021), which, due to their complex lifestyle, have a variety of spatial and temporal interactions in varied environments (Perombelon, 2002; Charkowski, 2007, 2018).

Plant pathogenic SRP (consisting of *Pectobacterium* spp., *Dickeya* spp., and *Musicola* spp., formerly characterized as pectinolytic *Erwinia* spp.) are a useful model for studying the environmental role of tailocins. SRP bacteria are widespread in various ecological niches, including rain and surface water, natural and agricultural bulk and rhizosphere soils, sewage, the exterior and interior of host and non-host plants as well as the surface and interior of insects (Van Gijsegem et al., 2021). Because of the diverse environments in which SRP bacteria may be found, these pathogens may encounter various other bacteria with whom they must effectively compete.

This study aimed to assess the presence and activity of tailocins induced and isolated from pectinolytic *Dickeya dadantii* strain 3937 (Kotoujansky et al., 1982). This strain (formerly *Erwinia chrysanthemi* and *Pectobacterium chrysanthemi*; Samson et al., 2005) is a well-known necrotrophic plant pathogen that causes soft rot disease in a variety of crop, ornamental, and other nonfood plants worldwide, causing losses in agriculture (Kotoujansky, 1987). Strain 3937 has been widely used as a potent model system for research on the molecular biology and pathogenicity of bacteria belonging to Soft Rot *Pectobacteriaceae* for several decades (Reverchon and Nasser, 2013; Reverchon et al., 2016). While this strain continues to be the most studied strain of all *Dickeya* species its production of tailocins has not been previously described. Here, we characterize for the first time the tailocin produced by *D. dadantii* strain 3937.

Results

Dickeya dadantii 3937 produces tailocins

Cells of *D. dadantii* 3937 treated with mitomycin C produced syringe-like macromolecular structures resembling bacteriophage tails. Following convention, the tailocins produced by *D. dadantii* were named dickeyocins. Imaging using TEM and AFM revealed that these structures consist of a central rod-shaped core (tube) wrapped in a contractable sheath (Figure 1). Both the tube and the sheath were built of multiple protein subunits, with a clearly visible helical arrangement of the subunits in the sheath. The average length of the dickeyocins produced by strain 3937 was 166 ± 7 nm. When the sheath entirely covered the tube (in the extended, "loaded" form), the individual dickeyocin has a diameter of 23 ± 2 nm. Fibers were visible at the distal end of the sheath. When the sheath contracts, it revealed an internal tube of a length of 92 ± 7 nm with an attached spike at the distal end (Figure 1).

The yield of tailocins purified from mitomycin-induced cells of *D. dadantii* 3937 equaled approx. 10^{11} particles mL^{-1} of culture. The particles could also be isolated from non-treated cells, indicating a low basal production level. However, induction with mitomycin C increased the yield approx. 10–100-fold. We employed three different methods to estimate the concentration of phage-tail-like particles in the tested preparations. The results were consistent for the independently obtained batches of purified dickeyocins (Supplementary Table S2). The average concentration from three independently obtained samples was 10^6 relative units (AU) mL^{-1}, 10^{11} killing particles mL^{-1}, and 10^{11} particles mL^{-1}, according to the spot test, the

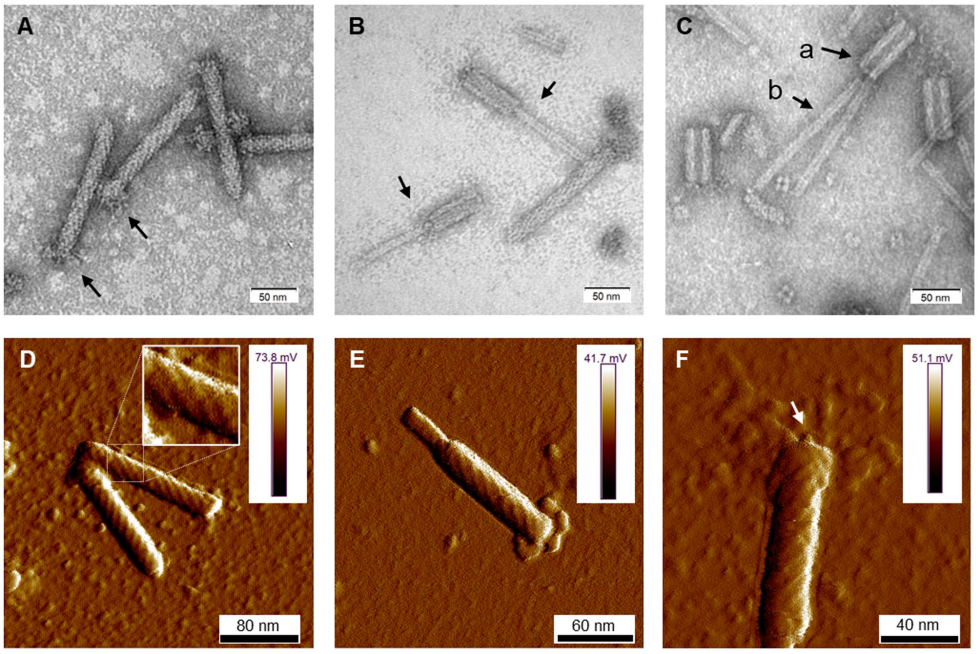

FIGURE 1
The morphology of tailocins from *Dickeya dadantii* 3937. Images were obtained using TEM **(A–C)** and AFM **(D–F)**. The individual panels show: **(A)** particles in an extended (active) form. Arrows indicate the positioning of fibers; **(B)** contracted particles indicated by arrows; **(C)** tailocins disintegrated by heat treatment (50°C); Arrows indicate: **(a)** empty sheath with a hollow inner channel and **(b)** tube (core) separated from the sheath; **(D)** two extended particles. A fragment of the photo was magnified to display the helical arrangement of protein subunits in the sheath; **(E)** a contracted molecule in an AFM image; and **(F)** an extended particle with an arrow pointing at the presumed tube-attached spike.

Poisson distribution killing method, and the NanoSight measurements, respectively. Furthermore, comparing the results from the activity-based Poisson method and the direct particle count indicated that most phage-like particles purified from the cultures of *D. dadantii* strain 3937 were undamaged and in the extended ("loaded") form. This was in line with the microscopic observations done with TEM and AFM.

Dickeyocins from *Dickeya dadantii* strain 3937 are phylogenetically related to the tail of *Peduovirus* P2

Proteins in the dickeyocins were separated by SDS-PAGE and sequenced. Eight cleary distinguishable bands were excised from the gel, and the digested peptides were analyzed by MS. The peptides could be readily mapped to six *D. dadantii* proteins with annotations implying their phage relationship: phage baseplate assembly protein (encoded in locus Dda3937_00029/DDA3937_RS12055), baseplate assembly protein J (Dda3937_00030/DDA3937_RS12060), tail fiber protein (Dda3937_04606/DDA3937_RS12070), putative side tail fiber protein (Dda3937_03808/DDA3937_RS12100), major sheath protein (Dda3937_03810/DDA3937_RS12110), and major tail tube protein (Dda3937_03811/DDA3937_RS12115; Figure 2). Genes encoding these six proteins were mapped to a single *ca.* 22-kb region in the genome of *D. dadantii* strain 3937 (GenBank accession number: NC_014500.1: genome location: 2,734,508–2,757,061; Figure 2C). This genomic region contained 28 genes, from which 16 genes encoded various bacteriophage structural proteins.

The nucleotide sequence of the 22-kb putative dickeyocin-encoding fragment in *D. dadantii* strain 3937 showed the highest similarity to a prophage region in *D. dadantii* strain XJ12 [100% query coverage (qc), 99.35% identity]. Highly similar regions were also found in the genomes of several other strains of *D. dadantii*, as well as *D. solani*, *D. dianthicola*, and *D. fangzhongdai* (Supplementary Data S1). Strains of *D. zeae* showed lower similarity, with query coverage between 54 and 76% and identity of 83–84%. A much lower score was observed for the next best hit—*Musicola paradisiaca* (formerly *Dickeya paradisiaca*). Other non-*Dickeya* microorganisms with regions showing some degree of homology included *Serratia* sp. ATCC 39006 (Supplementary Data S1; with 10% query coverage and 81.45% identity).

Importantly, at the nucleotide level, the dickeyocin region of *D. dadantii* strain 3937 showed no significant similarity to the known carotovorocin Er cluster of *P. carotovorum* Er (Genbank accession: AB045036; Yamada et al., 2006). Moreover, the amino acid sequence of the sheath protein of tailocin from strain 3937 showed only a 34% identity to that of carotovorocin Er, with a query coverage of 83%.

The *ca.* 22-kb cluster encoding dickeyocin from *D. dadantii* strain 3937 was also surveyed against the collection of viral sequences (NCBI taxid: 10239), yielding only low similarity scores (7% query coverage, 78% identity for the best hit).

Furthermore, in an attempt to find undomesticated phages related to dickeyocin we searched the viral protein database, using three structural proteins of dickeyocin as queries: sheath protein (WP_013318223), tail tube protein (WP_013318224), and baseplate assembly protein J (WP_013318212). Based on this search, proteins in dickeyocin exhibited homology to proteins of *Salmonella* phages

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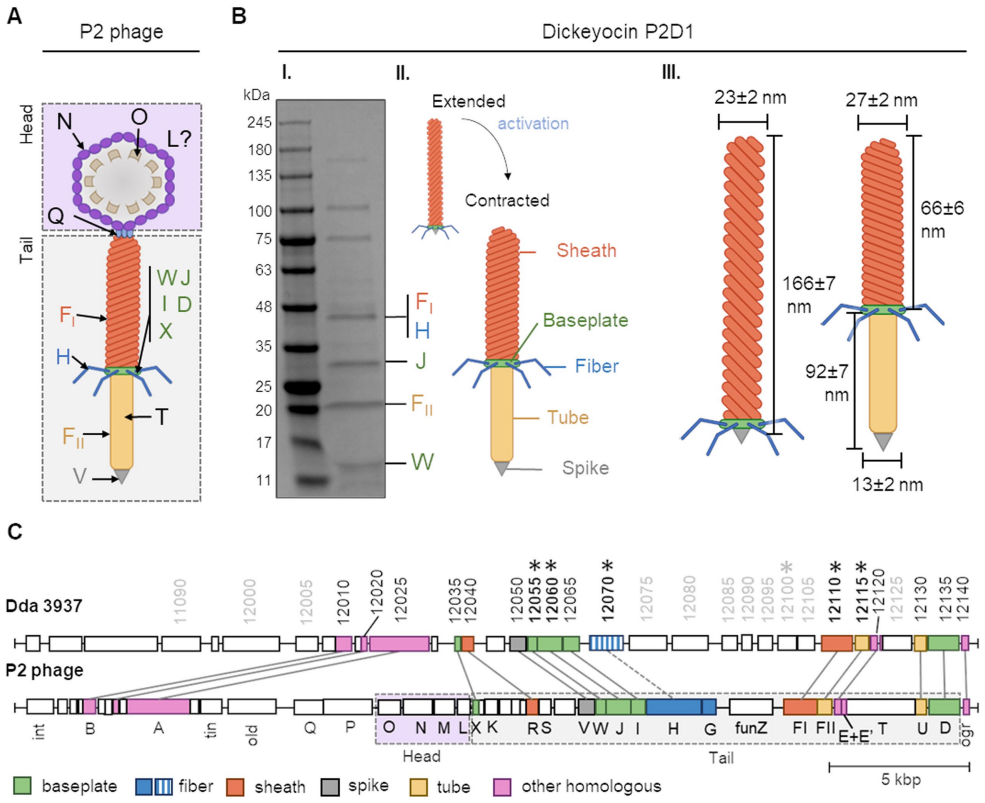

FIGURE 2
The building blocks of tailocins of *Dickeya dadantii* strain 3937 and their encoding genes in relation to those of the *Peudovirus* P2. Panel **(A)** shows a schematic representation of the structure of the P2 phage, together with the nomenclature of the building proteins. The graph was prepared based on Christie and Calendar (2016) (PMID: 27144088, https://pubmed.ncbi.nlm.nih.gov/27144088/). WJIDX—baseplate proteins, F_I—sheath, H—fiber, F_{II}—tube, V—spike, T—tape measure protein, Q—portal protein, N—major capsid protein, O—capsid scaffold, and L—head completion protein. Panel **(B)** shows: **(I)** an SDS-PAGE separation of proteins from mitomycin-induced cultures of *D. dadantii* 3937. Bands containing proteins homologous to those of the P2 phage are provided with the respective protein designations; **(II)** schematic representation of extended and contracted forms of dickeyocin P2D1; and **(III)** dimensions of extended and contracted forms of dickeyocin P2D1. Panel **(C)** shows the alignment between the complete genome sequence of the P2 phage (NC_001895; 33,593 bp) and a region of the same length in the genome of *D. dadantii* 3937 (NC_014500.1; range: 2,723,487–2,757,061). The numbering of genes in *D. dadantii* corresponds to the numbering of loci in the genome (locus tag prefix DDA3937_RS). Homologous proteins are marked with the same color.

SW9 and PSP3, *Erwinia* phage Etg, *Enterobacteria* phage fiAA91-ss, *Peduovirus* P2, several *Escherichia* and *Yersinia* phages, as well as to multiple poorly-characterized phages of bacteria in class *Caudoviricetes*, derived from the human metagenome (Supplementary Data S1). The best-characterized phage having a high homology to dickeyocin was *Peduovirus* P2—a phage that infects *Escherichia coli* and other hosts, including *Salmonella* and *Klebsiella* (Bertani, 1951; Christie and Calendar, 2016). For the three investigated dickeyocin proteins, their amino acid identity toward their P2 homologs ranged from 70 to 79%, with 100% query coverage (Supplementary Data S2). Therefore, we used the *Peduovirus* P2 bacteriophage as a reference to assign functions to proteins in dickeyocin, as well as to investigate the genetic rearrangements in the dickeyocin cluster in relation to the fully-functional phage P2 (Figure 2C). The dickeyocin cluster in the genome of *D. dadantii* strain 3937 lacked sequences associated with the phage head (proteins O, M, N, L), as well as the tape measure protein. There was also a difference in the genetic content of the intergenic region of the tail, as well as low homology of proteins building the tail fibers (31% query coverage, 54% identity). Following the present naming convention, we named the newly characterized tailocins from *D. dadantii* 3937 dickeyocin P2D1 (P2-like Dickeyocin 1).

Dickeyocin P2D1 expresses bactericidal activity exclusively against members of soft rot *Pectobacteriaceae*

Fifty-two bacterial strains were surveyed for their sensitivity to tailocins induced from *D. dadantii* strain 3937. These included 41 strains of different species and subspecies of *Dickeya* and *Pectobacterium*, as well as six other bacterial strains belonging to the *Enterobacteriaceae* family, a single strain of *Serratia marcescens* (family *Yersiniaceae*), three *Pseudomonas* spp., and a single strain of *Staphylococcus aureus* representing Gram-positive bacteria (Supplementary Table S1). Bactericidal activity was observed against eight strains [*D. dadantii* subsp. *dieffenbachie* strain NCPPB 2976, *D. dianthicola* strains NCPPB 3534 and IPO 980, *D. undicola* CFBP 8650, *D. zeae* strains NCPPB 3532 and 3531, *D. oryzae* strain CSL RW192, and *Musicola paradisiaca* strain NCPPB 2511 (old name:

Dickeya paradisiaca strain NCPPB 2511)], but not against any of the *Pectobacterium* spp. tested (Supplementary Table S1; Figure 3). Likewise, dickeyocin P2D1 was inactive against any of the non-SRP strains included in the screening assay: six *Enterobacteriaceae* strains (*Citrobacter freundii* ATCC 8090, *Escherichia coli* ATCC 8739, *Escherichia coli* ATCC 25922, *Escherichia coli* OP50, *Klebsiella quasipneumoniae* ATCC 700603, and *Klebsiella aerogenes* ATCC 51697), three *Pseudomonas* spp.: (*Pseudomonas aeruginosa* PA14, *Pseudomonas aeruginosa* PAO1, and *Pseudomonas donghuensis* P482), *Serratia marcescens* ATCC 14756 and *S. aureus* ATCC 25923 indicating their limited range of bactericidal activity (Figure 3).

P2D1 killing efficiency is bacterial species-dependent

We determined the kinetics of killing of eight susceptible strains treated with P2D1. The share of intact cells at two time points: 20 and 120 min post treatment is shown in Supplementary Figure S1. In all the cases, the killing of the susceptible cells was fast; in the first 20 min, the most killing effect was observed for *D. dianthicola* strain IPO 980 (*ca.* 60% reduction of cell numbers). In contrast, the slowest killing caused by dickeyocin P2D1 was observed for *D. dianthicola* strain NCPP 3534 (*ca.* 22% reduction of cell numbers). For the other six strains tested, on average, a 45–60% reduction in cell numbers was observed after this same time (20 min.). About 50–60% killing of all strains by dickeyocin P2D1 was seen after 120 min-incubation (Supplementary Figure S1).

P2D1 dickeyocin directly punctures the cell envelope of susceptible bacterial strains

We assessed the interaction of dickeyocin P2D1 on cells of the susceptible *M. paradisiaca* strain at the single-cell level using transmission electron microscopy (TEM). Images clearly showed that

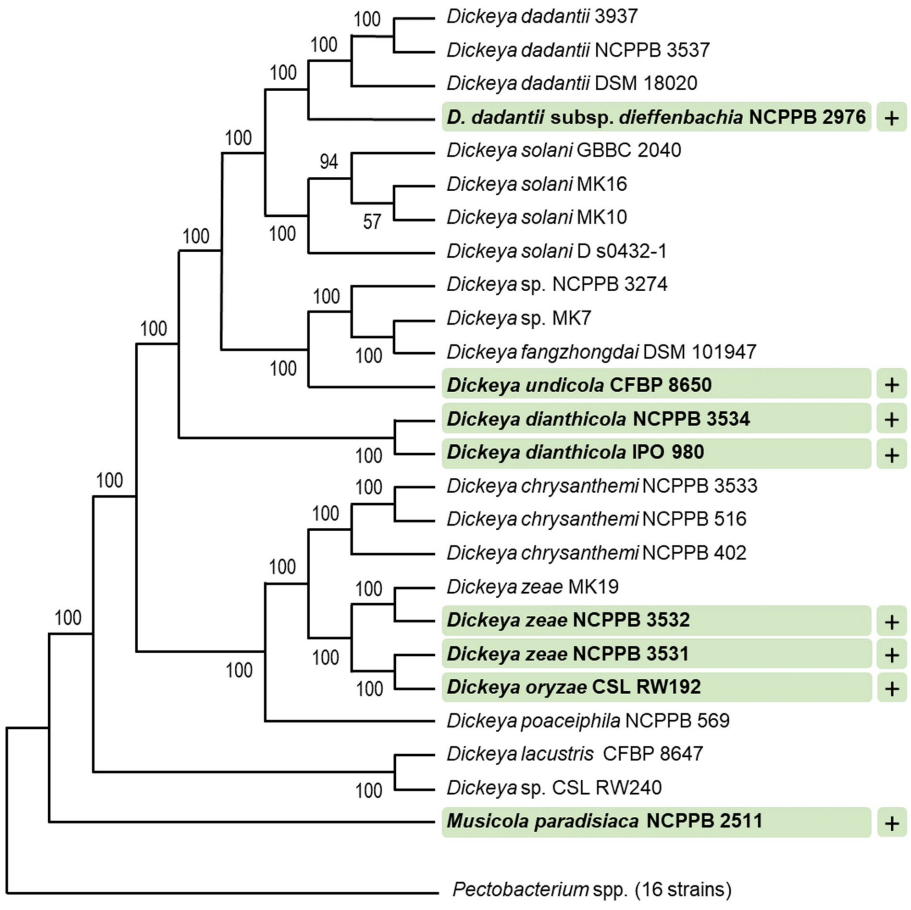

FIGURE 3
Target range of tailocins from *Dickeya dadantii* 3937. Strains susceptible to P2D1 dickeyocin were marked with a plus (+). A phylogenetic tree for 41 SRP strains was obtained using EDGAR 3.2 Fasttree 2.1 algorithm. The tree was built out of a core of 1,616 genes per genome (nucleotide sequences), 66,256 genes in total. The core had 1,560,951 bp per genome, 63,998,991 in total. The lengths of the branches do not reflect the phylogenetic distance. All analyzed *Pectobacterium* spp. were collapsed. This included 16 strains: *Pectobacterium cacticida* CFBP 3628, *Pectobacterium fontis* CFBP 8629, *Pectobacterium betavasculorum* CFBP 2122, *Pectobacterium peruviense* CFBP 5834, *Pectobacterium atrosepticum* SCRI1043, *Pectobacterium atrosepticum* NCPPB 549, *Pectobacterium parmentieri* CFBP 8475, *Pectobacterium parmentieri* SCC3193, *Pectobacterium polonicum* DPMP 315, *Pectobacterium punjabense* CFBP 8604, *Pectobacterium actinidiae* LMG 26003, *Pectobacterium brasiliense* LMG21371, *Pectobacterium polaris* NCPPB 4611, *Pectobacterium versatile* CFBP 6051, *Pectobacterium carotovorum* CFBP 2046, and *Pectobacterium aroidearum* NCPPB 929. The numbers at the tree branches represent Shimodaira-Hasegawa (SH) branch support values (Shimodaira, 2002).

dickeyocin P2D1 adsorbed to the bacterial cell envelope (Figures 4A,B), followed by puncturing the outer cell membrane (resulting from the conformational change of dickeyocin P2D1 from an extended "loaded" to a contracted form; Figures 4C,D), creating a pore connecting the cytoplasm of the cell with the external environment and leading eventually to the death of the attacked cell.

Dickeyocin P2D1 is able to bind to nonviable (dead) bacterial cells

A cell adsorption assay was employed to investigate whether the dickeyocin P2D1 can adsorb to nonviable (dead) and viable bacterial cells of the susceptible or resistant bacterial species. Dickeyocin P2D1 was incubated either with viable or chloramphenicol-killed cells of susceptible *M. paradisiaca* or with viable or antibiotic-killed cells of resistant *D. dadantii* for 40 min and the remaining dickeyocin P2D1 in the medium was measured. Incubation of dickeyocin P2D1 both with the dead and viable cells of *M. paradisiaca* resulted in the total loss of the tailocin activity, indicating that it bound equally efficiently to viable as well as nonviable cells of the susceptible bacterium. No adsorption of dickeyocin P2D1 to either dead or alive cells of the resistant *D. dadantii* cells was observed (Supplementary Figure S2).

Production of dickeyocin P2D1 is not associated with T6SS

To determine whether dickeyocin P2D1 production in *D. dadantii* depends on the type VI secretion system (T6SS), dickeyocin P2D1 production was induced in *D. dadantii* mutant A5587 (Golanowska, 2015) that carries an insertional mutation in the *tssK* gene (type VI secretion system baseplate subunit TssK)—an essential subunit of the type VI secretion apparatus (Nguyen et al., 2017). The tailocins obtained from the *D. dadantii* T6SS mutant using standard mitomycin induction methods were morphologically indistinguishable and similarly abundant compared to dickeyocin P2D1 induced in the wild-type *D. dadantii* strain 3937 (Supplementary Figure S3). These results suggest that at least *tssK* gene, essential for the proper function of T6SS, does not affect dickeyocin P2D1 production, morphology, or release from the cell.

Activity of P2D1 dickeyocins is modulated by environmental conditions and enzyme treatments

Dickeyocin P2D1 was stable at temperatures between 4 and 42°C, showing no significant loss in activity following 24 h incubation at these temperatures. Temperatures above 50°C, however, led to a 32-fold loss in activity while incubation at 65 and 80°C (Figure 5A) led to a complete loss of activity. Likewise, a single freeze–thaw cycle negatively affected the stability of dickeyocin P2D1. Freezing at −20°C resulted in a 128-fold reduction in activity, while no bactericidal effect remained after freezing at −80°C (Figure 5A). We tested the stability of P2D1 under seven different pH conditions (2, 3.5, 5, 7, 9, 10.5, and 12). The particles remained active after 24 h incubation in pHs ranging from 3.5 to 12. However, their bactericidal properties were lost at pH 2 (Figure 5B). To determine whether dickeyocin P2D1 that was inactivated at low pH could be restored to an active (extended, "loaded") form, we diluted the pH 2-treated, inactive dickeyocin P2D1 in PBS buffered to pH 12 to achieve a neutral pH environment. Dickeyocin P2D1 did not regain bactericidal activity at pH 7. Osmotic

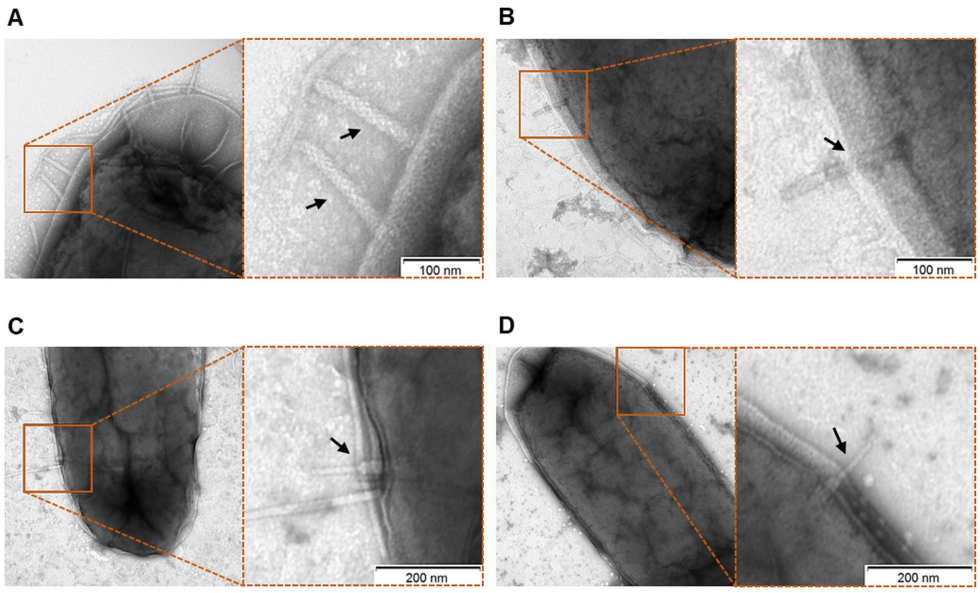

FIGURE 4
Interaction of P2D1 dickeyocins with the cells of a susceptible strain *Musicola paradisiaca* NCPPB strain 2511, evaluated using transmission electron microscopy (TEM). Bacterial cells of *M. paradisiaca* NCPPB 2511 from overnight culture in TSB were harvested and washed with PBS. Such prepared cells were incubated with PEG-purified dickeyocins (approximate concentration in bacterial suspension 10^6 particles mL^{-1}) for 20 min, followed by obtaining images with TEM. For all panels, the TEM images on the right are the enlarged sections of the original micrographs on the left. Arrows indicate P2D1 attached to the surface of bacterial cells, either in extended **(A,B)** or contracted **(C,D)** form.

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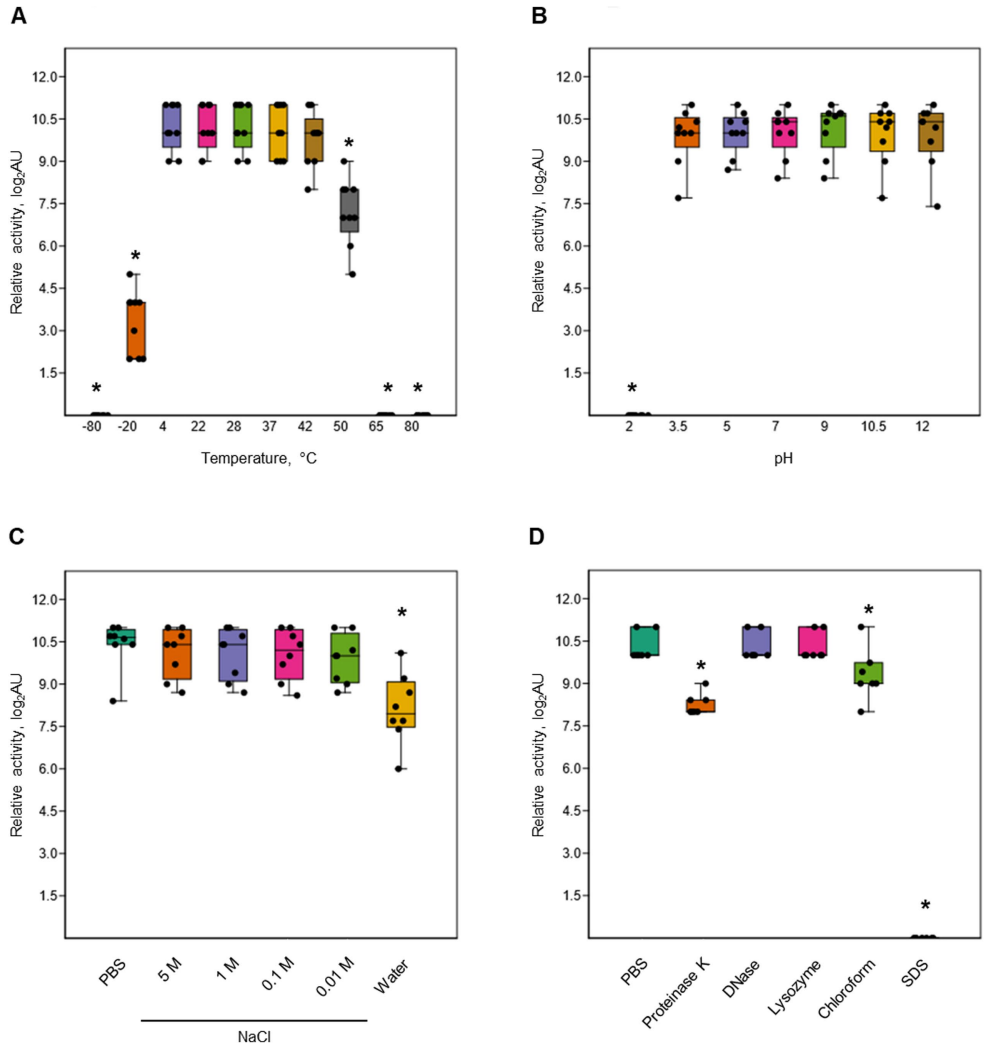

FIGURE 5
Stability of P2D1 dickeyocins from *Dickeya dadantii* strain 3937 upon different treatments. The graphs show the influence of temperature **(A)**, pH **(B)**, salinity **(C)**, as well as enzymatic digestion (proteinase K, DNase, and lysozyme), chloroform treatment, and SDS detergent **(D)** on bactericidal activity of P2D1 against *Musicola paradisiaca* NCPPB 2511. For temperature, pH/osmolarity, and enzyme/detergent/organic solvent treatments, dickeyocins stored in PBS pH 7, were used as a control. The results are shown as a box plot; whiskers reflect the maximum and minimum, box sides reflect the first and third quartile, and the bars reflect medians. Points indicate particular measurements ($n = 9$). The asterisks (*) represent statistically significant differences ($p < 0.05$) between the treatment and the control using the Mann–Whitney test.

conditions generated by NaCl added to water at concentrations between 0.01 and 5 M had no adverse effect on the activity of dickeyocin P2D1 within 24 h when compared to controls (dickeyocin P2D1 in PBS containing 0.137 M NaCl and 0.0027 mM KCl). In contrast, incubation of dickeyocin P2D1 in deionized water led to a 4-fold loss in activity (Figure 5C). Dickeyocin P2D1 activity was significantly ($p < 0.05$) reduced by treatment with proteinase K and chloroform for 1 h (4-fold and 2-fold loss of activity, respectively). Complete loss of activity was seen after incubation in 1% SDS. In contrast, neither lysozyme nor DNase influenced dickeyocin P2D1 activity (Figure 5D).

Dickeyocin P2D1 is nontoxic for *Caenorhabditis elegans*

No inhibition of the survival of *C. elegans* was seen after its exposure to high concentrations of purified dickeyocin P2D1 (Supplementary Figure S4). The average survival rate of *C. elegans* cultivated in the presence of P2D1 dickeyocins was *ca.* 98–99% and was not significantly statistically different from that of nematodes grown without dickeyocin P2D1. The survival of *C. elegans* was not dickeyocin P2D1-concentration-dependent, as similar survival rates were recorded for all dickeyocin concentrations tested (Supplementary Figure S4). Furthermore, dickeyocin P2D1 did not exhibit bactericidal activity against *E. coli* OP50—the food source for *C. elegans*.

Discussion

Our understanding of the mechanisms underlying bacterial competitive behaviors remains limited. Although numerous bacterial species are known to produce and exploit tailocins for their competitive advantage, little is known of how commonly such

entities are employed by plant-pathogenic bacteria, including pectinolytic, necrotrophic SRP pathogens, to enable their environmental fitness (Scholl, 2017; Patz et al., 2019). This study revealed the presence of tailocins in one of the broadest characterized and economically important member of the Soft Rot *Pectobacteriaceae* family—strain *D. dadantii* 3937. We identified and characterized in detail a novel phage tail-like particle—dickeyocin P2D1, produced by this strain not only upon mitomycin C treatment but also constitutively in culture. Although tailocins were initially noted in a limited number of *Dickeya* spp. (strains of *Erwinia chrysanthemi* dissimilar from strain 3937) isolated from several crop and ornamental plants in the past (1960 and 1970s; Echandi, 1979), these particles were not characterized in detail, probably due to the lack of appropriate molecular techniques at that time.

Dickeyocin P2D1 has a typical morphology and expresses features similar to those of other R-type tailocins described so far for a large group of bacteria, including *Escherichia coli* (Mennigmann, 1965), *Burkholderia cenocepacia* (Yao et al., 2017), *Pseudomonas aeruginosa* (Scholl, 2017), *Proteus vulgaris* (Coetzee et al., 1968), and *Yersinia enterocolitica* (Strauch et al., 2001). Likewise, tailocins were also identified in *Pectobacterium* spp. (Kamimiya et al., 1977; Choi and Kim, 1988; Yamada et al., 2006)—plant pathogens closely related to *Dickeya* spp. The tailocin of SRP bacteria best characterized to date is carotovorocin Er isolated from *P. carotovorum* Er (Kamimiya et al., 1977; Nguyen et al., 2002). Interestingly, as shown in this study, despite the morphological similarities of dickeyocin P2D1 and carotovorocin Er and the rather close phylogenetic relationship and the lifestyle shared by the producer strains, these tailocins show little sequence homology. This suggests that dickeyocin P2D1 and carotovorocin Er originated from different phage ancestors and that the ability to produce tailocins was acquired several times and independently by various members of the SRP family.

The cluster encoding dickeyocin P2D1 exhibits high homology with that encoding the tail of phage *Peduovirus* P2, indicating that these two probably had a common ancestor. Phage P2 is a temperate bacteriophage commonly found in genomes of strains belonging to the *Pseudomonadota* phylum (Christie and Calendar, 2016). Its presence has been noted in at least 127 genera in 32 *Pseudomonadota* families (Casjens and Grose, 2016), indicating its extreme ubiquitousness in the bacterial world. Accordingly, R-type tailocins resembling the tail of phage P2 have been extensively studied and are now the best characterized to date (Scholl, 2017; Granato et al., 2019). To explore the occurrence of R-type tailocins in SRP, we searched available *Dickeya* spp. and *Pectobacterium* spp. genomes for regions carrying phage tail-like genes homologous to those encoding dickeyocin P2D1 and having organizational similarity. Our analysis revealed the presence of P2D1-like clusters in several *Dickeya* spp. strains, including *D. solani*, *D. dianthicola, D. zeae*, and *D. fangzhongdai*. The P2D1-like clusters were, however, absent in all *Pectobacterium* spp. genomes as well as in genomes of the other bacterial species unrelated to SRP analyzed in our study. The lack of the dickeyocin P2D1 cluster both in *Pectobacterium* spp. and in bacteria phylogenetically distant to SRP may indicate a strong and sustained phylogenetic association between dickeyocin P2D1and *Dickeya* spp. This is somewhat unexpected given that various *Dickeya* and *Pectobacterium* are often present in the same environment (e.g., soil, water, and plant surface; Van Gijsegem et al., 2021). Under such conditions, horizontal gene transfer would frequently occur between these phylogenetically related SRP species (Toth et al., 2021).

The restricted occurrence of genes encoding dickeyocin P2D1 in the genomes of *Dickeya* spp. aligns with its narrow bactericidal activity. In our study, dickeyocin P2D1 exclusively targeted *Dickeya* species as well as *Musicola paradisiaca* (former *Dickeya paradisiaca*) but not any other bacteria tested. It is noteworthy, however, that these *Dickeya* strains differed somewhat in the susceptibility to dickeyocin P2D1 while these differences were unrelated to the phylogenetical distance between them. This is in line with other studies (Chopra et al., 2015; Fagundes et al., 2016; Principe et al., 2018) that showed that the host range of pyocins, tailocins produced by *Pseudomonas* spp., is usually restricted to kin strains (Patz et al., 2019). Likewise the tailocins of *Escherichia coli* (Tantoso et al., 2022) and *Yersinia enterocolitica* (Strauch et al., 2001) also killed only related taxa. Contrarily, some recent reports have revealed tailocins that target different species of the same genus and/or even different genera. For example, *Burkholderia cenocepacia* tailocins were active against other *Burkholderia* species (Yao et al., 2017) and those of *P. fluorescens* were active against *Xanthomonas vesicatoria* (Principe et al., 2018). The quite narrow target range of dickeyocin P2D1 is somewhat surprising given that multiple species of SRP bacteria are often found together in the same infected plant (Perombelon, 1988; Pérombelon, 1992; Ge et al., 2021). In such a setting, these various species would be expected to experience intense competition and would be expected to benefit from a promiscuous tailocin (Shyntum et al., 2019). It thus appears that in such mixed infections, *Dickeya* spp. and *Pectobacterium* spp. compete using mechanisms unrelated to phage-tail-like particles, as reported previously (Ma et al., 2007; Czajkowski et al., 2013), but this conjecture requires more experimental support. Similarly, the presumed bacterial receptor for P2D1 remains unidentified. A number of tailocins that engage with *Pseudomonas* spp. bacteria rely on lipopolysaccharide (LPS) components as their receptors (Scholl, 2017). It is plausible to consider that P2D1 may also engage with the bacterial LPS of susceptible hosts, but further research is required to investigate this interaction on a molecular level.

Our study revealed that dickeyocin P2D1 is produced even without induction with mitomycin C, although such induction boosts the tailocin production 10 to 100-fold. To precisely estimate the concentration of P2D1 particles, we used a well-established Poisson distribution killing method (Kageyama et al., 1964; Williams et al., 2008) as well as a new approach we developed. The Poisson distribution killing method is an indirect enumeration method as it employs the number of survivors in tailocin-treated bacterial suspensions. Advantage of indirect methods is that they provide the number of active ("loaded") tailocin particles in a sample. In this study, we have complemented the indirect counting approach by direct enumeration of tailocin particles with NanoSight NS300. Direct particle counts avoid the need for culturing and, when combined with killing-based methods, enables an estimation of the fraction of active tailocins in preparation. This appears to be a novel application of NanoSight to evaluate the quality and the active fraction of tailocin preparations.

One of the immediate applications of dickeyocin P2D1 would be in controlling SRP infections in crops (Becker et al., 2022). A major practical limitation of tailocins as therapeutic agents is their narrow bactericidal range. This can, however, be at least partially overcome by using cocktails of tailocins having different bactericidal ranges,

similarly as in the use of bacteriophages (Abedon et al., 2021). Despite this limitation, tailocins have shown to be effective antibacterial agents in various applications (Scholl and Martin, 2008; Behrens et al., 2017). They have several benefits compared to other biological control agents evaluated to date (Becker et al., 2022). One of the biggest advantages of tailocins is their incapacity to proliferate on-site after application. Likewise, tailocins themselves cannot spread via transduction or transformation mechanisms because only the protein products and not the encoding nucleic acids are employed. The high target selectivity of tailocins also prevents disruption of other, often beneficial bacteria present in the same niche. Likewise, since dickeyocin P2D1 was nontoxic to *C. elegans*, a model eukaryotic organism, such tailocins are unlikely to impact other eukaryotic organisms present in soil and/or on plants (Balciunas et al., 2013). Additional studies are needed to fully explore the potential of dickeyocin P2D1 to achieve plant disease control, such as targeting production, the long-term effectiveness, and consistency of control under field conditions, including stability, formulation, and eco-toxicological risks. Studies of the *in planta* expression of dickeyocin P2D1 should provide great insight into the interactions of *Dickeya* species with each other and with other bacteria under natural and agricultural conditions.

Materials and methods

Bacterial strains and culture conditions

All bacterial strains included in this study are listed in Supplementary Table S1. Bacteria were routinely propagated in liquid Trypticase Soy Broth (TSB, Oxoid) or on solid Trypticase Soy Agar (TSA, Oxoid) at 28°C for 24 h. Liquid cultures were agitated during incubation (120 rpm). When required, bacterial cultures were supplemented with kanamycin (Sigma-Aldrich) to a final concentration of 50 μg mL^{-1}.

Induction, purification, and concentration of tailocins from *Dickeya dadantii* 3937 culture

Dickeya dadantii strain 3937 was grown overnight (*ca.* 16 h) in TSB at 28°C with shaking (120 rpm). The cultures were then rejuvenated by diluting them 1: 40 in 250 mL of fresh TSB medium. The diluted culture grew for 2.5 h under the same conditions. Such prepared 3937 cultures were then supplemented with mitomycin C (Abcam, Poland) to a final concentration of 0.1 μg mL^{-1} to induce the production of tailocins. Following mitomycin C treatment, the cultures were incubated for another 24 h at 28°C with shaking (120 rpm). Bacterial cells were then removed by centrifugation (10 min, 8,000 RCF), and the supernatant containing putative particles was collected and filtered through a sterile 0.2 μm PES (polyether sulfone) membrane filter using the Nalgene Rapid-Flow Sterile Disposable Filter Units (Thermo Fisher Scientific). Finally, to precipitate tailocins, PEG-8000 (Promega) was added to the filtrate to a final concentration of 10%, and the sample was incubated at 4°C on a magnetic stirrer for the next 16–24 h. The tailocins were collected by centrifugation (1 h, 16,000 RCF, 4°C) and resuspended in 5 mL of Phosphate Buffered Saline (PBS, pH 7.2, Sigma-Aldrich). The resulting pellet was resuspended in 1/50 of the initial volume of the initial sample. The purified particles were stored at 4°C for future use.

Initial qualitative screen of tailocins for the bactericidal activity

The activity of the purified and concentrated particles was initially tested qualitatively on a limited panel of bacterial strains (=17 strains, Supplementary Table S1) using a spot test assay as described before (Hockett and Baltrus, 2017; Yao et al., 2017).

Microscopic imaging

Tailocins were imaged using both transmission electron microscopy (TEM) and atomic force microscopy (AFM), as described earlier (Vacheron et al., 2021). TEM analyses were done at the Laboratory of Electron Microscopy (Faculty of Biology, University of Gdansk, Poland). For TEM analysis, particles obtained as described above were adsorbed onto carbon-coated grids (GF Microsystems), stained with 1.5% uranyl acetate (Sigma-Aldrich), and directly visualized with an electron microscope (Tecnai Spirit BioTWIN, FEI) using a previously described protocol (Bartnik et al., 2022). At least 10 images were taken for each preparation to estimate the diameters of the particles. For the AFM analysis, purified and PEG-concentrated particles were used directly without further preparations. AFM imaging was conducted in air mode using the Bioscope Resolve microscope (Bruker), in ScanAsyst (Peak Force Tapping) mode, employing the SCANASYST-AIR probes (f0 7.0 kHz, diameter < 12 nm, k: 0.4 N/m) as described earlier (Sokolova et al., 2023). Similarly, as described for TEM analysis, for AFM, at least 10 images were taken for each preparation to estimate the diameters of the particles.

Determination of the concentration of tailocins

To estimate the concentration of tailocins, three independent methods were applied.

Direct particle count with NanoSight NS300

NanoSight NS300 instrument (Malvern Panalytical), equipped with an sCMOS camera and a Blue488 laser, was used to directly assess the concentration and size distribution of particles obtained after induction and purification, as described above. The tailocin samples were diluted 1,000 times in sterile PBS buffer pH 7.2 (Sigma-Aldrich) to achieve the optimal concentration for observation. The camera gain was set to 14, the number of captures per sample equaled 5, each lasting 60 s, and the detection threshold was set to 5 as suggested by the manufacturer. Measurements were conducted at room temperature (*ca.* 22–23°C). Three biological replicates were used to determine the concentration and size distribution of the obtained tailocins, and the results were averaged for further analysis.

Semiquantitative estimation by a spot test

Samples interrogated for tailocins were serially 2-fold diluted in PBS pH 7.2 (Sigma-Aldrich; Yao et al., 2017). Two μL of each dilution

were spotted onto TSA plates overlayed with 15 mL of soft top agar [Nutrient Broth (NB, Oxoid) with 7 g L^{-1} agar]. Before pouring, the soft top agar was cooled to 45°C and inoculated with 250 µL of an overnight culture of a tailocin indicator strain (susceptible strain *M. paradisiaca*). Plates were incubated overnight at 28°C. The highest dilutions of particles capable of cell lysis, visible as plaques (halos) in the bacterial lawn in soft top agar, were determined. Each tailocin dilution was tested in triplicates and the entire experiment was repeated two times with the same setup. The reciprocal of the highest dilution causing a visible plaque was defined as the value of the relative activity in arbitrary units (=1 AU).

Poisson distribution killing method

Tailocins were also quantified using the Poisson distribution killing method based on the protocol described by Yao et al. (2017) and initially introduced by Kageyama et al. (1964) and Williams et al. (2008). This method is based on the number of bactericidal events, determined from the number of bacterial survivors in a population with a known number of initial viable cells (Yao et al., 2017). To determine this, 10 µL of undiluted and 10-fold diluted samples of tailocins were added to 100 µL of an overnight TSB (Oxoid) culture of a susceptible strain *Musicola paradisiaca* NCPPB 2511 (10^8 CFU mL^{-1}) and incubated for 40 min at 28°C with shaking (120 rpm). As a negative control, PBS pH 7.2 was used instead of the tailocins suspension. Each combination was tested in triplicates. After incubation, the suspensions were serially diluted up to 10^{-7} in PBS and plated in triplicate on TSA plates. The colonies that emerged following overnight incubation at 28°C were enumerated to calculate the bacterial survival ratio (S) in the treated samples compared to the negative control. S was calculated as the number of viable bacteria in a sample incubated with tailocins divided by the number of viable bacteria in the negative control. The number of lethal events per bacterial cell (m) was calculated as $m = -\ln(S)$. The total number of active killing particles per milliliter (based on the assumption that tailocins adsorption to bacterial cells in each sample was quantitative within the first 40 min incubation period) was calculated by multiplying m by the initial number of bacterial cells per milliliter (CFU mL^{-1}).

Target range of the tailocins isolated from *Dickeya dadantii* 3937

Overnight cultures of the investigated strains were prepared in 1 mL aliquots of TSB in 2 mL microcentrifuge tubes (Eppendorf) which were incubated horizontally with shaking (120 rpm) at 28°C for 16–24 h. The overnight cultures were used as an inoculum in a spot test carried out on 48-well plates (Greiner). Briefly, 10 µL of the inoculum was transferred to each well of the plate and mixed with 500 µL of liquified soft top agar [Nutrient Broth, NB, (Oxoid) with 7 g L^{-1} agar], precooled to 45°C in a water bath. Plates were gently stirred (20 rpm) to ensure an even distribution of bacterial cells in the inoculated wells. After the agar had solidified, plates were left to dry for 10 min. in a laminar flow hood. 2 µL of 10-fold diluted tailocins (approximately 10^{10} particles mL^{-1}) purified from mitomycin-induced cultures of *D. dadantii* 3937 were spotted on the surface of the inoculated soft-top agar in the wells of the multitier plate. Inoculated plates were incubated for 24 h at 28°C and then inspected for the presence of a growth inhibition. A spot of lack of growth was interpreted as the susceptibility of the given strain to the tailocins (positive reaction). 2 µL of sterile PBS was spotted on a lawn of a susceptible strain as a negative control. The susceptibility of each bacterial strain was tested in triplicate, and the entire experiment was repeated twice.

Determination of the bacterial killing rate

The rapidity by which tailocins killed bacteria was measured in 96-well plates (Nest) using an Epoch 2 microplate reader (BioTek). 25 µL of a suspension containing tailocins (approximately 10^{11} particles mL^{-1}) in PBS pH 7.2 were added to 100 µL of 5 McF (approximately 10^8 CFU mL^{-1}) bacterial suspension in PBS. An overnight culture of a tested strain in TSB were harvested by centrifugation (5 min, 8,000 RCF). The OD_{600} of the PBS suspensions was measured each minute for 2 h. The plate was incubated at 28°C with shaking. The susceptible strain *M. paradisiaca* strain NCPPB 2511 was used as a positive control, and sterile PBS pH 7.2 without tailocins was used as a negative control. The log-transformed values of OD_{600} at each time point were normalized to the log-transformed starting OD_{600} and regressed against time. The regression coefficient [$\Delta Log_{10}(OD_{600})min^{-1}$] was calculated for each of the obtained curves. The killing proportion was estimated at two representative time points (20 and 120 min) as the average % of the initial OD_{600} of the selected bacterial culture compared to controls ($n = 10$).

Analysis of the tailocins with SDS-PAGE and ESI LC–MS/MS

Proteins within tailocins were separated using a 4–20% sodium dodecyl sulfate-polyacrylamide gradient gel (Mini-PROTEAN TGX Stain-Free Precast, Bio-Rad Hercules, United States) electrophoresis (SDS-PAGE) using previous methods (Sambrook et al., 1989). Protein bands were excised from the gel using a sterile scalpel, and the excised gel pieces were placed in separate 1.5 mL Eppendorf tubes for amino acid sequencing. In-gel digestion was performed according to a standard protocol consisting of gel de-coloration and removal of Coomassie staining, reduction/alkylation with dithiothreitol (DTT), and iodoacetamide (IAA), respectively (Golebiowski et al., 2022). First, digestion was carried out overnight with trypsin (Promega Mass Spectrometry Grade Gold) at 37°C. The tryptic peptides were then eluted from the gel with sequential washing of gel pieces with 50 mM ammonium bicarbonate buffer, 5% formic acid in 50% acetonitrile, and 100% acetonitrile (Goldman et al., 2019). All samples were then concentrated (SpeedVac), and the final cleanup was carried out using the StageTips method on the C18 phase to a 50% acetonitrile solution with 1% acetic acid (Schmidt and Sinz, 2017). After concentrating the samples to 30 mL using the SpeedVac, fragmentation mass spectra were recorded for analysis. ESI LC–MS/MS analysis was performed on Triple Tof 5600+ mass spectrometer with DuoSpray Ion Source (AB SCIEX, Framingham, MA, United States) connected to the Eksigent microLC (Ekspert MicroLC 200 PLus System, Eksigent, Redwood City, CA, United States) equipped

with the ChromXP C18CL column (3 µm, 120 Å, 150 mm × 0.5 mm). The microLC−MS/MS system was controlled by the AB SCIEX Analyst TF 1.6 software. Chromatographic separation was carried out for 30 min in a gradient program: (1) 0–1 min—20% solvent B, (2) 1–26 min—20 − 60% solvent B, (3) 26–28 min—98% solvent B, and (4) 29–30 min—20% solvent B, where solvent A was 0.1% formic acid in water and solvent B 0.1% formic acid in acetonitrile. The identification of proteins present in the examined gel bands was carried out based on the obtained fragmentation spectra using the ProteinPilot software (v 4.5) or Peaks Studio and the appropriate protein database (UniProt *Dickeya dadantii 15.02.2023, unreviewed*) with an automated false discovery rate (1% FDR).

Bioinformatic analyses

Prediction of prophage regions in the genome of *D. dadantii* 3937 (Genbank accession: NC_014500.1) was conducted using PHASTER (Arndt et al., 2016). The tailocin cluster and individual proteins it encodes were analyzed against NCBI databases using BLASTn and BLASTp (Altschul et al., 1990). The topology of the tailocin cluster in *D. dadantii* 3937 was investigated using BioCyc (Karp et al., 2019). Phylogenomic analysis of the Soft Rot *Pectobacteriaceae* (SRP) genomes was based on core genome sequences and was performed using EDGAR ver. 3.0 (Dieckmann et al., 2021) accessed via https://edgar3.computational.bio.uni-giessen.de.

Stability of tailocins

Tailocins were incubated for 24 h at several different temperatures (−80, −20, 4, 22, 28, 37, 42, 50, 65, and 80°C), pH values (2, 5, 3.5, 7, 9, 10.5, and 12), and NaCl concentrations (0.01, 0.1, 1, and 5 M), after which their bacterial growth inhibitory activity was assessed by a spot test using *M. paradisiaca* as described above. All tested samples had a total volume of 200 µL, and the initial concentration of PEG-purified tailocin of approximately 10^{10} particles mL^{-1}. To obtain the test samples, tailocins were diluted by mixing a volume of 20 µL of PEG-purified samples with 180 µL of either PBS (temperature stability), PBS with pH modified by addition of either HCl or NaOH to the desired values (pH stability), or in water containing different concentrations of NaCl. The experiments were repeated three times, each with three technical replications per tested condition.

Effect of enzyme/detergent/organic solvent treatment on the activity of tailocins

Tailocins were incubated for 1 h at 37°C with the following enzymes: proteinase K (Sigma-Aldrich, final concentration: 0.5 mg mL^{-1}), DNase (Sigma-Aldrich, final concentration: 10 U mL^{-1}), or lysozyme (Sigma-Aldrich, final concentration: 0.5 mg mL^{-1}), and at room temperature (22°C) with sodium dodecyl sulfate (SDS, Sigma Aldrich, final concentration: 1%), or chloroform (POCH, 50% v/v). The remaining activity of treated tailocins was then assessed in a spot test as described above. The processed samples had a total volume of 200 µL, and a tailocin concentration of approximately 10^{10} particles mL^{-1}. The samples were prepared by mixing 20 µL of PEG-purified samples (about 10^{11} particles mL^{-1}) with the appropriate volume of the factor stock to a final volume of 200 µL with PBS. The exception were the chloroform-treated samples where 200 µL of sample were mixed on a shaker with 200 µL of chloroform. Prior to testing, the chloroform-treated sample was centrifuged (5 min at 4000 RCF), and the aqueous phase was removed for assay. The experiments were repeated three times, each with three technical repetitions per tested condition.

Binding of tailocins to nonviable bacterial cells

Overnight bacterial cultures of *M. paradisiaca* NCPPB 2511 and *D. dadantii* strain 3937 grown in TSB were washed twice with PBS buffer and then killed by incubation with 5 mg mL^{-1} chloramphenicol (Sigma-Aldrich) for 60 min, with shaking (120 rpm, 28°C; Gonzalez and Kunka, 1987). The killing of the cells after 1 h was confirmed by plating 4 aliquots (10 µL) of treated culture on TSA plates, incubating at 28°C for 24 h, and verifying last of bacterial growth. After killing, the nonviable bacterial cells were again washed three times with PBS to remove the remaining antibiotic. The PEG-purified tailocin samples were then added to suspension of viable and nonviable susceptible and nonsusceptible bacteria to a final concentration of approximately 10^{10} particles mL^{-1} and incubated for 40 min with shaking at 120 rpm at 28°C. After incubation, samples were filtered through a 0.2 µm PES (polyether sulfone) membrane filter, and the remaining tailocins was assessed by a spot test using *M. paradisiaca*. The experiments were repeated three times, each with three technical replication per tested condition.

Testing the influence of pH on the bactericidal activity of tailocins

The PEG-purified tailocins (approximately 10^{11} particles mL^{-1}) were diluted 10-fold in PBS buffered to pH 2, 7, and 12 and incubated for 24 h at room temperature. The pH of each sample was then adjusted to neutral pH (pH 7) using an equal volume of the buffered PBS. The solutions were then incubated for 4 h at room temperature and tested for growth inhibitory activity using a spot test in three replicates as described above.

Caenorhabditis elegans toxicity assay

Sensitivity of *Caenorhabditis elegans* to tailocins produced by *D. dadantii* was tested as described before (Kirienko et al., 2014) with slight modifications. Briefly, wild-type Bristol N2 strain of *C. elegans* nematode obtained from the *Caenorhabditis* Genetic Center (CGC, University of Minnesota, Minneapolis, United States) was maintained as described before (Stiernagle, 2006; Krzyzanowska et al., 2019) on Nematode Growth Medium (NGM) plates with *Escherichia coli* strain OP50 as a food source. The toxicity of the tailocins was tested on nematode cultures synchronized as described earlier (Porta-de-la-Riva et al., 2012). Briefly, nematode eggs were harvested from cultures treated with a mixture of 5 M NaOH and 5.25% NaOCl (1,

3, v:v)—a treatment that eliminates adult worms. Recovered eggs were hatched in an S-complete medium, and the nematodes were grown to the L4 stage using *E. coli* OP50 as a food source. The synchronized cultures were then transferred into wells of a 96-well plate. The worms were counted under the microscope (Leica MZ10f stereomicroscope, Leica) and *ca.* 30 worms placed in each well supplemented with PEG-purified and ultracentrifugation concentrated (1 h, 26,000 RCF, 4°C) tailocin in an S-complete medium at a concentration of 10^{12} particles mL^{-1}. Nematode cultures grown without tailocins were used as control. The plates were incubated for 24 h in the dark at 25°C. The survival of nematodes in tailocin-supplemented cultures was compared to the control. The experiment was repeated twice with the same setup, and the results were averaged for analysis.

Statistical analysis

All statistical tests were conducted using either Past 4.13 software (Hammer et al., 2001) or Microsoft Office Excel.[1] The Shapiro–Wilk (Shapiro and Wilk, 1965) and F-tests (Shen and Faraway, 2004) were used to test for normality and variance equality of data, respectively. For pairwise testing, the *t*-test (Semenick, 1990) was applied for data having a normal distribution and equal variances, the Welch test (Welch, 1947) was used for samples with a normal distribution but unequal variances, while the U Mann–Whitney test (Mann and Whitney, 1947) was applied for data that were not normally distributed. One-way ANOVA (Ross and Willson, 2017) was used to compare more than two data groups. Levene's test (Schultz, 1985) was used to test the homogeneity of variance, and the normality of the residuals was conducted using the Shapiro–Wilk test. Welch's one-way ANOVA, followed by the Games-Howell *post hoc* test (Mégevand, 2022), was used for the data groups with non-homogeneous variance and normally distributed residuals. For groups with a non-homogeneous variance and without normally distributed residuals Kruscal-Wallis's one-way ANOVA (McKight and Najab, 2010) followed by Dunn's *post hoc* test (Ruxton and Beauchamp, 2008) was applied.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Author contributions

MB: Conceptualization, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. DK: Conceptualization, Investigation, Supervision, Visualization, Writing – original draft, Writing – review & editing. MN: Investigation, Methodology, Visualization, Writing – original draft. MS: Investigation, Methodology, Writing – original draft. MR: Investigation, Methodology, Writing – original draft. PC: Methodology, Writing – original draft. KW: Investigation, Methodology, Writing – original draft. RC: Conceptualization, Data curation, Funding acquisition, Resources, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer AJ-K declared a shared affiliation with the authors to the handling editor at the time of review.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1307349/full#supplementary-material

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Chapter 2

Stress-driven temporal production of phage tail-like particles (tailocins) in *Dickeya dadantii* strain 3937

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Stress-driven temporal production of phage tail-like particles (tailocins) in *Dickeya dadantii* strain 3937

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Tailocins are bacteriocins resembling bacteriophage tails. Previously, we reported the production of tailocins in the plant pathogen *Dickeya dadantii* 3937, the synthesis of which was upregulated upon treatment with mitomycin C. In this study, we investigated how mitomycin treatment over time influences the expression of tailocin-related genes, the accumulation of tailocin particles, and the survival of producer cells. The expression of tailocin P2D1 structural genes peaks two hours after the addition of mitomycin C as measured with an RT-qPCR assay. Simultaneous measurements of tailocin titer revealed that the concentration of the particles in the culture supernatant peaked 6 h after induction and remained stable for at least 18 h. Progressive accumulation of P2D1 that occurred from 2 to 6 h after mitomycin C treatment was associated with a substantial decrease in viable cells of the tailocin-producing strain (ca. 100,000-fold). Decreased cell viability upon tailocin production indicates that they are released from the cells upon cell lysis. Likewise, we found new potent inducers, viz., hydrogen peroxide and antibiotics affecting DNA replication and repair (viz. norfloxacin and ciprofloxacin), that can increase tailocin yield in *D. dadantii* 3937.

Keywords Real-time qPCR, Phage tail-like particles, *Erwinia chrysanthemi*, Bacteriophage

Approximately 70–80% of sequenced bacterial genomes contain genes of viral origin, suggesting that these viral genes contribute to important phenotypes in the bacterial hosts[1,2]. Some of these genetic elements can encode phage tail-like particles known as tailocins[3]. Tailocins are nanomolecular structures resembling syringes that share evolutionary and morphological features with bacteriophage tails, type VI secretion systems, and extracellular contractile injection systems[4]. These phage tail-like particles are used in both defense mechanisms as well as offensive weapons by bacteria[5]. They are released from bacteria in response to environmental stresses or competitive pressure, acting as precision weapons, targeting and killing closely related bacterial competitors[6]. By eliminating those closely related strains, the producers improve their chances of survival and resource acquisition in various ecological niches[7,8].

Tailocin production has been described both in Gram-negative and Gram-positive bacteria[9–12]. The producing strains encompass a range of human, animal, and plant pathogens as well as saprophytic bacteria found in diverse environments, including the rhizosphere, bulk soil, within plants, aquatic habitats, as well as in insects, animals, and humans (reviewed in:[13]. We recently described a novel tailocin dickeyocin P2D1 that is produced by the plant pathogenic bacterium *Dickeya dadantii* strain 3937. P2D1 tailocins resemble the tail structures of bacteriophage *Peduovirus* P2. They exhibit bactericidal activity specifically against phylogenetically related bacterial strains without adversely affecting eukaryotic cells in a *Caenorhabditis elegans* killing model[14].

The genetic cluster encoding the P2D1 tailocin seems to be widely distributed in the genomes of *Dickeya* spp. In our recent screen of 74 complete and high-quality *Dickeya* spp. genomes deposited in the NCBI database, 52 (70% of the genomes) contained tailocin clusters having high homology to the P2D1 cluster (> 70% threshold for coverage and identity)[15]. The widespread presence of P2D1 tailocins in this bacterial group suggests that they provide a competitive advantage during interactions with closely related strains.

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Production of tailocins is known to be triggered by the SOS response associated with DNA damage[16]. Still, the exact mechanism of induction and production of phage tail-like particles remains poorly understood for most analyzed strains. Under laboratory settings, the most commonly used inducers of phage tail-like particles are UV radiation and mitomycin C. UV radiation primarily induces reversible thymine dimers in DNA. This activation triggers the cleavage of the LexA repressor, leading to the derepression of SOS-regulated genes, including those involved in the production of tailocins[17]. In contrast, mitomycin C is a DNA crosslinking agent that induces severe and irreversible DNA damage, activating the SOS response[18]. Mitomycin C can cause a stronger and more prolonged SOS response than UV radiation and, therefore, probably stimulates more production of tailocins than UV radiation in vitro[19].

Among the current knowledge gaps in tailocin biology are details of the environmental conditions under which the production of tailocins is triggered and how these conditions may contribute to the overall success of a particular strain in a given niche[6]. It is plausible that many factors contributing to cellular stress in natural environment may induce tailocin production. However, no comprehensive studies have yet been conducted to explore the in situ induction of tailocins. Additionally, there is limited data on the temporal relationships between the induction of tailocin production and the accumulation of active particles. Such insights are needed to better understand how tailocins can mediate the outcomes of interactions between bacterial strains in natural settings. Therefore, this study aimed to determine the conditions that trigger P2D1 tailocin production in *D. dadantii* strain 3937. Specifically, we explored the mitomycin C concentration-dependent as well as the subsequent time-dependent production of P2D1 tailocin. In turn, the accumulation of P2D1 tailocin was linked to the expression of P2D1 genes upon induction in real time using qRT-PCR. We also assessed other triggers of P2D1 induction and discussed how these inducers could modulate the concentration of P2D1 tailocins under natural environmental conditions.

Materials and methods

Bacterial strains, chemical compounds, and growth media

Dickeya dadantii strain 3937[20] and *Musicola paradisiaca* strain IFB 0117 (NCPPB 2511)[21,22] were grown at 28 °C on either trypticase soya agar (TSA; Oxoid), in trypticase soy broth (TSB; Oxoid), in potato dextrose broth (PDB; Biocorp) or in M9 minimal medium (MP Biomedicals) supplemented with glucose (Sigma-Aldrich) to a final concentration of 0.4%. Liquid cultures were agitated during incubation (120 rpm). To solidify the media, 15 g L^{-1} bacteriological agar (Oxoid) was added. Soft top agar (STA) was prepared using 30 g of trypticase soy broth (Oxoid) and 7 g of bacteriological agar (Oxoid) per liter. When required, the growth media were supplemented with various concentrations of mitomycin C (Abcam), chloramphenicol (A&A Biotechnology), ampicillin (A&A Biotechnology), ciprofloxacin (Sigma-Aldrich), norfloxacin (Abcam) or hydrogen peroxide (Laboratorium Galenowe Olsztyn, Poland) as described below.

Induction, purification, and Estimation of Tailocin titer after induction

P2D1 tailocins were induced, purified, concentrated, and quantified as described in[14]. Briefly, *Dickeya dadantii* strain 3937 was grown overnight (ca. 16 h) in TSB at 28 °C with shaking (120 rpm). The cultures were then rejuvenated by diluting them 1:40 in 10 or 100 mL of fresh TSB medium. The diluted culture grew for 2.5 h under the same conditions, reaching approximately 7.5 log colony-forming units (CFU) per milliliter, with a turbidity of 0.8 to 1.0 on the McFarland scale. Such prepared *D. dadantii* cultures were then supplemented with mitomycin C (Abcam, Poland) to a final concentration of 1 μg mL^{-1} to induce the production of P2D1 tailocins. Following mitomycin C treatment, the cultures were incubated for another 24 h at 28 °C with shaking (120 rpm). 2.2 mL of each post-induction bacterial culture was cleared from bacterial cells through centrifugation (10 min., 8000 RCF, 22 °C). The resulting supernatant was filtered (0.2 μm, PES membrane, Googlab), and 2 mL of the filtrate was transferred to tubes containing polyethylene glycol (PEG-8000, Promega) to achieve the final PEG concentration of 10%. The samples were incubated for 16–20 h at 4 °C with gentle horizontal shaking to precipitate tailocins. The precipitant was harvested by centrifugation (1 h, 16000 RCF, 4 °C). After removing the supernatant, the samples were dried under laminar flow until the remaining liquid evaporated. The resulting tailocins were resuspended in 200 μL of phosphate-buffered saline (PBS, pH 7.2), yielding a tenfold concentration relative to the initial volume. The purified tailocins were stored at 4 °C until further analysis.

The concentration of tailocin particles was determined using a semi-quantitative spot test assay, as described earlier[9]. Briefly, Petri dishes containing TSA were overlaid with soft top agar (STA). Before pouring, the soft top agar was cooled to 48 °C and inoculated (1:60) with an overnight culture of tailocin-sensitive strain *M. paradisiaca* IFB 0117 (NCPPB 2511)[14]. The samples tested for tailocin titer were serially twofold diluted in PBS (pH 7.2). Then, in duplicates, 2 μL of each tested suspension was spotted on the plates inoculated with *M. paradisiaca*. After overnight incubation at 28 °C, the highest dilution of tailocins causing the formation of clear zones (plaques) on the bacterial lawn was determined. Two-fold tailocin dilutions were prepared and tested to enhance resolution. Since the tested method for determining tailocin concentration is based on assessing their activity, the results were expressed as relative activity in arbitrary units (AU), with 1 AU being defined as equal to the highest dilution that still caused a visible plaque on the lawn of sensitive bacterial strain (*Musicola paradisiaca* strain IFB 0117 (NCPPB 2511)). Consequently, the results in the graphs are presented as $\log_2$AU.

Effect of mitomycin C concentration on P2D1 yield

To determine whether the concentration of mitomycin C affects the induction and the final yield of the P2D1 tailocins in *D. dadantii* strain 3937, we tested eleven concentrations of mitomycin C. *D. dadantii* strain 3937 was grown for 16 h in 10 mL of TSB at 28 °C with shaking (120 rpm). The cultures were then diluted 1:40 in fresh TSB, incubated under the same conditions for 2.5 h (to approx. 7.5 CFU mL^{-1}, turbidity 0.8-1.0 McFarland), and then spiked with 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, or 10 μg mL^{-1} of mitomycin C (Table S1). Following

the addition of the inducer, the resulting bacterial cultures were further incubated at 28 °C for 6 h with shaking (120 rpm). In each case, tailocin titer was estimated as described above. The experiment was independently conducted four times with two replicates each, and the results were averaged.

Effect of mitomycin C on the viability of *D. dadantii* 3937 cells

D. dadantii strain 3937 was cultured in TSB medium for 16 h at 28 °C with shaking (120 rpm). The overnight cultures were rejuvenated (1:40) in 250 mL of fresh TSB medium, and the incubation was continued under the same conditions for 2.5 h (to approx. 7.5 CFU mL^{-1}, turbidity 0.8-1.0 McFarland). Subsequently, mitomycin C was added to a final concentration of 1 μg mL^{-1}, and the cultures were continued under the same conditions. Samples were collected at various time points during the incubation: 2.5 h before mitomycin C addition, at the time of inducer administration (T = 0), and at 0.5, 1, 2, 4, 6, 8, and 24 h post-treatment. The control sample consisted of bacterial cultures not subjected to mitomycin C treatment. To quantify the number of cells, all samples were serially diluted tenfold in PBS (Sigma-Aldrich, pH 7.2), and 10 μl aliquots of the dilutions were plated on TSA plates by spotting as described earlier[23]. The plates were dried under a laminar flow until the liquid was fully absorbed and incubated at 28 °C for 20 h. Following incubation, the number of colonies was counted, and the average colony-forming units (CFU) per milliliter of the original culture were calculated. The experiment used two biological replicates, with each containing three technical replicates.

Yield of P2D1 Tailocins at different time points after induction

To examine the changes in P2D1 concentration following the addition of the inducing agent, strain *D. dadantii* 3937 was grown for 16 h in 10 mL of TSB at 28 °C with shaking (120 rpm). The cultures were then diluted 1:40 in fresh TSB and incubated under the same conditions for an additional 2.5 h. To induce the production of tailocins, bacterial cultures were spiked with 1 μg mL^{-1} of mitomycin C, and the resulting culture was incubated under the same conditions for another 24 h. Culture samples were collected at T = 0 (addition of mitomycin C to *D. dadantii* strain 3937 culture) and after 0.5, 1, 2, 4, 6, 8, and 24 h after induction. In each case, tailocin titer was estimated as described above using the semi-quantitative spot test with *M. paradisiaca* IFB 0117 (NCPPB 2511) as a reporter strain. The experiment was repeated three times, and the results were averaged.

Expression of genes involved in P2D1 synthesis with RT-qPCR

Expression of the fiber, tube, sheath, and reference genes *lpxC* and *rplU* (Fig. S1) was analyzed by RT-qPCR at 0, 1, 2, and 4 h after mitomycin C induction, alongside untreated samples collected at the same time points. Three independent biological replicates were analyzed for each condition and time point.

Isolation of RNA

Total RNA from bacterial cultures was isolated using the RNeasy Protect Bacteria Mini Kit (QIAGEN) according to the manufacturer's protocol involving proteinase K treatment. The processed samples included the cultures of *D. dadantii* 3937 grown as described for determining the activity of tailocin inducers, harvested immediately before the addition of mitomycin C (T = 0), and at 1, 2, and 4 h after treatment. Samples untreated with mitomycin C were processed in the same way as a control. For time points 0, 1, and 2 h post-induction, 5 ml of the culture was mixed with RNAprotect Bacteria Reagent (QIAGEN) at a 1:2 ratio, and the cells were pelleted (10 min., 5000 RCF, 22 °C) for isolation of RNA. For mitomycin-treated samples collected after 4 h, due to the loss in viable cell count, a larger volume (50 ml) of the culture was harvested, pelleted (3 min, 8000 RCF, 22 °C), resuspended in 500 μl of PBS buffer, and immediately mixed with 1 ml of the RNAprotect Bacteria Reagent. The increased culture volume processed for timepoint T = 4 h did not apply to the control (untreated) samples. The purified RNA was treated with the TURBO DNA-free Kit (Thermo Fisher Scientific) to remove potential genomic DNA contamination. The integrity of RNA was verified by agarose gel electrophoresis, and its concentration was determined using NanoDrop 2000 (Thermo Fisher Scientific). The samples were stored at -80 °C for further use.

cDNA synthesis, real-time qPCR, and expression analysis

RNA was reverse transcribed into cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche) with random hexamer primers and with the optional denaturation step. For each reaction, 500 ng of RNA was used as a template. Real-time qPCR was performed on the CFX96 instrument (Bio-Rad) using Power SYBR Green PCR Master Mix (Thermo Fisher Scientific), as described before[24]. The template cDNA was diluted 1:4. All primers used in the experiment were designed using Primer3Plus[25] (Fig. S2, Fig. S3, and Table S2). Primer specificity was verified by agarose gel electrophoresis and melt curve analysis (Fig. S1, Fig S2). Primer efficiency was established based on serial dilutions of post-PCR products as a template (Fig. S3). Each biological replicate was analyzed in two technical replicates. A sample maximization design was employed, in which all samples for a given gene were run on the same qPCR plate to minimize inter-run variability. Relative gene expression was quantified using the ΔΔCq method[26]implemented in the gene expression analysis module of CFX Maestro 2.3 software (Bio-Rad). Target gene expression was normalized to the reference genes *lpxC* and *rplU*, previously validated as stable in *D. dadantii*[27]. Expression levels for each target gene were compared to the 0-hour control sample (collected immediately prior to inducer addition). Samples collected at 1, 2, and 4 h included both mitomycin C-treated and untreated conditions, enabling evaluation of time-dependent changes in tailocin structural gene expression and comparison between treated and untreated cells. Gene expression results relative to the control group (prior to induction) were presented both as $\log_2$ fold changes and as values converted to linear scale, to facilitate interpretation.

Effect of antibiotics on P2D1 Tailocin yield

To test whether chemicals other than mitomycin C can induce the production of P2D1 tailocins in *D. dadantii* strain 3937, four antibiotics with different modes of action were selected and used in a model experiment: chloramphenicol – inhibiting protein synthesis, ampicillin – that inhibits bacterial cell wall synthesis, ciprofloxacin, and norfloxacin – both inhibiting DNA replication and repair. *D. dadantii* strain 3937 was grown for 16 h in 10 mL of TSB at 28 °C with shaking (120 rpm). The cultures were then diluted 1:40 in fresh TSB and incubated under the same conditions for an additional 2.5 h. To induce the production of tailocins, bacterial cultures were spiked with either chloramphenicol (final concentration: 4 μg mL^{-1}), ampicillin (final concentration: 0.004 μg mL^{-1}), ciprofloxacin (final concentration: 0.016 μg mL^{-1}) and norfloxacin (final concentration: 0.016 μg mL^{-1}) (Table S1). As a positive control and known P2D1 inducer, mitomycin C (final concentration: 1 μg mL^{-1}) was used. After adding putative inducers, the resulting bacterial cultures were further incubated at 28 °C for 6 h with shaking (120 rpm). In each case, tailocin titer was estimated as described above using the semi-quantitative spot test with *M. paradisiaca* IFB 0117 (NCPPB 2511) as a reporter strain. The obtained results were expressed as relative activity in arbitrary units (AU) as described above. The experiment was independently conducted four times, containing two technical replicates each, and the results were averaged.

Effect of hydrogen peroxide on P2D1 Tailocin yield

To assess the effect of hydrogen peroxide on tailocin induction in *D. dadantii* 3937 and to compare it with the bacterial cell response induced by mitomycin C, an experiment was conducted following similar conditions as those described above to test the effect of varying concentrations of mitomycin C. The only difference in treatment was that four concentrations of hydrogen peroxide (0.5, 1, 5, and 10 mM) were tested instead of mitomycin C, at 6 h post induction (Table S1). A single concentration of mitomycin C (1 μg mL^{-1}) and an untreated bacterial culture were positive and negative controls, respectively. The yield of P2D1 particles obtained following each treatment was expressed in relative units (AU) as described above. The experiment was conducted three times, each time with two technical replicates.

Effect of the type of growth medium on P2D1 Tailocin yield

To test whether the type and composition of the growth medium affect the production of P2D1 tailocins, three different media were tested: M9 minimal medium with 0.4% glucose, PDB (rich medium with potato extract and glucose), and TSB (rich medium with peptone and glucose). *D. dadantii* strain 3937 was grown for 16 h in 10 mL of TSB at 28 °C with shaking (120 rpm). The cultures were diluted 1:40 in fresh TSB, PDB, or M9 + 0.4% glucose medium and incubated under the same conditions for 2.5 h. To induce the production of tailocins, bacterial cultures were spiked with 1.0 μg mL^{-1} of mitomycin C. Following the addition of the inducer, the resulting bacterial cultures were further incubated at 28 °C for 6 h with shaking (120 rpm). In each case, tailocin titer was estimated as described above using the semi-quantitative spot test with *M. paradisiaca* IFB 0117 (NCPPB 2511) as a reporter strain. The experiment was independently repeated three times, with two replicates each, and the results were averaged.

Results

P2D1 Tailocin production is dependent on the concentration of mitomycin C inducer

D. dadantii 3937 was reported to produce P2D1 tailocins at a baseline level of only about ca. 6.5 log_2AU when grown in TSB in the absence of an inducer[14]. However, production was greatly stimulated following treatment with 0.1 μg mL^{-1} of mitomycin C[14]. Here, we established the effectiveness of mitomycin C induction over a range of concentrations (0.1–10 μg mL^{-1}). Although mitomycin C significantly upregulated P2D1 tailocin production at all concentrations tested, the highest yield was observed at concentrations between 0.5 and 1.5 μg mL^{-1}. At these concentrations, activity levels approached 12.5 log_2AU, an approximately 64-fold increase relative to that in untreated cells (Fig. 1A).

Mitomycin C causes a decline in viable *D. dadantii* 3937 cells that is caused by the accumulation of P2D1 Tailocins

The impact of mitomycin C (1 μg mL^{-1}) on the growth and survival of *D. dadantii* strain 3937 in TSB medium was evaluated. In the control culture, which was not exposed to mitomycin C, the number of viable bacterial cells increased over 34-fold during a 24-hour incubation period (rising from 7.7 to 9.2 log CFU mL^{-1}) (Fig. 2A). However, in the presence of mitomycin C, bacterial survival dropped sharply. Within 4 h after the addition of mitomycin, the number of viable cells decreased approximately 800-fold (from 7.6 to 4.7 log CFU mL^{-1}). This decline was even more pronounced by 6 h after mitomycin addition, with viable cell numbers dropping 3.6 million-fold to just 10 CFU mL^{-1}. By the end of the 24-hour incubation with mitomycin C, the bacterial population was nearly eradicated, with only about 4 CFU mL^{-1}.

P2D1 Tailocin production reaches a maximum level six hours after induction with mitomycin C

Significant increases in P2D1 production in mitomycin C-treated samples over that in the untreated control were seen within 2 h after mitomycin treatment: tailocin abundance increased 9-fold (to 8.86 log_2AU) relative to the control (5.67 log_2AU) (Fig. 2B). The highest P2D1 titer was observed 6 h after mitomycin C addition, reaching 13.9 log_2AU, representing about a 123-fold increase compared to the untreated control. No further increases in P2D1 tailocin yield upon mitomycin C -treatment were observed in samples collected from 6 to 24 h after induction. This indicates that after reaching its maximum titer, the number of P2D1 tailocin particles remained stable (Fig. 2B).

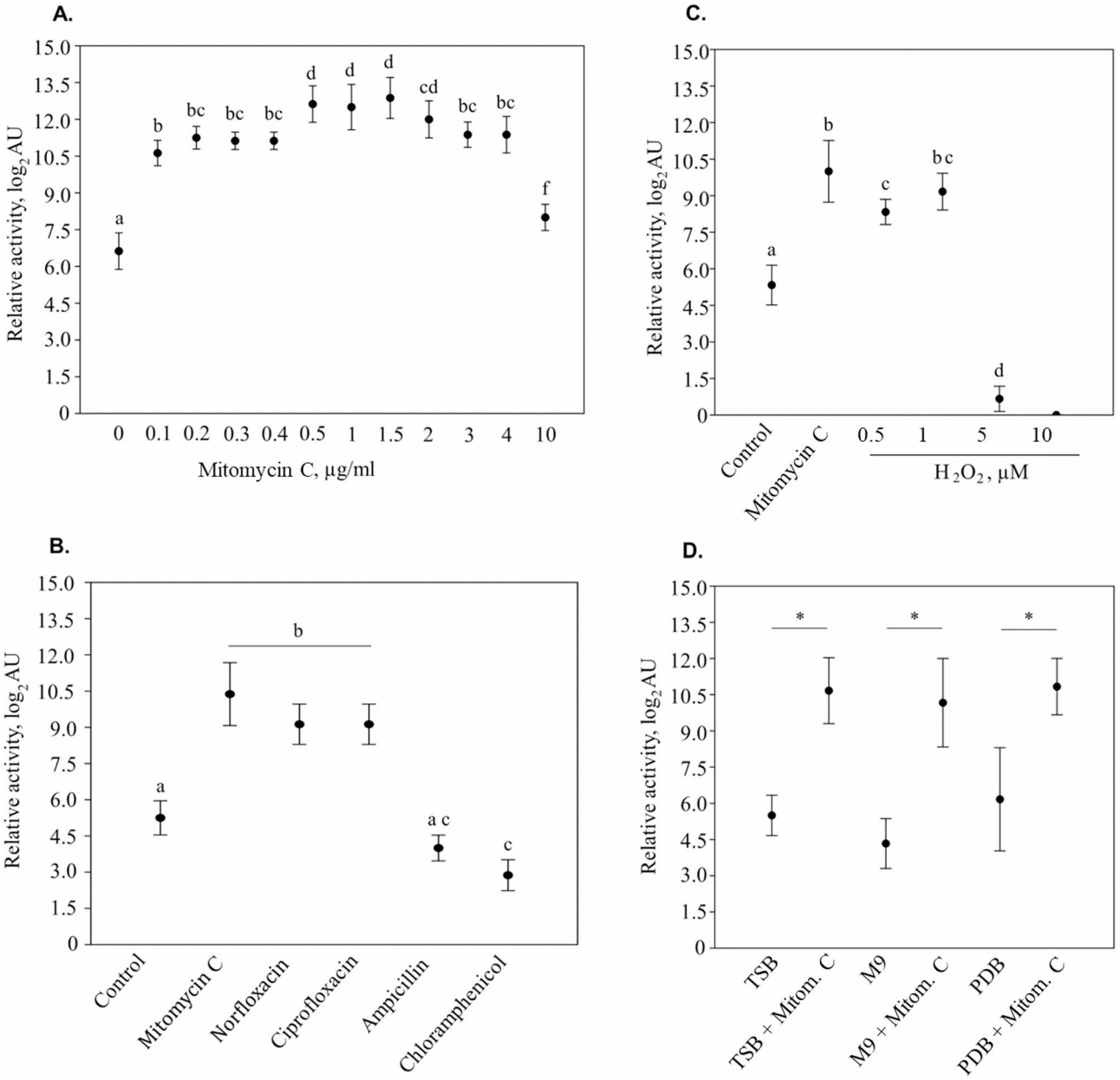

Fig. 1. Production of tailocins by *D. dadantii* 3937 depending on the applied concentration of mitomycin C, the growth medium, and the type of inducer. Tailocin titer was expressed as relative tailocin activity in arbitrary units (AU), with 1 AU defined as the reciprocal of the highest dilution that caused a visible plaque on a lawn of susceptible strain *M. paradisiaca* IFB 0117 (NCPPB 2511). In all panels, data points show mean values, and error bars represent standard deviations. Statistically significant differences between groups for analyses in panels A and C were determined using Tukey's pairwise comparison[43]followed by Copenhaver Holland post hoc analysis[44]while for analyses in panels B and D, these were determined by Kruskal-Wallis test[45]followed by Dunn's post hoc test[46]. In all panels, groups labeled with the same letter are not significantly different ($\alpha = 0.05$). Panel (**A**) depicts the titer of P2D1 tailocins depending on the applied concentration of mitomycin C. Panel (**B**) shows tailocin titer when testing the inducive potential of selected antibiotics: norfloxacin 0.016 µg mL^{-1}; ciprofloxacin 0.016 µg mL^{-1}; ampicillin 0.004 µg mL^{-1}; chloramphenicol 4 µg mL^{-1}. Treatment with mitomycin C (1 µg mL^{-1}) was used as a reference. Control – culture with no potential inducer added. Panel (**C**) shows tailocin yield following induction with different concentrations of hydrogen peroxide: 0.5, 1, 5, and 10 mM, with mitomycin treatment as reference (1 µg mL^{-1}). **Control** – culture with no potential inducer added. Panel (**D**) shows tailocin yield in different growth media when induced with mitomycin C (1 µg mL^{-1}): TSB – Trypticase Soya Broth; M9 – M9 minimal medium with 0.4% glucose; PDB – Potato Dextrose Broth.

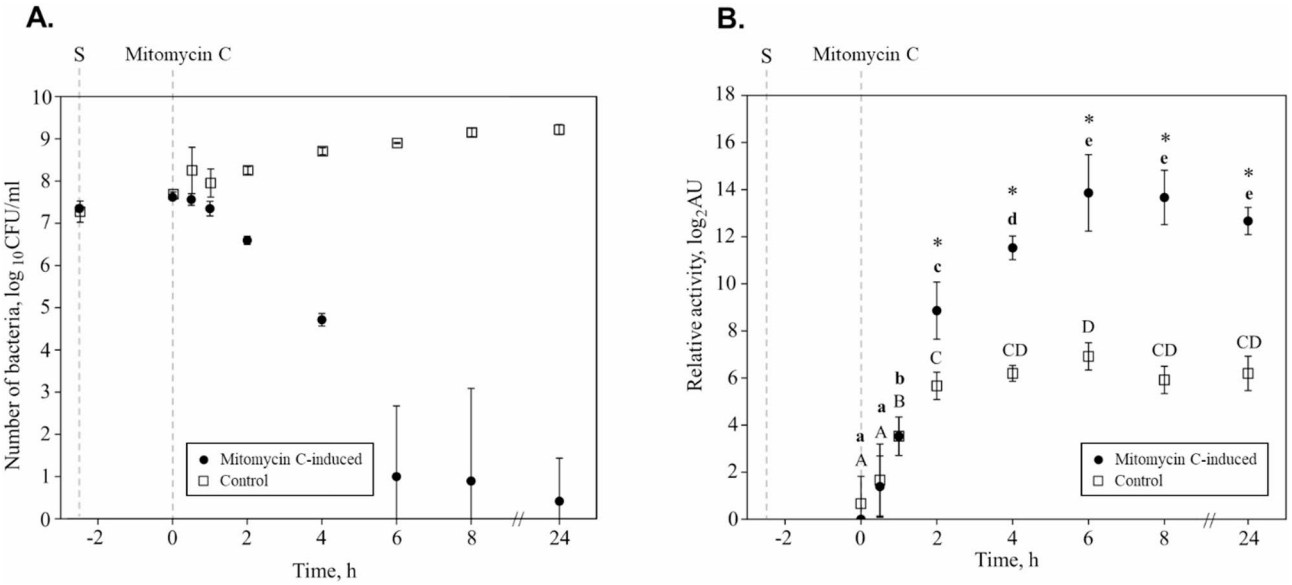

Fig. 2. Tailocin yield and viable bacterial cell count as a function of time following the addition of inducer. (**A**) Survival of *D. dadantii* strain 3937 over time, measured in colony-forming units (CFU). Data points represent the viable bacterial cell count in cultures treated with mitomycin C at a concentration of 1 μg mL^{-1}. The counts are shown at various intervals, illustrating the decline in bacterial viability as the cells produce and release tailocins.(**B**) The titer of P2D1 tailocins isolated from the culture of *D. dadantii* strain 3937 at different time points (0–24 h) post induction with mitomycin C (1 μg mL^{-1}). The x-axis indicates the incubation time of bacterial cells with the inducer, after which tailocins were harvested for activity against *M. paradisiaca* IFB 0117 (NCPPB 2511). Cultures unexposed to mitomycin C were used as controls. Points represent the mean activity (mean AU) of measurements from 3 independent experiments. Error bars indicate standard deviation ranges. S represents the start of the culture, and Mitomycin C indicates the time of spiking the culture with mitomycin C. In panel (B), significant differences between Mitomycin C-treated samples collected at different time points were determined using the Mann-Whitney U test[47]. Groups labeled with the same letter are not significantly different ($\alpha = 0.05$). Small letters are used for Mitomycin C-treated samples and capital letters for control. Asterisks (*) indicate statistically significant differences ($p < 0.05$; Mann-Whitney U test[47] between experimental and control samples collected at the same time points.

Expression of genes encoding P2D1 structural genes peaks two hours after induction

The expression of three genes encoding structural proteins of P2D1 tailocin (responsible for forming the sheath, tube, and tail fiber) was analyzed using RT-qPCR to monitor the temporal pattern of tailocin gene expression after induction. Gene expression was compared between *D. dadantii* cells treated with mitomycin C (1 μg mL^{-1}) and untreated control cells. In the control cells to which no inducer was added, no significant changes in gene expression were observed over time, except for a slight increase in the expression of the sheath protein gene at 4 h (Fig. 3). In contrast, cells exposed to mitomycin C showed a marked increase in the expression of all tailocin genes. After 1 h, the expression of the genes encoding the sheath, tube, and fiber proteins increased by approximately 15-, 9-, and 4-fold, respectively. Peak expression was seen 2 h after induction, with the tube protein gene exhibiting a 655-fold increase, while the sheath and fiber genes increased by 295- and 191-fold, respectively, compared to the control (Fig. 3, Table S3). By 4 h, the expression of all three tailocin genes had numerically declined, with the decrease being statistically significant for the fiber and sheath genes (Fig. 3).

P2D1 Tailocins are induced by antibiotics that affect the replication and repair of bacterial genomic DNA

We examined the impact of selected antibiotics (viz. ampicillin, ciprofloxacin, norfloxacin, and chloramphenicol) on the production of P2D1 tailocins. Notably, norfloxacin and ciprofloxacin significantly increased tailocin production, with tailocin activity levels rising 14-fold compared to the control (untreated culture). These results were statistically comparable to the induction of P2D1 tailocins conferred by mitomycin C. In contrast, chloramphenicol caused a five-fold decrease in P2D1 production, whereas ampicillin addition did not affect P2D1 tailocin production (Fig. 1B).

P2D1 Tailocins are inducted by hydrogen peroxide

Supplementation of *D. dadantii* 3937 culture with hydrogen peroxide significantly affected P2D1 tailocin production, as evidenced by changes in bactericidal activity against *M. paradisiaca* IFB 0117 (NCPPB 2511) (Fig. 1C). The highest mean induction was observed after adding H_2O_2 to the culture to a concentration of 1 mM, increasing bactericidal activity to an average of 9.2 log_2AU, making it 14.3 times higher than the control (5.3 log_2AU). Adding H_2O_2 to a concentration of 1 mM conferred a similarly high level of tailcin production

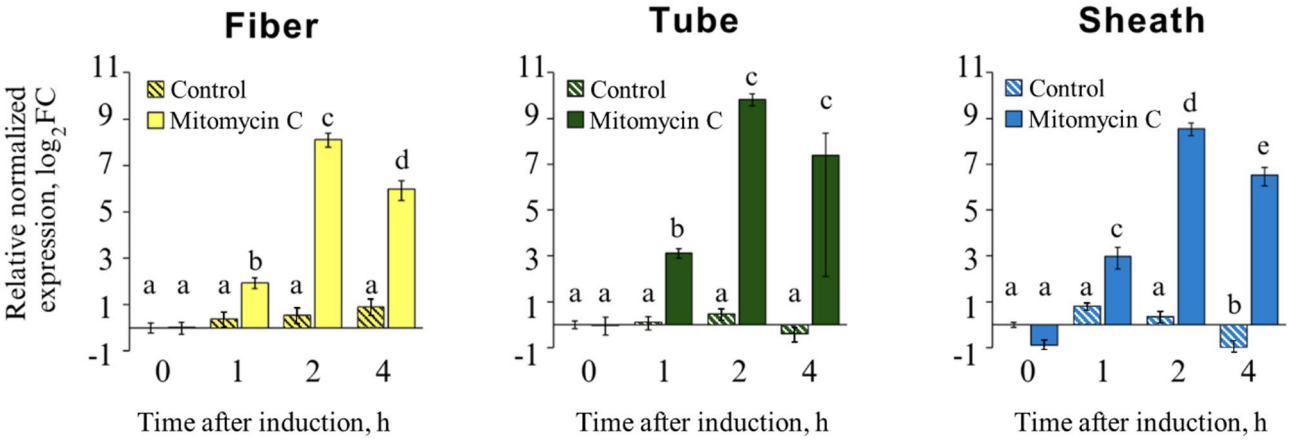

Fig. 3. Expression of P2D1-encoding structural genes in *D. dadantii* 3937 at different time points following induction with mitomycin C. The graphs depict the relative expression levels of three target genes: fiber (DDA3937_RS12070), tube (DDA3937_RS12115), and sheath (DDA3937_RS12110), in samples collected after specified incubation times following the addition of mitomycin C (1 μg mL[- [1]). Control samples consist of bacteria that were not treated with the antibiotic. The fold change (log2) in gene expression for the target genes was calculated relative to the control samples collected at time 0. Statistically significant differences among the experimental groups were determined using one-way ANOVA[48], followed by Tukey's Honest Significant Difference post hoc analysis[49]. Groups marked with the same letter do not differ significantly (α = 0.05).

as 1 μg mL^{-1} of mitomycin C. H_2O_2 at 0.5 mM increased activity 8-fold (to 8.3 log_2AU). In contrast, tailocin production in cultures treated with the highest concentrations of hydrogen peroxide (5 and 10 mM H_2O_2) was negligible (Fig. 1C). Cell viability measurements revealed that treatment with 1 mM hydrogen peroxide caused only a modest reduction in viable cell counts in comparison to the control (approx. 3.5-fold) 15 min after exposure, followed by an increase in viability at the 6- and 24-hour time points. In contrast, exposure to 10 mM hydrogen peroxide led to a tenfold decrease in viability within 15 min (from approximately 7.7 to 6.6 log CFU mL-1), with cell counts remaining 4 log units lower than those observed for 1 mM treatment at both 6 and 24 h post-treatment (poor recovery). Treatment with 5 mM H_2O_2 had an intermediate effect, causing a sharp initial decline in viable cell counts – comparable to that observed with 10 mM – followed by a partial recovery, albeit slower than that seen in the 1 mM condition.

The type of the growth medium affects neither the basal level nor the induction of P2D1 Tailocins

We found that the type of microbiological medium used during the cultivation of the *D. dadantii* 3937 strain did not affect the production level of P2D1 tailocins (mean 5.3 log_2AU). This was true both for the constitutive tailocin production in the absence of any inducers as well as upon the addition of mitomycin C (mean 10.6 log_2AU) (Fig. 1D), as no statistically significant differences (α = 0.05) were observed within the cells in the various unmodified media or between multiple media to which mitomycin C had been added.

Discussion

Although multiple studies have investigated the genetic factors associated with the production of phage tail-like particles (tailocins), wide-ranging analyses examining the interplay between tailocin inducers, induction timing, structural gene expression, and the viability of the producing bacterial cells remain scarce. Likewise, most studies have addressed the induction of tailocins only with mitomycin C and have not assessed how promiscuous tailocin production might be in the presence of other compounds that assault bacteria in natural habitats. This study has thus addressed the dynamics of the production of P2D1 tailocin and the various toxicants that would induce its production in plant-pathogenic *Dickeya* spp[28]. using the model strain *D. dadantii* 3937 [20] (see Fig. 4).

The marked upregulation of P2D1 production was observed across a range of inducer concentrations, indicating that the regulation of tailocin production is a quantitative rather than qualitative trait. This observation confirmed the importance of DNA damage as an inducer of this potent phage tail-like particle in *D. dadantii* 3937, as we have demonstrated earlier[14]. It also extends these findings by characterizing the dose-dependent effects of mitomycin C. Our observations align with findings on other bacteriocins, where moderate stress levels maximize bacteriocin synthesis[29]. However, the highest concentrations of mitomycin C used in our study (above 1.5 μg mL^{-1}) resulted in significantly decreased P2D1 yields. This is consistent with other studies of the impact of mitomycin on cells, which show that excessive DNA damage from mitomycin C treatment overwhelms cellular repair machinery, resulting in impaired cellular functions and even death[30]thereby reducing tailocin yield.

The induction of P2D1 tailocin with mitomycin C caused a remarkable decline in viable *D. dadantii* cells (ca. 3.5-million-fold reduction observed by 6 h after induction). Such an observation aligns well with the known canonical mechanism of tailocin production, where the synthesis and release of these particles disrupt the cell membranes and/or negatively affect other cellular processes, resulting in the rapid death of the producers[4,31].

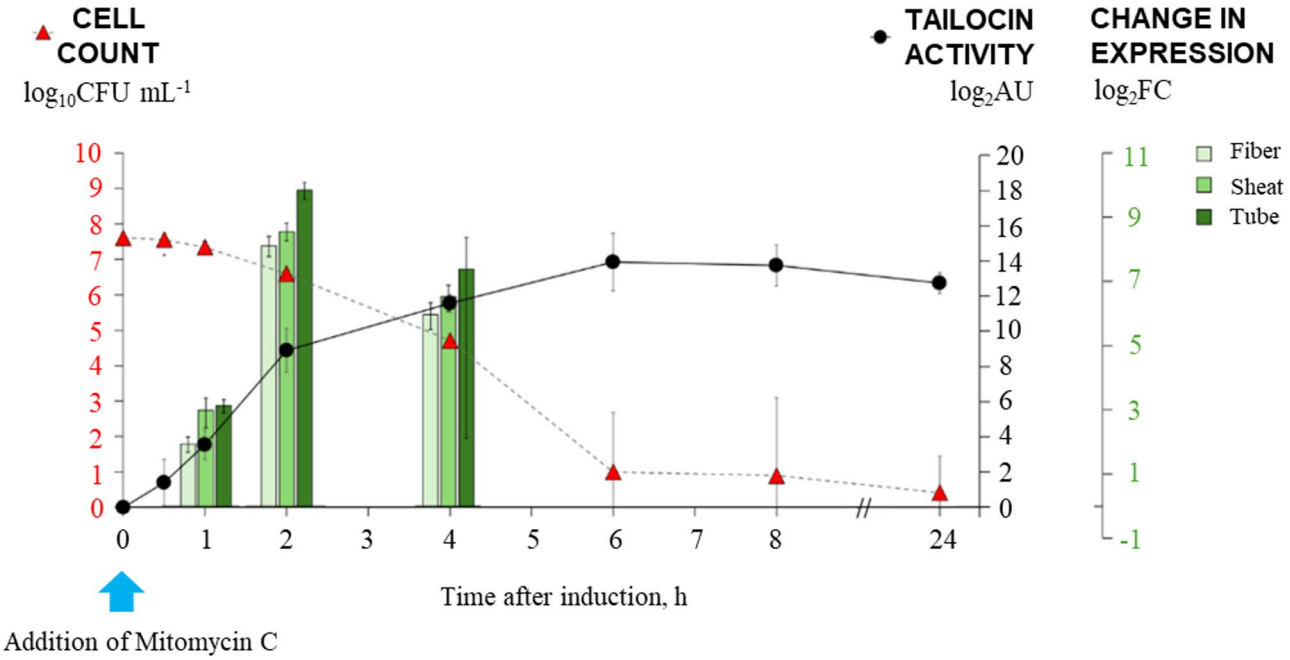

Fig. 4. Temporal response of *D. dadantii* 3937 to mitomycin C. The figure illustrates the dynamics of strain 3937 in response to stress induced by mitomycin C. The graph presents the number of viable bacterial cells producing P2D1 tailocins, the relative activity of the produced tailocins against *M. paradisiaca* IFB 0117 (NCPPB 2511), and the relative expression levels of genes encoding structural proteins of the tailocins. The cultures were treated with mitomycin C at a concentration of 1 μg mL[- [1], with the inducer added to the bacterial culture at time 0.

The dynamics of tailocin production is, therefore, correlated with the overall number of viable cells within the population capable of producing tailocins. In *Pseudomonas aeruginosa*, it has been shown that under natural conditions, less than 1% of the population spontaneously produces tailocins and undergoes lysis, whereas under artificial induction with mitomycin C, tailocin production and cell lysis become widespread and frequent[31]a phenomenon that we also observed in our study. However, it is also notable that a small fraction of induced cells in our study survived. This might be attributed to a limit on the number of cells within the population that are capable of producing tailocins. Such a limit is likely driven by a heterogeneous response to stress factors within a cell population[31]. This heterogeneous response suggests that not all cells activate tailocin production, resulting in a subset of cells that do not undergo lysis and thus survive. We speculate that our observations mirror the situation observed in colicin production, where a self-limiting regulation loop ensures efficient resource allocation during stress[32].

Furthermore, the constant levels of tailocin abundance six hours or more after induction suggests that their production may cease once a certain threshold concentration is reached. The transcriptional activity of structural tailocin genes peaked 2 h post-induction. Then, a marked decline in mRNA levels appeared by 4 h, indicating that transcription is temporally limited. However, the concentration of tailocin particles continued to rise until 6 h, indicating that translation and particle assembly persisted transiently after the peak in transcription and that tailocin particles accumulated progressively during this period. After 6 h, particle levels plateaued. The constant levels of tailocin activity from 6 to 24 h imply that P2D1 particles are highly stable once assembled. It can be predicted that their stability in the natural environment would depend on factors such as high temperature, freeze-thaw cycles, highly acidic pH, and the presence of proteases, all of which we have shown to decrease the activity of P2D1 [14].

Simultaneously with the peak in tailocin production, we observed a sharp decline in cell viability between 4 and 6 h after mitomycin C treatment, with over 99.9% of the population dead at the 6-hour time point compared to T = 0. Analysis of the temporal expression of P2D1 structural genes revealed that peak transcription also occurred two hours after induction - with a significant upregulation of all P2D1 tailocin genes encoding tube, fiber, and sheath. This fast transcriptional response is characteristic of bacteriocin systems regulated by the SOS response[33]where such genes are among those induced in response to DNA-damaging agents[32,34]. Similar temporal patterns as seen here have been reported in other bacterial species, where gene expression peaks early in the SOS stress response, followed by a decline as the cells transit into a survival state[6,35]. The declining expression of tailocin genes observed 4 h or more after exposure to DNA-damaging agents may suggest that their transcriptional activation is tightly regulated. Such a synchronized response ensures that the synthesis of tailocins, such as the P2D1, does not compromise cellular integrity during prolonged stress[36]. In other microorganisms, the regulation of tailocin synthesis involves complex mechanisms that balance positive and negative regulatory factors. For instance, in *Pseudomonas aeruginosa*, tailocin production is controlled by the

positive regulator PrtN and the negative regulator PrtR, with additional oversight provided by the LexA protein, which acts as a gatekeeper for the SOS response[37]. In *Stenotrophomonas maltophilia*, MpsA and MpsH function as positive regulators, while MpsR acts as a repressor under normal conditions, preventing unnecessary tailocin production[38]. The proteins and mechanisms involved in regulating the production of P2D1 tailocin in *Dickeya dadantii* and any buffering system that allows a portion of the population to survive despite the high cost of tailocin production under inducing conditions remain to be elucidated.

The induction of P2D1 by compounds other than mitomycin C (i.e., antibiotics and hydrogen peroxide) provides valuable insights into the versatility of tailocin regulation in *D. dadantii*. Perhaps not surprisingly, antibiotics such as ciprofloxacin and norfloxacin, which inhibit bacterial replication, apparently mimic the effect of mitomycin C by inducing the SOS response, as seen in other studies[39,40]. Similarly, oxidative stress caused by hydrogen peroxide conferred tailocin production in *D. dadantii* to levels similar to that mediated by mitomycin C. This latter observation supports the hypothesis that tailocin synthesis is under the control of the generalized stress response[4,13,31]. However, the significantly decreased production of P2D1 tailocins at higher hydrogen peroxide concentrations highlights the importance of stress insensitivity in modulating tailocin output[10]. Here, 1 mM hydrogen peroxide induced the highest tailocin production, likely generating sufficient stress to trigger synthesis without severely compromising cell viability. In contrast, the sharp reduction observed at 10 mM likely reflects rapid and extensive cell death. The reduced tailocin yield at 5 mM is more difficult to interpret but may result from a combination of decreased viability and – based on studies in *Escherichia coli* – suppressed metabolic activity, potentially associated with a transition into a persister-like state[41]. Lastly, as expected, chloramphenicol inhibited P2D1 production, likely by impairing protein synthesis and interference with cell wall synthesis, respectively[42].

The lack of differences in P2D1 tailocin accumulation in several different growth media highlights the robustness and apparent environmental insensitivity of tailocin synthesis in *D. dadantii* strain 3937. These findings contrast with studies on other phage tail-like particles, where medium composition strongly influenced tailocin yield due to the differences in nutrient availability or pH[6,9]. The medium independence of P2D1 production suggests that tailocin regulation is predominantly governed by the activation of cellular stress pathways rather than other factors.

Our study gives new insights into the events during the assembly and release of P2D1 tailocin in *D. dadantii* and probably to tailocin synthesis in other closely related plant pathogenic bacteria. Other studies have yet to provide detailed insights into the sequential events of tailocin assembly and release under various inducing conditions. The tight linkage between stress and response and tailocin production seen in this study supports the need for further studies of the regulation of tailocin production to better link it to inter- and intraspecies competition in natural habitats where such stresses are common.

Data availability

Data generated or analyzed during this study are included in this article (including its Supplementary Information files). These data can also be obtained from the corresponding author and shared freely upon reasonable request. Correspondence and requests for materials should be addressed to Robert Czajkowski (robert.czajkowski@ug.edu.pl) and/or to Dorota M. Krzyzanowska (dorota.krzyzanowska@ug.edu.pl).

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Author contributions

MS: Conceptualization, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing, DMK: Conceptualization, Investigation, Supervision, Visualization, Writing – original draft, Writing – review & editing, MB: Investigation, Methodology, Writing – original draft, Writing – review & editing, RC: Conceptualization, Data curation, Funding acquisition, Resources, Supervision, Writing – original draft, Writing – review & editing.

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The authors declare no competing interests.

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Chapter 3
Tailocin-mediated interactions among Soft Rot *Pectobacteriaceae*

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Tailocin-Mediated Interactions Among Soft Rot Pectobacteriaceae

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ABSTRACT

Bacteria carry phage-derived elements within their genomes, some of which can produce phage-like particles (tailocins) used as weapons to kill kin strains in response to environmental conditions. This study investigates the production and activity of tailocins by plant-pathogenic bacteria: *Pectobacterium*, *Dickeya,* and *Musicola* genera, which compete for niche, providing an attractive model to study the ecological role of tailocins. Microscopy revealed that most analysed strains (88%) produced tailocins. Tailocin-mediated killing interactions were assessed across 351 strain pairs, showing that *Dickeya* spp. had a higher likelihood of killing neighbours (57.1%) than *Pectobacterium* spp. (21.6%). Additionally, *Dickeya* spp. strains exhibited broader phylogenetic killing, targeting both *Pectobacterium* spp. and *Musicola* sp., while *Pectobacterium* spp. tailocins were genus-specific. The mutual (bilateral) killing was observed in 33.9% of interactions, predominantly within *Dickeya* spp. Although tailocins were morphologically indistinguishable between producers, genomic analyses identified conserved clusters having diverse structural and organisational differences between *Pectobacterium* spp. and *Dickeya* spp. tailocins. This suggests different origins of these particles. Induction experiments demonstrated that tailocin production was boosted by hydrogen peroxide, supporting the role of these particles in bacteria–bacteria competition during plant infection when plants produce ROS to protect themselves from pathogens. Tailocins were detectable in infected potato tissue but not in river water, highlighting the particular ecological relevance of tailocins in these studied environments.

1 | Introduction

In highly competitive and resource-limited environments that bacteria occupy, their survival and fitness often depend on their ability to outcompete neighbouring rival cells (Bauer et al. 2018; Wagner 2022). To gain a competitive edge, bacteria have evolved diverse strategies, including producing and utilising tailocins (syn. phage tail-like particles) that can be lethal to other bacteria. Tailocins directly resemble the tails of bacteriophages but function independently from viral infection and replication

(Booth et al. 2023). Tailocins are highly specialised killing instruments that, by puncturing the cell envelope, lead to the disruption of the cell membrane and the death of the targeted cell (Scholl 2017). Until recently, the utilisation of tailocins has been primarily studied in *Pseudomonas* species (Carim et al. 2021). However, there are also reports of tailocins isolated from various other Gram-negative and Gram-positive bacterial species, including human, animal and plant pathogens, as well as saprophytic bacteria that inhabit a wide range of environments (Backman, Burbano, et al. 2024).

We previously characterised a new type of tailocin produced by the plant-pathogenic bacterium *Dickeya dadantii* reference strain 3937 (Borowicz et al. 2023). This R-type tailocin (Carim et al. 2021), dickeyocin P2D1, was stable under most environmental conditions tested and exhibited broad killing potential against members of different *Dickeya* species but was harmless to *Pectobacterium* species and *Caenorhabditis elegans*. We postulated that the dickeyocin P2D1 targets were restricted to phylogenetically closely related strains. Within the SRP species complex, (consisting of *Pectobacterium* spp., *Dickeya* spp., and *Musicola* spp., defined below), only one other R-type tailocin, carotovoricin Er from *Pectobacterium carotovorum* Er, was described in detail (Yamada et al. 2006). Still, the extent of its host range is unknown. The sequence of carotovoricin Er differs from that of dickeyocin P2D1, indicating a distinct evolutionary origin of these two tailocins (Yamada et al. 2006). Besides these limited studies, little attention has been paid to the presence and ecological role of tailocins in SRP bacteria.

SRPs are Gram-negative bacteria formerly collectively named pectinolytic *Erwinia* spp. They are a particularly appropriate group of bacteria for studying the environmental role of R-type tailocins in the environment. *Pectobacterium* spp., *Dickeya* spp., and *Musicola* spp. belong to the same species complex and are often found together in infected hosts. Consequently, due to their metabolic similarities and overlapping niches, they are known to compete against each other (Toth et al. 2021). Furthermore, these bacteria occupy various ecological niches, including host and nonhost plants, natural and agricultural soils, rainwater, surface water, sewage and insects (Van Gijsegem et al. 2021). In these diverse environments, SRP bacteria often encounter a diverse array of other species, both closely and distantly related, but their competitive strategies in these alternative biomes remain unknown (Bellieny-Rabelo et al. 2019).

Our understanding of the mechanisms of competition in SRP bacteria mediated by tailocins remains poorly understood (Marquez-Villavicencio et al. 2011; Hugouvieux-Cotte-Pattat 2016; Hugouvieux-Cotte-Pattat et al. 2023). Specifically, no systematic studies have been conducted to assess whether the production of tailocins is common in this group of bacteria and how it contributes to their overall ecological success (Borowicz et al. 2023). Addressing this gap remains important for understanding how plant-pathogenic bacteria establish and thrive in diverse environments, particularly in those niches where microbial competition between kin strains is expected to be common.

This study aimed to assess the expression and killing potential of tailocins in a large number of SRP strains obtained from one selected environment—the Durance River in France (Ben Moussa et al. 2022). Although river water is not recognised as a primary environment where SRP bacteria thrive, this niche links the several distinct ecosystems that support the growth and survival of SRP. Because water connects ecosystems that support the growth and survival of SRP, the SRP strains found in water are highly diverse. Therefore, water-isolated strains belong to different species encountered on different host plants, but also, interestingly, they belong to SRP species not found on plants, which have probably evolved to occupy alternative niches (Ben Moussa et al. 2023). Likewise, the tested SRP strains could potentially interact in water or be transmitted to a common plant host, where they might interact during infection (Van Gijsegem et al. 2021). We aimed to characterise interactions among SRP bacteria guided by tailocins to understand better how these pathogens share the environment and whether they compete for niches during infection. For this, we characterised the SRP tailocins and identified gene clusters associated with their synthesis, thus determining the prevalence of tailocin production among environmental SRP strains and their apparent role in intragenus competition among the diverse strains that coexist in this aquatic habitat.

2 | Materials and Methods

2.1 | Bacterial Strains and Culture Conditions

Bacterial strains included in this work are listed in Table S1. The pool comprised 25 SRP isolates from the waters of Durance River (France), collected between 2015 and 2017 (Ben Moussa et al. 2022), as well as two reference strains: *Dickeya dadantii* 3937 (Pritchard et al. 2013) and *Musicola paradisiaca* IFB 0117 (NCPPB 2511) (Hugouvieux-Cotte-Pattat et al. 2021). The latter strains have been previously studied (Borowicz et al. 2023). Routine propagation of bacterial cells was performed at 28°C in liquid Trypticase Soy Broth (TSB; Oxoid), with agitation (120 rpm), or on solid Trypticase Soy Agar (TSA; Oxoid) under the same growth conditions (Czajkowski et al. 2010).

2.2 | Induction and Purification of Tailocins

Tailocin particles were purified as described previously (Borowicz et al. 2023). Briefly, an overnight culture (ca. 16 h) of each strain was rejuvenated (1:40) in a fresh aliquot of TSB and cultured for 2.5 h. Next, tailocin production was induced by the addition of 0.1 μg/mL of mitomycin C (Abcam, Poland). Twenty-four hours after induction, tailocins were purified using PEG from 10 mL volumes of filtered (0.2 μm, PES membrane, GoogLab) culture supernatants. Purified particles were stored in phosphate-buffered saline (PBS), pH 7.2, at 4°C.

2.3 | Imaging

The morphology of purified tailocin particles was investigated by transmission electron microscopy (TEM) and atomic force microscopy (AFM). TEM imaging was performed as described previously (Borowicz et al. 2023) using a Tecnai Spirit BioTWIN microscope (FEI). AFM imaging was performed

using JPK NanoWizard 4 (NanoScience), in nondestructive quantitative imaging (QI) force spectroscopy mode, employing the SCANASYST-AIR probes ($f0$ 7.0 kHz, diameter < 12 nm, k: 0.4 N/m) and the SCANASYST-FLUID+ probes ($f0$ 150 kHz, diameter < 12 nm, k: 0.7 N/m). Spring constants and sensitivity of the probes were calibrated using the thermal calibration method. Before imaging, the samples were deposited on freshly cleaved mica surfaces and air-dried. Images were processed using JPK data processing software. Dimensions of tailocins were calculated based on TEM images. Tailocins from a given strain were considered for measurement only if more than four individual particle images were available for analysis. Analysis was accomplished on 13 out of 18 *Pectobacterium* spp. strains, with a median of 10 particles measured per strain, and 7 *Dickeya* spp., with a median of 17 particles measured per strain.

2.4 | Killing and Sensitivity Assays

Twenty-seven SRP strains, including 25 water isolates (Table S1), were tested in pairs to determine the target range (killing spectrum) of the tailocin produced by each group member. Likewise, the sensitivity of strains to the tailocins produced by all other strains in the analysed pool was assessed. Both the killing spectrum and sensitivity were tested using the spot assay as previously described (Hockett and Baltrus 2017; Yao et al. 2017; Borowicz et al. 2023). Each combination was tested in duplicate, and the entire experiment was performed twice using independently obtained batches of tailocins.

2.5 | SDS-PAGE, ESI LC–MS/MS and Mapping of Identified Proteins to Genomic *Loci*

Proteins in PEG-purified tailocin preparations were separated by SDS-PAGE, fragmented, and the resulting peptides sequenced using mass spectrometry, as described in detail previously (Golebiowski et al. 2022; Borowicz et al. 2023). Briefly, bands were excised from the gel and subjected to in-gel trypsin digestion, and the peptides were eluted, cleaned up and concentrated (Schmidt and Sinz 2017; Goldman et al. 2019). The peptides were then analysed by ESI LC–MS/MS. This included separating peptides on an Eksigent microLC column ChromXP C18CL (3 μm, 120 Å, 150 × 0.3 mm). The samples were placed onto the column with a CTC Pal Autosampler (CTC Analytics AG, Zwinger, Switzerland), and the injection volume was 5 μL. Solvents A and B consisted of 0.1% (v/v) formic acid (FA) in water and acetonitrile, respectively. LC gradient parameters: 18%–50% solvent B in 30 min (Fiołka et al. 2019). The mass spectra with the Triple Tof 5600+ mass spectrometer with DuoSpray Ion Source (AB SCIEX, Framingham, MA, USA) enabled the identification of proteins based on the obtained fragmentation spectra using Peaks Studio 11 (Bioinformatic Solutions) and the appropriate protein database (Table S1) (1% FDR). The annotations of the identified proteins were searched for phage-associated functions, and the encoding genes were mapped to the genomes of the tested strains (Dataset S4). Tailocin-encoding regions were identified based on the clustering of the identified phage/tailocin-related genes within the genomes of the analysed strains and previous studies concerning the structure of the P2D1 tailocin cluster (Borowicz et al. 2023). As the full annotated sequence of the carotovoricin Er cluster is unavailable, the originally uploaded sequence (accession: AB017338.2) (Yamada et al. 2006) annotated by us using RAST (https://rast.nmpdr.org/rast.cgi) (Brettin et al. 2015) was used for cluster synteny analysis.

2.6 | Phylogenetic and Bioinformatic Analyses

2.6.1 | Synteny of Tailocin Clusters

Gene clusters encoding tailocins were extracted from the genomes of *Pectobacterium*, *Dickeya*, and *Musicola* species except for *Pectobacterium odoriferum* A122-S21-F16, where the putative cluster was split across multiple contigs. For *Pectobacterium* spp., the genomic region containing the tailocin cluster was defined as the region between the genes annotated as *tolC* (locus tag in *P. versatile* A69-S13-O15: EG331_00440) and *ybiB* (locus tag in *P. versatile* A69-S13-O15: EG331_00565). In contrast, for *Dickeya* species and a single *Musicola* sp. strain, the genomic region containing the tailocin cluster was identified as the region between the genes annotated as *methyl-accepting chemotaxis protein* (locus tag in *D. dadantii* 3937: DDA3937_RS1200) and *guaD* (locus tag in *D. dadantii* 3937: DDA3937_RS12145).

Accession numbers of genomes from which the tailocin regions were extracted are listed in Table S1 and Dataset S4. The extracted sequences were then subjected to synteny analysis and visualisation using the Clinker tool (Gilchrist and Chooi 2021). The analysis was performed separately for *Pectobacterium* and *Dickeya* species, along with a single strain of *Musicola* sp. Genes within the clusters were colour-coded based on their putative roles in tailocin production. In the visualisation, links between genes indicate sequence identity percentages greater than 50%. The order of strains in the visualisation was determined according to their phylogenetic relationships. Synteny analysis was based on *Pectobacterium versatile* A73-S18-O15 for *Pectobacterium* spp. and *Dickeya dadantii* 3937 for *Dickeya* spp.

2.6.2 | Multilocus Sequence Analysis

A subset of strains was used for phylogenetic analysis. The selected strains possessed the following features: (1) The tailocin cluster had to be present on a single genomic contig; (2) genes annotated as fibre and sheath had to be present within the region identified as the tailocin cluster; and (3) the strain had to demonstrate the ability to target at least one other analysed strain through tailocin interaction. Orthologous sequences were clustered into homologous families using the SiLix software package v1.2.9 (Miele et al. 2011) with an 80% identity threshold and at least 80% overlap. Orthologous sequences (658 coding sequences) were concatenated, aligned using MUSCLE (Edgar 2004) software v5.1 and filtered using the GBLOCK tool (Castresana 2000). The alignments were used to build a phylogenetic tree with the BioNJ algorithm and SeaView software v5.0.5 (Gouy et al. 2010), with 200 bootstrap replications.

2.6.3 | Phenotype-Based Dendrogram

Agglomerative clustering (Nielsen 2016) was applied to prepare a dendrogram for tested strains based on the similarity of their killing spectra. The ability to kill a given strain was assigned the value of 1, and the lack of this ability was noted as 0. The scikit-learn (Virtanen et al. 2020) and SciPy (Virtanen et al. 2020) libraries were used for clustering and dendrogram plotting, respectively. A Python script for implementing the above libraries was written with the assistance of ChatGPT-4o (OpenAI) (Dataset S3).

2.6.4 | Prevalence of Carotovoricin and Dickeyocin Clusters in SRP Complete Genomes

The carotovoricin cluster was blasted against 116 *Pectobacterium* spp. complete, high-quality genomes available on the NCBI database in June 2024 using the NCBI Megablast algorithm with default parameters (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The presence of the cluster was defined according to the 70% sequence coverage and identity thresholds. The available sequence of the originally described carotovoricin Er cluster was incomplete (accession: AB017338.2). Therefore, as a reference carotovoricin sequence for *Pectobacterium* spp., we used the full nucleotide sequence of the carotovoricin cluster from *P. versatile* A73-S18-O15 (19,528 bp). For *Dickeya* spp. and the single *Musicola* sp. strain, the full nucleotide sequence of the dickeyocin cluster of *D. dadantii* 3937 strain (26,580 bp) was blasted against a single *Musicola* sp. and 74 *Dickeya* spp. complete genomes available on the NCBI database in June 2024. Results were interpreted based on analogous thresholds as described for P2D1 tailocin.

2.7 | Detection of Tailocins in River Water and Potato Tubers

To determine whether SRPs produce detectable levels of tailocins in river water and potato tuber tissue, we inoculated these environments with bacteria. After incubation, the tailocins were purified and spot tests were conducted as outlined below. Each experiment was performed twice, and detection was considered positive when all replicates were tailocin-positive. To prepare the inoculation suspensions, the six strains, grown separately viz., *P. versatile* A69-S13-O15, *P. quasiaquaticum* A411-S4-F17, *P. aquaticum* A212-S19-A16, *D. dadantii* 3937, *D. chrysanthemi* A604-S21-A17 and *D. zeae* A586-S18-A17, were grown in TSB (Oxoid) for 24 h at 28°C. The cells were harvested by centrifugation (4200 RCF, 5 min) and resuspended in PBS buffer. Turbidity of the suspension was adjusted to 0.06 McFarland (approx. 10^6 CFU/mL). Five millilitre of filter-sterilised (0.2 μm, PES membrane) and autoclaved Durance River water was inoculated with 50 μL of bacterial suspension, three replicates per strain. Uninoculated water was used as a control. Samples were incubated for 72 h at 15°C with shaking (120 rpm) to mimic natural conditions. Following incubation, 2 mL of water from each sample was centrifuged to remove bacterial cells (8000 RCF, 10 min), and the supernatant was processed for purification and detection of tailocins. Tubers cv. Gala were surface sterilised by immersion for 20 min in 5% commercial bleach (ACE; Procter and Gamble), followed by a double rinse in distilled water and air drying under laminar flow. Each tuber was inoculated by inserting a pipette tip containing 50 μL of the test suspension (Krzyzanowska et al. 2019). Tubers inoculated with PBS buffer alone were used as a negative control. Three potato tubers were tested per treatment. Inoculated tubers were placed in humid boxes (85% to 90% relative humidity). Samples were incubated at 28°C for 72 h to enable the development of soft rot symptoms. The tubers were then cut at the inoculation site. Tissue macerated by bacteria was excised, weighed, and placed in homogenisation bags (Bioreba). PBS buffer supplemented with 0.02% diethyldithiocarbamic acid (DIECA; Sigma-Aldrich) was added to the samples (1:2, w/v) (Perombelon and Van Der Wolf 2002). The samples were homogenised, and 1 mL aliquots were diluted 1:1 with PBS 0.02% DIECA in new tubes. Bacteria and plant debris were pelleted by centrifugation (8000 RCF, 10 min, RT), and the supernatant was processed for purification and detection of tailocins. The supernatants from both experimental setups were filtered (0.2 μm, PES membrane) and transferred to new tubes containing PEG-8000 (final concentration 10% m/v). Samples were incubated overnight at 4°C with shaking. The putative tailocins were collected by centrifugation (1 h, 16,000 RCF, 4°C) and resuspended in 1/20 volume of the initial sample in PBS. Tailocins were detected using a spot test (Hockett and Baltrus 2017; Yao et al. 2017). Five microlitres aliquots of purified tailocins were spotted on soft top agar plates, each inoculated with a strain sensitive to tailocins of the six investigated SRPs (Table S2).

2.8 | Semiquantitative Assessment of Tailocin Production Depending on the Type of Inductive Factor

We investigated whether hydrogen peroxide can induce the production of tailocins in SRPs in a way similar to mitomycin C. The experiment was conducted on the same group of six strains tested for tailocin production in river water and potato tuber tissue. Tailocin production was induced in rejuvenated cells by the addition of 0.1 μg/mL mitomycin C (Abcam, Poland) or 0.003% (0.88 mM) hydrogen peroxide (Laboratorium Galenowe Olsztyn Sp. z o.o., Poland) and cultures with no inductive factor were used as controls. Twenty-four hours postinduction, tailocins were PEG-purified from 2 mL volumes of filtered (0.2 μm, PES membrane) culture supernatants and resuspended in 1/10 of the initial volume in PBS (Borowicz et al. 2023). The concentration of tailocins was estimated by a semiquantitative spot test (Yao et al. 2017; Borowicz et al. 2023) using tailocin-susceptible strains (Table S2). The reciprocal of the highest dilution causing a visible plaque was defined as the relative activity in arbitrary units (= 1 AU), and the AU per mL of culture was calculated. The experiment was conducted twice, with three replicates per combination.

2.9 | Competition Assay In Vitro

Competition assays were performed using a spontaneous rifampicin-resistant mutant of *D. solani* A623-S20-A17 selected on TSA with 50 μg/mL of rifampicin (Sigma-Aldrich) (Glandorf et al. 1992; Berg et al. 2007) and designated *Ds* RIF. This strain was tested in an in vitro competition assay against three sets of competitor strains. Each set comprised two *Dickeya* spp. strains which either: (1) were killed by the tailocins of *D. solani* A623-S20-A17 (*D. dianthicola* A260-S21-A16 and *D. zeae* A661-S21-A17), (2) produced tailocins

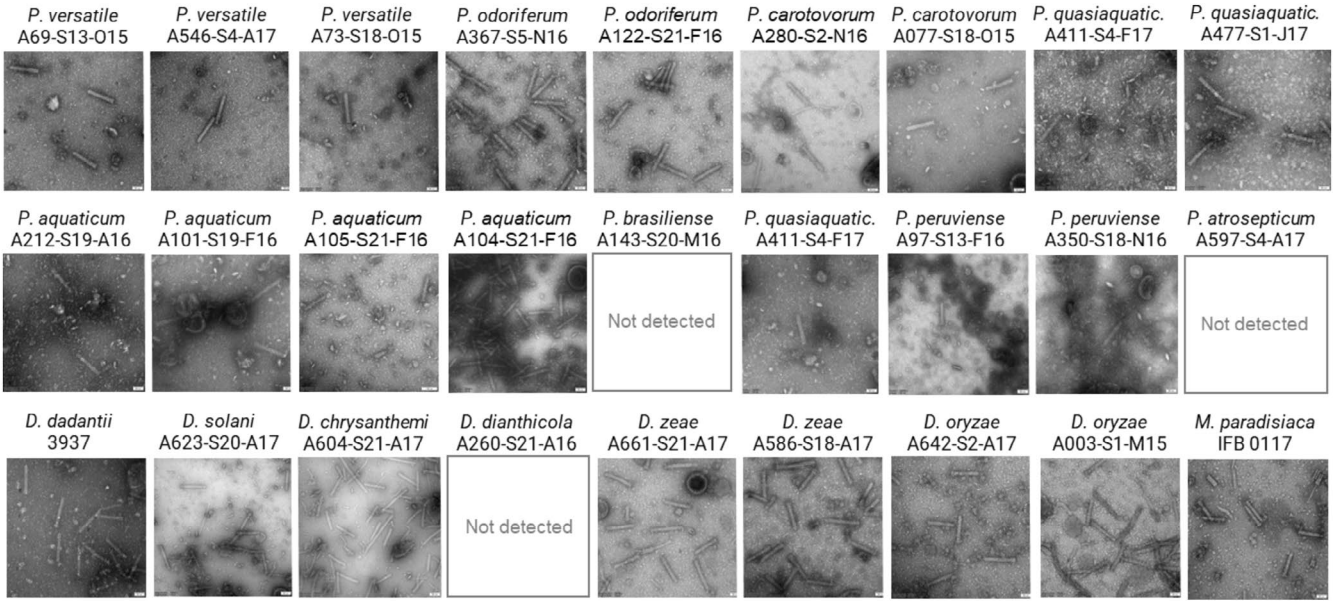

FIGURE 1 | TEM images picturing tailocins isolated from the 27 analysed SRP strains.

killing *D. solani* A623-S20-A17 (*D. zeae* A586-S18-A17 and *D. oryzae* A3-S1-M15) or (3) were killed by the tailocins of *D. solani* A623-S20-A17 but at the same time produced tailocins targeting this strain (mutual killing) (*D. dadantii* 3937 and *D. chrysanthemi* A604-S21-A17). All strains were grown overnight in 0.1 TSB medium. Next, the cells were harvested by centrifugation (3500 RCR, 5 min), and the pellet was resuspended in a fresh medium to obtain a turbidity of 0.5 McF. This equaled, on average, 1.4×10^8 CFU/mL ($\pm 4.5 \times 10^7$), verified by dilution plating. One hundred and fifty microlitres of 0.1 TSB in a single well of a 96-well plate was inoculated (1:1, 25:25 μL) with the suspension of *Ds* RIF and one of the competing strains to ensure the strains had an even start and avoid tailocin carryover from the initial overnight cultures. Each co-culture was tested under two conditions: 0.1 TSB alone or 0.1 TSB with 0.003% (0.88 mM) H_2O_2 (tailocin-inductive stress). The cultures were incubated for 22 h at 28°C, with orbital shaking, in the EPOCH2 reader (BioTek). Culture from each well was dilution-plated on two media types: TSB agar (for counting the total number of CFUs) and TSB agar with 50 μg/mL of rifampicin (for counting the fraction of *Ds* RIF). *Ds* RIF was tested against the wild-type (*wt*) variant of the strain as a control of the mutant's fitness under the applied experimental conditions. Moreover, the ability of *Ds* RIF to produce tailocins was verified in a spot assay, as described above. The competition experiment was performed twice, with two separate co-cultures (replicates) per experiment.

3 | Results

3.1 | Most *Pectobacterium* spp. and *Dickeya* spp. Strains Produce Phage Tail-Like Particles

We assessed tailocins production, diameters and morphology using transmission electron microscopy (TEM) and atomic force microscopy (AFM). TEM imaging of preparations obtained from mitomycin C-induced cultures revealed that 22 out of 25 studied SRP isolates (88%) produce phage tail-like particles (tailocins) (Figure 1). The three exceptions were *Pectobacterium brasiliense* A143-S20-M16, *Pectobacterium atrosepticum* A597-S4-A17 and *Dickeya dianthicola* A260-S21-A16. All tailocins had similar morphologies in TEM and AFM images, regardless of whether they were derived from *Dickeya/Musicola* or *Pectobacterium* spp. (Figures 1 and 2). The average length of tailocins produced by *Pectobacterium* spp. was 163 ± 15 nm. Tailocins produced by *Dickeya/Musicola* spp. were significantly shorter, measuring 153 ± 20 nm (Mann–Whitney test, $p < 0.05$). Similarly, tailocins produced by *Pectobacterium* spp. were, on average, 2 nm wider than those of *Dickeya/Musicola* species, with dimensions of 26 ± 2 and 24 ± 2 nm, respectively, the difference being statistically significant (Mann–Whitney test, $p < 0.05$). Three-dimensional images generated based on AFM scans for seven representative tailocin particles are shown in Figure 2.

3.2 | The Susceptible Targets of SRP Phage Tail-Like Particles Vary Depending on the Producing Strain, With Several Exhibiting Intergenus Action

We evaluated the breadth of strains killed by phage tail-like particles produced by the SRP strains. In total, 702 unique tailocin-mediated interactions (producer–target combinations) were examined for the 351 unique pairs (individual producer vs. individual target) of SRPs tested (Figure 3). The incidence of a strain being susceptible to a particular tailocin was 16.8% (Table 1). This killing incidence was higher among *Dickeya* strains than among *Pectobacterium* strains (57.1% and 21.6%, respectively) (Table 1). Strains *P. versatile* A73-S18-015 and *P. polaris* A641-S16-A17 were particularly susceptible to tailocins, being killed by those from eight different strains. In contrast, *P. carotovorum* A280-S2-N16 was resistant to tailocins produced by all strains tested. 21 of the 25 strains produced tailocins that killed at least one strain from the 27 SRPs. Of the four strains not exhibiting any killing activity, three

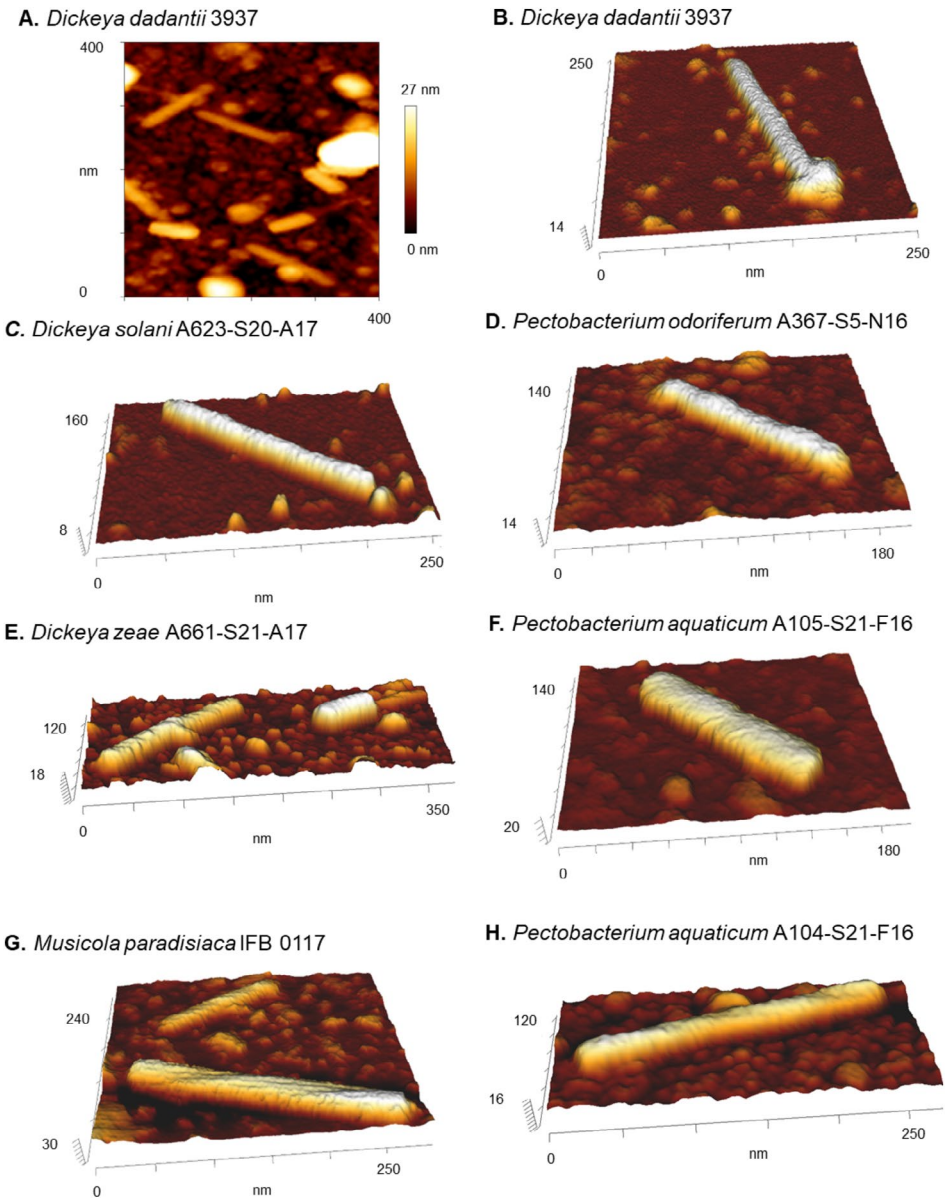

FIGURE 2 | Images of the representative tailocins generated based on AFM scans. AFM imaging was performed using the JPK NanoWizard 4 (NanoScience) in QI in the quantitative imaging (QI) force spectroscopy mode. Images were processed using JPK Data Processing software. Panel (A) shows the AFM scan of Dickeyocin P2D1 produced by *D. dadantii* 3937; panels (B–H) show the highest quality 3D visualisations of representative tailocin particles, generated based on AFM scans, for the following strains: *D. dadantii* 3937, *D. solani* A623-S20-A17, *D. zeae* A661-S21-A17, *M. paradisiaca* IFB 0117, *P. odoriferum* A367-S5-N16, *P. aquaticum* A105-S21-F16, *P. aquaticum* A104-S21-F16.

(*P. brasiliense* A143-S20-M16, *P. atrosepticum* A597-S4-A17 and *D. dianthicola* A260-S21-A16) lacked visible tailocins in TEM analyses (Figure 1), while *P. peruviense* A350-S18-N16 produces tailocins (as evidenced by TEM microscopy); these phage tail-like particles did not target any of the 26 SRPs tested (Figure 3). In contrast, *D. oryzae* A3-S1-M15 and *P. odoriferum* A122-S21-F16 produced tailocins with the broadest target range, each killing 9 out of the 26 strains (33.3%).

We also investigated the ability of tailocins to kill strains in genera different from the producer. *Pectobacterium* strains produced tailocins targeting only other members of the genus. In contrast, five out of eight tested *Dickeya* spp. (62.5%) could kill at least one representative of *Pectobacterium* sp., either a *P. versatile* strain or the closely related *P. odoriferum*. *M. paradisiaca* IFB 0117, the single representative of *Musicola* spp., strongly resembled *Dickeya* spp. both in tailocin susceptibility and the specificity of its tailocins (Figure 3; Table 1).

3.3 | Several SRP Strains Produced Tailocins That Targeted Other Tailocin-Producing Strains, Resulting in a Phenomenon Known as 'Mutual Killing'

Analysis of tailocin-mediated interactions among the collection of SRPs revealed that 33.9% of the killing events were retaliated (mutual)—in which strains could kill each other (Table 1). For 20 of the 351 pairs of SRPs tested (5.7%), a given strain pair was mutually inhibitory—a phenomenon we designate 'mutual killing'.

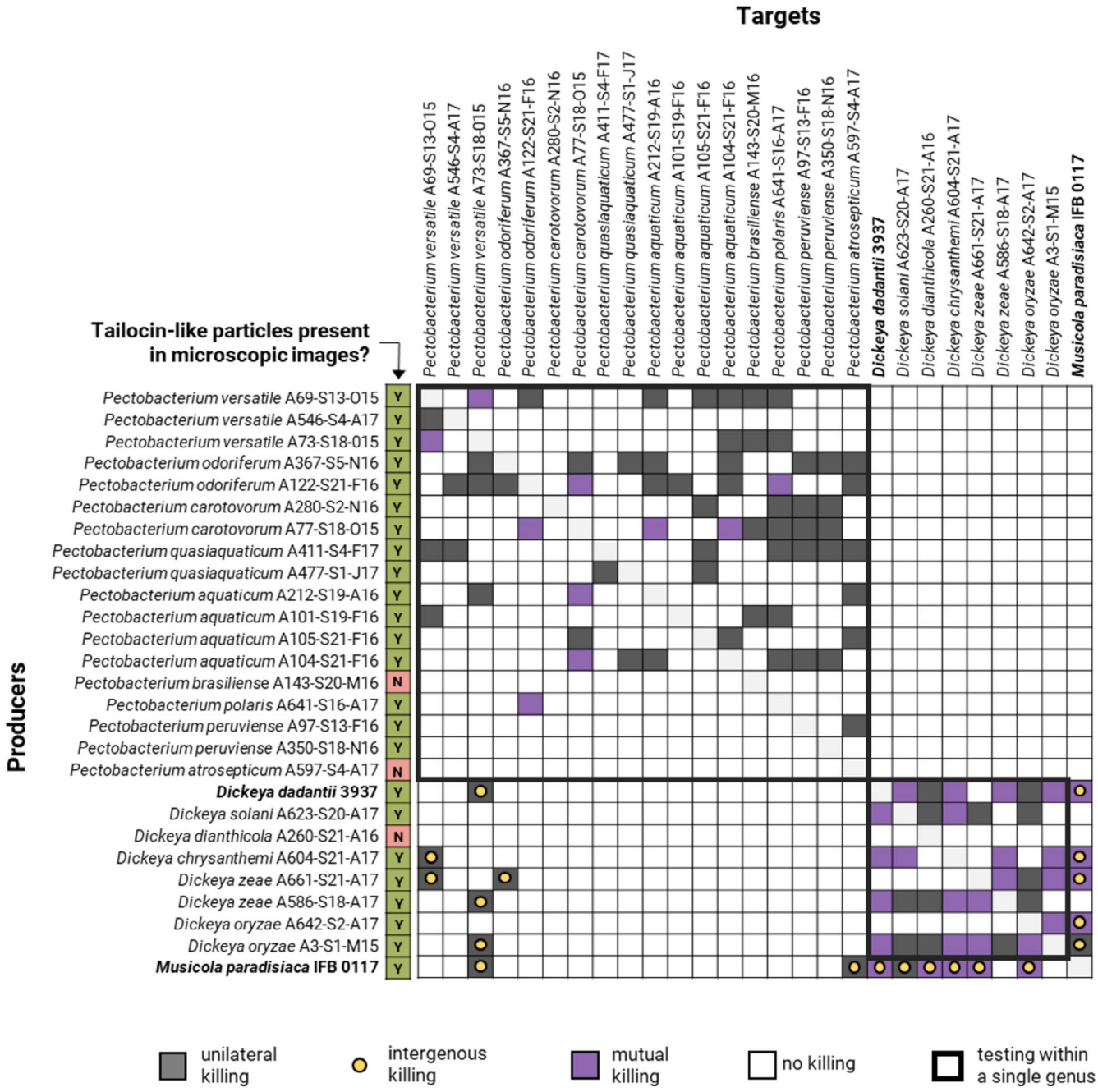

FIGURE 3 | Matrix of tailocin-mediated interactions within the group of tested SRP strains. The tested pool comprised 27 strains: 25 isolates from the Durance River water and two type strains: *D. dadantii* 3937 and *M. paradisiaca* IFB 0117 (in bold). Grey-shaded cells indicate the susceptibility of a target strain to the tailocins of a given producer strain (unilateral killing). Unilateral killing where the producer and the target belong to different genera, is additionally marked by a yellow dot. Lillac cells also indicate the susceptibility of the target strain to the investigated tailocin, but in particular situations where the target strain was observed to produce "retributive" tailocins against the producer (mutual killing). A dedicated column indicates whether tailocins were (Y) or were not (N) detected by microscopic imaging in culture supernatants of the tested strains following treatment with mitomycin C.

Mutual killing was more common within *Dickeya* spp. (65.6% of killing events were mutual) than within *Pectobacterium* spp. (15.2%), and we observed no mutual killing between members of *Dickeya* and *Pectobacterium* (Table 1). The mutual killing was very common (80% of killing events) in mixed pairs of *Dickeya* strains and *M. paradisiaca* IFB 0117, reflecting the clustering between strain IFB 0117 and *Dickeya* spp.

3.4 | Tailocins of *Dickeya* spp. and *Pectobacterium* spp. Are Phylogenetically Distinct

Based on the tailocin-associated protein identified by mass spectrometry (Dataset S4), we identified gene clusters responsible for tailocin production in the genomes of the SRP strains. Among the 25 strains examined, tailocin-encoding clusters were absent

TABLE 1 | Tailocin-mediated killing events for the tested pool of 27 SRP strains.

Group	Total interactions[a]	Killing events		Mutual killing		
		No.	% Total	No.	% Total	% Killing events
All SRPs	702	118	16.8	40	5.7	33.9
Pectobacterium spp.[b]	306	66	21.6	10	3.3	15.2
Dickeya spp.[c]	56	32	57.1	21	37.5	65.6
Mixed genera[d]	340	19	5.6	9	2.6	47.37
Psp vs. *Dsp*	162	0	0	0	0	0
Dsp vs. *Psp*	162	6	3.7	0	0	0
Dsp vs. *M. par*	8	5	62.5	4	50	80
M. par vs. *Dsp*	8	6	75	5	62.5	83.3
M. par vs. *Psp*	18	2	11.1	0	0	0
Psp vs. *M. par*	18	0	0	0	0	0

[a]Total interactions—the number of combinations where each strain from a subset was tested both for the potential to produce tailocins and for tailocin susceptibility (potential target).
[b]Both strains in the tested interaction assigned to *Pectobacterium* spp. (total of 18 strains tested).
[c]Both strains in the tested interaction assigned to *Dickeya* spp. (total of 8 strains tested).
[d]Each strain of the pair representing a different genus; *Psp*—*Pectobacterium* spp.; *Dsp*—*Dickeya* spp.; *M. par*—*Musicola paradisiaca* IFB 0117. The first strain in each combination is the tailocin producer.

only in two strains—*Pectobacterium brasiliense* A143-S20-M16 and *Pectobacterium atrosepticum* A597-S4-A17. These two strains, together with *Dickeya dianthicola* A260-S21-A16 (that possesses tailocin cluster), also lacked tailocin production by imaging.

We also performed gene synteny analyses of the tailocin clusters within the *Pectobacterium* and *Dickeya* (including *Musicola*) genera (Figure 4). The cluster in *Pectobacterium* sp. exhibited homology with that encoding carotovoricin Er (Nguyen et al. 2002) while those in *Dickeya* species and *Musicola* sp. were genetically similar to those encoding dickeyocin P2D1 (Borowicz et al. 2023). Furthermore, no SRP strain possessed both carotovoricin Er and P2D1 encoding clusters. *Pectobacterium* spp. strains exclusively contained the carotovoricin Er-like cluster, while *Dickeya* spp. strains solely harboured the cluster encoding P2D1-like tailocin. Thus, no strain that contains both Er and P2D1 tailocins has been identified.

The overall genomic organisation of the tailocin clusters was conserved within each genus (Figure 4). In *Pectobacterium* species (Figure 4A), the most prominent difference was the presence of a highly variable gene annotated as encoding a tail fibre protein/pyocin knob domain-containing protein that appeared to contain segments shared among different strains (Figure 4). Additional, albeit less pronounced, variability was observed in the sequence of the gene encoding a tail tape measure protein. In *P. brasiliense* A143-S20-M16, this cluster was flanked by genes conserved across other strains; however, instead of a complete tailocin cluster, it contained only the single gene annotated as a mobile element protein gene. In *P. atrosepticum* A597-S4-A17, this region included not only conserved flanking genes but also seven genes present in other clusters and one strain-specific gene. In *Dickeya* species (and *Musicola* sp.) (Figure 4B), the main differences between strains included (1) the presence of strain-specific genes likely unrelated to tailocin production, (2) the orientation of genes not directly involved in tailocin structural components (such as aromatic acid/H+ transporters and phytase family proteins), (3) sequence variations in intergenic regions (data not shown) and (4) sequence differences in tail fibre genes. Notably, *D. dianthicola* A260-S21-A16, which did not produce tailocins, did not exhibit substantial differences in the genomic region encoding tailocins in other strains. Specifically, the tailocin cluster was present in the genome and showed high homology to that of other *Dickeya* spp. tailocin clusters.

3.5 | Carotovoricin and Dickeyocin Clusters Are Common in the Genomes of *Pectobacterium* spp. and *Dickeya* spp.

Given that not all SRPs examined produced tailocins nor harboured the tailocin cluster, we determined the prevalence of tailocin clusters across a broader range of SRP strains to assess the frequency of this trait better. We examined the complete genomes of 116 *Pectobacterium* spp. for the presence of the carotovoricin cluster and 74 genomes of *Dickeya* and 1 *Musicola* spp. genome for the presence of genes encoding dickeyocin. Within the *Pectobacterium* clade, 96 genomes (83%) harboured homologous tailocin regions, having >70% coverage and identity. Twelve *Pectobacterium* genomes had partial alignment (10% of strains), and 9 (8%) showed no evidence of the tailocin cluster (Dataset S1). Within the many *Dickeya* spp. and the single *Musicola* spp. genome examined, 52 (69%) had a cluster that aligned well with the dickeyocin cluster, while 16 (21%) genomes had coverage below 70%, and 7 (9%) genomes had no apparent tailocin cluster (Dataset S2). None of the six complete genomes

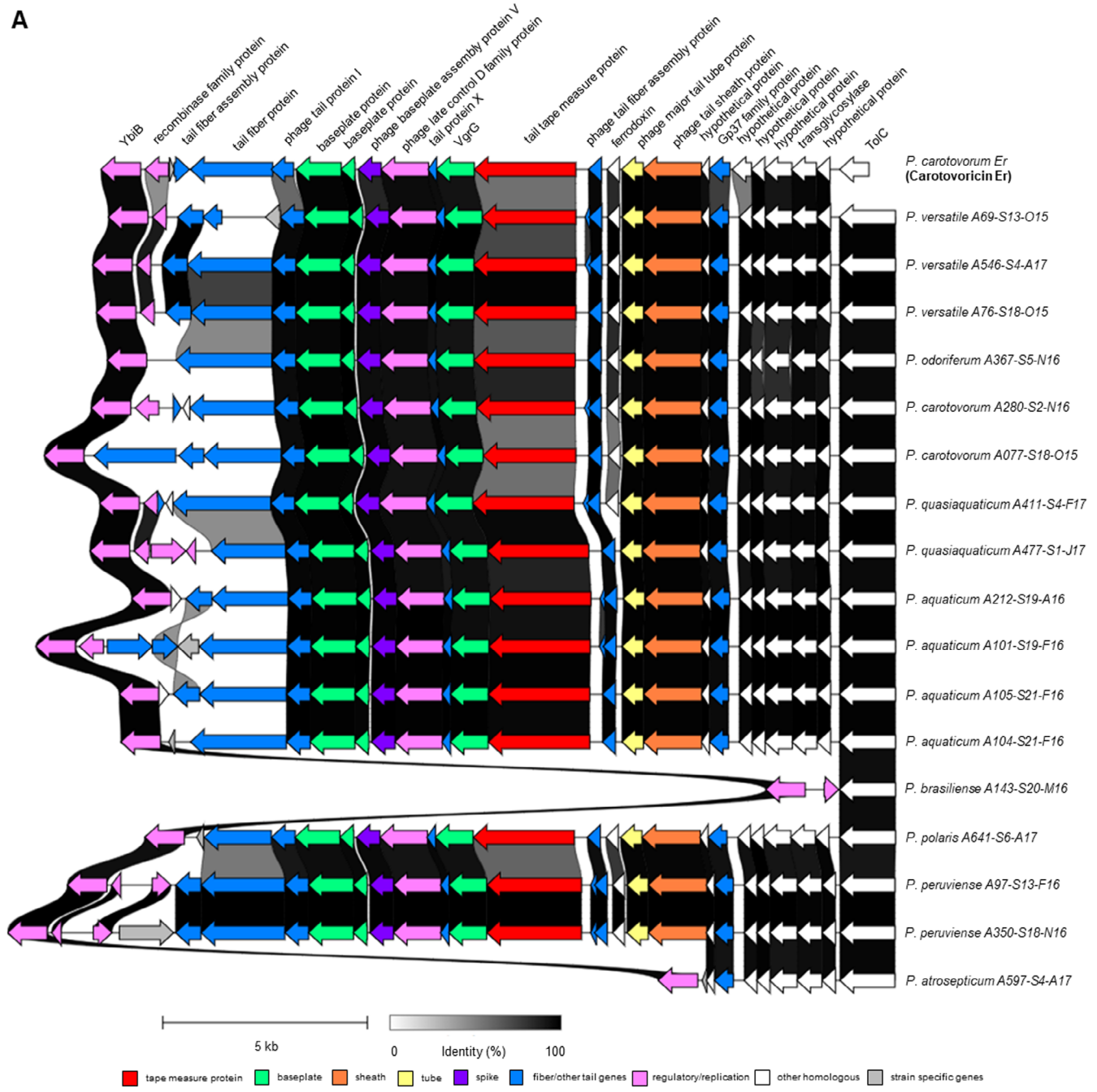

FIGURE 4 | Comparison of tailocin clusters in the analysed group of SRPs. The upper panel (A) depicts regions homologous to the carotovoricin-encoding cluster typical for *Pectobacterium* spp. (reference: Carotovoricin Er accession: AB045036.1, *P. versatile* A69-S13-O15 RMBQ01000001.1, positions in the genome: 94,551–113,781). The lower panel (B) shows regions homologous to dickeyocin P2D1, typical for *Dickeya* spp. and also present in *Musicola paradisiaca* IFB 0117 (reference: *D. dadantii* 3937 NC_014500.1, positions in the genome 2,730,482–2,757,061).

of the species *P. atrosepticum* present in the NCBI database had a tailocin cluster.

3.6 | Tailocin-Killing Specificity Is Not Aligned With Phylogenetic Distance in SRP Strains

The topology of the dendrograms of the phylogenetic tree of SRP based on MultiLocus Sequence Analysis (MSLA) was not well aligned with that based on the patterns of tailocin-killing position (Figure S1). This suggests that tailocin activity may be influenced by factors other than taxonomic relationships, as reported earlier (Warring et al. 2022).

3.7 | Constitutive and Induced Production of Tailocins in *Dickeya* and *Pectobacterium* spp.

Given that our previous study revealed the surprising constitutive basal level of tailocin production in *D. dadantii* 3937

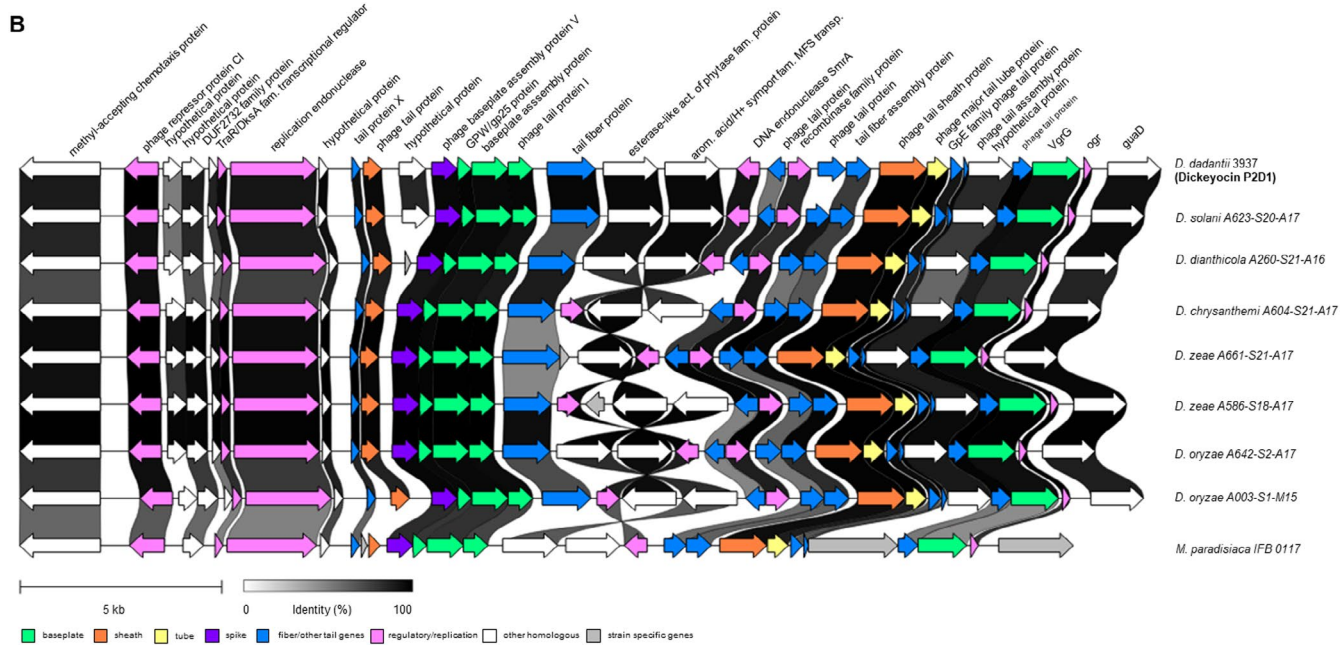

FIGURE 4 | (Continued)

(Borowicz et al. 2023), we explored whether this was also the case in other strains. Tailocin production was thus assessed in six strains (three *Pectobacterium* and three *Dickeya* spp.) (*P. versatile* A69-S13-O15, *P. quasiaquaticum* A411-S4-F17, *P. aquaticum* A212-S19-A16, *D. dadantii* 3937, *D. chrysanthemi* A604-S21-A17, *D. zeae* A586-S18-A17). Furthermore, we wished to establish whether reactive oxygen species (ROS), such as H_2O_2, often associated with plant infection (Torres et al. 2006; Reverchon et al. 2016), could induce the production of tailocins in SRPs. Basal levels of tailocin production were observed for all tested strains even in the absence of ROS. However, the basal level of tailocins produced varied substantially between strains, with the highest basal production observed for the model strain *D. dadantii* 3937 (7×10^4 AU/mL) and the lowest for *P. aquaticum* A411-S4-F17 (5×10^2 AU/mL) (Figure 5). The average abundance of tailocin was slightly (43%) higher for *Dickeya* than for *Pectobacterium* spp. (Mann–Whitney test, $p<0.05$) (2.8×10^4 and 1.6×10^4 AU/mL of culture, respectively).

Mitomycin C (0.1 μg/mL, control) caused a significant induction (approx. 5- to 20-fold) of tailocin production in all strains except *P. quasiaquaticum* A411-S4-F17 (Figure 5). The highest induction was observed for *D. zeae* A586-S18-A17 (19-fold) and *D. chrysanthemi* A604-S21-A17 (20-fold). Meanwhile, the induction of tailocins by mitomycin C in *D. dadantii* 3937 was relatively low (5-fold) due to its high production in the absence of mitomycin.

Hydrogen peroxide also induced tailocin production in all strains (approx. 15- to 81-fold) (Figure 5). For most strains, hydrogen peroxide (0.88 mM, 0.003%) conferred a higher yield of tailocins than that induced by mitomycin C (0.1 μg/mL). Notably, while no induction of tailocins by mitomycin in *P. quasiaquaticum* A411-S4-F17 was observed, the addition of hydrogen peroxide resulted in the highest increase compared to basal production levels of all strains (81-fold) (Figure 5).

3.8 | Detectable Levels of Phage Tail-Like Particles Are Produced in Rotting Potato Tuber Tissue but Not in River Water

Tailocin production was explored in SRP in a variety of natural settings, in addition to cultural media. Three *Pectobacterium* spp.: *P. versatile* A69-S13-O15, *P. quasiaquaticum* A411-S4-F17, *P. aquaticum* A212-S19-A16, and three *Dickeya* spp.: *D. dadantii* 3937, *D. chrysanthemi* A604-S21-A17, *D. zeae* [very recently reclassified as *D. parazeae* (Hugouvieux-Cotte-Pattat and Van Gijsegem 2021)] (A586-S18-A17) were inoculated into river water (the source of the strains) as well as into potato tubers. Tailocin production was not detected in any strain in river water (Table 2) but was abundant in macerated potato tuber tissue. Tailocins were found not only in the symptomatic tissues caused by those strains that were pathogenic to potato (*P. versatile* A69-S13-O15, *D. dadantii* 3937, *D. chrysanthemi* A604-S21-A17, *D. zeae* A586-S18-A17) but also in healthy tuber tissue surrounding the site where nonpathogenic strains (*P. aquaticum* A212-S19-A16 and *P. quasiaquaticum* A411-S4-F17) were inoculated (Table 2; Figure S2).

3.9 | Tailocin Production Is Not Always Sufficient to Ensure Competitive Success Against Susceptible Neighbours

We explored the extent to which tailocin production by *D. solani* A623-S20-A17 (Ds) influenced the outcome of two-strain co-cultures with strains (1) susceptible to its tailocins or (2) targeted by the competitor's tailocins (Figure S3). To monitor changes in the proportion of *Ds* in mixed cultures, we selected a spontaneous rifampicin-resistant mutant of *Ds* (*Ds* RIF), which could be selectively recovered on rifampicin-containing media. Ds RIF retained tailocin production and did not differ from the parental strain in growth rate in 0.1 TSB or ability to compete

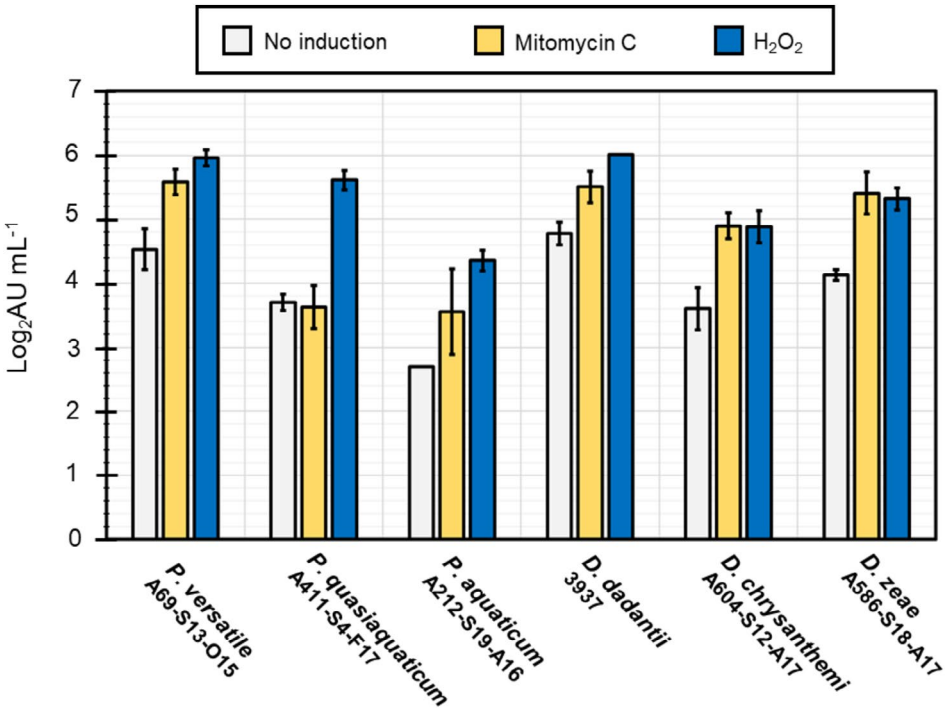

FIGURE 5 | Basal and induced tailocin production levels in six *Pectobacterium* spp. and *Dickeya* spp strains. The graph shows the logarithm of the arbitrary tailocin units per mL of culture (Log AU/mL) for three strains of *Pectobacterium* spp. (*P. versatile* A69-S13-O15, *P. quasiaquaticum* A411-S4-F17, *P. aquaticum* A212-S19-A16) and three strains of *Dickeya* spp. (*D. dadantii* 3937, *D. chrysanthemi* A604-S21-A17, *D. zeae* A586-S18-A17). The basal production levels (no induction) are compared to those with mitomycin C or hydrogen peroxide after induction. Averages from two experiments, each consisting of three separate inductions, are shown, and error bars represent the standard deviation.

TABLE 2 | Production of tailocins in river water and potato tubers.

Strain	Tailocins production			
	In Durance River water	In infected potato tuber	Mitomycin C induction in TSB	H_2O_2 induction in TSB
P. versatile A69-S13-O15	–	+	+	+
P. quasiaquaticum A411-S4-F17	–	+[a]	+	+
P. aquaticum A212-S19-A16	–	+[a]	+	+
D. dadantii 3937	–	+	+	+
D. chrysanthemi A604-S21-A17	–	+	+	+
D. zeae A586-S18-A17	–	+	+	+

Note: Qualitative tailocin production in different conditions for various bacterial strains. The strains tested include *P. versatile* A69-S13-O15, *P. quasiaquaticum* A411-S4-F17, *P. aquaticum* A212-S19-A16, *D. dadantii* 3937, *D. chrysanthemi* A604-S21-A17, and *D. zeae* A586-S18-A17. Tailocin production was assessed in infected potato tubers, in Durance River water, and as a positive control following Mitomycin C and H_2O_2 induction in TSB (Tryptic Soy Broth). The symbols represent the following: "+" indicates detectable tailocins in the sample with the applied method; "–" indicates undetectable tailocins in the sample with the applied method.
[a]Indicates that tailocins were detectable if tuber tissue in direct contact with inoculated bacteria was pooled from three potato tubers.

with the wt in vitro (Figure S4) These results indicate that the rifampicin-resistant *Dickeya* spp. strains are not compromised in competitiveness under the applied experimental conditions (Figure 6). The competitive interactions were explored with three types of competitors: (1) those susceptible to the tailocins of *Ds* RIF, (2) those producing a tailocin inhibitory to *Ds* RiF, or (3) those for which mutual killing with Ds RIF occurs in vitro. The success of a strain was defined as the extent of its end-point dominance in the co-culture (>90% of total CFU) following its initial inoculation in equal proportions (Figure S3). Two competitors that could kill *Ds* RIF with their tailocins prevailed over *Ds* RIF, suggesting that tailocins contributed to this success. However, Ds RIF outcompeted only a single strain that was susceptible to its tailocin and only in the presence of H_2O_2. This suggests that tailocins alone do not guarantee the success of a producer against a susceptible rival.

4 | Discussion

Our study revealed that most SRP strains produce phage tail-like particles. While *P. brasiliense* A143-S21-A16 and *P.*

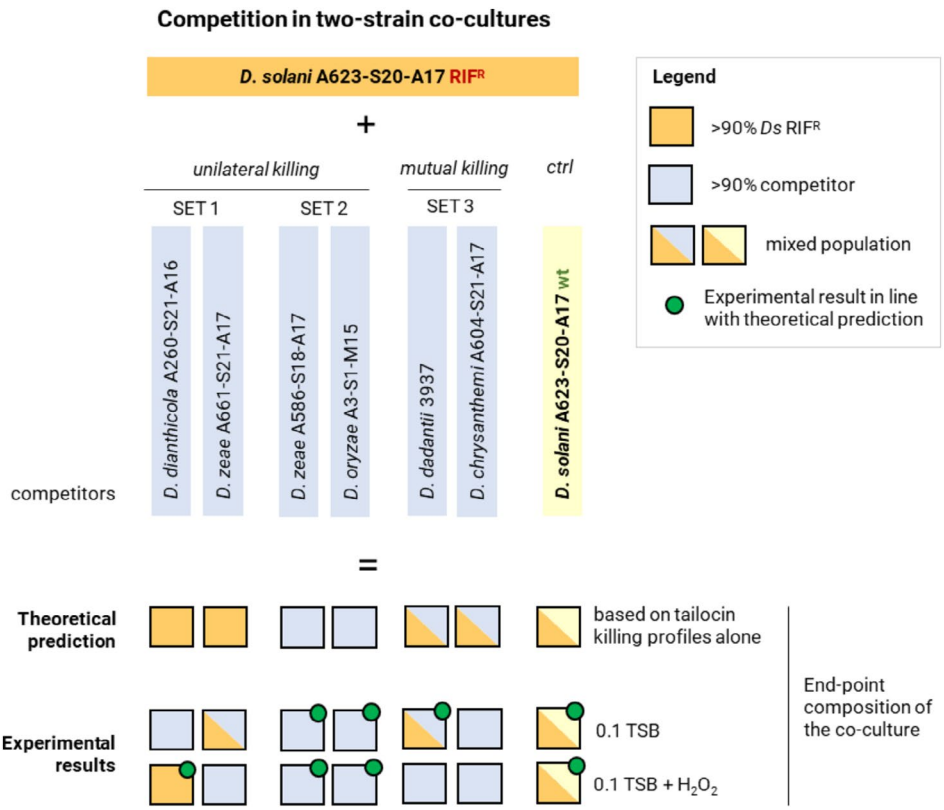

FIGURE 6 | Competition between *D. solani* A623-S20-A17 RIF^R (*Ds* RIF) and other SRP strains in co-cultures. The success was defined as the end-point dominance of a given strain in the co-culture (> 90% of total CFU), with both strains ensured an even start (1:1 mix; ca. 10^7 CFU/mL/strain). The theoretical winners, predicted based on tailocin-mediated interactions, were compared with those that were experimentally established. Co-cultures were conducted for 22 h in 0.1 TSB alone or supplemented with 0.88 mM H_2O_2 (tailocin-inductive conditions). SET 1: Strains sensitive to tailocins of *Ds* RIF. SET 2: Strains producing tailocins against *Ds* RIF. SET 3: Strains sensitive to tailocins of *Ds* RIF and producing tailocins targeting this strain (mutual killing). *ctrl*: control experiment; competition between *Ds* RIF and the *wt* variant of this strain.

atrosepticum A597-S4-A17 lacked tailocin genomic clusters, *D. dianthicola* A260-S21-A16 had a complete cluster but did not produce tailocins. This could be due to reliance on other competitive strategies (Hibbing et al. 2010) or regulation by specific environmental factors not present in the experiment (Bellieny-Rabelo et al. 2019). Furthermore, we assessed the prevalence of tailocin clusters in 190 high-quality, complete genomes of SRP bacteria and found that 83% of *Pectobacterium* spp. and 69% of *Dickeya* spp. genomes contain clusters encoding tailocins. This indicates that tailocins are a very common feature among SRP and may suggest that their production contributes to the ecological success of these bacteria (Ma et al. 2007). The prevalence of tailocins in SRP aligns with the previous observations documenting their presence in various bacterial species, including *Pseudomonas* spp. (Carim et al. 2021), *Pantoea* spp. (Stice et al. 2023) and *Streptomyces* spp. (Nagakubo et al. 2021).

Most tailocins can kill only closely related strains. This finding has, however, usually been based on studies of a small set of strains either at the species or genus level (Granato et al. 2019). Some observations suggest that some phage tail-like particles can kill different species than the producer (Yao et al. 2017; Principe et al. 2018). Our work interrogated a large group of strains belonging to three genera for their mutual tailocin-based interactions. While some SRP strains produce tailocins with a broad range of activity, killing more than 30% of other strains of different species, caution, therefore, should be taken to accept the current dogma of a narrow killing range of tailocins.

Interestingly, while some *Dickeya* spp. produced tailocins able to kill *Pectobacterium* spp. cells, the opposite was not observed in our study. Due to their evolutionary association with bacteriophage tails (Ghequire and De Mot 2015), it has been suggested that tailocins might retain some degree of flexibility in their host range, such as known phage–host interactions (Backman, Burbano, et al. 2024). Intergenus activity may remain an important trait in competition as mixed bacterial communities involving both genera are frequently observed in the field, and the ability to target a broader range of competitors could confer a selective advantage (Perombelon 1988; Charkowski 2009, 2018; Barny et al. 2024).

Likewise, the observation of mutual killing, where two strains reciprocally target each other with tailocins, has not been extensively documented. The higher incidence of mutual killing within *Dickeya* spp. compared to *Pectobacterium* spp. suggests some (yet unknown) ecological factors may select for this behaviour more often in *Dickeya* spp. than in *Pectobacterium* spp. This is somewhat surprising, given that multiple species of SRP bacteria are frequently found together in the same environments (Toth et al. 2021; Van Der Wolf et al. 2021). In such settings, these various species would be expected to experience similar

ecological pressure and benefit from reciprocal tailocin production (Shyntum et al. 2019).

Tailocins isolated from both SRP genera exhibited similar morphological characteristics despite the apparent phylogenetic differences in the origins of tailocin clusters in *Pectobacterium* spp. and *Dickeya* spp. These findings suggest that these phage tail-like particles have been acquired independently in those genera. All of the clusters present in *Dickeya* spp. exhibit homology to that encoding P2D1 tailocin of *D. dadantii* 3937 (Borowicz et al. 2023), whereas all clusters present in *Pectobacterium* spp. express homology to the cluster encoding carotovoricin Er (Yamada et al. 2006). The fact that these phage tail-like particles were observed in all *Dickeya* spp. and most of the *Pectobacterium* spp. strains tested suggests that the tailocin clusters were acquired before species diversification in both genera and were subsequently maintained rather unaltered. As tailocins are large protein complexes and their synthesis should be a burden for a producer cell, their conservation suggests that tailocin production plays an important role in the ecology of these bacteria (Glasner et al. 2008; Hugouvieux-Cotte-Pattat 2016).

The genes encoding tailocin fibre proteins exhibited the lowest homology in the tailocin clusters both in *Dickeya* spp. and *Pectobacterium* spp. This confirms that fibre proteins play a pivotal role in determining the specificity of tailocins and their killing range, as reported earlier (Dams et al. 2019). Fibre proteins are the most important determinants of target recognition in bacterial cells (Backman, Burbano, et al. 2024). This specificity is crucial for the success of tailocins as killing agents, enabling them to discriminate between susceptible and resistant bacterial strains. In bacteriophages, receptor-binding proteins are known to undergo positive selection to adapt to variations in surface receptors of target bacteria, leading to high variability even among closely related strains (Bertozzi Silva et al. 2016). In SRP, the killing spectrum of tailocins, determined by the sequence of fibre proteins, appears to be more dependent on ecological factors and the pressure of host–pathogen interactions encountered by the individual strains than on the evolutionary relationships between them.

Although mitomycin C is widely used to trigger tailocin induction under laboratory conditions, in natural and agricultural environments, the likely inducers of tailocins remain unknown. It seemed likely that other factors that cause DNA damage and thus activate the SOS pathway could also serve as inducers of tailocins. In the proof-of-concept experiment, we found that hydrogen peroxide was a potent inducer of SRP phage tail-like particles. Like mitomycin C, hydrogen peroxide is known to activate the ROS response (Storz et al. 1990; Erill et al. 2007). During infection, SRP bacteria often encounter oxidative stress in plant hosts during the immune response of the plant (Reverchon and Nasser 2013; Jiang et al. 2016). Therefore, the induction of tailocins by reactive oxygen species may serve as a mechanism for bacterial survival in their natural settings, aiding in eliminating competing bacteria under stressful conditions. Indeed, the highest levels of competition might be expected during the infection process, where the change in the plant niche during injection leads to very high bacterial numbers. The ability of plant-pathogenic bacteria, including SRP, to constitutively produce tailocins, as well as under stressful conditions, such as exposure to ROS, may lead to important ecological consequences. The basal tailocin production by a fraction of the SRP populations may be seen as an altruistic behaviour in which a small number of bacterial cells sacrifice themselves to confer an advantage for the entire population over other microorganisms residing in the same location (Griffin et al. 2004). Alternatively, basal tailocin production may be understood as a division of labour in which subpopulations of bacteria are 'assigned' to different tasks to grant success to the entire population in a given niche (Lee et al. 2010; West and Cooper 2016; Zhang et al. 2016). The ability to express tailocins in response to such oxidative stress might give SRP a competitive edge, allowing them to eliminate competitors in the plant environment when they must compete with all other microorganisms (Backman, Latorre, et al. 2024).

The variable level of response to mitomycin C and hydrogen peroxide we observed in SRP strains highlights the still unravelled complexity of tailocin regulation. This suggests that in SRP, depending on the strain and type of inducer, the production of tailocins is regulated differently in different strains. Similar observations were made for prophages, where various environmental factors induced phages with different efficiencies (Casjens 2003; Varani et al. 2013).

The SRP strains addressed in this study were isolated from a single habitat. Despite this, this collection comprised both aquatic and nonaquatic strains, most of which (except *P. aquaticum* and *P. quasiaquaticum* strains known to be avirulent on plants) infect plants in agricultural settings (Ben Moussa et al. 2022). We found that tailocins are produced in infected potato tubers but not in river water. This aligns with the other studies in which tailocins were found to be produced primarily in nutrient-rich, competitive environments (Carim et al. 2021). Decaying plant material would be expected to be a much more nutrient-rich environment than river water. The environmental conditions in potato tubers might provide several stimuli for tailocin production, including ROS, plant exudates, cell wall degradation products and microbial competition. In contrast, water is neither the common niche nor the environment where competition is likely to be common. Instead, water rather serves as a translocation medium from one host to another (Pédron and Van Gijsegem 2019; Van Der Wolf et al. 2021). This observation may be particularly pertinent for SRP bacteria, which compete with each other and other microorganisms for resources present within the plant tissues (Reverchon and Nasser 2013). The potato tuber tissue represents a primary ecological niche for SRP pathogens (Perombelon and Kelman 1980; Pérombelon and Salmond 1995). Consequently, the presence of tailocins in both rotting tissue and the healthy surrounding tissue suggests that tailocin production may be considered to be part of the virulence strategy to minimise the risk of competitive infections. These assumptions, however, still need to be experimentally assessed.

Surprisingly, SRP strains belonging to species that are not found on plants (Portier et al. 2020) and which do not provoke disease symptoms (*vide*: *P. quasiaquaticum* A411-S4-F17 and *P. aquaticum* A212-S19-A16) did produce tailocins in the plant environment but not in river water. The low nutrient availability, the cost of production of this complex machinery, and the few direct competitors of SRP bacteria in river water likely explain the absence of tailocin production in this environment (Lamichhane

and Bartoli 2015; Bradford et al. 2013; Toth et al. 2021). The fact that these tailocin clusters were maintained in these water-associated species and were induced by the same stress trigger as plant-associated species suggests that tailocin production is not solely tied to SRP virulence on plants but could also play a role in other niches where these bacteria encounter stress. For example, it has been suggested that these water-associated species could be associated with insect larvae in the water stream (Ben Moussa et al. 2022). Whether tailocin production is triggered in such an environment and/or in other environments remain to be determined.

Multiple factors play roles in the competition between microbes, with success being the sum of factors/traits of varying importance depending on the environmental context (Hibbing et al. 2010). In our study of mixed populations of tailocin producers and targets, tailocins were not always sufficient to ensure the dominance of producers over susceptible strains. The widespread occurrence of tailocin clusters among SRP led us to conclude that tailocin-mediated success is often conditional. It is well known that SRP bacteria use diverse weaponry for intragenus and intragenus interactions; therefore, tailocin production may be just one possibility to compete with kin microbes.

Several factors, such as environmental conditions, strain-specific interactions, and the relative abundance of producers versus susceptible strains, can influence the outcome of competitive interactions. This study explored an 'even start' scenario, where tailocin-producing and susceptible competitors were initially mixed in equal cell numbers. In bacterial populations, even when one strain has an antagonistic advantage (like producing phage tail-like particles), an equal start condition can neutralise this advantage because the susceptible strain is not immediately overwhelmed by that trait (Ross-Gillespie and Kummerli 2014). Moreover, tailocin production, unlike the secretion of most bacterial antimicrobials, requires the lysis of the producer cells. Ergo, it can negatively influence the growth rate of the producer's population. This burden can be assumed to be negligible when the producer-to-target ratio is high. Therefore, we speculate that tailocin production may be useful to SRP bacteria to protect already conquered niches from being invaded by competitors, a process named niche exclusion (Bauer et al. 2018).

Author Contributions

Marcin Borowicz: conceptualisation, investigation, methodology, visualisation, writing – original draft, writing – review and editing. **Dorota M. Krzyżanowska:** conceptualisation, investigation, supervision, visualisation, writing – original draft, writing – review and editing. **Marta Sobolewska:** investigation, methodology, visualisation. **Magdalena Narajczyk:** investigation, methodology, visualisation, writing – original draft. **Inez Mruk:** investigation, methodology, writing – original draft. **Paulina Czaplewska:** methodology, writing – original draft. **Jacques Pédron:** conceptualisation, investigation, methodology, writing – review and editing. **Marie-Anne Barny:** conceptualisation, investigation, methodology, writing – review and editing. **Pierre Yves Canto:** investigation, methodology. **Joanna Dziadkowiec:** conceptualisation, investigation, methodology. **Robert Czajkowski:** conceptualisation, data curation, funding acquisition, resources, supervision, writing – original draft, writing – review and editing.

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Ethics Statement

The authors have nothing to report.

Consent

The authors have nothing to report.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All data from this study are presented in the manuscript or its attached Supporting Information.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Chapter 4

Beyond kin killing: *Dickeya*-derived phage-tail-like bacteriocin P2D1 targets phylogenetically distant *Pseudomonas* spp.

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Original Article

Beyond kin killing: *Dickeya*-derived phage-tail-like bacteriocin P2D1 targets phylogenetically distant *Pseudomonas* spp.

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Abstract

Tailocins, phage-tail-derived bacteriocins, are increasingly recognized as potent mediators of microbial antagonism, yet their ecological scope beyond kin-targeting remains poorly understood. Here, we investigated whether P2D1, a tailocin produced by the plant pathogen *Dickeya dadantii* 3937, can act against environmental bacteria phylogenetically distant from *Dickeya* spp. Screening 480 soil and rhizosphere isolates from three distinct plant-associated habitats in Poland, we identified nine *Pseudomonas* spp. strains susceptible to tailocin P2D1. Whole-genome sequencing and phenotype profiling revealed that these isolates spanned multiple clades, including taxa related to *P. germanica*, *P. tensinigenes*, and *P. parakoreensis*. The *D. dadantii* mutant lacking genes encoding tailocin sheath and tube proteins lost antagonistic activity against *Pseudomonas* isolates, confirming that tailocins alone mediate the observed killing. Plant tissue assays revealed that six of the P2D1-susceptible strains were nonpathogenic and could mitigate *D. dadantii*–induced soft rot on potato. In contrast, three isolates related to *P. tensinigenes* were able to cause rot on their own under permissive conditions. Together, these findings demonstrate that P2D1 tailocin extends its activity to ecologically co-occurring but taxonomically distant *Pseudomonas*, suggesting that conserved receptors underline cross-genus targeting. More broadly, our results add to the limited evidence for tailocin activity beyond kin killing and therefore challenge the prevailing paradigm of kin-restricted tailocin specificity. They further suggest that tailocins may influence microbial community assembly across taxonomic boundaries, while their *in vivo* roles remain understudied.

Keywords **phage tail-like particles, tailocins, microbe–microbe interactions, environmental microbiology, soil, rhizosphere**

Introduction

Microbial communities in natural environments are shaped by intense competition for resources, with bacteria deploying a diverse array of strategies to outcompete their neighbors [1, 2]. Among these competitive factors, particularly intriguing yet poorly understood players are tailocins [3]. These phage tail-like bacteriocins are contractile nanomachines that, due to their structural similarities, are believed to be evolutionarily associated with bacteriophage tails [4]. Tailocins employ a single-hit killing mechanism facilitated by high-affinity recognition and membrane penetration in target bacterial cells, resulting in the rapid depolymerization of the susceptible cell and its ultimate death [5]. While tailocins production occurs across diverse bacterial species, their ecological role remains to be fully elucidated.

Tailocins are currently regarded as mediators of intraspecific competition, promoting kin killing through their specificity toward closely related strains, a context in which their activity has predominantly been characterized to date [6]. However, several studies have reported tailocins capable of acting across taxonomic boundaries [7–11], suggesting that their ecological role may be broader than previously assumed. This shift in perspective is particularly relevant in densely populated, taxonomically diverse microbial environments, where microorganisms continuously compete for limited space and resources [12, 13].

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Soft rot *Pectobacteriaceae* (SRP), including *Dickeya* spp., are well-characterized plant-associated bacteria known to produce tailocins [14]. We recently described a novel tailocin, dickeyocin P2D1, produced by *D. dadantii* strain 3937. Genetic clusters, such as those that encode the P2D1 tailocin, have been found to be widely distributed across *Dickeya* spp. [14, 15]. This suggests that P2D1 may play a significant ecological role in these bacteria, potentially conferring a competitive advantage within infected plant tissues and in other niches from which these bacteria are commonly isolated, such as soil, the rhizosphere, and aquatic environments [16, 17]. To date, SRP tailocins, including P2D1, have primarily been examined in the context of intraspecific competition, while their capacity to target nonkin bacterial taxa has not yet been systematically explored.

Here, we hypothesize that P2D1 tailocins can target phylogenetically distant environmental bacteria outside SRP, extending their ecological function beyond intraspecies competition. To demonstrate this, we independently screened for P2D1 susceptibility in a pool of environmental isolates and constructed deletion mutants of *D. dadantii* 3937 that lacked genes encoding core structural components of the P2D1 tailocin to test their interaction with these isolates. With this approach, we assessed for the first time the potential widespread ecological role of tailocins, specifically P2D1 produced by *Dickeya* spp. strains.

Materials and Methods

Bacterial strains and culture conditions

All strains used in this study are listed in Table 1. Unless otherwise specified, routine cultivation of strains was carried out at 28°C in Trypticase Soy Broth (TSB; Oxoid) with agitation at 120 rpm, or on Trypticase Soy Agar plates (TSA; Oxoid). *Escherichia coli* strains were cultured on Luria-Bertani (LB) agar supplemented with diaminopimelic acid (DAP) for the MFDpir strain. Chloramphenicol or ampicillin was added as required to maintain plasmids (Table S1).

Isolation of environmental bacterial strains

To obtain environmental isolates for subsequent testing for tailocin sensitivity, soil and rhizosphere samples were collected from three locations in Poland: tulip flower bed soil in Białogóra (54.81533° N, 17.96580° E), raspberry bush rhizosphere in Gdynia (54.50774° N, 18.54757° E), and cornfield soil in Częstochowa (50.95110° N, 19.22670° E). Samples used for microbial isolation were collected from environments with a potential presence of SRP bacteria [17]. Two grams of each sample were suspended in 4 ml of one-fourth-strength Ringer's buffer (BioMaxima), shaken at 140 rpm for 30 min at room temperature, diluted 100× in the same buffer, and plated on 10% TSB agar. After incubation for 48 h at 28°C, morphologically distinct colonies were collected, and the resulting 480 bacterial isolates were subcultured onto TSB agar plates under the same conditions to obtain pure cultures [19].

Purification of P2D1 tailocins

P2D1 tailocin particles from *D. dadantii* 3937 were purified from mitomycin C–treated cultures using a previously described protocol [15]. The purified preparations were stored at 4°C until use.

Modified spot test for high-throughput P2D1 tailocin susceptibility screening

A fast and robust screening method involving 48-well plates (Greiner) was designed to test the new bacterial isolates for sensitivity to P2D1 tailocins. Each well was first filled with 20 μl of TSB medium. Next, a single bacterial colony of each tested strain was picked from a solid medium using a wooden toothpick and suspended evenly in the TSB droplet within the well. After inoculating all wells, 500 μl of molten soft-top agar [containing 30 g TSB and 7 g bacteriological agar (Oxoid) per 1 l], cooled to $\sim$ 45°C, was added to each well. To facilitate immediate mixing of the inoculum with the freshly added medium, the multi-well plate was continuously agitated on a mini orbital shaker (Mini-Shaker, Biosan) (150 rpm) throughout the addition of the soft top agar. Once the agar had solidified, 2 μl of the purified P2D1 tailocins was spotted at the center of each well. Following 24 h of incubation at 28°C, the wells were inspected for zones of growth inhibition (clearance) indicative of tailocin activity. Wells inoculated with a P2D1-susceptible strain IFB 0117 [14] served as the positive control, while the negative control comprised the resistant P2D1 producer strain *D. dadantii* 3937. Isolates identified as sensitive during the screening were further tested using a classical spot assay [7].

Genomic sequencing

Whole-genome sequencing of P2D1-susceptible isolates was performed on a MinION platform (Oxford Nanopore Technologies) using the wf-bacterial-genomes Nextflow workflow (v1.4.1). Genomic DNA was extracted with the Wizard Genomic DNA Purification Kit (Promega). Sequencing libraries were prepared according to the Oxford Nanopore ligation sequencing protocol and loaded onto R9.4.1 flow cells. Basecalling was performed with Guppy (v6.x, Oxford Nanopore). Reads were assembled *de novo* using Flye (v2.9.5) and polished with Medaka (v2.0.0). Assemblies were annotated with the NCBI Prokaryotic Genome Annotation Pipeline (PGAP, February 2025 release).

Bioinformatic analyses

Taxonomic identification

Genome-based taxonomic identification and phylogenetic placement of the P2D1-sensitive isolates were performed using the JSpeciesWS web server [20] with FASTA genome sequences as input. First, the most closely related type strains in the GenomeDB database were identified for each isolate based on the highest *Z*-score correlation coefficient in pairwise tetra-nucleotide correlation (Tetra) analysis. Next, five type strains showing the highest Z-scores in Tetra-nucleotide analysis were subjected to pairwise Average Nucleotide Identity (ANI) calculations against the genomes of the strains under investigation. Both BLAST-based (ANIb) and MUMmer-based (ANIm) algorithms were applied. Additionally, the phylogenetic placement of the strains within the target genus was assessed based on 16S ribosomal RNA (rRNA) gene sequences (details are provided in Supplementary Dataset S1).

Dendrograms

To investigate the phylogenetic relationships between bacterial isolates, dendrograms were generated based on two independent datasets: genomic similarity and phenotypic profiles. Pairwise ANIm values (expressed as percent identity) were used to quantify genomic relatedness, and hierarchical clustering (agglomerative approach) was

Table 1 Bacterial strains used in this study.

Bacterial strain	Description	Source
***Escherichia coli* K12**		
DH5α	*supE44 lacU169 (Φ80lacZ*△ M15) *hsdR17 (rK mK) recA1 endA1 gyrA96 thi-1 relA1*	Laboratory collection
DH5α λpir	λpir phage lysogen of DH5α	Laboratory collection
MFD*pir*	*RP4-2-Tc::(ΔMu1::aac(3)IV-ΔaphA-·Δnic35-ΔMu2::zeo) ΔdapA::erm-pir) ΔrecA*	[18]
***Dickeya dadantii* 3937**		
D. dadantii 3937	Wild type, a representative member of Soft Rot *Pectobacteriaceae,* produces P2D1 tailocins	Laboratory collection
△3810	Δ*Dda3937_03810,* deficient in tailocin sheath*;* collection number (Lyon): D643	This study
△3811	Δ*Dda3937_03811,* deficient in tailocin tube*;* collection number (Lyon): D644	This study
△3810-11	Δ*Dda3937_03810* Δ*Dda3937_03811* (double mutant); collection number (Lyon): D646	This study
△3810-11 [pSJG]	Double mutant △3810-11 carrying the complementation vector pSJG	This study
Enviromental isolates		
MC6 MD6 ME6 MB7	A set of strains isolated from raspberry bush rhizosphere in Białogóra, Poland, 2024 (5 481 533°N, 1796580°E); susceptible to P2D1 tailocins from *D. dadantii 3937*	This study
Tul1A2 Tul2A7	A set of strains isolated from tulip flower bed rhizosphere in Gdynia Poland, 2024, (5 450 774° N, 1854757° E); susceptible to P2D1 tailocins produced by *D. dadantii 3937*	This study
G3-3 G3-19 G3-39	A set of strains isolated from cornfield soil in Częstochowa, Poland, 2024 (5 095 110° N, 1922670° E); susceptible to P2D1 tailocins produced by *D. dadantii 3937*	This study
Other		
Dickeya zeae NCPPB 3532	Member of the Soft Rot *Pectobacteriaceae,* sensitive to P2D1 talocins and used as an indicator strain for their detection	Laboratory collection

applied to group the strains accordingly. The same clustering method was used to analyze phenotypic data obtained from BIOLOG plate-based assays (described below), where positive and negative reactions were encoded as binary values (1 or 0). All clustering and dendrogram visualizations were performed using scikit-learn, SciPy, pandas, matplotlib, and numpy libraries [21, 22]. Python scripts supporting the analysis were developed with assistance from ChatGPT-4o (OpenAI) (Supplementary Script S1).

Comparative genomic analysis

To assess the genetic similarity among the nine P2D1-susceptible strains, we compared their genomes with respect to shared, accessory, and unique genes. The analysis was performed using the Pangenome Explorer platform (https://panexplorer.southgreen.fr/cgi-bin/home.cgi) [23]. To assess the distribution of core and accessory genes, the PanACoTA pipeline was employed. Gene clusters predicted to be present or absent in individual environmental genomes were extracted and visualized using Venn diagrams generated in PAleontological STatistics (PAST) software [24].

Strain profiling with BIOLOG phenotypic microarrays

The ability of bacterial strains to utilize different carbon sources and their tolerance to various chemical stressors were assessed using the GEN III MicroPlate™ (94 phenotypic traits, including carbon utilization, chemical sensitivity, and physiological properties) and EcoPlate™ (31 carbon-source utilization traits) (Biolog) [25]. Plates were inoculated according to the manufacturer's protocol and incubated at 28°C. At 24 and 48 h postinoculation, absorbance in each well was measured at 595 nm in the Epoch 2 microplate reader (BioTek). Results were normalized to the negative control and averaged across three biological replicates. A positive result was defined as an absorbance value at least twice that of the negative control.

Assessment of phenotypic traits in P2D1-sensitive bacterial isolates

Bacterial isolates susceptible to P2D1 were investigated for selected traits on agar-solidified media plates: colony morphology was assessed on TSA and M9 0.4% glucose (MP Biomedicals), pectinolytic activity was evaluated on Crystal Violet Pectate (CVP) [26], siderophore production was assessed using Chrome Azurol S (CAS) agar [27, 28], and pyoverdine production was evaluated in King's B [29].

Microscopic imaging

The morphology of tailocin particles purified from the cultures of the wild-type *D. dadantii* 3937 and its mutants was investigated by transmission electron microscopy (TEM). TEM imaging was performed as described earlier [15] using the Tecnai Spirit BioTWIN microscope (FEI).

Construction of P2D1-deficient mutants

P2D1 production in *D. dadantii* 3937 was abolished by generating in-frame deletion mutants in the loci *Dda3937_03810* (alternative locus designation *DDA3937_RS12110*) and *Dda3937_03811* (*DDA3937_RS12115*). The two genes encode the tail sheath protein (ADM98779.1) and tail tube protein (ADM98780.1) of the tailocin, respectively [15]. Additionally, a double mutant deprived of both genes was constructed. The deletions were performed using the

pRE112 suicide plasmid (Cm^R) carrying the *sacB* gene to enable counter-selection [30] (Supplementary Table S1). Procedures were analogous to those described earlier for *D. solani* [30]. Two polymerase chain reaction (PCR) fragments corresponding to the upstream and downstream 0.5 kbp DNA of the gene(s) to be deleted in *D. dadantii* 3937 were amplified using the Primestar master mix (Takara) and cloned into SacI/KpnI digested pRE112 using the T5 exonuclease DNA assembly (TEDA) method [31] (list of oligonucleotides available in Supplementary Table S2). Chemical ultracompetent DH5α λpir cells were prepared with the Mix & Go! *E. coli* Transformation Kit using standard procedures (Zymo Research). Transformants were selected onto an LB plate supplemented with chloramphenicol (20 μg ml^{-1}) and screened for the presence of the target construct by colony PCR with primers L762/L763. Constructs were extracted, confirmed by restriction map and Sanger sequencing, and then transferred into the competent *E. coli* strain MFDpir [18] prepared with the TSS method [31]. *Escherichia coli* MFDpir produces the RP4 conjugation machinery, which allows the transfer of the suicide plasmid into *D. dadantii 3937* by conjugation. For conjugation, colonies of *D. dadantii* 3937 and MFDpir were mixed in the same proportion in 500 μl LB and centrifuged for 2 min at 8000 rpm. The pellet was resuspended in 90 μl LB with 5 μl diaminopimelic acid at 57 mg ml^{-1} and deposited onto an LB agar plate. After an overnight incubation at 30°C, the bacteria were resuspended in 1 ml LB, diluted in a 10-fold series from 10^{-1} to 10^{-4}, and spread onto LB agar supplemented with chloramphenicol at 4 μg ml^{-1} to select the first event of recombination. Transconjugants re-isolated on this medium were then spread onto LB agar without NaCl, supplemented with 5% sucrose, and incubated at 20°C for 2–3 days to allow for the second recombination event. Sucrose-resistant colonies were then patched on LB-Cm plates to check for plasmid loss and streaked onto LB agar plates. The successful in-frame deletions were verified by colony PCR on purified colonies.

Engineering a complementation construct for the P2D1 mutation

Previous RNAseq experiments [32] indicate that transcription of the Dda3937_03810 gene initiates at position 2 751 779, which is 16 bases upstream of its start codon. Furthermore, a strong transcription termination signal is located at position 2 753 545—39 bases downstream of the Dda3937_03811 gene. These findings suggest that *Dda3937_03810* and *Dda3937_03811* function together as an operon. Based on these data, we constructed a complementation plasmid designed to co-express both *Dda3937_03810* and *Dda3937_03811*. We cloned these genes, along with the 200 bp region upstream of *Dda3937_03810,* into the low-copy mobilizable plasmid pEGL332. This arrangement ensures that the native promoter for *Dda3937_03810* is aligned in the same orientation as the *plac* promoter within pEGL332. As a result, in the complementation plasmid, *Dda3937_03810* and *Dda3937_03811* are transcribed under the control of both their native promoter and the plac promoter, which increases the likelihood of transcription of both genes. The PCR fragment containing *Dda3937_03810* and *Dda3937_03811* were amplified with oligonucleotide pairs L1802/L1803 and then cloned into HindIII-linearized pEGL332 by TEDA [31]. The hybrid plasmid, designated as pSJG, was verified by restriction mapping and DNA sequencing. Then, it was transferred to *D. dadantii 3937* strains by biparental mating using MFDpir cell as the donor strain.

Phenotypic comparison between wild-type *D. dadantii* 3937 and mutant strains

Phenotypes of the *D. dadantii* 3937 tailocin-deficient mutants, with and without complementation (Table 1), were compared to the wild-type strain. The comparisons included tailocin production, pathogenicity on potato tubers, and metabolic profiles assessed using GEN III MicroPlates and EcoPlates (Biolog), as described above. In addition, growth rates of the strains were evaluated at 28°C in TSB and in M9 medium supplemented with 0.4% glucose.

Antibiosis assay

The inhibitory activity of *D. dadantii* 3937 against environmental *Pseudomonas* spp. strains and *vice versa* was evaluated in an *in vitro* antibiosis assay on TSA plates, following the protocol [33]. After overnight incubation at 28°C, cultures were examined for the presence of inhibition zones. The assay was performed twice, with three technical replicates each time.

Virulence assays on potato tubers

Whole-tuber injection assay

To prepare the bacterial inoculum, cells from an overnight culture in TSB were harvested by centrifugation (4200 RCF, 5 min) and resuspended in PBS buffer. The turbidity of the suspension was adjusted to 0.06 McF (ca. 2×10^6 CFU ml^{-1}). To prepare plant material, tubers cv. Gala were surface sterilized by immersion for 20 min in 5% commercial bleach (ACE, Procter and Gamble), followed by a double rinse in distilled water and air drying under laminar flow. Each tuber was inoculated by inserting a pipette tip containing 50 μl of the test suspension into the tuber (up to the level of liquid within the tip) [34]. Tubers inoculated with PBS buffer alone were used as a negative control. Ten tubers per treatment were used to assess the potential virulence of P2D1-sensitive environmental isolates and to compare the *D. dadantii* 3937 wild-type strain with its mutants. Inoculated tubers were placed in humid boxes (85%–90% relative humidity). Samples were incubated at 28°C to enable the development of soft rot symptoms. After 72 h, the tubers were cut at the inoculation site, and the tissue macerated by bacteria, if present, was spooned out and weighed. The experiment was conducted twice under the same conditions.

Potato tuber slices assay

Virulence and biocontrol assay on potato tuber slices was performed according to a modified protocol from Krzyzanowska *et al.* [35]. Surface-sterilized potato tubers cv. Gala were sliced, and three wells (5 mm in diameter) were created in each slice. The slices were then placed in large glass Petri dishes (18 cm in diameter) lined with Whatman 3 filter paper disks, cut to fit, and moistened with 5 ml of sterile distilled water. To prepare bacterial inocula, cells were collected from overnight TSB cultures (4200 RCF for 5 min) and resuspended in 0.85% NaCl. Biocontrol activity was assessed by mixing equal volumes of pathogenic strain suspensions (adjusted to 0.03 McFarland, $\approx 10^6$ CFU ml^{-1}) and candidate biocontrol strains (3 McFarland, $\approx 10^8$ CFU ml^{-1}). For pathogenicity assessment and single-strain controls, treatments consisted of a single strain mixed 1:1 with

Table 2 Genome characteristics and ANI values relative to the closest type strain.

Isolate	Source	Genome size (Mbp)	CDS	GenBank accession no.	Closest related type strain (GenomesDB)	ANIb (aligned sequence), (%)	ANIm (aligned sequence), (%)
MC6	Rabsberry bush rhizosphere	6.53	5761	CP186504	*P. tensinigenes ZA 5.3*	91.86 (87.22)	93.04 (86.76)
MD6		6.49	5759	CP186503	***P. germanica* FIT28**	**98.51 (93.80)**	**98.83 (94.35)**
ME6		6.49	5755	CP186502	***P. germanica* FIT28**	**98.49 (93.80)**	**98.83 (94.35)**
MB7		6.49	5752	CP186501	***P. germanica* FIT28**	**98.51 (93.79)**	**98.83 (94.35)**
Tul1A2	Tulip flower bed soil	6.63	5788	CP186500	***P. tensinigenes* ZA 5.3**	**95.13 (90.36)**	**95.72 (91.22)**
Tul2A7		6.63	5787	CP186499	***P. tensinigenes* ZA 5.3**	**95.16 (90.18)**	**95.72 (91.22)**
G3-3	Cornfield soil	6.12	5451	CP186498	*P. parakoreensis* BML-PP030	92.42 (84.64)	**93.45 (85.62)**
G3-19		6.19	5491	JBMPIQ000000000	*P. parakoreensis* BML-PP030	92.38 (83.89)	93.36 (85.78)
G3-39		6.12	5448	CP186497	*P. parakoreensis* BML-PP030	92.45 (84.51)	93.45 (85.83)

sterile 0.85% NaCl. Each well was filled with 30 μl of the corresponding mixture, with nine wells inoculated per treatment using three slices from different tubers. The slices were incubated in a humid chamber at 28°C for 48 h, after which the diameter of tissue maceration around each well was measured. The experiment was performed twice.

Results

P2D1 tailocin-sensitive isolates were detected across all sampled locations

Out of 480 environmental isolates tested, originating from three locations, 9 were susceptible to P2D1: 4 from raspberry bush rhizosphere soil (MC6, MD6, ME6, MB7), 2 from tulip flower bed soil (Tul1A2, Tul2A7), and 3 from cornfield soil (G3-3, G3-19, G3-39) (Fig. 1A).

P2D1-sensitive isolates are members of *Pseudomonas* spp.

Analysis of 16S rRNA gene sequences classified all nine P2D1-sensitive isolates within the genus *Pseudomonas* spp. (Fig. 1B; Supplementary Dataset S1). Whole-genome sequencing enabled higher-resolution taxonomic assignment based on ANI, the accepted standard for species delineation (species-level cutoff: ∼95% to 96%).

Three of the four raspberry rhizosphere isolates (MD6, ME6, MB7) were assigned to *P. germanica*, while the two tulip flower bed isolates (Tul1A2, Tul2A7) aligned with *P. tensinigenes* (ANI >95%) (Table 2). Strain MC6 also clustered closest to the *P. tensinigenes*–type strain, but its ANIm (93%) and ANIb (92%) values fell below the species threshold. Cornfield soil isolates (G3-3, G3-19, G3-39) were most closely related to *P. parakoreensis* yet similarly failed species assignment (ANIb 92%, ANIm 93%). Thus, while several isolates could be confidently assigned to known *Pseudomonas* species, others (MC6, G3-3, G3-19, G3-39) likely represent previously undescribed taxa. Several isolates: G3-3 and G3-39 from cornfield soil; MD6, ME6, and MB7 from raspberry; and Tul1A2 and Tul2A7 from tulip—exhibited ANIb values exceeding 99% with >99% alignment (Supplementary Dataset S2). This suggests that, although independently recovered from environmental samples, isolates within each source are in most cases clonal or near-clonal, with the notable exceptions of strains MC6 and G3-19.

Hierarchical clustering of ANIm values revealed three robust clades (Fig. 1C). Generally, isolates from a given sampling location clustered together, demonstrating phylogenetic consistency within habitats. An exception was strain MC6, which, although isolated from raspberry, clustered with the two tulip flower bed isolates. Despite this close clustering, ANIm values below 90% indicated that MC6 was conspecific with neither its environmental group nor the tulip isolates.

Within each environmental clade, the remaining strains exhibited ANI values >95%, consistent with species-level similarity (Fig. 1C; Supplementary Dataset S2).

P2D1-susceptible *Pseudomonas* spp. isolates form distinct groups based on genomic and phenotypic traits

Genome sizes of the analyzed *Pseudomonas* isolates ranged from 6.12 to 6.63 Mbp (Table 2). The collective gene pool of the nine strains comprised 8121 genes, including 4181 (51.4%) core genes shared by all strains and 3062 (37.7%) accessory genes present in at least two genomes (Fig. 1D). Strain-specific genes (10.9% of the total) were largely contributed by MC6 (493 genes) and G3-19 (369). In contrast, the tulip flower bed isolates carried zero, and the remaining five isolates harbored only 2–12 each (Fig. 1D). The gene count for G3-19 may be less reliable than for the other strains, as its genome is represented by contigs rather than a closed assembly.

Genomic diversity varied across clades: the tulip flower bed isolates were nearly identical, with only three unique genes in total, whereas the raspberry rhizosphere and cornfield soil clades displayed greater heterogeneity, each comprising two or more distinct P2D1-susceptible *Pseudomonas* strains (Fig. 1E). Out of 125 traits assayed across both BIOLOG plates, 21 showed variation among the isolates. Overall, strain-relatedness inferred from biochemical profiles was consistent with that inferred from genomic sequence analysis (Fig. 1F).

In plate-based growth assays, all nine isolates grew on TSA and on M9 with glucose as the sole carbon source. Colonies appeared glossy and mucoid, with abundant exopolysaccharide production most pronounced on TSA—a trait not universally observed among *Pseudomonas* spp. (Fig. S1A and B). All strains fluoresced on King's B medium (Fig. S1C), consistent with pyoverdine production typical of fluorescent *Pseudomonas*. Only the three cornfield soil isolates (G3-3, G3-19, G3-39) grew and produced siderophores on CAS medium after 96 h, whereas the remaining six isolates showed no growth (Fig. S1D). No pectinolytic activity was detected on CVP medium at 24 h. Still, cavities appeared after 96 h in the tulip isolates Tul1A2 and Tul2A7 and in the raspberry isolate MC6—the three strains that also cluster

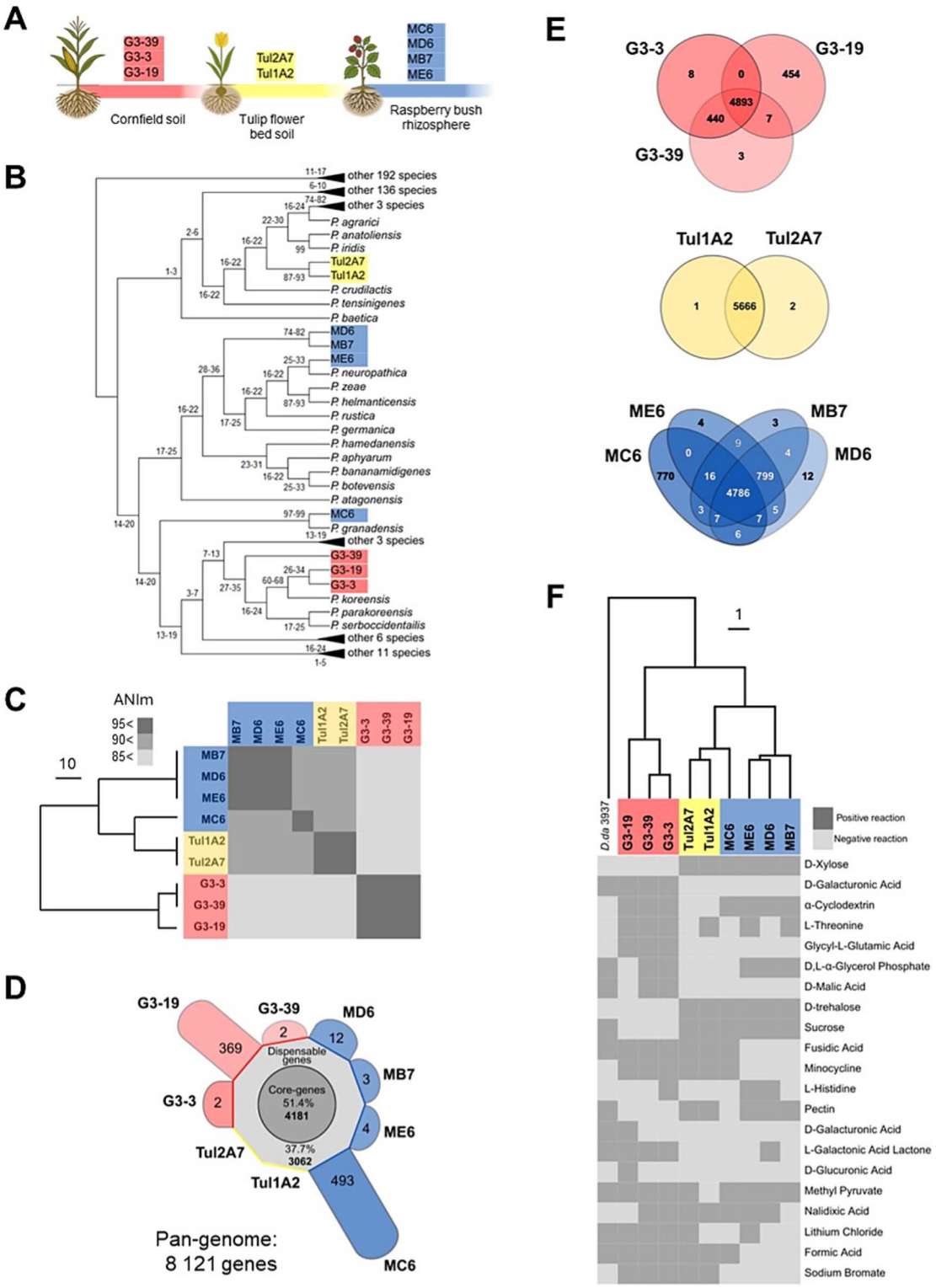

Figure 1 Genomic and phenotypic characterization of P2D1 tailocin-susceptible *Pseudomonas* isolates. (A) Summary of isolation sources with the number of isolates obtained from each environment and the corresponding colour coding: red—cornfield soil; yellow—tulip flowed bed soil; blue—raspberry rhizosphere. (B) Phylogenetic tree of *Pseudomonas*-type strains based on 16S rRNA gene sequences. The tree was inferred using the maximum likelihood method with adaptive bootstrap support. For clarity, some branches are collapsed, while those containing the strains of interest are shown in full detail. The complete tree is available in Supplementary Dataset S1. (C) Heatmap of pairwise ANIm values with hierarchical clustering. Higher values (darker shading) indicate greater genomic similarity, and the dendrogram illustrates the relationships among isolates. (D) Distribution of core, accessory, and strain-specific (unique) genes in the pangenome. (E) Venn diagrams showing: the shared gene clusters between isolates from distinct environmental origins; gene cluster overlap between strains from the same environment; and a comparison of gene content between a rhizosphere isolate from raspberry and isolates from tulip. (F) Phenotype-based dendrogram derived from BIOLOG metabolic profiles (125 individual assays), illustrating phenotypic diversity among susceptible strains. For clarity, only traits showing variation across *Pseudomonas* spp. strains are displayed in the heatmap. *D. dadantii* 3937 was used as an outgroup. Complete results are provided in Supplementary Dataset S3.

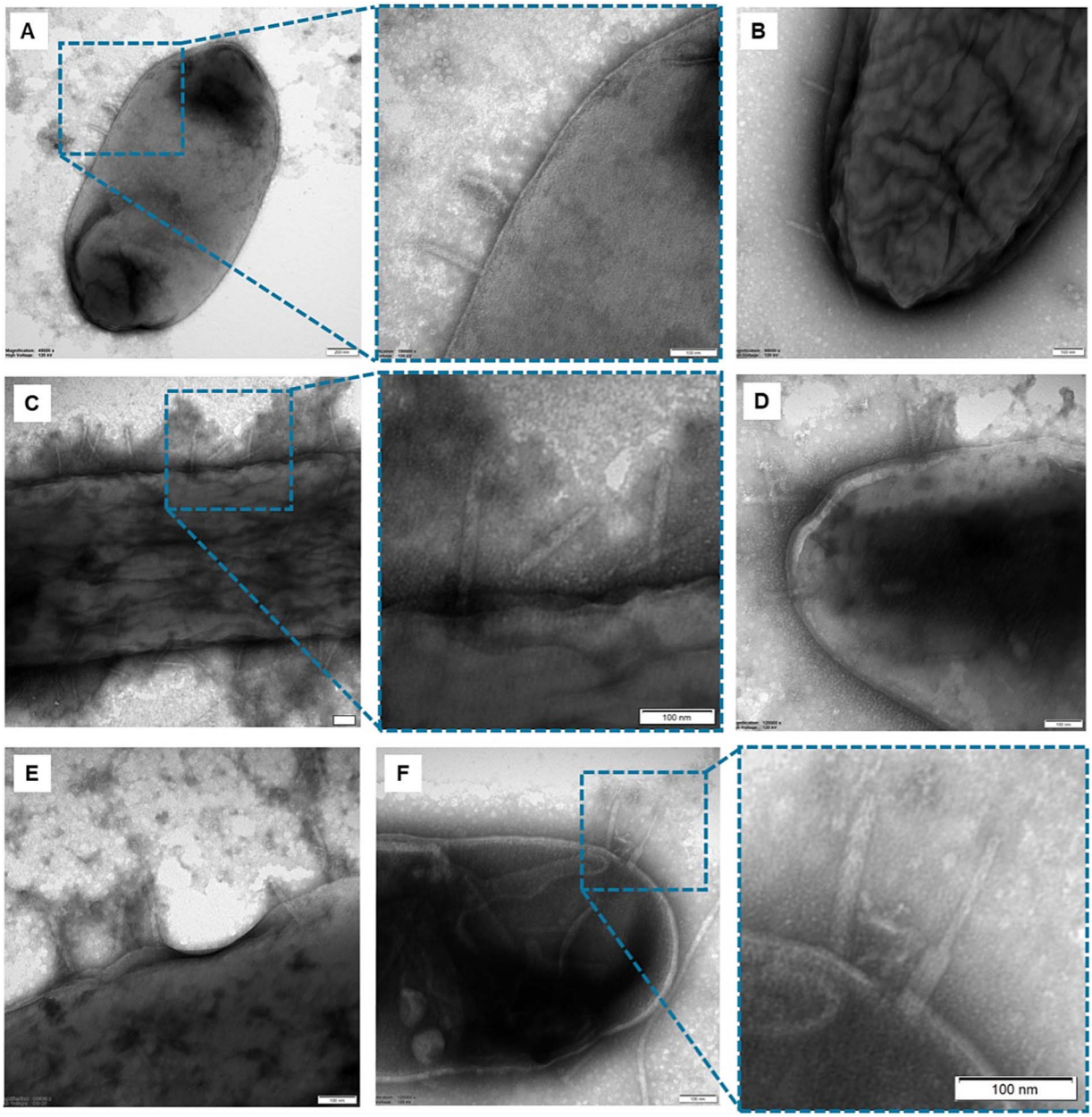

Figure 2 P2D1 tailocins of *D. dadantii* 3937 attached to the cells of susceptible environmental *Pseudomonas* spp. panels: (A) Tul1A2; (B) G3-39; (C) ME6; (D, E) MD6; (F) *D. zeae* NCPPB3532 (positive control). Scale bar: 100 nm.

together in both genomic and biochemical analyses (Fig. S1E and F). These findings indicate genomic and phenotypic heterogeneity among the P2D1-susceptible *Pseudomonas* spp. isolates.

D. dadantii 3937 tailocins attach to *Pseudomonas* cells, leading to cell puncture

Using transmission electron microscopy (TEM), we observed that P2D1 tailocins from *D. dadantii* 3937 attach to the surfaces of susceptible *Pseudomonas* cells, similarly to their binding on the positive control strain *D. zeae* NCPPB 3532 (Fig. 2A–F). These results provide direct ultrastructural evidence that *D. dadantii* tailocins physically bind to and interact with environmental isolates not related to SRP.

Tailocin-deficient *D. dadantii* mutants fail to kill environmental *Pseudomonas* spp.

To confirm that the inhibition of environmental strains by *D. dadantii* 3937 was mediated solely by tailocins, we constructed single (Δ3810, Δ3811) and double (Δ3810-11) mutants of *D. dadantii*, lacking genes encoding the sheath, the tube, or both genes, respectively (Table 1). None of these mutants produced complete (functional) tailocin particles (Fig. S2) or inhibited growth of the tailocin-susceptible SRP strain in soft-top agar overlay assays (Fig. 3). Aside from the absence of tailocin production, the mutants were indistinguishable from the wild type in growth rate (Fig. S3), virulence on potato tubers (Fig. S4, Fig. 4A), and biochemical profiles (Fig. S5). The double mutant (Δ3810-11) was selected for subsequent experiments.

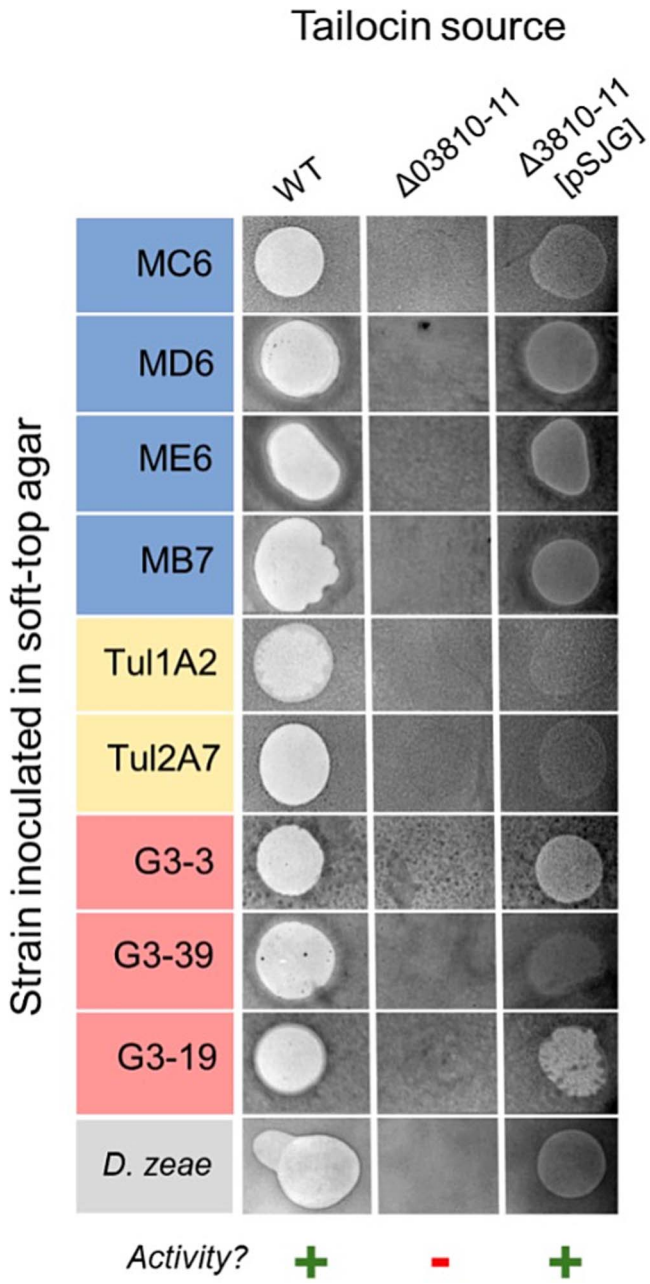

Figure 3 Activity of P2D1 tailocins isolated from *D. dadantii* 3937 WT, its P2D1-deficient mutant Δ3810-11, and a mutant with complementation plasmid pSJG. Tailocins were isolated from mitomycin C–induced cultures of the three tested strains and evaluated for activity against susceptible *Pseudomonas* isolates, as well as a control susceptible strain, *D. zeae* NCPPB 3532. The presence of a clearance zone indicates the presence of active tailocins in the tested preparation.

Tailocin preparations from induced Δ3810-11 cultures failed to inhibit any of the nine *Pseudomonas* isolates, in contrast to the wild type; however, complementation with plasmid pSJG (Table S2), which carries both disrupted genes under the operon's native promoter, restored tailocin production and activity (Fig. 3, Fig. S2). Transformation with the complementation plasmid pSJG, most likely due to metabolic cost, resulted in a some unsignificant reduced growth rate of the transformed strains in M9 medium supplemented with 0.4% glucose, both in the mutant and in the wild-type host (Fig. S3).

Additionally, direct antibiosis was assessed using a plate assay to evaluate the production of antimicrobial metabolites. For both the wild type and Δ3810-11, only contact inhibition of *Pseudomonas* spp. isolates at colony borders was observed (Fig. S6A). These results indicate that, under the tested conditions, diffusible secondary metabolites of *Dickeya* do not contribute to antagonism against environmental *Pseudomonas*, supporting the conclusion that tailocins are the primary inhibitory factor. In a reciprocal setup, no inhibition of *Dickeya* strains by the tested *Pseudomonas* spp. was observed (Fig. S6B).

Pseudomonas strains sensitive to tailocin P2D1 either cause potato decay or suppress *D. dadantii* in biocontrol assays

In the potato slice assay, three *Pseudomonas* spp. strains—Tul1A2, Tul2A7, and MC6—caused tuber tissue maceration (Fig. 4A). These included two isolates originating from tulip flower bed soil and one raspberry isolate (MC6), all most closely related to *P. tensinigenes*. In the potato injection assay, which provides more microaerophilic conditions, only MC6 displayed mild pathogenicity within the experimental timeframe (72 h postinoculation). In contrast, six isolates (MD6, ME6, MB7, G3-3, G3-19, and G3-39) not only lacked pathogenicity when inoculated alone (Fig. 4A) but also exhibited a biocontrol effect, significantly reducing soft rot symptoms caused by co-inoculated *D. dadantii* 3937 (Fig. 4B; with *Pseudomonas* isolates applied in excess, as typical for biocontrol assays). Together, these findings highlight the contrasting outcomes among P2D1-susceptible *Pseudomonas* isolates, ranging from independent pathogenicity to biocontrol of soft rot.

Discussion

This study is the first to demonstrate that P2D1 tailocins produced by *the Dickeya* spp. can kill soil-associated *Pseudomonas* spp., which are phylogenetically distant from bacteria belonging to the Soft Rot *Pectobacteriaceae* (SRP) family. So far, tailocins have generally been described as possessing narrow host ranges, restricting their ecological role to competition with closely related strains [3, 36]. Only a few studies have demonstrated broader killing spectra, including *Pseudomonas fluorescens* tailocins suppressing *Xanthomonas vesicatoria* [8], a *Burkholderia cenocepacia* tailocin killing *P. aeruginosa* [7], and *P. syringae* targeting *Erwinia amylovora*, *X. perforans,* and the human pathogen *Salmonella enterica* [10]. Our present findings, derived from a study explicitly designed to search for such "off-target" effects, suggest that previously reported cases may not be rare exceptions but instead part of a broader and underexplored phenomenon with potentially significant ecological implications.

Among 480 environmental isolates tested, only nine (1.9%) were sensitive to P2D1 tailocins, yet these isolates were found across distinct environments and geographical locations. This so-called rare-but-widespread sensitivity pattern is consistent with results obtained in other studies, where tailocin-susceptible bacterial populations were typically low in frequency but widely distributed. For example, Yao and coworkers demonstrated that BceTMilo was active against 76 *Burkholderia* isolates, including both clinical and environmental samples, indicating its efficacy across multiple geographic and environmental contexts [7]. In other studies, maltocin P28 was active against 38 clinical and environmental *Stenotrophomonas maltophilia* strains [37], and tailocin from *P. syringae* USA011R targeted strains from

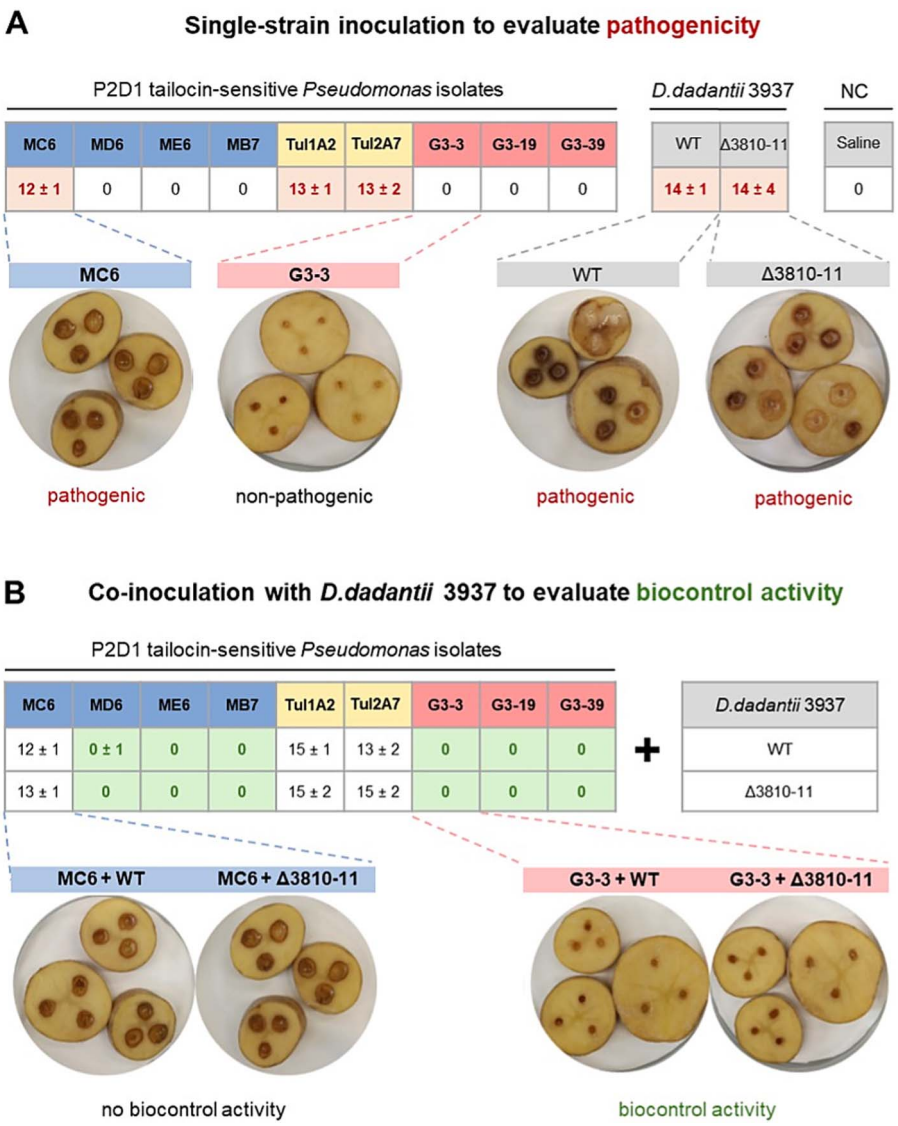

Figure 4 Effect of *Pseudomonas* isolates on potato tuber tissue in a slice assay. (A) shows the ability of *pseudomonas* isolates, *D. dadantii* 3937, and the 3937 tailocin mutant (Δ3810-11) to cause disease symptoms on tuber tissue when inoculated alone (pathogenicity). (B) shows the potential of the *pseudomonas* isolates to protect potato tissue from maceration when co-inoculated with the known pectinolytic pathogen *D. dadantii* 3937 (biocontrol activity). Median diameter of rotten tissue (mm; ± half interquartile range) is provided in the tables for all tested combinations. Alongside the tabulated results, the figure shows representative images depicting the characteristic appearance of healthy and diseased samples.

distinct genera sourced from plant, clinical, and laboratory environments [10]. Likewise, R-type pyocins of *P. aeruginosa* targeted only a small fraction of clinical and environmental isolates; however, sensitive strains are found in different cystic fibrosis patients [38]. Furthermore, from an ecological perspective, finding susceptible isolates in geographically distant soils supports the idea that tailocins may act as selective forces in diverse plant microbiomes. Rhizosphere studies demonstrate that pyocins can significantly influence strain competition and community assembly, particularly under conditions of nutrient limitation [3].

Microscopic imaging revealed that P2D1 tailocins attach directly to the cell surface of susceptible *Pseudomonas* spp. isolates, resembling the interaction observed in the known susceptible strain *D. zeae* NCPPB 3532 [15]. This observation was supported by experiments with a P2D1-defective *D. dadantii* 3937 mutant, which failed to kill susceptible *Pseudomonas* spp. cells, thereby demonstrating that P2D1 tailocins alone are sufficient to mediate the observed killing. However, the specific surface determinants underlying P2D1 susceptibility in both *Dickeya* and *Pseudomonas* remain unidentified. Studies of *Pseudomonas* strains susceptible to tailocins produced by other *Pseudomonas* (pyocins) have shown that their binding specificity and target sensitivity are primarily governed by the structure, composition, and proper presentation of the lipopolysaccharide (LPS) O-antigen on the bacterial surface [39]. In the same model, conserved LPS chemotypes promote cross-species binding and susceptibility to tailocins among *Pseudomonas* strains [39]. In *Dickeya* spp. and the related *Pectobacterium* spp., LPS has been implicated in sensitivity to certain, but not all, bacteriophages [40, 41]. By contrast, the determinants of tailocin susceptibility in these bacteria remain uncharacterized. Identifying the surface features, particularly those shared between *Dickeya* spp.

and *Pseudomonas* spp. that enable P2D1 cross-reactivity, may represent an important direction for future research, as described in other bacterial systems [42, 43].

In plant assays, P2D1-susceptible *Pseudomonas* spp. showed contrasting effects on plant tissue, both when inoculated alone and when co-inoculated with *D. dadantii* 3937. Three environmental isolates behaved as opportunistic soft-rot pathogens, whereas six others exhibited attenuation of *D. dadantii*-induced soft rot in potato tubers. These dynamics mirror the known dual roles of *Pseudomonas* spp.: some species (e.g. *P. marginalis*, *P. palleroniana*) cause soft rot [44, 45], whereas others serve as biocontrol and plant-beneficial agents [46]. Importantly, *Pseudomonas* spp. are known to co-occur with *Dickeya* spp. and *Pectobacterium* spp. in rotting potato tissues as part of a polymicrobial "spoilage microbiota" [47]. Several pectinolytic *P. fluorescens* strains have been identified alongside *Pectobacterium* spp. in diseased potato tubers in Kenya, confirming that both strains can coexist in rotten potato tissue [48]. At the same time, other *Pseudomonas* spp. are known biocontrol organisms: *P. chlororaphis* suppresses *Dickeya* spp. virulence by quenching quorum-sensing signals [49], while *P. fluorescens* eliminates competing *Pectobacterium* through a Type VI-secreted amidase, thereby protecting potato tubers [50]. Consistently, co-inoculation of antagonistic *P. fluorescens*, *P. putida,* or *P. donghuensis* strains has been shown to significantly reduce potato blackleg/soft-rot severity caused by SRP pathogens [19, 33, 35, 51].

It is therefore evident that P2D1-susceptible *Pseudomonas* spp. isolates can compete with SRP, including *D. dadantii,* on different planes—either as alternative pathogens of potato tissue or as antagonists mitigating soft rot. Our results, therefore, indicate that by targeting *Pseudomonas* with divergent ecological functions, P2D1 tailocins could function as potent modulators of both microbial competition and plant health.

Taken together, our findings indicate that tailocins produced by plant-associated bacteria can act against ecologically co-occurring competitors across genera, rather than being restricted to genetically closely related strains. Clusters encoding P2D1-like tailocins are widespread across *Dickeya* spp., suggesting positive selection for this trait [14]. Such an antagonistic capacity may be particularly advantageous for soft-rot pathogens, which cycle between insect, aquatic, soil, and plant niches and repeatedly encounter diverse resident microbiota [16, 17, 52]. Likewise, tailocin production is inherently costly, as it requires cell lysis and can leave producing populations vulnerable to competition by insensitive community members in mixed habitats [53]. Consequently, the net benefit of tailocin deployment likely depends on spatial structure, timing, and the fraction of cells committing to production [54]. At the same time, the precise contribution of P2D1 to *Dickeya* success during niche invasion remains unresolved. Future *in vivo* studies will be crucial to determining the ecological relevance of tailocins and their role in shaping microbial community structure.

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Author contributions

Marcin Borowicz (Supervised laboratory experiments by undergraduate students, Performed experimental work, Carried out bioinformatic analyses, Contributed to figure preparation, Analyzed data, Drafted the manuscript, (Contributed to funding acquisition), Jan Styn (Collected samples, Performed the screening for tailocin susceptibility, Participated in laboratory experiments), Kacper Tomasik (Collected samples, Performed the screening for tailocin susceptibility, Participated in laboratory experiments), Łukasz Rąbalski (Performed DNA sequencing on the isolates), Magdalena Narajczyk (Performed transmission electron microscopy imaging), Erwan Gueguen (Designed and supervised the construction of mutants), Sylwia Jafra (Generated mutant and complementation strains), Julie Baltenneck (Generated mutant and complementation strains), Dorota M. Krzyżanowska (Contributed to writing the original draft and the review editing, Analyzed data, Contributed to figure preparation, Provided supervision and conceptual input), and Robert Czajkowski (Contributed to conceptualization, writing the original draft and the review editing, Supervised the research, Responsible for funding acquisition)

Supplementary material

Supplementary material is available at *ISME Communications* online.

Conflicts of interest

None declared.

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Data availability

The *Pseudomonas* spp. genome sequences have been deposited in NCBI GenBank under accession numbers CP186497 - CP186504 and JBMPIQ000000000.

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Original Article

Summary and outlook

Prior to the work presented in this thesis, tailocin-mediated interactions in Soft Rot Pectobacteriaceae had received little experimental attention. The only R-type tailocin described in detail from this group of bacteria was carotovoricin Er from *Pectobacterium carotovorum* (Nguyen et al., 1999; Yamada et al., 2006), and no tailocin had been characterised in any *Dickeya* species. Four publications resulting from my doctoral research project have changed this picture. I established that *Dickeya dadantii* produces a distinct R-type tailocin, dickeyocin P2D1, whose production can be triggered by ecologically relevant stress; that tailocin production is widespread among environmental SRP strains; that killing interactions among these strains form complex networks that cross genus boundaries; and that the killing range of P2D1 extends to phylogenetically distant *Pseudomonas* species that share the same habitats as *Dickeya*. Together, these findings move tailocins from a molecular curiosity observed in single reference strains to a widespread and potentially consequential component of the SRP competitive arsenal.

The characterisation of dickeyocin P2D1 (Chapter 1) revealed a particle whose structural and genetic features place it firmly within the R-type tailocin family but whose evolutionary origin is distinct from that of the only other SRP tailocin known at the time. P2D1 is stable across the pH and temperature ranges typical of plant surfaces and rhizosphere soil, but is irreversibly inactivated by freezing and strong acidity (<3.5 pH) (Borowicz et al., 2023). These physicochemical boundaries define the conditions under which P2D1 could function as a competitive weapon, from seasonal and geographic constraints at the landscape scale to local variation within the microniches that bacteria occupy on plant surfaces and in the rhizosphere. The P2D1 gene cluster shares homology with the tail of *Peduovirus* P2, whereas the carotovoricin Er cluster of *Pectobacterium* derives from a different phage lineage (Yamada et al., 2006). That two closely related genera, which frequently co-occur in the same infected plant tissue, carry tailocins of independent phage origin indicates that the selective pressure favouring tailocin acquisition has operated independently in each lineage.

Resolving when and how P2D1 is produced was the focus of Chapter 2. The temporal profile of P2D1 production follows a tightly coordinated sequence: structural gene expression peaks within two hours of induction, particle accumulation in the supernatant reaches its maximum at six hours, and particle levels remain stable for at least eighteen hours thereafter

in a laboratory setting (Sobolewska, Krzyżanowska, Borowicz et al., 2025). The six-hour delay between transcription and peak particle release reflects the time required for translation, assembly, and lysis of the producing cells – a process that kills over 99.9% of the induced population. P2D1 is also produced constitutively, without deliberate induction although at levels approximately 60-fold lower than the induced maximum (Borowicz et al., 2023; Borowicz et al., 2025; Sobolewska, Krzyżanowska, Borowicz et al., 2025), consistent with observations in other systems where a small fraction of cells undergoes spontaneous lysis and tailocin release while the majority continues to grow (Sigal et al., 2024; Vacheron et al., 2021). Beyond confirming the lethal cost of tailocin deployment, this temporal profile has a practical implication: once released, P2D1 particles persist in the extracellular environment for hours, which means that even a brief burst of production can generate a lasting pool of bactericidal particles. The demonstration that hydrogen peroxide, ciprofloxacin, and norfloxacin all trigger P2D1 production at levels comparable to mitomycin C, which strongly suggests that tailocin induction is linked to the DNA damage response rather than to a mitomycin C-specific pathway. Of these inducers, H_2O_2 is the most pertinent to natural conditions, because SRP bacteria encounter plant-derived reactive oxygen species during the apoplastic oxidative burst that accompanies infection (Bolwell et al., 2002; Van Gijsegem et al., 2017).

Whether tailocin production is a property of individual reference strains or a common trait of SRP populations in natural habitats was addressed in Chapter 3. The survey of 27 strains isolated from the Durance River showed that 24 produce tailocin particles detectable by electron microscopy, and genomic screening of 190 complete SRP genomes confirmed that tailocin clusters are present in 83% of *Pectobacterium* and 69% of *Dickeya* genomes (Borowicz et al., 2025). Genomic comparison further revealed that all *Dickeya* tailocin clusters are homologous to the P2D1 locus, whereas all *Pectobacterium* clusters are homologous to the carotovoricin Er locus, confirming that the two genera acquired their tailocins independently from different phage donors and have maintained them since before species diversification (Borowicz et al., 2025). The mapping of 351 pairwise killing interactions among the Durance River strains revealed that *Dickeya* tailocins kill more frequently and across broader taxonomic boundaries than *Pectobacterium* tailocins, and that *Dickeya* tailocins can target *Pectobacterium* strains whereas the reverse was not observed. The asymmetry of these interactions, together with the high incidence of mutual killing among *Dickeya* strains,

indicates that tailocin-driven competition within SRP is not a simple producer-versus-target dynamic but a network of reciprocal and inter-genus antagonism whose outcome depends on the specific combination of strains present.

Chapter 4 extended this picture beyond SRP by demonstrating that P2D1 can kill soil-associated *Pseudomonas* species that are phylogenetically distant from its producer. Among 480 environmental isolates screened, nine *Pseudomonas* strains spanning several phylogenetic clades were susceptible, and deletion of the P2D1 sheath and tube genes in *D. dadantii* 3937 abolished killing, confirming that the observed activity depends on the tailocin particle (Borowicz et al., 2026). The susceptible strains were found across geographically distinct habitats, which is consistent with the rare-but-widespread sensitivity pattern reported for tailocins in other systems (Weaver et al., 2022; Yao et al., 2017). Six of the nine susceptible *Pseudomonas* are nonpathogenic and suppress *D. dadantii* soft rot on potato, which means that P2D1 has the capacity to eliminate, at least under laboratory conditions, bacteria that would otherwise function as natural antagonists of its producer. The remaining three cause soft rot themselves under permissive conditions. P2D1 can therefore target bacteria with contrasting ecological roles relative to its producer, consistent with the co-occurrence of SRP and *Pseudomonas* in plant-associated environments, where both genera are commonly found (Barny et al., 2024; Borowicz et al., 2026; Ge et al., 2021; Toth et al., 2011). That a tailocin from a strain isolated in 1970 retains killing activity against contemporary environmental *Pseudomonas* implies that the surface epitopes recognised by P2D1 are conserved over decades.

These findings raise a question that runs through the entire thesis: if tailocin production requires the death of the producing cell, why are tailocin gene clusters maintained in the vast majority of SRP genomes, and why have they been independently acquired from different phage donors in *Pectobacterium* and *Dickeya*? Several lines of evidence from the evolutionary and genomic record point toward a selective advantage that outweighs the cost. The genomic prevalence itself is an argument: tailocin clusters have been retained across species diversification in both genera, which implies sustained positive selection over evolutionary time rather than neutral drift (Borowicz et al., 2025; Ghequire & De Mot, 2015). The fiber protein genes – the primary determinants of target specificity – are the most variable component of the tailocin cluster in both genera, a pattern consistent with ongoing

diversifying selection driven by coevolution with target bacteria (Borowicz et al., 2025; Fautt et al., 2025). That tailocin loci of independent phage origin have been maintained in parallel in two genera that share the same hosts and habitats reinforces the conclusion that the fitness benefit is robust and not contingent on a single ecological context (Ghequire & De Mot, 2015).

The ecological data generated in this thesis complement this evolutionary picture. When SRP strains were inoculated into potato tissue and into river water under laboratory conditions, tailocin particles were detected in the infected tissue but not in the water (Borowicz et al., 2025), linking tailocin production to the nutrient-rich, high-density conditions of plant infection – precisely the conditions under which theoretical models predict that interference competition is most effective (Booth et al., 2023; Cornforth & Foster, 2013). H_2O_2 – a compound that SRP encounter during every plant infection – triggers tailocin production at concentrations comparable to mitomycin C (Borowicz et al., 2025; Sobolewska, Krzyżanowska, Borowicz et al., 2025), providing a plausible mechanism by which the plant immune response could activate interbacterial competition among colonising bacteria. The killing range demonstrated in Chapter 4 means that the competitive benefit of tailocin production is not limited to eliminating close relatives but extends to a broader pool of organisms encountered in soil and plant tissue, including *Pseudomonas* strains with biocontrol activity against *Dickeya* spp. itself.

Against these arguments, however, stands a limitation that this thesis shares with most studies of tailocin ecology published to date: all evidence for killing comes from laboratory assays. Although tailocin particles were detected in experimentally infected potato tissue (Chapter 3), this demonstrates that particles are present during infection under laboratory conditions, not that they kill competitors in that tissue. The competition experiments in Chapter 3 showed that tailocin producers do not always outcompete susceptible strains in mixed culture. In Chapter 4, although P2D1 killed *Pseudomonas* in spot assays, co-inoculation of susceptible *Pseudomonas* with wild-type *D. dadantii* and its tailocin-deficient mutant on potato tissue – designed to test whether the tailocin provides a competitive advantage during plant infection – yielded no measurable difference in disease outcome. This null result may reflect the design of the biocontrol assay, in which *Pseudomonas* was applied in excess – a ratio at which even complete tailocin release may not generate enough particles to shift the competitive balance – or it may indicate that the potato slice assay does not capture the

spatial or temporal dynamics under which tailocin activity affects competition *in planta*. Resolving this will likely require experimental systems that more closely replicate the conditions of natural infection. For example, this could include whole-plant inoculation models in which bacterial colonisation proceeds over days rather than hours, or microfluidic co-culture systems that allow real-time observation of tailocin release and target cell death at the single-cell level, as has been demonstrated for pyocins in *Pseudomonas* (Vacheron et al., 2021). Such work would also benefit from addressing several molecular unknowns that remain unresolved: 1) the identity of the P2D1 receptor on susceptible cells – presumably an LPS component (Kohler et al., 2010), but not yet demonstrated in SRP; 2) the regulatory cascade linking the SOS response to tailocin gene expression in *Dickeya*, where homologues of the PrtR/PrtN regulators known from *Pseudomonas aeruginosa* have not been characterised (Penterman et al., 2014); and 3) the mechanism that determines which fraction of a population commits to tailocin production – and death – under a given level of stress. Whether the H_2O_2 concentrations generated during the apoplastic oxidative burst are sufficient to activate tailocin production in planta also awaits direct measurement, and spatiotemporal tracking of tailocin gene expression during infection – using fluorescent reporters or genetically encoded H_2O_2 biosensors – would establish when and where in the infection process the transition from genomic potential to active deployment occurs. Beyond their ecological role, whether tailocins could be harnessed as targeted biocontrol agents against SRP – exploiting their narrow specificity and inability to self-replicate – remains an open avenue that the characterisation work in this thesis now makes possible to explore.

This thesis began with a single particle from a single strain and ends with a system: tailocins are produced by the majority of tested environmental SRP, are triggered by a stress that these bacteria encounter during every plant infection, form complex killing networks that extend across genera, and target ecologically relevant competitors in habitats where SRP live. Together, these findings indicate that tailocins are a functional component of interbacterial competition in SRP. How their deployment translates into competitive outcomes across the diverse environments that these bacteria occupy – from infected plant tissue to soil, water, and insect-associated niches – remains an open question, and answering it will require moving from laboratory assays to the habitats in which SRP compete and cause disease.

Summary and outlook references

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"*Let us depart. No matter how difficult it is... we must live."*

Hayao Miyazaki, *Nausicaä of the Valley of the Wind, Vol. 7*

About the author

Marcin Borowicz

Education

Intercollegiate Doctoral School of Biotechnology of the University of Gdansk and Medical University of Gdansk, Poland

PhD student in Biotechnology *2022 - ongoing*

Identification and characterization of functional prophages present in *Pectobacterium* spp. and *Dickeya* spp. genomes.

Intercollegiate Faculty of Biotechnology of the University of Gdansk and Medical University of Gdansk, Poland

Master of Science in Biotechnology *2020 - 2022*

Molecular determinants of bacterial adhesion to biotic and abiotic surfaces – a case study of the *Ochrobactrum* genus.

Intercollegiate Faculty of Biotechnology of the University of Gdansk and Medical University of Gdansk, Poland

Bachelor of Science in Biotechnology *2017 - 2020*

Potato tuber microbiome in the context of susceptibility to soft rot – metagenomic analysis and the formation of a collection of isolates

Scientific projects participation

Intercollegiate Faculty of Biotechnology UG & MUG, Poland

PhD Student

NCN project SONATA BIS 10 PI: Robert Czajkowski
10.2020 - ongoing

University of Gdańsk, Poland

International Genetically Engineered Machine (iGEM) team Instructor

Providing day to day support to IFB-Gdansk team **(Gold medal)**
01.2023 - 11.2023 (11 months)

Bio Laboratorium of Pomeranian Science and Technology Park Gdynia, Poland

Research team leader

InnovaBio Pomorze project, project title: "UV germicidal lamp"
06.2020 - 11.2020 (5 months)

Bio Laboratorium of Pomeranian Science and Technology Park Gdynia, Poland

Research team member

InnovaBio Pomorze project, project title "Analysis of the siderophores produced by strain of *Pseudomonas* sp. using the isoelectric focusing technique".
06.2018 - 11.2018 (5 months)

Internships

Institute of Ecology and Environmental Sciences of Paris, Sorbonne University, France

France Excellence (SSHN), Campus France

From core weapons to accessory systems: understanding competition within in Soft Rot *Pectobacteriaceae*
11.2025 - 12.2025 (1 month)

MAP Labaratory, University Claude Bernard Lyon 1, CNRS, INSA, France

Polish National Agency for Academic Exchange Internship

Molecular biology techniques for Soft Rot *Pectobacteriacea* mutagenesis
11.2024 - 12.2024 (1 month)

University of Oslo, Norway

Erasmus+ Internship

Atomic Force Microscopy training
02.2024 - 03.2024 (2 weeks)

University of Turku, Finland

Baltic Science Network Mobility Programme for Research Internships

Action spectrum of light-harvesting antenna phosphorylation in Arabidopsis thaliana
07.2021 - 10.2021 (3 months)

Teaching Experience

University of Gdańsk (2022–2025)
Individual Research Project – Biotechnology (BSc, 3rd year)
Teaching Assistant (laboratory classes)
- Mentored students in experimental design, data analysis, and scientific reporting

University of Gdańsk (2023–2025)
Practical Applications of Microorganisms (BSc, 3rd year)
Teaching Assistant (laboratory classes)
- Conducted laboratory classes focused on applied microbiology

University of Gdańsk (2025)
Fundamentals of the Prokaryotic Cell (BSc, 1st year)
Teaching Assistant (tutorial classes)
- Led discussion-based classes on laboratory safety and microbial visualization

University of Gdańsk (2026)
Bacterial Model Organisms (BSc, 1rd year)
Teaching Assistant (tutorial and laboratory classes)
- Taught basic concepts of bacterial physiology using model organisms

Grants

UGrants-start Fellowship – University of Gdańsk 2025
Competitive internal research grant supporting doctoral projects aimed at publication in high-impact journals.
Project: Ecological impact of tailocins produced by a model Soft Rot *Pectobacteriaceae* bacterium
Funding: 15,000 PLN. Principal Investigator: Marcin Borowicz.

PLGrid Computational Grant – Poland's national supercomputing infrastructure 2024 - 2025
Project: Structure of the P2D1 tailocin and other particles produced by *D. dadantii* 3937
Awarded 5,000 GPU hours on NVIDIA A100 and 30 TB HPC storage on the Athena supercomputer Polish high-performance computing infrastructure PLGrid; grant no. PLG/2024/017352
Principal Investigator: Marcin Borowicz.

Scientific publications

Borowicz M, *Styn J, Tomasik K, Rąbalski Ł, Narajczyk M, Gueguen E, Jafra J, Baltenneck J, Krzyżanowska D, Czajkowski R*
Beyond kin killing: Dickeya-derived phage-tail-like bacteriocin P2D1 targets phylogenetically distant *Pseudomonas* spp.
ISME Communications, *2026*

Sobolewska M, Krzyżanowska D, ***Borowicz M****, Czajkowski R*
Production of P2D1 tailocins by *Dickeya dadantii* 3937: a temporal relationship between the stressor onset, gene expression, and the concentration of active particles
Scientific Reports, *2025*

Borowicz M*, Krzyżanowska D, Sobolewska M, Narajczyk, M, Mruk I, Czaplewska P, Pedron, J, Barny, MA, Canto, P Dziadkowiec, J, Czajkowski R*
Tailocin-mediated interactions among Soft Rot *Pectobacteriaceae.*
Molecular Ecology, *2025*

Jafra S, Jabłońska M, Maciąg T, Matuszewska M, ***Borowicz M****, Prusiński M, Żmudzińska W, Thiel M, Czaplewska P, Krzyżanowska D, Czajkowski R.*
An iron fist in a velvet glove: the cooperation of a novel pyoverdine from *Pseudomonas donghuensis* P482 with 7-hydroxytropolone is pivotal for its antibacterial activity.
Environmental Microbiology, 2024

Borowicz M*, Krzyżanowska D, Narajczyk M, Sobolewska M, Rajewska M, Czaplewska P, Węgrzyn K, Czajkowski R.*
Soft rot pathogen *Dickeya dadantii* 3937 produces tailocins resembling the tails of *Peduovirus* P2.
Frontiers in Microbiology, 2023

Borowicz M*, Krzyżanowska D, Jafra S.*
Crystal violet-based assay for the assessment of bacterial biofilm formation in medical tubing.
Journal of Microbiological Methods, 2023

Selected scientific conferences

Invited speech
Borowicz M*, Krzyżanowska D, Czajkowski R*
Ecological Role of Tailocins in Soft Rot *Pectobacteriaceae* and Their Impact on Microbial Community Structure
Mini Symposium on Tailocins, *Danish Society for Viruses of Microbes, 2025, Kopehnagen, Denmark*

Oral presentation - best presentation award
Borowicz M*, Krzyżanowska D, Czajkowski R.*
Repurposed Phage Tails in Environmental Interactions:
Tailocins of Soft Rot Pectobacteriaceae
IV Bacteriophage Symposium 2025, Gdańsk, Poland

Oral poster presentation - best poster award
Borowicz M*, Krzyżanowska D, Czajkowski R.*
Repurposed Phage Tails in Environmental Interactions:
Tailocins of Soft Rot Pectobacteriaceae
Plant Security and Food Safety: Microbiology, Soil Science, Food Quality and Agricultural Genetics, 2025 Toruń, Poland

Oral presentation
Borowicz M*, Krzyżanowska D, Czajkowski R.*
Decoding the ecological impact of tailocins in Soft Rot Pectobacteriaceae
XVII Meeting of the Working Group 'Biological and integrated control of plant pathogens', 2025, Torino, Italy

Oral and poster presentation
Borowicz M*, Krzyżanowska D, Narajczyk M, Sobolewska M, Mruk I, Czaplewska P, Rajewska M, Dziadkowiec J, Pedron J, Barny MA, Czajkowski R.*
Phage tail-like particles (tailocins) as weapons in fratricidal competition among Soft Rot *Pectobacteriaceae*
miCROPe2024, Vienna, Austria

Awards and scholarships

2025
Best Oral Presentation at IV Bacteriophages Symposium, Poland

2025
Best Poster Presentation at Plant Security and Food Safety: Microbiology, SoilScience, Food Quality and Agricultural Genetics, 2025 Toruń, Poland

2025
III place at Univentum Labs Ideas – Young Fahrenheit competition

2024
NAWA - STER scholarship for research internship at Univeristy of Lyon

2024
Best Oral Presentation at III Bacteriophages Symposium, Poland

2022
Best Master's Thesis in Genetics, Polish Genetic Society Gdańsk Division

2017, 2018, 2019, 2021
Rector’s Scholarship for outstanding Academic Performance

2018, 2019, 2021
Congratulatory letter of Dean of the IFB UG & MUG for outstanding academic performance

Positions of trust

2025
PhD students’ representative in the Biotechnology Discipline Council Intercollegiate Faculty of Biotechnology UG & MUG

2019 - 2021
Vice Chairman of the Faculty Council of Students Government Intercollegiate Faculty of Biotechnology UG & MUG

2019 - 2022
Students’ representative in the Faculty Council Intercollegiate Faculty of Biotechnology UG & MUG

Languages

Polish - native
English - fluent (C1)

Interests

Synthetic biology
Comic books
Science popularization

Running
Swimming
Chess

Appendix 1: Supplementary information to publications in Chapters 1-4

Supplementary information for Chapter 1

Soft rot pathogen *Dickeya dadantii* 3937 produces tailocins resembling the tails of *Peduovirus* P2

Borowicz, M., Krzyżanowska, D. M., Narajczyk, M., Sobolewska, M., Rajewska, M., Czaplewska, P., Węgrzyn, K., & Czajkowski, R. (2023).
Frontiers in Microbiology, 14, 1307349.
https://doi.org/10.3389/fmicb.2023.1307349

Supplementary Materials for

Soft rot pathogen *Dickeya dadantii* 3937 produces tailocins resembling the tails of *Enterobacteria* bacteriophage P2

Marcin Borowicz *et al.*

Corresponding author: Robert Czajkowski. Email: robert.czajkowski@ug.edu.pl

This PDF file includes:

Figs. S1 to S4
Tables S1 to S2
References

Other Supplementary Materials for this manuscript include the following:

Data S1 to S2

Fig. S1

Host-dependent killing rate of P2D1 dickeyocins. The killing rate was calculated as the percentage (%) of the remaining viable bacterial cells of the susceptible *Dickeya* spp. strains (measured by OD_{600}) after 20 min (A) and after 120 min (B) of their incubation with P2D1 dickeyocins. The results are shown as a box plot; whiskers reflect the maximum and minimum, box sides reflect the first and third quartile, and the bars reflect medians. Points indicate particular measurements (n=10). Statistically significant differences between the treatments were obtained using Welch's one-way analysis of variance followed by the Games-Howell post hoc test Groups with the same letter are not significantly different. ($p<0.05$).

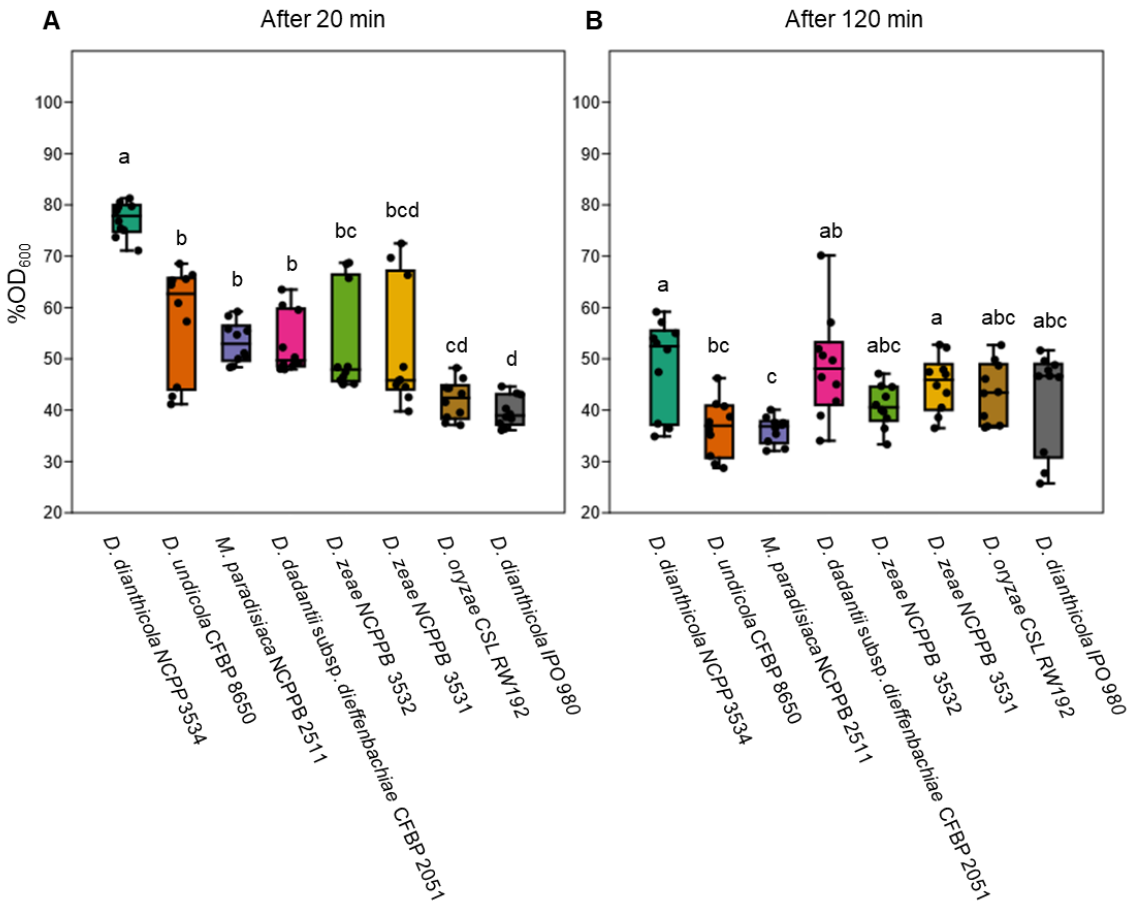


Fig. S2

Remaining activity of dickeyocins P2D1 after their binding to viable and nonviable (dead) cells of the susceptible (*M. paradisiaca* NCPPB 2511) and nonsusceptible (*D. dadantii* 3937) strains. Results are shown as box plots; the whiskers reflect the maximum and minimum, the box sides reflect the first and third quartile, bars reflect the medians. Points indicate particular measurements (n=9). Statistically significant differences between the treatment and the control were obtained using Kruscal-Wallis's one-way analysis of variance followed by Dunn's post hoc test. Groups with the same letter are not significantly different ($p<0.05$). AU – relative units.

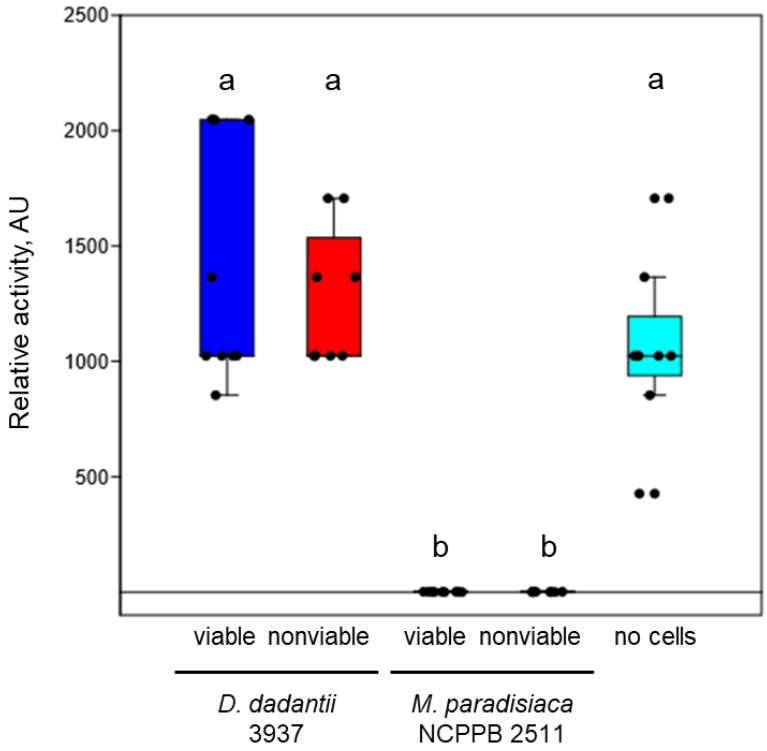


Fig. S3

Morphologic comparison of phage tail-like particles produced by T6SS defective mutant (A5587) and P2D1 dickeyocins produced by a wild type *D. dadanti* strain 3937. Representative TEM images of tailocins produced by the *Dickeya dadantii* wild-type strain and mutant A5587 (insertion in the *tssK* gene) are shown. The diameters (weight and length) were compared using TEM, with *n* representing the number of particles measured to obtain the average.

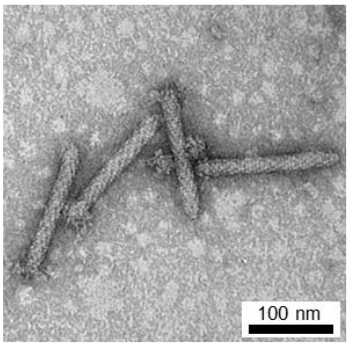
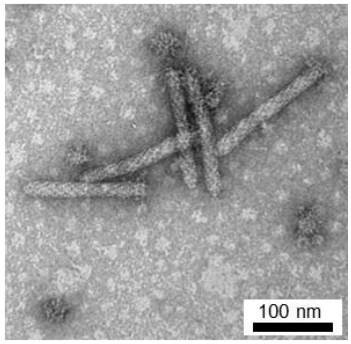
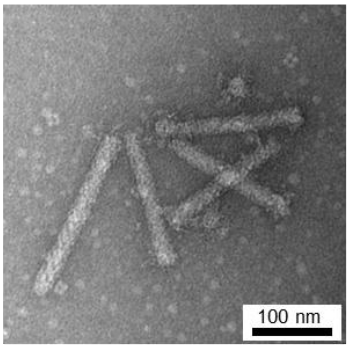
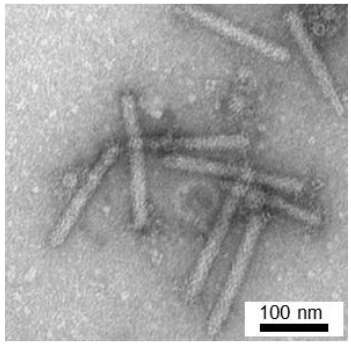
	Dimensions (nm ± SD)		Morphology in TEM	
	Length	**Width**		
Wild type *D. dadanti* 3937 *n=50*	166 ± 7	23 ± 2		
T6SS defective mutant (A5587) *n=25*	171 ± 10	25 ± 2		

Fig. S4

Survival of *C. elegans* in the presence of P2D1 dickeyocins in the liquid killing assay. *C. elegans* growth medium supplemented with PBS without tailocins served as a control. Eight different concentrations of P2D1 were tested. Results are shown as box plots; the whiskers reflect the maximum and minimum, the box sides reflect the first and third quartile, bars reflect the medians. Points indicate particular measurements (n=9).

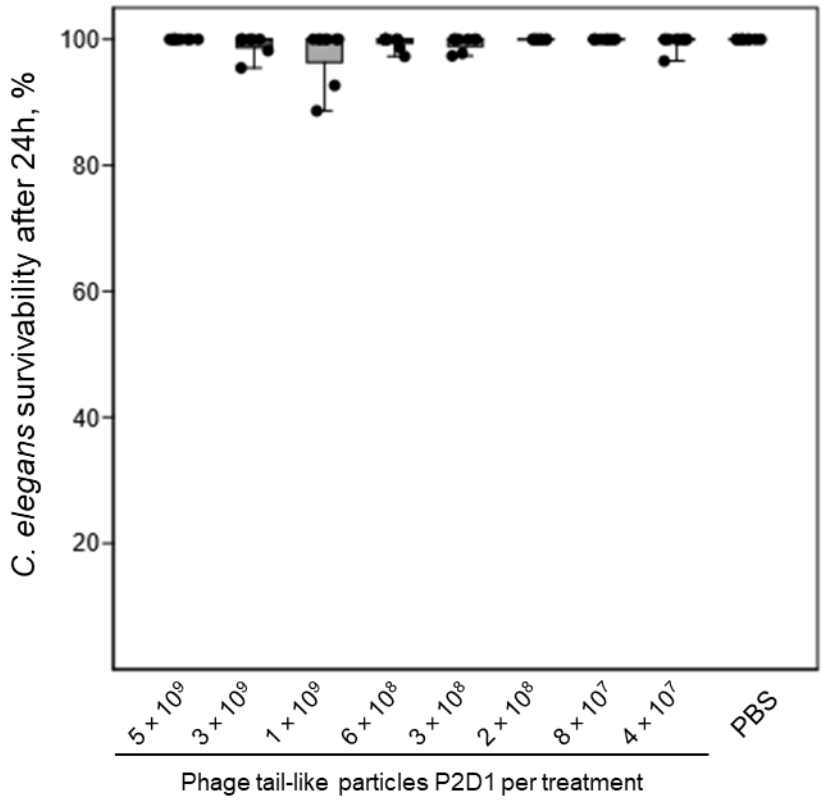


Table S1

List of strains used in this study.

Strain	Host plant/origin of isolation	Geographical origin, year of isolation	Other collection numbers	Ref.
Soft Rot *Pectobacteriaceae*				
Dickeya chrysanthemi NCPPB 3533	*Solanum tuberosum*	United States, 1987	IFB0139	*(1)*
Dickeya chrysanthemi NCPPB 402	*Chrysanthemum morifolium*	United States, 1956*	IFB0055, CFBP 2048, ATCC 11663	*(1, 2)*
Dickeya chrysanthemi NCPPB 516	*Parthenium argentatum*	Denmark, 1957*	IFB0724, CFBP 1270	*(1)*
Dickeya dadantii 3937	*Saintpaulia sp.*	France, 1972	IFB0016	*(3)*
Dickeya dadantii DSM 18020	*Pelargonium capitatum*	Comoros, 1961*	IFB0010, CFBP 1269, NCPPB 898, SCRI 1269	*(2)*
Dickeya dadantii NCPPB 3537	*Solanum tuberosum*	Peru, 1987	IFB0127	*(1)*
Dickeya dadantii subsp. *dieffenbachiae* NCPPB 2976	*Dieffenbachia*	United States, 1977*	IFB0718, CFBP 2051	*(1, 2)*
Dickeya dianthicola IPO 980	*Solanum tuberosum*	Netherlands	IFB0140	*(4)*
Dickeya dianthicola NCPPB 3534	*Solanum tuberosum*	Netherlands, 1987	IFB0126	*(4)*
Dickeya fangzhongdai DSM 101947	*pear tree (bleeding cancer)*	China, 2009	IFB0716, CFBP 8607	*(5)*
Dickeya lacustris CFBP 8647	*water*	France, 2017	IFB0715, LMG 30899	*(6)*
Dickeya oryzae CSL RW 192	*river water*	England	IFB0220	*(1)*
Dickeya poaceiphila NCPPB 569	*Saccharum officinarum*	Australia, 1958	IFB0717, CFBP 8731	*(7)*
Dickeya solani D s0432-1	*Solanum tuberosum*	Finland, 2004	IFB0135, IPO 3295, LMG 27551	*(8)*
Dickeya solani GBBC 2040	*Solanum tuberosum*	Belgium, 2007*	IFB0484, LMG 25865	*(4)*
Dickeya solani MK10	*Solanum tuberosum*	Israel	IFB0723	*(4)*
Dickeya solani MK16	*river water*	United Kingdom	IFB0272, IPO 3494	*(1, 4)*
Dickeya sp. CSL RW 240	*river water*	England	IFB0721	*(1)*
Dickeya sp. MK7	*river water*	Scotland	IFB0275	*(1)*
Dickeya sp. NCPPB 3274	*Aglaonema*	St. Lucia, 1983	IFB0722	*(1)*
Dickeya undicola CFBP 8650	*water*	Malaysia, 2014	IFB0714, LMG 30903	*(9)*
Dickeya zeae MK19	*river water*	Scotland	IFB0719	*(1)*
Dickeya zeae NCPPB 3531	*Solanum tuberosum*	Australia, 1987*	IFB0138	*(1)*
Dickeya zeae NCPPB 3532	*Solanum tuberosum*	Australia, 1987*	IFB0720	*(1)*

Musicola paradisiaca NCPPB 2511	*Musa paradisiaca*	Colombia, 1973*	IFB0117, ATCC 33242, LMG 2542	*(1, 10)*
Pectobacterium actinidiae LMG 26003	*Actinidia chinensis*	Korea	IFB5641	*(11)*
Pectobacterium aroidearum NCPPB 929	*Zantedeschia aethiopica*	South Africa, 1959	IFB5514, LMG 2417	*(12)*
Pectobacterium atrosepticum NCPPB 549	*Solanum tuberosum*	United Kingdom, 1957	IFB5399, CFBP1526, ATCC 33260	*(13)*
Pectobacterium atrosepticum SCRI 1043	*Solanum tuberosum*	Scotland, 1985	IFB5102	*(14)*
Pectobacterium betavasculorum CFBP 2122	*Beta vulgaris cv. Saccharata*	USA, 1971	IFB5269, CFBP 2122, NCPPB 2795	*(13)*
Pectobacterium brasiliense LMG 21371	*Solanum tuberosum*	Brazil, 1999	IFB5390, ATCC BAA-417	*(15)*
Pectobacterium cacticida CFBP 3628	*Carnegiea gigantea*	USA, 1944	IFB5644, ATCC 49481, CIP 105191	*(16)*
Pectobacterium carotovorum CFBP 2046	*Solanum tuberosum*	Denmark, 1952	IFB5263, NCPPB 312, ATCC 15713	*(16)*
Pectobacterium fontis CFBP 8629	*water*	Malaysia, 2015	IFB5645, LMG30744	*(17)*
Pectobacterium parmentieri CFBP 8475	*Solanum tuberosum*	France, 2008	IFB5648, LMG 29774	*(18)*
Pectobacterium parmentieri SCC3193	*Solanum tuberosum*	Finland, 1980s	IFB5395	*(19)*
Pectobacterium peruviense CFBP 5834	*Solanum tuberosum*	Peru, 1979	IFB5232, LMG 30269; PCM 2893; SCRI 179	*(20)*
Pectobacterium polaris NCPPB 4611	*Solanum tuberosum*	Norway, 2010	IFB5646, CFBP 8603	*(21)*
Pectobacterium polonicum DPMP 315	*vegetable field*	Poland, 2016	IFB5673, LMG 31077	*(22)*
Pectobacterium punjabense CFBP 8604	*Solanum tuberosum*	Pakistan, 2017	IFB5642, LMG30622	*(23)*
Pectobacterium versatile CFBP 6051	*Solanum tuberosum*	Netherlands, 2001	IFB5636, NCPPB 3387	*(15)*
Other strains				
Citrobacter freundii ATCC 8090		Unknown, 1928	NCTC 9750	*(24)*
Escherichia coli ATCC 25922	*clinical isolate*	USA, 1946	DSM 1103, NCIB 12210	*(25)*
Escherichia coli ATCC 8739	*feces*			*(26)*
Escherichia coli OP50				*(27)*
Klebsiella aerogenes ATCC 51697				*(28)*
Klebsiella quasipneumoniae ATCC 700603			K6, CCUG 45421, LMG 20218	*(29)*
Pseudomonas aeruginosa PA14	*clinical islate*		DSM 19882	*(30)*
Pseudomonas aeruginosa PAO1	*clincal isolate*	Australia, 1954	DSM 22644, ATCC 15692	*(31)*
Pseudomonas donghuensis P482	*Solanum lycopersicum*	Poland, 2012		*(32)*
Serratia marcescens ATCC 14756		USA	PCI 1107	*(33)*
Staphylococcus aureus ATCC 25923	*clinical isolate*			*(34)*

* – the year of addition to the collection
NCPPB – National Collection of Plant Pathogenic Bacteria
CFBP – French Collection for Plant Associated Bacteria
DSM – German Collection of Microorganisms and Cell Cultures GmbH
ATCC – American Type Culture Collection
LMG (BCCM) – Belgian Coordinated Collections of Micro-organisms

Table S2

Determination of the concentration of P2D1 dickeyocins induced from *D. dadantii* strain 3937. To determine the concentration of the dickeyocins, three independent methods were used: (i) semiquantitative estimation by a spot test, (ii) Poisson distribution killing method, and (iii) direct particle count with NanoSight NS300. For the estimation of the number of dickeyocins, three independent inductions of P2D1 dickeyocins (=3 biological replicates) were done. Likewise, the induction of phage tail-like particles from T6SS defective mutant of *D. dadantii* strain 3937 was done using the same protocol as used for P2D1 dickeyocins and described in the Materials and Methods section. The results are shown as relative units (AU) mL^{-1} ± standard deviation, killing particles mL^{-1} ± standard deviation, and particles mL^{-1} ± standard error, respectively.

Sample		Concentration determination method		
		Semiquantitative estimation (spot test, AU mL^{-1} ± standard deviation)	**Poisson distribution killing method,** (killing particles mL^{-1} ± standard deviation)	**Direct particle count with NanoSight NS300**, (particles mL^{-1} ± standard error)
Inductions from strain 3937	I	2.2±1.0 × $\mathbf{10^6}$	1.6±1.3 × $\mathbf{10^{11}}$	2.8±0.6 × $\mathbf{10^{10}}$
	II	7.2±5.1 × $\mathbf{10^6}$	2.7±1.9 × $\mathbf{10^{11}}$	1.5±0.2 × $\mathbf{10^{11}}$
	III	4.8±1.7 × $\mathbf{10^6}$	2.9±1.7 × $\mathbf{10^{11}}$	8.8±0.5 × $\mathbf{10^{10}}$
Induction from the T6SS defective 3937 mutant		3.8±0.8 × $\mathbf{10^6}$	2.1±1.4 × $\mathbf{10^{11}}$	8.2±1.0 × $\mathbf{10^{10}}$
PEG purification followed by ultracentrifugation		6.1±2.2 × $\mathbf{10^6}$	1.2±1.1 × $\mathbf{10^{12}}$	1.0±0.1 × $\mathbf{10^{12}}$

Data S1 (separate file)
NCBI database searches using blastp and blastn.

Data S2 (separate file)
Comparison of sequence homology between proteins of P2D1 and phage P2.

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Supplementary information for Chapter 2

Stress-driven temporal production of phage tail-like particles (tailocins) in *Dickeya dadantii* strain 3937

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SUPPLEMENTARY INFORMATION

to research article

Stress-driven temporal production of phage tail-like particles (tailocins) in *Dickeya dadantii* strain 3937

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Supplementary Figures

Figure S1. Specificity of primers applied in real-time qPCR. Agarose gel electrophoresis (2%) depicting single PCR products of the expected sizes for each of the analyzed target genes. L – GeneRuler™ 100bp DNA Ladder Plus (Thermo Fisher Scientific). nc – no template negative control; DNA staining was done using Novel Juice (GeneDireX). The image was captured with a ChemiDoc XSR (Bio-Rad) and processed using Image Lab software.

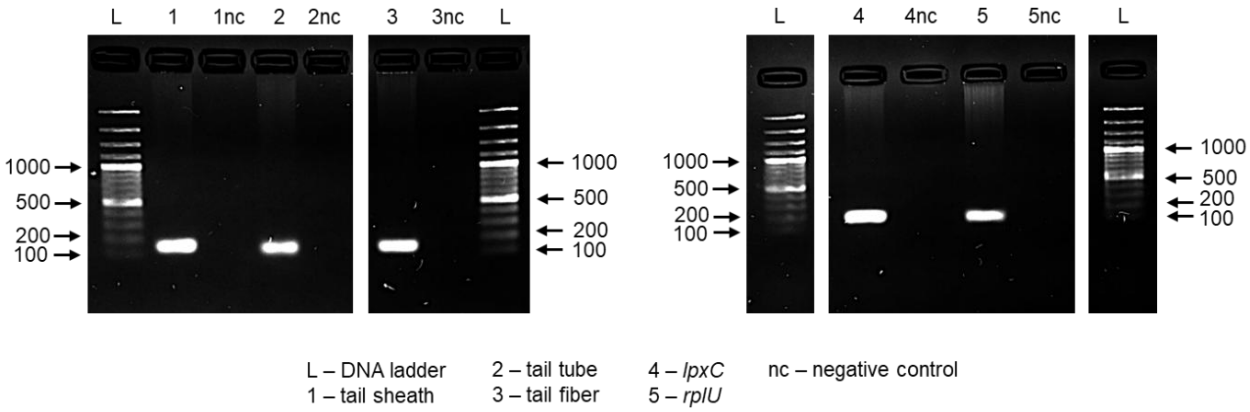


Figure S2. Melt curve analysis. Melt curve analysis (55-95 ºC with 0.5 ºC increment every 5 seconds) for the amplicons of each target gene, demonstrating that each pair of PCR primers produces a single peak. The gene loci corresponding to the designated targets are provided in Table S2.

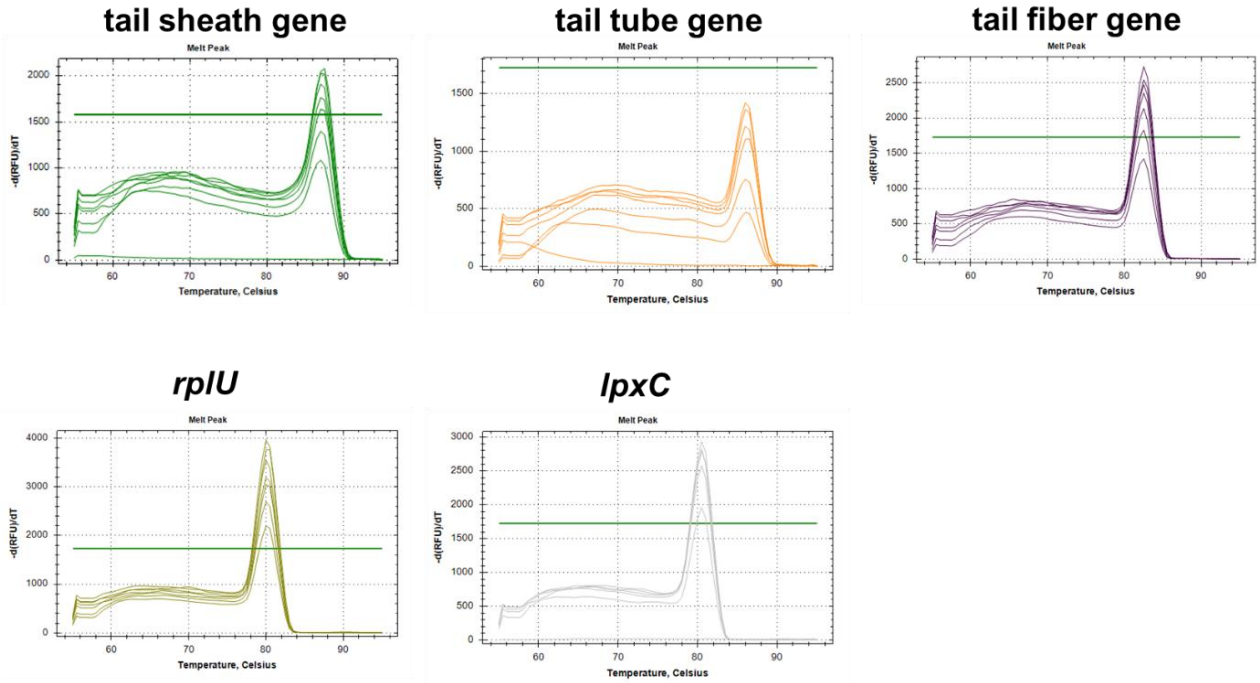
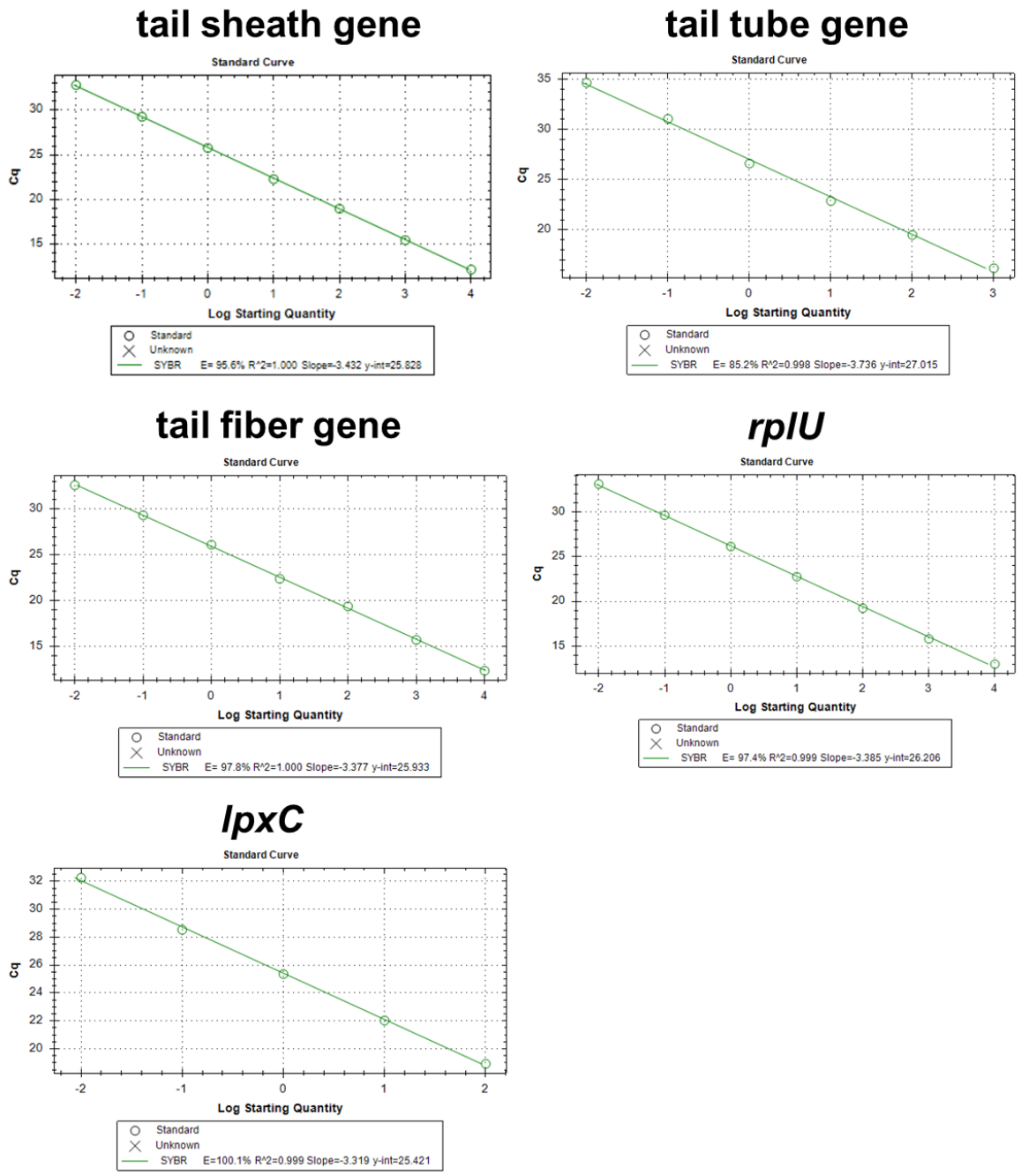


Figure S3. Standard curves for the estimation of PCR efficiency. Standard curves were used to determine PCR efficiency for the reference genes (*rplU* and *lpxC*) and the target genes (tail sheath gene, tail tube gene, and tail fiber gene). The gene loci corresponding to the designated targets are provided in Table S2.



Supplementary Tables

Table S1. Conditions for the induction of tailocin production.

Experiment	Inducer	Concentration	Unit	Incubation time, h
Effect of mitomycin C concentration on tailocin yield	Mitomycin C	0.1; 0.2; 0.3; 0.4; 0.5; 1; 1.5; 2; 3; 4; 10	μg mL^{-1}	24
Tailocin yield at different time points after induction	Mitomycin C	1	μg mL^{-1}	0; 0.5; 1; 2; 4; 6; 8; 24
Potential of selected inducers to induce tailocins	Mitomycin C	1	μg mL^{-1}	6
	Chloramphenicol	4	μg mL^{-1}	6
	Ampicillin	0.004	μg mL^{-1}	6
	Ciprofloxacin	0.016	μg mL^{-1}	6
	Norfloxacin	0.016	μg mL^{-1}	6
	Hydrogen peroxide	0.5; 1; 5; 10	mM	6

Table S2. Primers designed and used in this study.

Product of the target locus (gene name)	**GenBank locus tag (in genome NC_014500.1)**	**Primer name**	**Primer sequence**	**Amplicon length (bp)**	**Primer efficiency (%)**
Sheath	DDA3937_RS12110	Sht_3937_F	TGATCTGGCCGGACTTTATC	134	95.6
		Sht_3937_R	GACGTTGGACAGGGTTTTGT		
Tube	DDA3937_RS12115	Tub_3937_F	GGTGATGGAATGGAACATGG	116	85.2
		Tub_3937_R	TGTCGTCACGCTGGTAAGAG		
Fiber	DDA3937_RS12070	Fib_3937_F	GATCCTGATTGTCAGCACGA	125	97.8
		Fib_3937_R	TGAGTTGAGTGTCGGCGTAG		
UDP-3-O-acyl-N-acetylglucosamine deacetylase (*lpxC*)	DDA3937_RS18015	LpxC_3937_F	GCACCTGAAATTCCGATCAT	142	100.1
		LpxC_3937_R	CCCATTTGTCACCGTCTTCT		
50S ribosomal protein L21 (*rplU*)	DDA3937_RS02990	RplU_3937_F	ATGTACGCGGTTTTCCAAAG	124	105.5
		RplU_3937_R	CAACCATCAGAACCTGGTCA		

Table S3. Expression of structural genes encoding P2D1 at different time points following induction.

		Control			Mitomycin C treatment		
Gene (locus[A])	**time (h)**	**Exp.**	**Exp. SD**	**log_2 of Exp.**	**Exp.**	**Exp. SD**	**log_2 of Exp.**
Fiber	0	1.00	0.15	0.00	1.01	0.18	0.01
RS12070	1	1.30	0.31	0.38	3.85	0.62	1.94
	2	1.45	0.34	0.53	277.05	58.02	8.11
	4	1.87	0.48	0.90	62.66	17.56	5.97
Sheath	0	1.00	0.07	0.00	0.54	0.09	-0.89
RS12110	1	1.74	0.18	0.80	7.90	2.47	2.98
	2	1.27	0.22	0.35	374.81	70.33	8.55
	4	0.51	0.11	-0.96	91.84	24.95	6.52
Tube	0	1.00	0.12	0.00	0.99	0.26	-0.01
RS12115	1	1.07	0.21	0.10	8.78	1.25	3.13
	2	1.39	0.23	0.48	910.87	162.64	9.83
	4	0.76	0.16	-0.40	166.36	162.04	7.38

Exp. – normalized relative expression; Each value is averaged from 3 biological replicates; reference genes: *lpxC, rplU*

[A] Locus tag in the genome of *D. dadantii* 3937 (NC_014500.1); shared prefix: DDA393

Supplementary information for Chapter 3

Tailocin-mediated interactions among Soft Rot *Pectobacteriaceae*

Borowicz, M., Krzyżanowska, D. M., Sobolewska, M., Narajczyk, M., Mruk, I., Czaplewska, P., Pédron, J., Barny, M. A., Canto, P. Y., Dziadkowiec, J. & Czajkowski, R. (2025).
Molecular Ecology, 34(8), e17728.
https://doi.org/10.1111/mec.17728

Supplementary Materials for

Tailocin-mediated interactions among Soft Rot *Pectobacteriaceae*

Marcin Borowicz [a], Dorota M. Krzyżanowska [a], Marta Sobolewska [a], Magdalena Narajczyk [b], Inez Mruk [c], Paulina Czaplewska [c], Jacques Pédron [d], Marie-Anne Barny [d], Pierre Yves Canto [d], Joanna Dziadkowiec [e] and Robert Czajkowski [a,*]

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This PDF file includes:

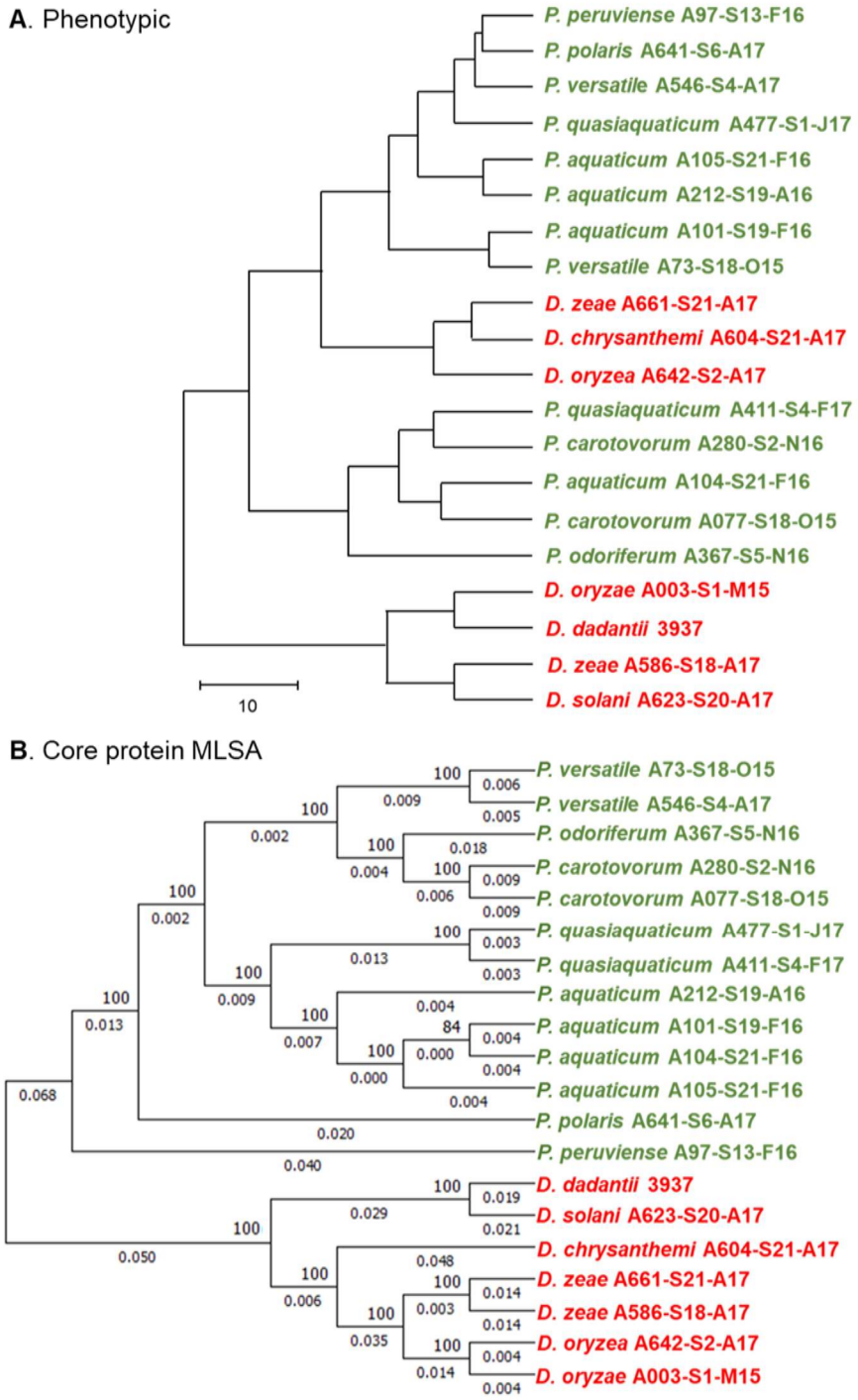
Figs. S1 to S4
Tables S1 to S2
References

Other Supplementary Materials for this manuscript include the following:

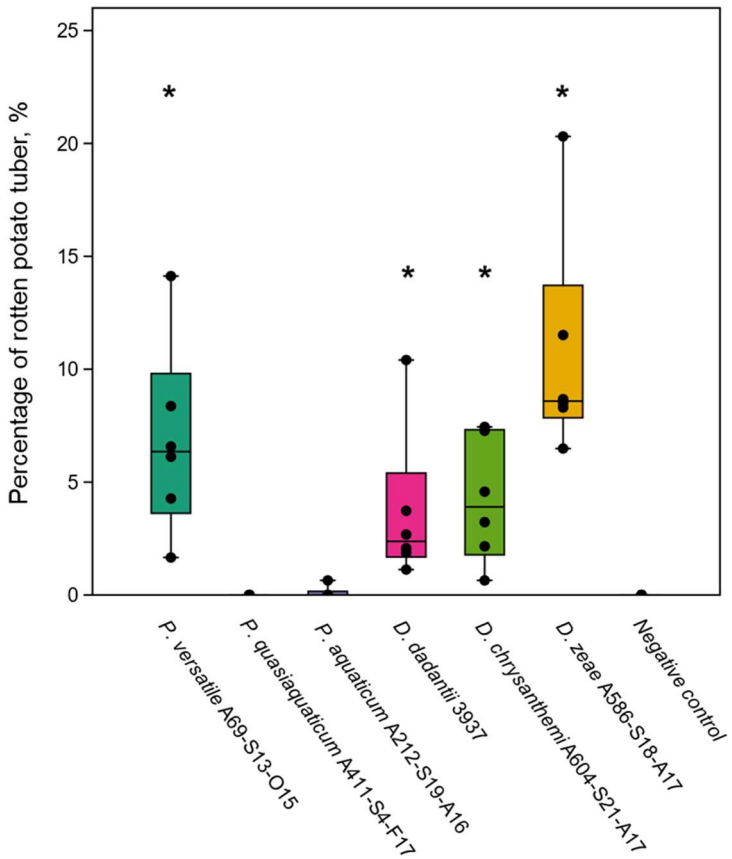
Supplementary Datasets S1 to S4

Supplementary Figures

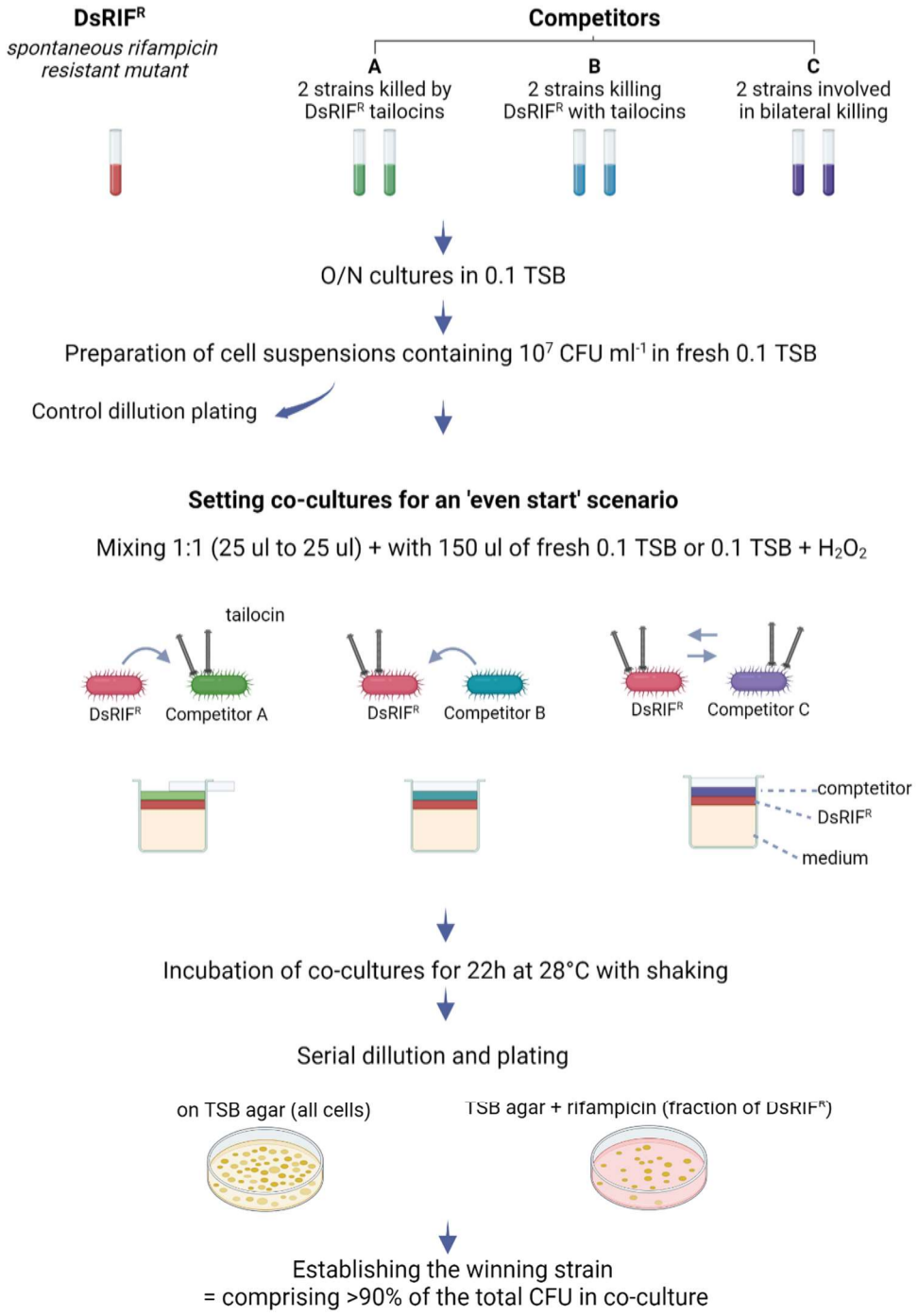
Supplementary Fig. S1. Comparison of phenotypic and phylogenetic trees. (A) dendrogram calculated based on the similarity of killing spectra of investigated strain; the tree is drawn to scale, with branch lengths measured in the distance; (B) multilocus sequence analysis of core nucleotide coding sequences using the BioNJ algorithm. Branch lengths and bootstrap values are shown. *Pectobacterium* spp. strains are marked in green and *Dickeya* spp. strains are marked in red.



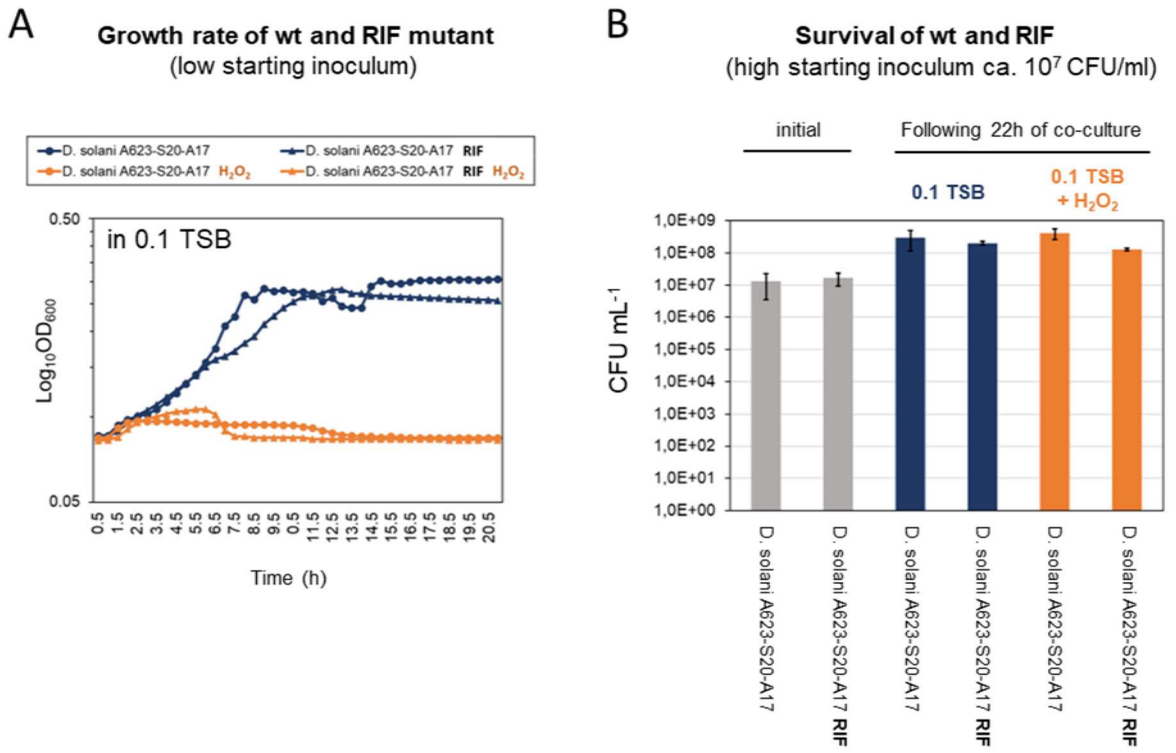
Supplementary Fig. S2. Severity of soft rot symptoms caused on potato tubers by six SRP strains tested for production of tailocins *in planta*. The results are expressed as a percentage of rotten tissue relative to the total mass of each potato tuber. In the box plot, whiskers reflect the maximum and minimum values; the box shows the interquartile range (Q1 to Q3), and the bars inside the boxes represent medians. Points indicate results for individual samples/tubers (n=6). The asterisks (*) denote statistically significant differences ($p < 0.05$) between a sample inoculated with the given strain and the negative control. Significance was calculated using the one-sample t-test for data sets with normal distribution and the Wilcoxon test for data not following a normal distribution. Negative control – tubers inoculated with sterile PBS buffer.



Supplementary Fig. S3 Schematic representation of the steps included in the competition experiment. Created with BioRender.com.



Supplementary Fig. S4 Comparison of growth rates (A) and the survival (B) of the *D. solani* A623-S20-A17 wt and the rifampicin-resistant mutant of this strain in 0.1 TSB alone and supplemented with 0.003 % hydrogen peroxide (H_2O_2). The test was performed in 96-well plates incubated at 28˚C, with shaking, in a multiwell plate reader (Biotek). To assess growth rates (A), 150 µl aliquots of 0.1 TSB medium were inoculated with 3 µl of suspension containing o/n culture diluted to 0.5 McF (low inoculum). Each time point is an average of two replicates. For cell survival (B), the medium was inoculated with a high initial number of cells (ca. 10^7 CFU/ml) applied in the co-culture competition experiments (Fig. 5). The results are the average of two separate experiments, 2 technical replicates each. The obtained results indicate that the wt and RIF^R variants of *D. solani* A623-S20-A17 have similar growth characteristics in the applied conditions and that 0.003% H_2O_2 prevents the growth of the strains when low initial inoculum is used but does not negatively affect the survival of cells in high-density cultures.



Supplementary Tables

Table S1. List of bacterial strains used in this study.

Strain	Isolation source, country, year	Reference	GenBank accession no. for genome
Dickeya chrysanthemi A604-S21-A17	Durance river. France. 2017	(*1*)	GCA_020406775.2
Dickeya dianthicola A260-S21-A16	Durance river. France. 2016	(*1*)	GCA_020406895.2
Dickeya oryzae A003-S1-M15	Durance river. France. 2015	(*1*)	GCA_020406815.2
Dickeya oryzae A642-S2-A17	Durance river. France. 2017	(*1*)	GCA_020406685.2
Dickeya solani A623-S20-A17	Durance river. France. 2017	(*1*)	GCA_020406975.1
Dickeya zeae A586-S18-A17 (recently reclassified to D. parazeae)	Durance river. France. 2017	(*1*)	GCA_020520245.1
Dickeya zeae A661-S21-A17	Durance river. France. 2017	(*1*)	GCA_020406575.2
Pectobacterium aquaticum A101-S19-F16	Durance river. France. 2016	(*1*)	GCA_003382625.2
Pectobacterium aquaticum A104-S21-F16	Durance river. France. 2016	(*1*)	GCA_003382595.2
Pectobacterium aquaticum A105-S21-F16	Durance river. France. 2016	(*1*)	GCA_003382585.2
Pectobacterium aquaticum A212-S19-A16	Durance river. France. 2016	(*1*)	GCA_003382565.3
Pectobacterium atrosepticum A597-S4-A17	Durance river. France. 2017	(*1*)	GCA_020406655.2
Pectobacterium brasiliense A143-S20-M16	Durance river. France. 2016	(*1*)	GCA_013449685.1
Pectobacterium carotovorum A077-S18-O15	Durance river. France. 2015	(*1*)	GCA_020520265.1
Pectobacterium carotovorum A280-S2-N16	Durance river. France. 2016	(*1*)	GCA_020406635.1
Pectobacterium odoriferum A122-S21-F16	Durance river. France. 2016	(*1*)	GCA_013450045.1
Pectobacterium odoriferum A367-S5-N16	Durance river. France. 2016	(*1*)	GCA_022507245.1
Pectobacterium peruviense A350-S18-N16	Durance river. France. 2016	(*1*)	GCA_003312355.2
Pectobacterium peruviense A97-S13-F16	Durance river. France. 2016	(*1*)	GCA_003312345.1
Pectobacterium polaris A641-S6-A17	Durance river. France. 2017	(*1*)	GCA_020406835.1
Pectobacterium quasiaquaticum A477-S1-J17	Durance river. France. 2017	(*1*)	GCA_014946775.2
Pectobacterium quasiaquaticum A411-S4-F17	Durance river. France. 2017	(*1*)	GCA_014946845.1
Pectobacterium versatile A546-S4-A17	Durance river. France. 2017	(*1*)	GCA_020406615.1
Pectobacterium versatile A69-S13-O15	Durance river. France. 2015	(*1*)	GCA_004296725.1
Pectobacterium versatile A73-S18-O15	Durance river. France. 2015	(*1*)	GCA_020865565.1
Dickeya dadantii 3937	*Saintpaulia* sp. France. 1972	(*2*)	GCA_000147055.1
Musicola paradisiaca IFB 0117 (NCPPB 2511)	*Musa paradisiaca*. Colombia. 1973 *	(*3*)	GCA_000400505.1

Table S2. List of sensitive strains used in a spot assay to detect tailocins from the given producer strains.

Tailocin producer	Sensitive strain
P. versatile A69-S13-O15	*P. aquaticum* A105-S21-F16
P. quasiaquaticum A411-S4-F17	*P. atrosepticum* A597-S4-A17
P. aquaticum A212-S19-A16	*P. aquaticum* A105-S21-F16
D. dadantii 3937	*M. paradisiaca* IFB 0117
D. chrysanthemi A604-S21-A17	*D. zeae* A586-S18-A17
D. zeae A586-S18-A17 (recently reclassified to *D. parazeae*)	*M. paradisiaca* IFB 0117

Supplementary datasets

Dataset S1 Results of a megablast search for carotovoricin clusters within 116 closed genomes of *Pectobacterium* spp. The carotovoricin sequence from *P. versatile* A73-S18-O15 was used as a query (genome accession: NZ_CP086369.1). (Separate xlsx file)

Dataset S2 Results of a megablast search for dickeyocin clusters within 75 closed *Dickeya* and *Musicola* spp. genomes. Dickeyocin P2D1 from *D. dadantii* 3937 was used as a query (genome accession: NC_014500.1). (Separate xlsx file)

Dataset S3 Python script for analyses (ChatGPT-4o) (Separate txt file)

Dataset S4 Tailocin supporting information (Separate xlsx file)

References

1. H. Ben Moussa *et al.*, The Diversity and Abundance of Soft Rot Pectobacteriaceae Along the Durance River Stream in the Southeast of France Revealed by Multiple Seasonal Surveys. *Phytopathology* **112**, 1676-1685 (2022).
2. A. Kotoujansky, M. Lemattre, P. Boistard, Utilization of a thermosensitive episome bearing transposon TN10 to isolate Hfr donor strains of Erwinia carotovora subsp. chrysanthemi. *J Bacteriol* **150**, 122-131 (1982).
3. N. Hugouvieux-Cotte-Pattat, C. J. des-Combes, J. Briolay, L. Pritchard, Proposal for the creation of a new genus Musicola gen. nov., reclassification of Dickeya paradisiaca (Samson et al. 2005) as Musicola paradisiaca comb. nov. and description of a new species Musicola keenii sp. nov. *Int J Syst Evol Microbiol* **71**, (2021).

Supplementary information for Chapter 4

Beyond kin killing: *Dickeya*-derived phage-tail-like bacteriocin P2D1 targets phylogenetically distant *Pseudomonas* spp.

Borowicz, M., Styn, J., Tomasik, K., Rąbalski, Ł., Narajczyk, M., Gueguen, E., Jafra, S., Baltenneck, J., Krzyżanowska, D. M., & Czajkowski, R. (2026).
ISME Communications, ycag012
https://doi.org/10.1093/ismeco/ycag012

Supplementary Information

to a research article

Beyond kin killing: *Dickeya*-derived phage-tail like bacteriocin P2D1 targets phylogenetically distant *Pseudomonas* spp.

Marcin Borowicz [a], Jan Styn [a, #], Kacper Tomasik [a, #], Łukasz Rąbalski [b], Magdalena Narajczyk [c], Erwan Gueguen [d], Sylwia Jafra [e], Julie Baltenneck [d], Dorota M. Krzyżanowska [e, *], Robert Czajkowski [a, *]

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[e] Laboratory of Plant Microbiology, Intercollegiate Faculty of Biotechnology of the University of Gdańsk and Medical University of Gdańsk, University of Gdańsk, A. Abrahama 58, 80-307 Gdańsk, Poland;

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Supplementary Tables

Supplementary Table S1 Plasmids used in this study

Plasmids	Description	Source
pRE112	Suicide vector for allelic exchange, Cm^R, *sacB*, *oriT* RP4, *ori*R6K	[1]
pEGL332	Amp^R, pSC101 ori, lacZp expression vector, mobilizable	[2]
pSJG	pEGL332-Δ*Dda3937_03810*-Δ*Dda3937_03811,* Amp^R ;	This study

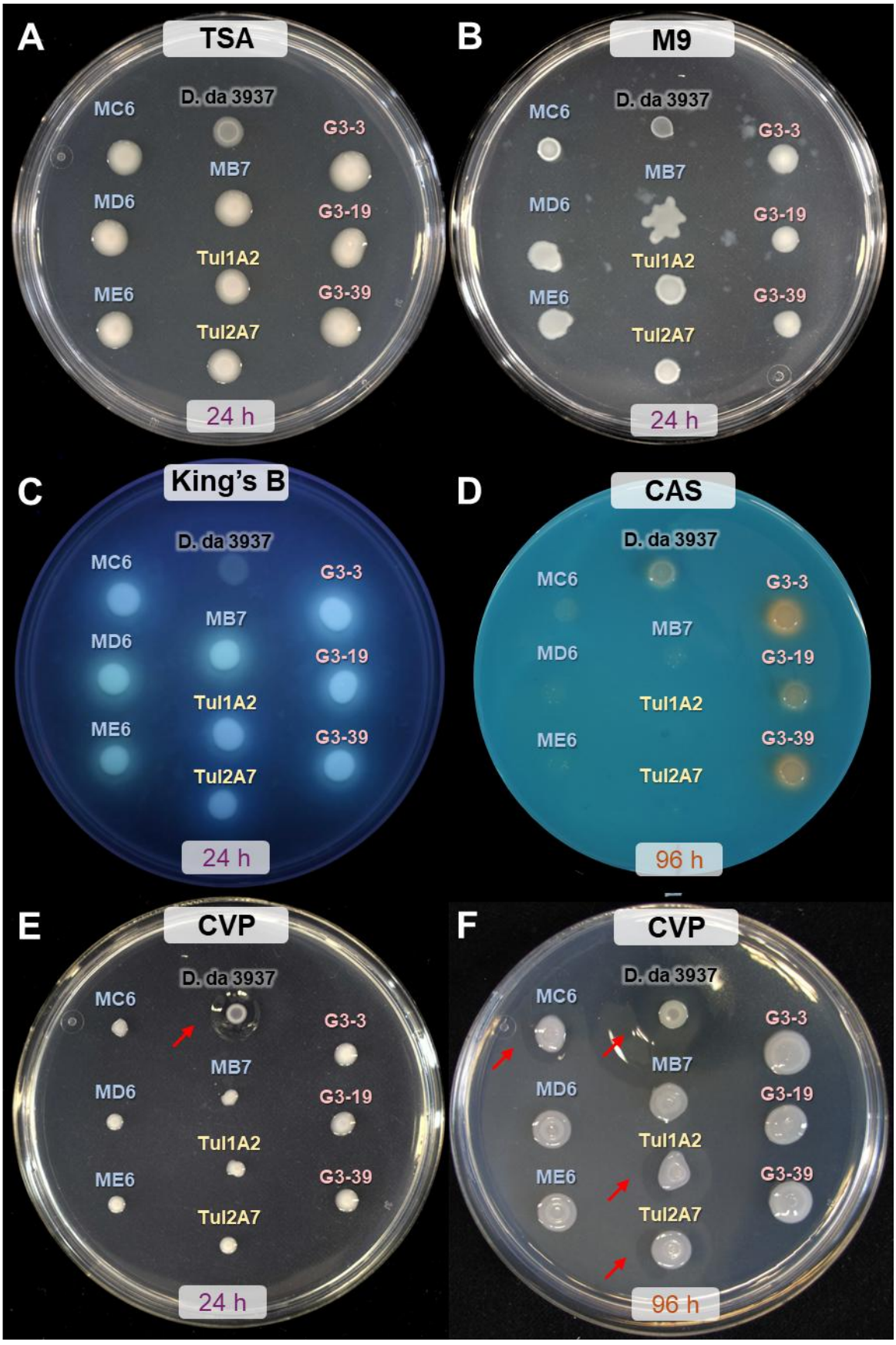
Supplementary Table S2 Oligonucleotides used in this study

Oligonucleotide	Sequence (5'-3')	Application
L6	gttttcccagtcacgac	Verification of cloning in the MCS of pEGL332
L7	caggaaacagctatgacc	
L762	gttattggtgcccttaaacg	Verification of correct cloning into pRE112
L763	gcatccaacgccattcatgg	
L1700	aactgcatgaattcccgggagagctcgtgacggatgccaaaacgcag	Amplification of the upstream and downstream 0.5-kb DNA fragments of *D. dadantii Dda3937_03810* and their cloning into suicide vector pRE112, in order to obtain a deletion mutant in the respective locus
L1701	ctccttagctgttatcactcatcaagaatgtctcctg	
L1702	ttgatgagtgataacagctaaggagcgacagac	
L1703	gatcccaagcttcttctagaggtaccggtcaacgccgttcactttc	
L1705	gatcccaagcttcttctagaggtaccagtcttgtccacttcgttgtag	Amplification of the upstream and downstream 0.5-kb DNA fragments of *D. dadantii Dda3937_03811* and their cloning into suicide vector pRE112, , to obtain a deletion mutant in the respective locus
L1706	aactgcatgaattcccgggagagctcgaccggcatctccgccag	
L1707	gtcgttacagacccagtgccatgagtctgtcg	
L1708	ctcatggcactgggtctgtaacgaccgcttcatg	
L1700	aactgcatgaattcccgggagagctcgtgacggatgccaaaacgcag	Amplification of the upstream 0.5-kb DNA fragment of *Dda3937_03810,* and 0.5-kb downstream fragment of *Dda3937_03811* and their cloning into suicide vector pRE112 (to delete both loci/genes – a double mutant)
L1701	ctccttagctgttatcactcatcaagaatgtctcctg	
L1704	ttgatgagtgataacagctaaggagggtctgtaacgaccgcttc	
L1705	gatcccaagcttcttctagaggtaccagtcttgtccacttcgttgtag	

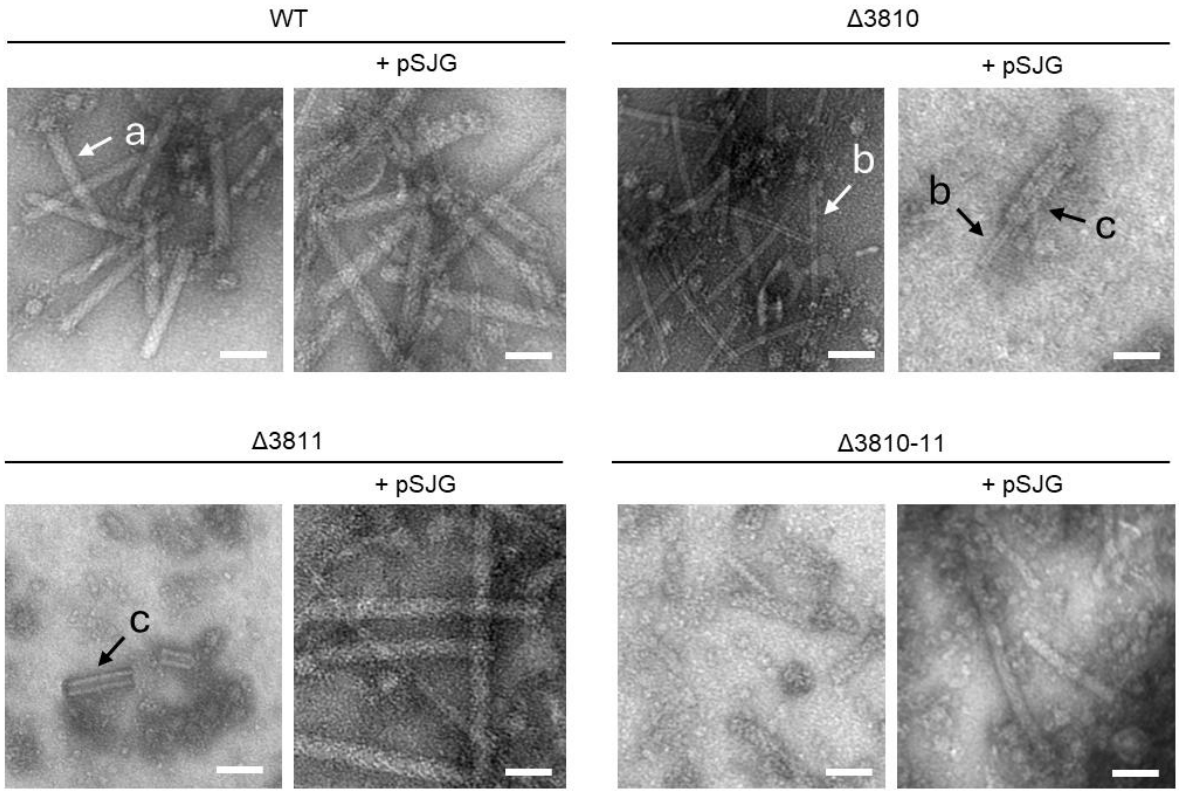
L1802	ccgggctgcaggaattcgatatcaagcttgcgccaaacctctacagatg	Amplification of the region comprising *Dda3937_03810* and *Dda3937_03811* of *D. dadantii 3937* with its native promoter and cloning into pEGL332 to obtain complementation vector
L1803	tcgaggtcgacggtatcgataagctttgaatggccagccgtagctgg	

Supplementary Figures

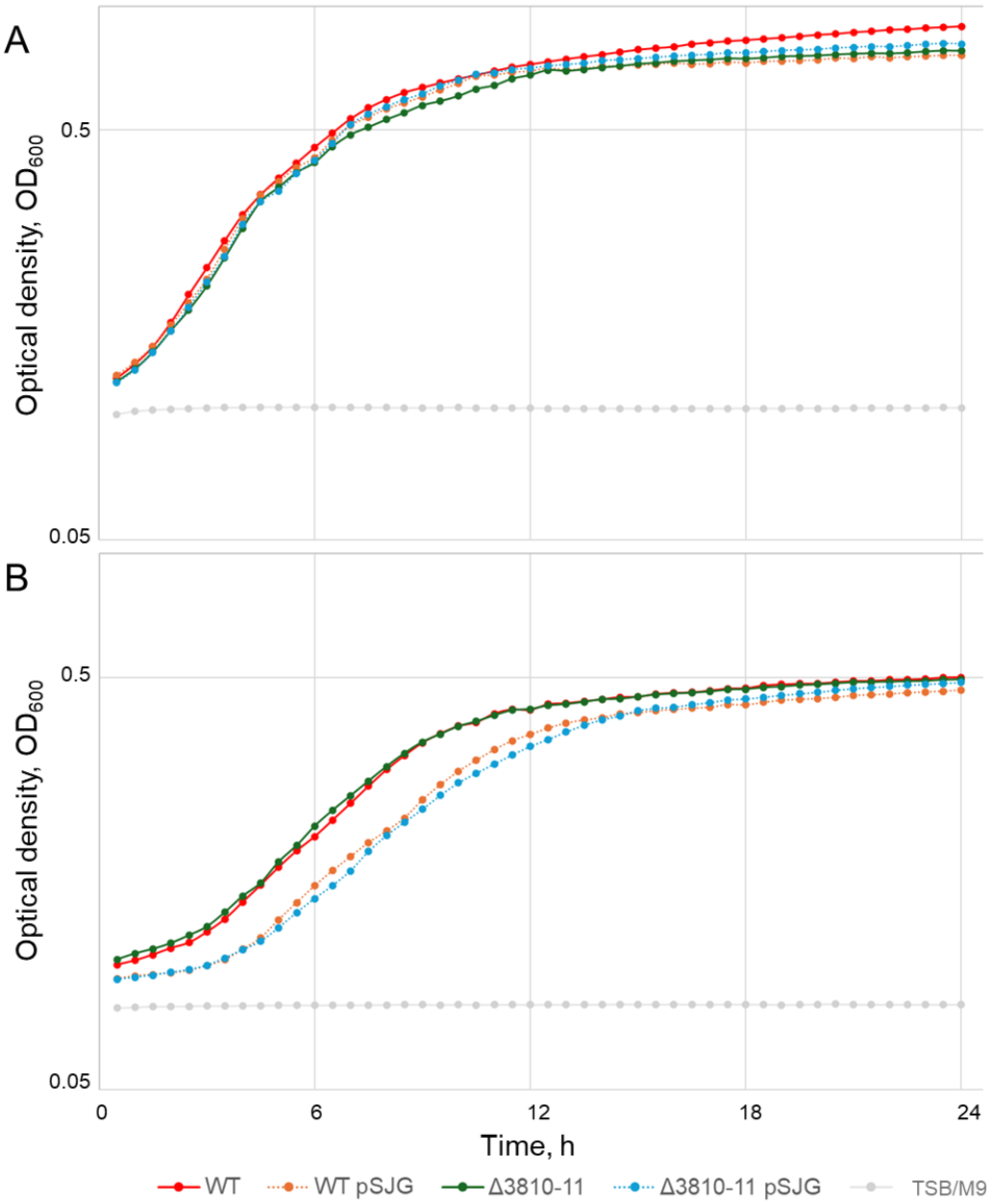
Supplementary Fig. S1 Morphology of P2D1 tailocin-susceptible *Pseudomonas* isolates grown on different media plates: TSA (A), M9 0.4% glucose (B), King's B (C), CAS agar for siderophore production (D), and crystal violet pectate (CVP) for the production of pectinases (E and F). Incubation time is indicated under each plate. On TSA, colonies of all tested *Pseudomonas* isolates exhibit a “slimy” morphology indicative of abundant extracellular matrix production. Images on King's B medium were taken under UV light (λ = 365 nm) to visualize pyoverdine fluorescence, characteristic of fluorescent pseudomonads. Where present, cavities on CVP medium are marked by arrows. *D. dadantii* 3937 was included as a reference for colony morphology and as a negative control for King’s B and a positive control on CVP.


A
TSA
D. da 3937
MC6
G3-3
MB7
MD6
G3-19
Tul1A2
ME6
G3-39
Tul2A7
24 h
B
M9
D. da 3937
MC6
G3-3
MB7
MD6
G3-19
Tul1A2
ME6
G3-39
Tul2A7
24 h
C
King's B
D. da 3937
MC6
G3-3
MB7
MD6
G3-19
Tul1A2
ME6
G3-39
Tul2A7
24 h
D
CAS
D. da 3937
MC6
G3-3
MB7
MD6
G3-19
Tul1A2
ME6
G3-39
Tul2A7
96 h
E
CVP
D. da 3937
MC6
G3-3
MB7
MD6
G3-19
Tul1A2
ME6
G3-39
Tul2A7
24 h
F
CVP
D. da 3937
MC6
G3-3
MB7
MD6
G3-19
Tul1A2
ME6
G3-39
Tul2A7
96 h

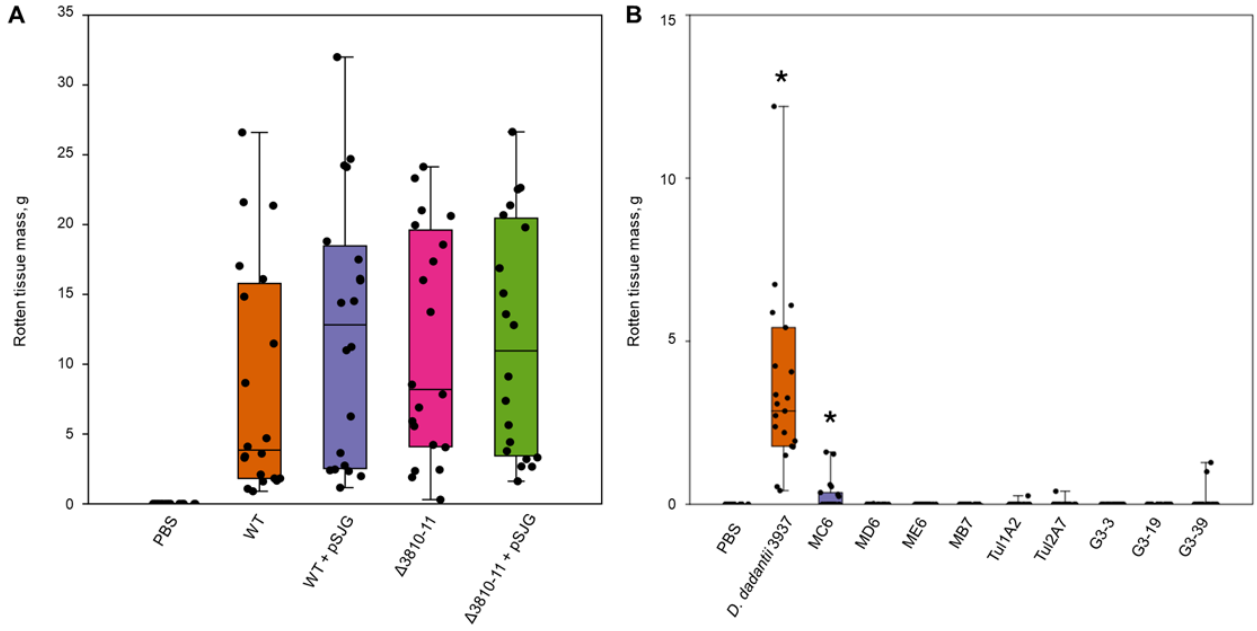
Supplementary Fig. S2 TEM images of tailocins purified from mitomycin C-induced cultures of the wild-type *D. dadantii* 3937 (WT) and its mutants. Only tubes are visible in the sheath mutant Δ3810, while only sheath fragments are present in the tube mutant Δ3811. No complete tailocin particles were detected in the preparations from the double mutant Δ3810-11. Scale bar 50 nm.



Supplementary Fig. S3 Comparison of growth curves for *D. dadantii* 3937 and its tailocin-defficient mutants. Overnight cultures were prepared in (A) tryptic soy broth (TSB, Oxoid), (B) M9 minimal medium (MP Biomedicals) supplemented with 0.4% glucose (Sigma-Aldrich) at 28 °C with shaking at 120 rpm. These cultures were diluted in a 96-well plate by mixing 10 µL of the overnight culture with 190 µL of fresh TSB. Each strain was tested in 12 technical replicates, and the experiment was performed in triplicate. The plates were incubated at 28 °C with continuous shaking at 237 rpm in an Epoch 2 microplate reader (BioTek). Optical density (OD) at 600 nm was measured every 30 minutes over 24 hours. Results are shown as average. pSJG – complementation plasmid. TSB/M9 – control with sterile medium.

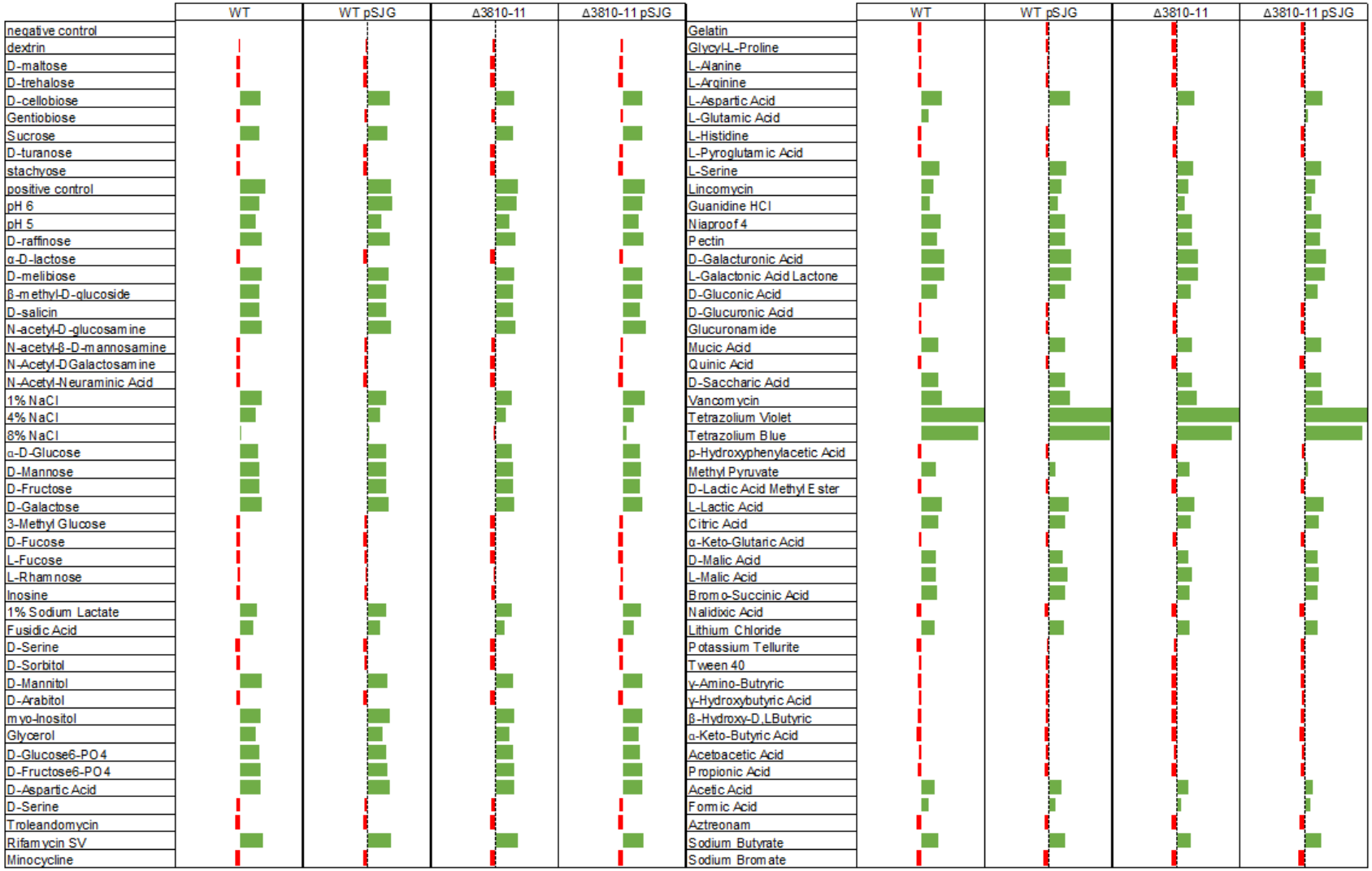


Supplementary Fig. S4 Virulence on potato tubers in a whole-tuber injection assay. (A) Virulence of *D. dadantii* 3937 and its tailocin-deficient double mutant Δ3810-11; pSJG denotes the complementation plasmid. No significant differences were detected between the wild type and its derivatives (Mann–Whitney test, $p > 0.05$). (B) Virulence of P2D1 tailocin-susceptible *Pseudomonas* isolates. Significant differences were observed between the negative control (PBS) and *D. dadantii* 3937, as well as isolate MC6 (one-sample Wilcoxon test, $p < 0.05$; marked with asterisks). Data are shown as box plots: whiskers indicate minimum and maximum values, boxes the interquartile range, and horizontal lines the medians. Each point represents an individual potato tuber (n = 20).



Supplementary Fig. S5 Phenotypic profiling of *D. dadantii* 3937 and its tailocin-deficient mutant using BIOLOG assays. (A) GEN III MicroPlate™ profiling (94 traits, including carbon utilization, chemical sensitivity, and physiological properties). (B) EcoPlate™ profiling (31 carbon-source utilization traits). Results were normalized to the negative control (positive reaction ≥2 × control) and averaged across three biological replicates. Data are presented as bar plots relative to the negative control baseline. pSJG denotes the complementation plasmid.

A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WT pSJG
Δ3810-11
Δ3810-11 pSJG
negative control
dextrin
D-maltose
D-trehalose
D-cellobiose
Gentiobiose
Sucrose
D-turanose
stachyose
positive control
pH 6
pH 5
D-raffinose
α-D-lactose
D-melibiose
β-methyl-D-glucoside
D-salicin
N-acetyl-D-glucosamine
N-acetyl-β-D-mannosamine
N-Acetyl-DGalactosamine
N-Acetyl-Neuraminic Acid
1% NaCl
4% NaCl
8% NaCl
α-D-Glucose
D-Mannose
D-Fructose
D-Galactose
3-Methyl Glucose
D-Fucose
L-Fucose
L-Rhamnose
Inosine
1% Sodium Lactate
Fusidic Acid
D-Serine
D-Sorbitol
D-Mannitol
D-Arabitol
myo-Inositol
Glycerol
D-Glucose6-PO4
D-Fructose6-PO4
D-Aspartic Acid
D-Serine
Troleandomycin
Rifamycin SV
Minocycline
Gelatin
Glycyl-L-Proline
L-Alanine
L-Arginine
L-Aspartic Acid
L-Glutamic Acid
L-Histidine
L-Pyroglutamic Acid
L-Serine
Lincomycin
Guanidine HCl
Niaproof 4
Pectin
D-Galacturonic Acid
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D-Gluconic Acid
D-Glucuronic Acid
Glucuronamide
Mucic Acid
Quinic Acid
D-Saccharic Acid
Vancomycin
Tetrazolium Violet
Tetrazolium Blue
p-Hydroxyphenylacetic Acid
Methyl Pyruvate
D-Lactic Acid Methyl Ester
L-Lactic Acid
Citric Acid
α-Keto-Glutaric Acid
D-Malic Acid
L-Malic Acid
Bromo-Succinic Acid
Nalidixic Acid
Lithium Chloride
Potassium Tellurite
Tween 40
γ-Amino-Butyric
γ-Hydroxybutyric Acid
β-Hydroxy-D,LButyric
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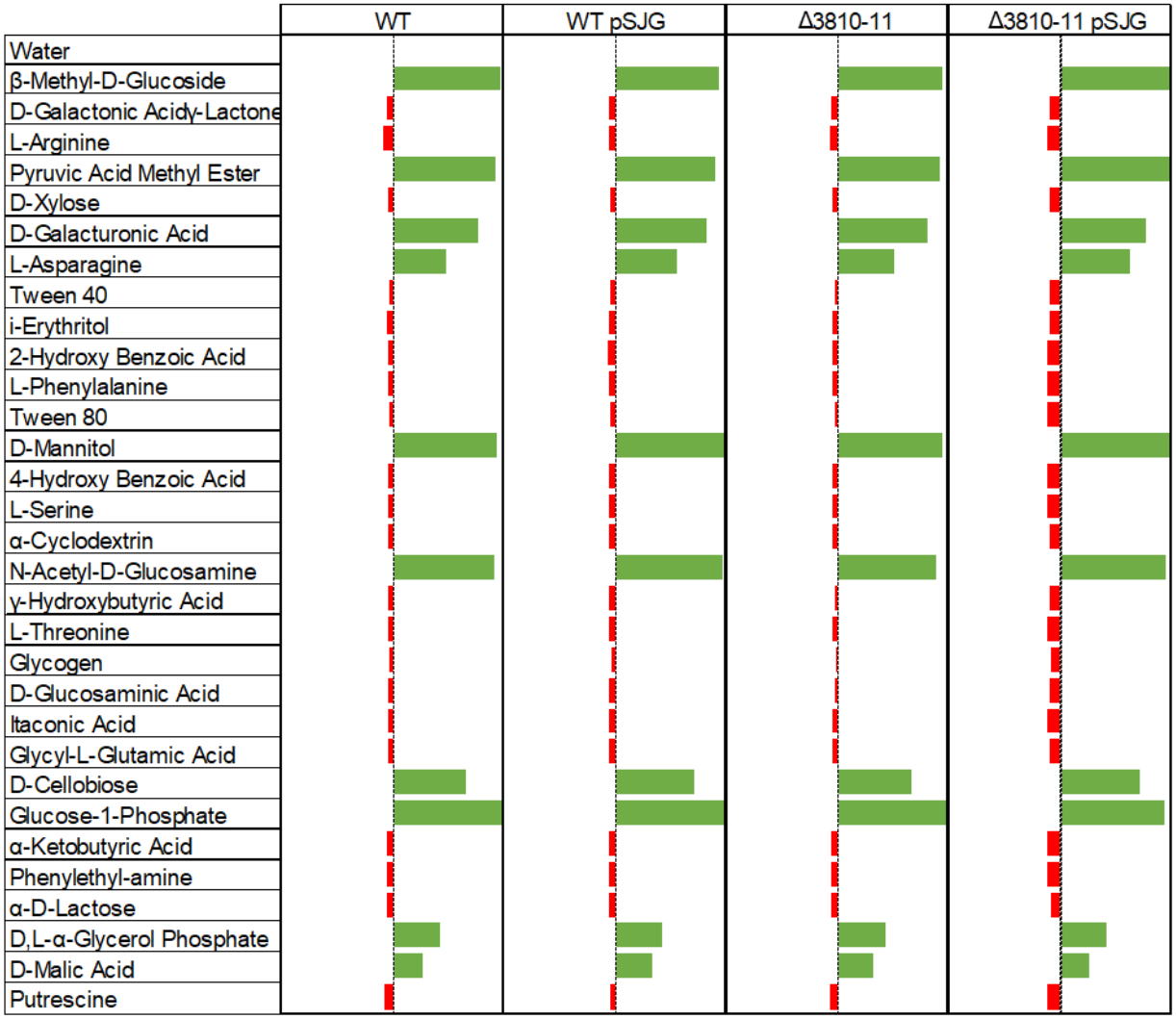
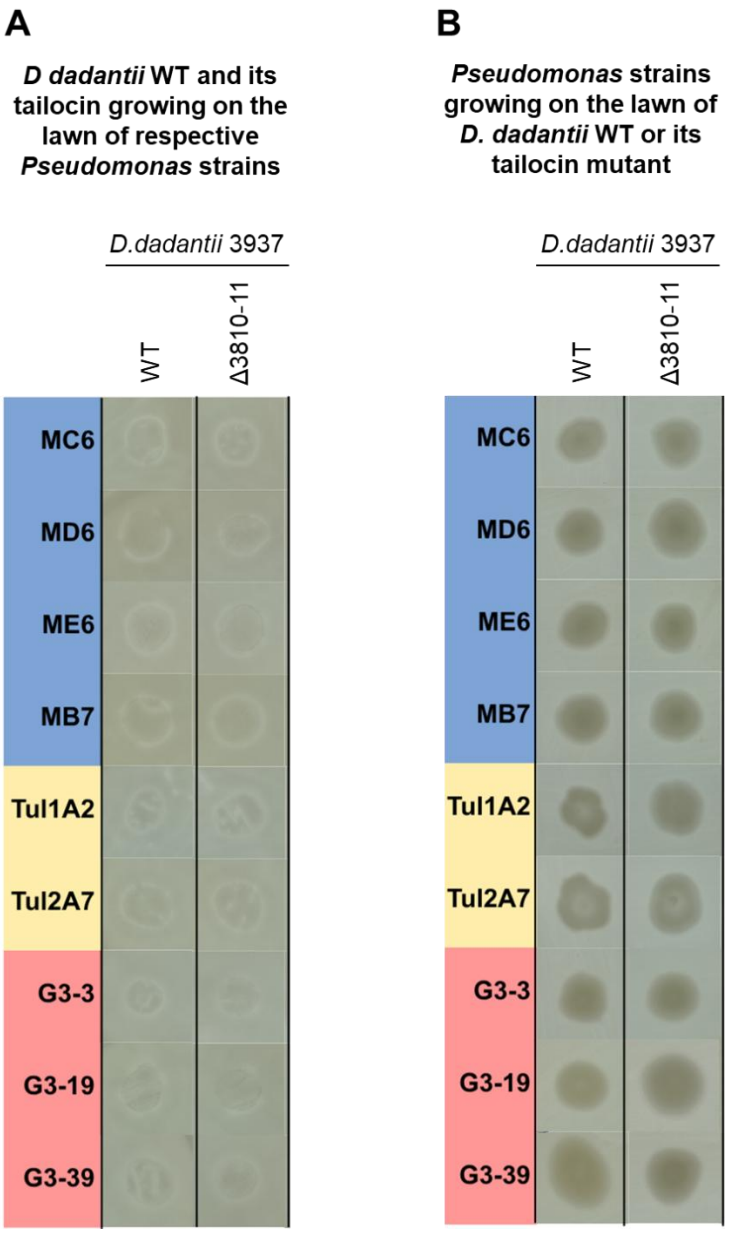
Propionic Acid
Acetic Acid
Formic Acid
Aztreonam
Sodium Butyrate
Sodium Bromate

B


WT
WT pSJG
Δ3810-11
Δ3810-11 pSJG
Water
β-Methyl-D-Glucoside
D-Galactonic Acidγ-Lactone
L-Arginine
Pyruvic Acid Methyl Ester
D-Xylose
D-Galacturonic Acid
L-Asparagine
Tween 40
i-Erythritol
2-Hydroxy Benzoic Acid
L-Phenylalanine
Tween 80
D-Mannitol
4-Hydroxy Benzoic Acid
L-Serine
α-Cyclodextrin
N-Acetyl-D-Glucosamine
γ-Hydroxybutyric Acid
L-Threonine
Glycogen
D-Glucosaminic Acid
Itaconic Acid
Glycyl-L-Glutamic Acid
D-Cellobiose
Glucose-1-Phosphate
α-Ketobutyric Acid
Phenylethyl-amine
α-D-Lactose
D,L-α-Glycerol Phosphate
D-Malic Acid
Putrescine

Supplementary Fig. S6 Reciprocal antibiosis assay on TSA media plates between *D. dadantii*, its tailocin-deficient mutant, and environmental *Pseudomonas* strains. *D. dadantii* wild-type and the mutant were spotted onto the lawns of environmental *Pseudomonas* strains (A) and *vice versa* (B). Results from one of two independent experiments yielding identical outcomes are shown.



Supplementary Datasets

Supplementary Dataset 1 Isolates Taxonomical Placement Among *Pseudomonas* Type Strains Based On 16S rRNA Gene – Full Tree (pdf file).

Supplementary Dataset 2 Average Nucleotide Identity Calculation (Excel file)

Supplementary Dataset 3 Isolates Full Phenotype Profiles (Excel file)

Supplementary Script 1 Hierarchical clustering and dendrogram visualization (txt file)

Uncategorized References

1. Brual T, Effantin G, Baltenneck J *et al.* A natural single nucleotide mutation in the small regulatory rna arcz of dickeya solani switches off the antimicrobial activities against yeast and bacteria. *PLOS Genetics*. 2023;**19**:e1010725 https://doi.org/10.1371/journal.pgen.1010725
2. Xia Y, Li K, Li J *et al.* T5 exonuclease-dependent assembly offers a low-cost method for efficient cloning and site-directed mutagenesis. *Nucleic Acids Res*. 2019;**47**:e15 https://doi.org/10.1093/nar/gky1169

Appendix 2: Author contribution statements

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Borowicz, M., Krzyżanowska, D., Narajczyk, M., Sobolewska, M., Rajewska, M., Czaplewska, P., Węgrzyn, K., & Czajkowski, R. (2023). Soft rot pathogen *Dickeya dadantii* 3937 produces tailocins resembling the tails of *Peduovirus* P2. *Frontiers in Microbiology*, 14. https://doi.org/10.3389/fmicb.2023.1307349

Conceptualization, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing:

Co-development of the study concept and experimental design; performance of the majority of laboratory experiments (tailocin induction, purification, and cross-species interaction assays); participation in preparation and visualization of figures; participation in interpretation of results; and participation in preparation of the initial draft of the manuscript and its subsequent revisions.

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Conceptualization, Investigation, Supervision, Visualization, Writing – original draft, Writing – review & editing:

Co-development of the study concept and experimental design; supervision of research activities; preparation and visualization of figures; interpretation of results; and contribution to the drafting and critical revision of the manuscript.

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Investigation, Methodology, Visualization, Writing – original draft.

Execution and optimization of sample visualization for transmission electron microscopy; and contribution to the preparation of the manuscript.

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Investigation, Methodology, Writing – original draft.

Participation in the design, optimization, and execution of experimental procedures related to tailocin induction, purification, and interaction assays; and contribution to the preparation of the manuscript.

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Investigation, Methodology, Writing – original draft.

Execution and optimization of sample visualization for atomic force microscopy; and contribution to the preparation of the manuscript.

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Methodology, Writing – original draft:

Execution of protein mass spectrometry analysis of tailocin samples, data processing, and protein identification; and contribution to the preparation of the manuscript.

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Investigation, Methodology, Writing – original draft:

Consultation and support in the optimization of experimental procedures related to tailocin purification; methodological advice; and contribution to the preparation of the manuscript.

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Conceptualization, Data curation, Funding acquisition, Resources, Supervision, Writing – original draft, Writing – review & editing:

Overall project supervision and co-development of the study concept; acquisition of funding and provision of research resources; guidance during data analysis and interpretation; and contribution to drafting, reviewing, and final editing of the manuscript.

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Conceptualization, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing:

Co-development of the study concept and experimental design; performance of the majority of laboratory experiments, including tailocin induction, purification, and quantitative PCR (qPCR) analyses; contribution to data analysis and figure preparation; contribution to the drafting and revision of the manuscript.

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Conceptualization, Investigation, Supervision, Visualization, Writing – original draft, Writing – review & editing:

Co-development of the study concept and experimental design; supervision of research activities; assistance in qPCR analyses; participation in data interpretation and figure preparation; contribution to the drafting and critical revision of the manuscript; and role as co-corresponding author.

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Investigation, Methodology, Writing – original draft, Writing – review & editing:

Participation in the design of experimental approaches; assistance with selected laboratory experiments (tailocin induction and quantification); and contribution to manuscript preparation and revision.

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Sobolewska, M., Krzyżanowska, D., Borowicz, M., & Czajkowski, R. (2025). Stress-driven temporal production of phage tail-like particles (tailocins) in *Dickeya dadantii* strain 3937. Scientific Reports, 15. https://doi.org/10.1038/s41598-025-13158-1

Conceptualization, Data curation, Funding acquisition, Resources, Supervision, Writing – original draft, Writing – review & editing:

Overall project supervision and co-development of the study concept and research framework; acquisition of funding and provision of laboratory resources; coordination of collaborative research and data curation; and contribution to the drafting, review, and final editing of the manuscript as the corresponding author.

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Conceptualisation, investigation, methodology, visualisation, writing – original draft, writing – review and editing:

Co-design of the experimental concept and study framework; performance of the majority of laboratory experiments (tailocin purification, AFM imaging, killing assays, and induction experiments); participation in preparation of selected figures; co-development and optimization of selected bioinformatic analyses (cluster synteny and phenotype-based dendrogram); participation in interpretation of results; and participation in preparation of the initial manuscript draft and its subsequent revisions.

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Conceptualisation, investigation, supervision, visualisation, writing – original draft, writing – review and editing:

Co-development of the study concept and experimental design; performance of competition assays; supervision of experimental work; participation in data interpretation and figure preparation; and contribution to the drafting and critical revision of the manuscript.

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Investigation, methodology, visualisation:

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Investigation, methodology, visualisation, writing – original draft:

Execution and optimization of sample visualization for transmission electron microscopy; and contribution to the preparation of the manuscript.

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Investigation, methodology, writing – original draft:

Execution of protein mass spectrometry analysis of tailocin samples, data processing, and protein identification; and contribution to the preparation of the manuscript.

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Methodology, writing – original draft:

Conceptualization of the protein mass spectrometry analyses; and contribution to the drafting of the manuscript.

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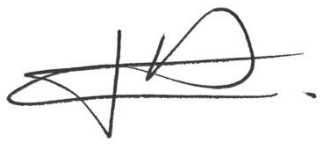
November 3, 2025



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Investigation, methodology:

Execution of experimental procedures related to genomic DNA extraction and preparation for sequencing; and methodological support during sample processing.

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Conceptualisation, investigation, methodology:

Provision of access to atomic force microscopy (AFM) facilities; methodological guidance and support during AFM imaging and data acquisition; and assistance in data analysis and interpretation.

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Conceptualisation, data curation, funding acquisition, resources, supervision, writing – original draft, writing – review and editing:

Overall project supervision and co-development of the study concept and research framework; acquisition of funding and provision of laboratory resources; coordination of collaborative efforts and data curation; and contribution to the drafting, review, and final editing of the manuscript as the corresponding author.

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Formal analysis; Investigation; Data curation; Visualization; Writing – original draft; Supervision of students; Funding acquisition:

Supervised laboratory experiments by undergraduate students, performed experimental work, carried out bioinformatic analyses, contributed to figure preparation, analyzed data, drafted the manuscript, and contributed to funding acquisition.

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Investigation:

Collected samples, performed the screening for tailocin susceptibility, and participated in laboratory experiments.

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Investigation:

Performed DNA sequencing on the isolates.

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Investigation; Visualization:

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Conceptualization; Supervision:

Designed and supervised the construction of mutants.

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February 15th 2026

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Author Contribution Statement

I hereby declare my contribution to the following manuscript:

Borowicz, M., Styn, J., Tomasik, K., Rąbalski, Ł., Narajczyk, M., Gueguen, E., Jafra, S., Baltenneck, J., Krzyżanowska, D. M., & Czajkowski, R. (2026). Beyond kin killing: *Dickeya*-derived phage-tail-like bacteriocin P2D1 targets phylogenetically distant *Pseudomonas* spp. ISME Communications. https://doi.org/10.1093/ISMECO/YCAG012

Investigation:

Generated mutant and complementation strains.

17.02.2026.....

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Conceptualization; Formal analysis; Investigation; Visualization; Writing – original draft; Writing – review & editing; Supervision:

Contributed to writing the original draft and the review editing, analyzed data, contributed to figure preparation, and provided supervision and conceptual input.

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Conceptualization; Data curation; Investigation; Resources; Supervision; Writing – original draft; Writing – review & editing; Funding acquisition:

Contributed to conceptualization, writing the original draft and the review editing, supervised the research, and was responsible for funding acquisition.

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