RESEARCH ARTICLE

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

Short title: Tailocins of *Dickeya dadantii* 3937

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Abstract

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 Enterobacteriophage P2 was identified. The *D. dadantii* tailocins, termed dickeyocin P2D1 (phage P2-like dickeyocin 1), were resistant to inactivation by pH (3.5 – 12), temperature (4 – 50 ºC), 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*.

Teaser

Tailocins are nanomolecular entities similar to syringes that are produced by various bacteria to fight other microorganisms present in the same environment.

Keywords:

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

46 **Introduction**

47

48 Under natural conditions, bacterial species inhabit shared environments, developing
49 spatial and temporal interspecies associations and communities having complex networks of
50 interactions (*1, 2*). In such communities, a particular member needs to continuously compete
51 for limited resources (i.e., scarce nutrients and limited space) with most other members of
52 the community to gain a competitive edge (*3, 4*). Given such challenging conditions, bacteria
53 have evolved diverse strategies to successfully coexist with both closely and distantly related
54 microbes (*5*). Such strategies, although employing a broad range of mechanisms, can be
55 distinguished as either 1) indirect, exploitative competition that occurs through the
56 consumption of resources and 2) direct, interference competition, where individual cells
57 directly kill one another, limiting their lifespan (*6*). Whereas exploitative competition depends
58 primarily on the utilization of limited resources by a strain, thereby restricting it from the
59 competitor, interference competition relies on producing various antimicrobial agents that aim
60 to kill other cells (*7*). These antimicrobials include, but are not limited to, broad-spectrum
61 antibiotics, toxins, contact-dependent inhibition, effectors transported via type VI secretion
62 system (T6SS effectors), low molecular weight bacteriocins, and tailocins (*5, 8*). A given
63 bacterial cell may often use several such systems to gain fitness advantages in the
64 environment (*7*). Among the systems bacteria exploit to fight competitive microbes, tailocins
65 are now receiving increasing attention (*9, 10*).

66 Tailocins are syringe-like nanomolecular entities that are evolutionary and
67 morphologically related to bacteriophage tails, type VI secretion systems, and extracellular
68 contractile injection systems (*10*). These particles, also known as high molecular weight
69 bacteriocins or phage tail-like particles, are chromosomally encoded and ribosomally
70 synthesized toxins that usually express a narrow killing range, interacting only with closely-
71 related bacterial species that typically would occupy the same niche (*9*). These agents
72 adsorp to the surface of susceptible cells, thereby puncturing the cell envelope, leading to
73 depolymerization of the cell membrane and, ultimately, the death of the attacked cell (*11*).

74 Tailocins are classified into two distinct families: rigid and contractile (R-type) and
75 noncontractile but flexible particles (F-type) (*12*). The R-type tailocins have feature of tails of
76 P2 or T-even bacteriophages of the family *Myovirideae* infecting *Escherichia coli*. In contrast,
77 the F-type tailocins resemble the flexible tails of bacteriophage lambda (λ) in the family
78 *Siphoviridae*. Although tailocins exhibit remarkable morphological similarity to bacteriophage
79 tails of the *Myoviridae* and *Siphoviridae* viruses, it is now believed that they have evolved
80 independently from bacteriophages and should not be considered exclusively as
81 domesticated prophages or phage remnants that bacteria harness for their advantage (*10*).
82 The production of tailocins has been demonstrated both in Gram-negative and Gram-
83 positive bacterial species. Producing strains include both human, animal, and plant
84 pathogens and saprophytic bacteria residing in various environments (*13-16*). Until recently,
85 tailocins have been best characterized in *Pseudomonas* species (*17*). There are, however,
86 reports of tailocins isolated from *Clostridioides* spp., *Serratia* spp., *Xenorhabdus* spp.,
87 *Burkholderia* spp., *Kosakonia* spp., *Budvicia* spp., *Pragia* spp., *Pectobacterium* spp. as well
88 as from other bacteria (*18*).
89 The omnipresence of tailocins in phylogenetically unrelated bacterial genera suggests
90 that these particles are important for fitness in various habitats (*10*). However, the ecological
91 role of tailocins in the natural environment of the producing strains has received little
92 attention, especially for plant-pathogenic bacteria residing in agricultural locations. This issue
93 is important given the diversity of conditions such bacteria encounter in such natural settings.
94 No comprehenisve studies have addressed tailocins produced by Soft Rot
95 *Pectobacteriaceae* (SRP) bacteria (*19*), which, due to their complex lifestyle, have a variety
96 of spatial and temporal interactions in varied environments (*20-22*).
97 Plant pathogenic SRP (consisting of *Pectobacterium* spp., *Dickeya* spp., and
98 *Musicola* spp., formerly characterized as pectinolytic *Erwinia* spp.) are a useful model for
99 studying the environmental role of tailocins. SRP bacteria are widespread in various
100 ecological niches, including rain and surface water, natural and agricultural bulk and
101 rhizosphere soils, sewage, the exterior and interior of host and non-host plants as well as the

102 surface and interior of insects (*19*). Because of the diverse environments in which SRP
103 bacteria may be found, these pathogens may encounter various other bacteria with whom
104 they must effectively compete.

105 This study aimed to assess the presence and activity of tailocins induced and isolated
106 from pectinolytic *Dickeya dadantii* strain 3937 (*23*). This strain (formerly *Erwinia*
107 *chrysanthemi* and *Pectobacterium chrysanthemi* (*24*)) is a well-known necrotrophic plant
108 pathogen that causes soft rot disease in a variety of crop, ornamental, and other nonfood
109 plants worldwide, causing lossess in agriculture (*25*). Strain 3937 has been widely used as a
110 potent model system for research on the molecular biology and pathogenicity of bacteria
111 belonging to Soft Rot *Pectobacteriaceae* for several decades *(26, 27)*. While this strain
112 continues to be the most studied strain of all *Dickeya* species its production of tailocins has
113 not been previously described. Here, we characterize for the first time the tailocin produced
114 by *D. dadantii* strain 3937.

115

116 ## *Results*

117 ### *D. dadantii 3937 produces tailocins*

118 Cells of *D. dadantii* 3937 treated with mitomycin C produced syringe-like
119 macromolecular structures resembling bacteriophage tails. Following convention, the
120 tailocins produced by *D. dadantii* were named dickeyocins. Imaging using TEM and AFM
121 revealed that these structures consist of a central rod-shaped core (tube) wrapped in a
122 contractable sheath (Fig. 1). Both the tube and the sheath were built of multiple protein
123 subunits, with a clearly visible helical arrangement of the subunits in the sheath. The average
124 length of the dickeyocins produced by strain 3937 was 166±7 nm. When the sheath entirely
125 covered the tube (in the extended, 'loaded' form), the individual dickeyocin has a diameter of
126 23±2 nm. When the sheath contracts, it revealed an internal tube of a length of 92±7 nm with
127 an attached spike at the proximal end. Fibers were visible at the distal end of the sheath (Fig.
128 1).

129 The yield of tailocins purified from mitomycin-induced cells of *D. dadantii* 3937
130 equaled approx. 10^{11} particles mL^{-1} of culture. The particles could also be isolated from non-
131 treated cells, indicating a low basal production level. However, induction with mitomycin C
132 increased the yield approx. 10-100 fold. We employed three different methods to estimate
133 the concentration of phage-tail-like particles in the tested preparations. The results were
134 consistent between these methods, as well as for the independently-obtained batches of
135 purified dickeyocins (Table S2). The average concentration from three independently
136 obtained samples was 10^6 relative units (AU) mL^{-1}, 10^{11} killing particles $mL^{-1,}$ and 10^{11}
137 particles mL^{-1}, according to the spot test, the Poisson distribution killing method, and the
138 NanoSight measurements, respectively. Furthermore, comparing the results from the activity-
139 based Poisson method and the direct particle count indicated that most phage-like particles
140 purified from the cultures of *D. dadantii* strain 3937 were undamaged and in the extended
141 ('loaded') form. This was in line with the microscopic observations done with TEM and AFM.

142

143 ***Dickeyocins from D. dadantii strain 3937 are phylogenetically related to the tail of P2***
144 ***bacteriophage***

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

156 genomic region contained 28 genes, from which 16 genes encoded various bacteriophage
157 structural proteins.
158 The nucleotide sequence of the 22-kb putative dickeyocin-encoding fragment in *D.*
159 *dadantii* strain 3937 showed the highest similarity to a prophage region in *D. dadantii* strain
160 XJ12 (100% query coverage (qc), 99.35% identity). Highly similar regions were also found in
161 the genomes of several other strains of *D. dadantii*, as well as *D. solani*, *D. dianthicola*, and
162 *D. fangzhongdai* (Data S1). Strains of *D. zeae* showed lower similarity, with query coverage
163 between 54-76% and identity of 83-84%. A much lower score was observed for the next best
164 hit – *Musicola paradisiaca* (formerly *Dickeya paradisiaca*). Other non-*Dickeya*
165 microorganisms with regions showing some degree of homology included *Serratia* sp. ATCC
166 39006 (Data S1) (with 10% query coverage and 81.45% identity).
167 Importantly, at the nucleotide level, the dickeyocin region of *D. dadatii* strain 3937
168 showed no significant similarity to the known carotovorocin Er cluster of *P. carotovorum* Er
169 (Genbank accession: AB045036) (*28*). Moreover, the amino acid sequence of the sheath
170 protein of tailocin from strain 3937 showed only a 34% identity to that of carotovorocin Er,
171 with a query coverage of 83%.
172 The ca. 22-kb cluster encoding dickeyocin from *D. dadantii* strain 3937 was also
173 surveyed against the collection of viral sequences (NCBI taxid: 10239), yieldingonly low
174 similarity scores (7% query coverage, 78% identity for the best hit).
175 Furthermore, in an attempt to find undomesticated phages related to dickeyocin we
176 searched the viral protein database, using three structural proteins of dickeyocin as queries:
177 sheath protein (WP_013318223), tail tube protein (WP_013318224), and baseplate
178 assembly protein J (WP_013318212). Based on this search, proteins in dickeyocin exhibited
179 homology to proteins of *Salmonella* phages SW9 and PSP3, *Erwinia* phage Etg,
180 *Enterobacteria* phage fiAA91-ss, *Peduovirus* (bacteriophage) P2, several *Escherichia* and
181 *Yersinia* phages, as well as to multiple poorly-characterized phages of bacteria in class
182 *Caudoviricetes*, derived from the human metagenome (Data S1). The best-characterized
183 phage having a high homology to dickeyocin was *Enterobacteria* phage P2 – a *Myoviridae*

184 phage that infects *Escherichia coli* and other hosts, including *Salmonella* and *Klebsiella* (*29,*
185 *30*). For the three investigated dickeyocin proteins, their amino acid identity towards their P2
186 homologs ranged from 70-79%, with 100% query coverage (Data S2). Therefore, we used
187 the *Enterobacteria* P2 bacteriophage as a reference to assign functions to proteins in
188 dickeyocin, as well as to investigate the genetic rearrangements in the dickeyocin cluster in
189 relation to the fully-functional phage P2 (Fig. 2C). The dickeyocin cluster in the genome of *D.*
190 *didantii* strain 3937 lacked sequences associated with the phage head (proteins O, M, N, L),
191 as well as the tape measure protein. There was also a difference in the genetic content of the
192 intergenic region of the tail, as well as low homology of proteins building the tail fibers (31%
193 query coverage, 54% identity). Following the present naming convention, we named the
194 newly characterized tailocins from *D. dadantii* 3937 **dickeyocin P2D1** (P2-like Dickeyocin 1).
195

196 ***Dickeyocin P2D1 expresses bactericidal activity exclusively against members of Soft***
197 ***Rot Pectobacteriaceae***

198 Fifty-two bacterial strains were surveyed for their sensitivity to tailocins induced from *D.*
199 *dadantii* strain 3937. These included 41 strains of different species and subspecies of
200 *Dickeya* and *Pectobacterium*, as well as 7 other bacterial strains belonging to the
201 *Enterobacteriaceae* family, 3 *Pseudomonas* spp., and a single strain of *Staphylococcus*
202 *aureus* representing Gram-negative bacteria (Table S1). Bactericidal activity was observed
203 against eight strains (*D. dadantii* subsp. *dieffenbachie* strain NCPPB 2976, *D. dianthicola*
204 strains NCPPB 3534 and IPO 980, *D. undicola* CFBP 8650, *D. zeae* strains NCPPB 3532
205 and 3531, *D. oryzae* strain CSL RW192, and *Musicola paradisiaca* strain NCPPB 2511 (old
206 name: *Dickeya paradisiaca* strain NCPPB 2511)), but not against any of the *Pectobacterium*
207 spp. tested (Table S1, Fig. 3). Likewise, dickeyocin P2D1 was inactive against any of the
208 non-SRP strains included in the screening assay: 7 *Enterobacteriaceae* strains (*Serratia*
209 *marcescens* ATCC 14756, *Citrobacter freundii* ATCC 8090, *Escherichia coli* ATCC 8739,
210 *Escherichia coli* ATCC 25922, *Escherichia coli* OP50, *Klebsiella quasipneumoniae* ATCC

211 700603, *Klebsiella aerogenes* ATCC 51697), 3 *Pseudomonas* spp.: (*Pseudomonas*
212 *aeruginosa* PA14, *Pseudomonas aeruginosa* PAO1, *Pseudomonas donghuensis* P482), and
213 *S. aureus* ATCC 25923 indicating their limited range of bactericidal activity (Fig. 3).

214

215 ***P2D1 killing efficiency is bacterial species-dependent***

216 We determined the kinetics of killing of eight susceptible strains treated with P2D1. The
217 share of intact cells at two time points: 20 and 120 minutes post treatment is shown in Fig.
218 S1. In all the cases, the killing of the susceptible cells was fast; in the first 20 minutes, the
219 most killing was observed for *D. dianthicola* strain IPO 980 (ca. 60% reduction of cell
220 numbers). In contrast, the slowest killing caused by dickeyocin P2D1 was observed for *D.*
221 *dianthicola* strain NCPP 3534 (ca. 22% reduction of cell numbers). For the other 6 strains
222 tested, on average, a 45 to 60% reduction in cell numbers was observed after this same time
223 (20 min.). About 50 to 60% killing of all strains by dickeyocin P2D1 was seen after 120 min-
224 incubation (Fig. S1).

225

226 ***P2D1 dickeyocin directly punctures the cell envelope of susceptible bacterial strains***

227 We assessed the interaction of dickeyocin P2D1 on cells of the susceptible *M. paradisiaca*
228 strain at the single-cell level using transmission electron microscopy (TEM). Images clearly
229 showed that dickeyocin P2D1 adsorbed to the bacterial cell envelope (Fig. 4 AB), followed by
230 puncturing the outer cell membrane (resulting from the conformational change of dickeyocin
231 P2D1 from an extended 'loaded' to a contracted form) (Fig. 4 CD), creating a pore
232 connecting the cytoplasm of the cell with the external environment and leading eventually to
233 the death of the attacked cell.

234

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

236 A cell adsorption assay was employed to investigate whether the dickeyocin P2D1 can
237 adsorb to nonviable (dead) and viable bacterial cells of the susceptible or resistant bacterial
238 species. Dickeyocin P2D1 was incubated either with viable or chloramphenicol-killed cells of
239 susceptible *M. paradisiaca* or with viable or antibiotic-killed cells of resistant *D. dadantii* for
240 40 min and the remaining dickeyocin P2D1 in the medium was measured. Incubation of
241 dickeyocin P2D1 both with the dead and viable cells of *M. paradisiaca* resulted in the total
242 loss of the tailocin activity, indicating that it bound equally efficiently to viable as well as
243 nonviable cells of the susceptible bacterium. No adsorption of dickeyocin P2D1 to either
244 dead or alive cells of the resistant *D. dadantii* cells was observed (Fig. S2).

245

246 ***Production of dickeyocin P2D1 is not associated with T6SS***

247 To determine whether dickeyocin P2D1 production in *D. dadantii* depends on the type VI
248 secretion system (T6SS), dickeyocin P2D1 production was induced in *D. dadantii* mutant
249 A5587 (*31*) that carries an insertional mutation in the *tssK* gene (type VI secretion system
250 baseplate subunit TssK) – an essential subunit of the type VI secretion apparatus (*32*). The
251 tailocins obtained from the *D. dadantii* T6SS mutant using standard mitomycin induction
252 methods were morphologically indistinguishable and similarly abundant compared to
253 dickeyocin P2D1induced in the wild-type *D. dadantii* strain 3937 (Fig. S3). These results
254 suggest that at least *tssK* gene, essential for the proper function of T6SS, does not affect
255 dickeyocin P2D1 production, morphology, or release from the cell.

256

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

259 Dickeyocin P2D1 was stable at temperatures between 4 and 42 °C, showing no significant
260 loss in activity following 24 h incubation at these temperatures. Temperatures above 50 °C,

261 however, led to a 32-fold loss in activity while incubation at 65 and 80 °C (Fig. 5 A) led to a
262 complete loss of activity. Likewise, a single freeze-thaw cycle negatively affected the stability
263 of dickeyocin P2D1. Freezing at -20 °C resulted in a 128-fold reduction in activity, while no
264 bactericidal effect remained after freezing at -80 °C (Fig. 5 A). We tested the stability of P2D1
265 under 7 different pH conditions (2, 3.5, 5, 7, 9, 10.5, and 12). The particles remained active
266 after 24 h incubation in pHs ranging from 3.5 to 12. However, their bactericidal properties
267 were lost at pH 2 (Fig. 5 B). To determine whether dickeyocin P2D1 that was inactivated at
268 low pH could be restored to an active (extended, 'loaded') form, we diluted the pH 2-treated,
269 inactive dickeyocin P2D1 in PBS buffered to pH 12 to achieve a neutral pH environment.
270 Dickeyocin P2D1 did not regain bactericidal activity at pH 7. Osmotic conditions generated
271 by NaCl added to water at concentrations between 0.01 to 5 M had no adverse effect on the
272 activity of dickeyocin P2D1 within 24 hours when compared to controls (dickeyocin P2D1 in
273 PBS containing 0.137 M NaCl and 0.0027 mM KCl). In contrast, incubation of dickeyocin
274 P2D1 in deionized water led to a 4-fold loss in activity (Fig. 5 C). Dickeyocin P2D1 activity
275 was significantly ($p<0.05$) reduced by treatment with proteinase K and chloroform for 1 hour
276 (4-fold and 2-fold loss of activity, respectively). Complete loss of activity was seen after
277 incubation in 1% SDS. In contrast, neither lysozyme nor DNase influenced dickeyocin P2D1
278 activity (Fig. 5 D).

279

280 ***Dickeyocin P2D1 is nontoxic for Caenorhabditis elegans***

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

288

289 **Discussion**

290 Our understanding of the mechanisms underlying bacterial competitive behaviors
291 remains limited. Although numerous bacterial species are known to produce and exploit
292 tailocins for their competitive advantage, little is known of how commonly such entities are
293 employed by plant-pathogenic bacteria, including pectinolytic, necrotrophic SRP pathogens,
294 to enable their environmental fitness (*9, 10*). This study revealed the presence of tailocins in
295 one of the broadest characterized and economically important member of the Soft Rot
296 *Pectobacteriaceae* family – strain *D. dadantii* 3937. We identified and characterized in detail
297 a novel phage tail-like particle – dickeyocin P2D1, produced by this strain not only upon
298 mitomycin C treatment, but also constitutively in culture. Although tailocins were initially
299 noted in a limited number of *Dickeya* spp. (strains of *Erwinia chrysanthem* dissimilar from
300 strain 3937) isolated from several crop and ornamental plants in the past (1960s and 1970s)
301 (*33*), these particles were not characterized in detail, probably due to the lack of appropriate
302 molecular techniques at that time.

303 Dickeyocin P2D1 has a typical morphology and expresses features similar to those of
304 other R-type tailocins described so far for a large group of bacteria, including *Escherichia coli*
305 (*34*), *Burkholderia cenocepacia* (*35*), *Pseudomonas aeruginosa (10)*, *Proteus vulgaris* (*36*)
306 and *Yersinia enterocolitica (37)*. Likewise, tailocins were also identified in *Pectobacterium*
307 spp. (*28, 38, 39*) – plant pathogens closely related to *Dickeya* spp. The tailocin of SRP
308 bacteria best characterized to date is carotovorocin Er isolated from *P. carotovorum* Er (*39,*
309 *40*). Interestingly, as shown in this study, despite the morphological similarities of dickeyocin
310 P2D1 and carotovorocin Er and the rather close phylogenetic relationship and the lifestyle
311 shared by the producer strains, these tailocins show little sequence homology. This suggests
312 that dickeyocin P2D1 and carotovorocin Er originated from different phage ancestors and
313 that the ability to produce tailocins was acquired several times and independently by various
314 members of the SRP family.

315 The cluster encoding dickeyocin P2D1 exhibits high homology with that encoding the
316 tail of *Enterobacteria* phage P2, indicating that these two probably had a common ancestor.
317 Phage P2 is a temperate bacteriophage belonging to the *Myoviridae* family, commonly found
318 in genomes of strains belonging to the *Proteobacteria* phylum (*30*). Its presence has been
319 noted in at least 127 genera in 32 *Proteobacteria* families (*41*), indicating its extreme
320 ubiquitousness in the bacterial world. Accordingly, R-type tailocins resembling the tail of
321 phage P2 have been extensively studied and are now the best characterized to date (*5, 10*).
322 To explore the occurrence of R-type tailocins in SRP, we searched available *Dickeya* spp.
323 and *Pectobacterium* spp. genomes for regions carrying phage tail-like genes homologous to
324 those encoding dickeyocin P2D1 and having organizational similarity. Our analysis revealed
325 the presence of P2D1-like clusters in several *Dickeya* spp. strains, including *D. solani*, *D.*
326 *dianthicola, D. zeae*, and *D. fangzhongdai*. The P2D1-like clusters were, however, absent in
327 all *Pectobacterium* spp. genomes as well as in genomes of the other bacterial species
328 unrelated to SRP analyzed in our study. The lack of the dickeyocin P2D1 cluster both in
329 *Pectobacterium* spp. and in bacteria phylogenetically distant to SRP may indicate a strong
330 and sustained phylogenetic association between dickeyocin P2D1and *Dickeya* spp. This is
331 somewhat unexpected given that various *Dickeya* and *Pectobacterium* are often present in
332 the same environment (e.g., soil, water, plant surface) (*19*). Under such conditions,
333 horizontal gene transfer would frequently occur between these phylogenetically related SRP
334 species (*42*).

335 The restricted occurence of genes encoding dickeyocin P2D1 in the genomes of
336 *Dickeya* spp. aligns with its narrow bactericidal activity. In our study, dickeyocin P2D1
337 exclusively targeted *Dickeya* species as well as *Musicola paradisiaca* (former *Dickeya*
338 *paradisiaca*) but not any other bacteria tested. It is noteworthy, however, that these *Dickeya*
339 strains differed somewhat in the suspectibiltiy to dickeyocin P2D1 while these differences
340 were unrelated to the phylogenetical distance between them. This is in line with other studies
341 (*43-45*) that showed that the host range of pyocins, tailocins produced by *Pseudomonas*

342 spp., is usually restricted to kin strains (*9*). Likewise the tailocins of *Escherichia coli* (*46*) and
343 *Yersinia enterocolitica (37)* also killed only related taxa. Contrarily, some recent reports have
344 revealed tailocins that target different species of the same genus and/oreven different
345 genera. For example, *Burkholderia cenocepacia* tailocins were active against other
346 *Burkholderia* species (*35*) and those of *P. fluorescens* were active against *Xanthomonas*
347 *vesicatoria (40)*. The quite narrow target range of dickeyocin P2D1 is somewhat surprising
348 given that multiple species of SRP bacteria are often found together in the same infected
349 plant (*47-49*). In such a setting these various species would be expected to experience
350 intense competition and would be expected to benefit from a promiscuous tailocin (*50*). It
351 thus appears that in such mixed infections, *Dickeya* spp. and *Pectobacterium* spp. compete
352 using mechanisms unrelated to phage-tail-like particles, as reported previously (*51, 52*), but
353 this conjecture requires more experimental support.

354 Our study revealed that dickeyocin P2D1 is produced even without induction with
355 mitomycin C, although such induction boosts the tailocin production 10 to 100-fold. To
356 precisely estimate the concentration of P2D1 particles, we used a well-established Poisson
357 distribution killing method (*53, 54*) as well as a new approach we developed. The Poisson
358 distribution killing method is an indirect enumeration method as it employs the number of
359 survivors in tailocin-treated bacterial suspensions. Advantage of indirect methods is that they
360 provide the number of active ('loaded') tailocin particles in a sample. In this study, we have
361 complemented the indirect counting approach by direct enumeration of tailocin particles with
362 NanoSight NS300. Direct particle counts avoid the need for culturing, and when combined
363 with killing-based methods, enables an estimation of the fraction of active tailocins in a
364 preparation. This appears to be a novel application of NanoSight to evaluate the quality and
365 the active fraction of tailocin preparations.

366 One of the immediate applications of dickeyocin P2D1 would be in controlling SRP
367 infections in crops (*18*). A major practical limitation of tailocins as therapudic agents is their
368 narrow bactericidal range. This can, however, be at least partially overcome by using

369 cocktails of tailocins having different bactericidal ranges, similarly as in the use of
370 bacteriophages (*55*). Despite this limitation, tailocins have shown to be effective antibacterial
371 agents in various applications (*56, 57*). They have several benefits compared to other
372 biological control agents evaluated to date (*18*). One of the biggest advantages of tailocins is
373 their incapacity to proliferate on-site after application. Likewise, tailocins themselves cannot
374 spread via transduction or transformation mechanisms because only the protein products
375 and not the encoding nucleic acids are employed. The high target selectivity of tailocins also
376 prevents disruption of sother, often beneficial bacteria present in the same niche. Likewise,
377 since dickeyocin P2D1 was nontoxic to *C. elegans*, a model eukaryotic organism, such
378 tailocins are unlikely to impact other eukaryotic organisms present in soil and/or on plants
379 (*58*). Additional studies are needed to fully explore the potential of dickeyocin P2D1 to
380 achieve plant disease control, such as targeting production, the long-term effectiveness, and
381 consistency of control under field conditions, including stability, formulation, and eco-
382 toxicological risks. Studies of the *in planta* expression of dickeyocin P2D1 should provide
383 great insight into the interactions of *Dickeya* species with each other and with other bacteria
384 under natural and agricultural conditions.

385

386 **Materials and Methods**

387 **Bacterial strains and culture conditions**

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

393

394 **Induction, purification, and concentration of tailocins from *Dickeya dadantii* 3937**
395 **culture**
396 *D. dadantii* strain 3937 was grown overnight (ca. 16 h) in TSB at 28 ºC with shaking (120
397 rpm). The cultures were then rejuvenated by diluting them 1:40 in 250 mL of fresh TSB
398 medium. The diluted culture grew for 2.5 hours under the same conditions. Such prepared
399 3937 cultures were then supplemented with mitomycin C (Abcam, Poland) to a final
400 concentration of 0.1 µg mL^{-1} to induce the production of tailocins. Following mitomycin C
401 treatment, the cultures were incubated for another 24 h at 28 ºC with shaking (120 rpm).
402 Bacterial cells were then removed by centrifugation (10 min, 8 000 RCF), and the
403 supernatant containing putative particles was collected and filtered through a sterile 0.2 µm
404 PES (polyether sulfone) membrane filter using the Nalgene Rapid-Flow Sterile Disposable
405 Filter Units (Thermo Fisher Scientific). Finally, to precipitate tailocins, PEG-8000 (Promega)
406 was added to the filtrate to a final concentration of 10%, and the sample was incubated at 4
407 °C on a magnetic stirrer for the next 16-24 h. The tailocins were collected by centrifugation (1
408 h, 16 000 RCF, 4 °C) and resuspended in 5 mL of Phosphate Buffered Saline (PBS, pH 7.2,
409 Sigma-Aldrich). The resulting pellet was resuspended in 1/50 of the initial volume of the initial
410 sample. The purified particles were stored at 4 °C.

411

412 **Initial qualitative screen of tailocins for the bactericidal activity**
413 The activity of the purified and concentrated particles was initially tested qualitatively on a
414 limited panel of bacterial strains (=17 strains, Supplementary Table S1) using a spot test
415 assay as described before (*35, 59*).

416

417 **Microscopic imaging**
418 Tailocins were imaged using both transmission electron microscopy (TEM) and atomic force
419 microscopy (AFM), as described earlier (*60*). TEM analyses were done at the Laboratory of

420 Electron Microscopy (Faculty of Biology, University of Gdansk, Poland). For TEM analysis,
421 particles obtained as described above were adsorbed onto carbon-coated grids (GF
422 Microsystems), stained with 1.5% uranyl acetate (Sigma-Aldrich), and directly visualized with
423 an electron microscope (Tecnai Spirit BioTWIN, FEI) using a previously described protocol
424 (*61*). At least ten images were taken for each preparation to estimate the diameters of the
425 particles. For the AFM analysis, purified and PEG-concentrated particles were used directly
426 without further preparations. AFM imaging was conducted in air mode using the Bioscope
427 Resolve microscope (Bruker), in ScanAsyst (Peak Force Tapping) mode, employing the
428 SCANASYST-AIR probes (f0 7.0 kHz, diameter <12 nm, k: 0.4 N/m) as described earlier
429 (*62*). Similarly, as described for TEM analysis, for AFM, at least ten images were taken for
430 each preparation to estimate the diameters of the particles.

431

432 **Determination of the concentration of tailocins**

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

434 *Direct particle count with NanoSight NS300*

435 NanoSight NS300 instrument (Malvern Panalytical), equipped with an sCMOS camera and a
436 Blue488 laser, was used to directly assess the concentration and size distribution of particles
437 obtained after induction and purification, as described above. The tailocin samples were
438 diluted 1000 times in sterile PBS buffer pH 7.2 (Sigma-Aldrich) to acheive the optimal
439 concentration for observation. The camera gain was set to 14, the number of captures per
440 sample equaled 5, each lasting 60 s, and the detection threshold was set to 5 as suggested
441 by the manufacturer. Measurements were conducted at room temperature (ca. 22-23 °C).
442 Three biological replicates were used to determine the concentration and size distribution of
443 the obtained tailocins, and the results were averaged for further analysis.

444 *Semiquantitative estimation by a spot test (35)*

445 Samples interrogated for tailocins were serially 2-fold diluted in PBS pH 7.2 (Sigma-Aldrich).
446 Two µl of each dilution were spotted onto TSA plates overlayed with 15 ml of soft top agar
447 (Nutrient Broth (NB, Oxoid) with 7 g L^{-1} agar). Before pouring, the soft top agar was cooled to
448 45 °C and inoculated with 250 µl of an overnight culture of a tailocin indicator strain
449 (susceptible strain *M. paradisiaca*). Plates were incubated overnight at 28 °C. The highest
450 dilutions of particles capable of cell lysis, visible as plaques (halos) in the bacterial lawn in
451 soft top agar, were determined. Each tailocin dilution was tested in triplicates and the entire
452 experiment was repeated two times with the same setup. The reciprocal of the highest
453 dilution causing a visible plaque was defined as the value of the relative activity in arbitrary
454 units (= 1 AU).

455 *Poisson distribution killing method (35)*

456 Tailocins were also quantified using the Poisson distribution killing method based on the
457 protocol described by (*35*) and initially introduced by others (*53, 54*). This method is based
458 on the number of bacteriocidal events, determined from the number of bacterial survivors in a
459 population with a known number of initial viable cells. To determine this, ten µl of undiluted
460 and 10-fold diluted samples of tailocins were added to 100 µL of an overnight TSB (Oxoid)
461 culture of a susceptible strain *Musicola paradisiaca* NCPPB 2511 (10^8 CFU mL^{-1}) and
462 incubated for 40 min at 28 °C with shaking (120 rpm). As a negative control, PBS pH 7.2 was
463 used instead of the tailocins suspension. Each combination was tested in triplicates. After
464 incubation, the suspensions were serially diluted up to 10^{-7} in PBS and plated in triplicate on
465 TSA plates. The colonies that emerged following overnight incubation at 28 °C were
466 enumerated to calculate the bacterial survival ratio (*S*) in the treated samples compared to
467 the negative control. S was calculated as the number of viable bacteria in a sample
468 incubated with tailocins divided by the number of viable bacteria in the negative control. The
469 number of lethal events per bacterial cell (*m*) was calculated as $m = -\ln(S)$. The total number
470 of active killing particles per milliliter (based on the assumption that tailocins adsorption to

471 bacterial cells in each sample was quantitative within the first 40 min incubation period) was
472 calculated by multiplying *m* by the initial number of bacterial cells per milliliter (CFU mL^{-1}).

473

474 **Target range of the tailocins isolated from *D. dadantii* 3937**

475 Overnight cultures of the investigated strains were prepared in 1 mL aliquots of TSB in 2 mL
476 microcentrifuge tubes (Eppendorf) which were incubated horizontally with shaking (120 rpm)
477 at 28 ºC for 16-24 h. The overnight cultures were used as an inoculum in a spot test carried
478 out on 48-well plates (Greiner). Briefly, 10 µL of the inoculum was transferred to each well of
479 the plate and mixed with 500 µl of liquified soft top agar (Nutrient Broth, NB, (Oxoid) with 7 g
480 L^{-1} agar), precooled to 45 ºC in a water bath. Plates were gently stirred (20 rpm) to ensure an
481 even distribution of bacterial cells in the inoculated wells. After the agar had solidified, plates
482 were left to dry for 10 min. in a laminar flow hood. Two µl of 10-fold diluted tailocins
483 (approximately 10^{10} particles mL^{-1}) purified from mitomycin-induced cultures of *D. dadantii*
484 3937 were spotted on the surface of the inoculated soft-top agar in the wells of the multitier
485 plate. Inoculated plates were incubated for 24 h at 28 ºC and then inspected for the presence
486 of a growth inhibition. A spot of lack of growth was interpreted as the susceptibility of the
487 given strain to the tailocins (positive reaction). Two µl of sterile PBS was spotted on a lawn of
488 a suseptical strain as a negative control. The susceptibility of each bacterial strain was tested
489 in triplicate, and the entire experiment was repeated two times.

490

491 **Determination of the bacterial killing rate**

492 The rapidity by which tailocins killed bacteria was measured in 96-well plates (Nest) using a
493 Epoch 2 microplate reader (BioTek). Twenty-five µl of a suspension containing tailocins
494 (approximately 10^{11} particles mL^{-1}) in PBS pH 7.2 were added to 100 µl of 5 McF
495 (approximately 10^{8} CFU mL^{-1}) bacterial suspension in PBS. An overnight culture of a tested
496 strain in TSB were harvested by centrifugation (5 min, 8000 RCF). The OD_{600} of the PBS

497 suspensions was measured each minute for 2 hours. The plate was incubated at 28 ºC with
498 shaking. The susceptible strain *M. paradisiaca* strain NCPPB 2511 was used as a positive
499 control, and sterile PBS pH 7.2 without tailocins was used as a negative control. The log-
500 transformed values of OD_{600} at each time point were normalized to the log-transformed
501 starting OD_{600} and regressed against time. The regression coefficient ($\Delta Log_{10}(OD_{600})min^{-1}$)
502 was calculated for each of the obtained curves. The killing proportion was estimated at two
503 representative time points (20 and 120 min) as the average % of the initial OD_{600} of the
504 selected bacterial culture compared to controls (n=10).

505

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

507 Proteins within tailocins were separated using a 4-20% sodium dodecyl sulfate-
508 polyacrylamide gradient gel (Mini-PROTEAN TGX Stain-Free Precast, Bio-Rad Hercules,
509 USA) electrophoresis (SDS-PAGE) using previous methods (*63*). Protein bands were
510 excised from the gel using a sterile scalpel, and the excised gel pieces were placed in
511 separate 1.5 mL Eppendorf tubes for amino acid sequencing. In-gel digestion was performed
512 according to a standard protocol consisting of gel de-coloration and removal of Coomassie
513 staining, reduction/alkylation with dithiothreitol (DTT), and iodoacetamide (IAA), respectively
514 (*64*). First, digestion was carried out overnight with trypsin (Promega Mass Spectrometry
515 Grade Gold) at 37 °C. The tryptic peptides were then eluted from the gel with sequential
516 washing of gel pieces with 50 mM ammonium bicarbonate buffer, 5% formic acid in 50%
517 acetonitrile, and 100% acetonitrile (*65*). All samples were then concentrated (SpeedVac),
518 and the final cleanup was carried out using the StageTips method on the C18 phase to a
519 50% acetonitrile solution with 1% acetic acid (*66*). After concentrating the samples to 30 ml
520 using the SpeedVac, fragmentation mass spectra were recorded for analysis. ESI LC-MS/MS
521 analysis was performed on Triple Tof 5600+ mass spectrometer with DuoSpray Ion Source
522 (AB SCIEX, Framingham, MA) connected to the Eksigent microLC (Ekspert MicroLC 200
523 Plus System, Eksigent, Redwood City, CA) equipped with the ChromXP C18CL column (3

524 µm, 120 Å, 150 × 0.5 mm). The microLC−MS/MS system was controlled by the AB SCIEX
525 Analyst TF 1.6 software. Chromatographic separation was carried out for 30 min in a
526 gradient program: (1) 0−1 min – 20% solvent B, (2) 1−26 min – 20−60% solvent B, (3) 26−28
527 min − 98% solvent B, (4) 29-30 min − 20% solvent B, where solvent A was 0.1% formic acid
528 in water and solvent B 0.1% formic acid in acetonitrile. The identification of proteins present
529 in the examined gel bands was carried out based on the obtained fragmentation spectra
530 using the ProteinPilot software (v 4.5) or Peaks Studio and the appropriate protein database
531 (UniProt *Dickeya dadanti 15.02.2023, unreviewed*) with an automated false discovery rate
532 (1% FDR).

533

534 **Bioinformatic analyses**

535 Prediction of prophage regions in the genome of *D. dadantii* 3937 (Genbank accession:
536 NC_014500.1) was conducted using PHASTER (42). The tailocin cluster and individual
537 proteins it encodes were analyzed against NCBI databases using BLASTn and BLASTp (43).
538 The topology of the tailocin cluster in *D. dadantii* 3937 was investigated using BioCyc (44).
539 Phylogenomic analysis of the Soft Rot *Pectobacteriaceae* (SRP) genomes was based on
540 core genome sequences and was performed using EDGAR ver. 3.0 (45) accessed via
541 https://edgar3.computational.bio.uni-giessen.de.

542

543 **Stability of tailocins (*43, 67, 68*)**

544 Tailocins were incubated for 24 hours at several different temperatures (-80, -20, 4, 22, 28,
545 37, 42, 50, 65, and 80 °C), pH values (2, 5, 3.5, 7, 9, 10.5 and 12), and NaCl concentrations
546 (0.01, 0.1, 1 and 5 M), after which their bacterial growth inhibitory activity was assessed by a
547 spot test using *M. paradisiaca* as described above. All tested samples had a total volume of
548 200 µl, and the initial concentration of PEG-purified tailocin of approximately 10^{10} particles
549 mL^{-1}. To obtain the test samples, tailocins were diluted by mixing a volume of 20 µL of PEG-

550 purified samples with 180 µl of either PBS (temperature stability), PBS with pH modified by
551 addition of either HCl or NaOH to the desired values (pH stability), or in water containing
552 different concentraitons of NaCl .The experiments were repeated three times, each with
553 three technical replications per tested condition.

554

555 **Effect of enzyme/detergent/organic solvent treatment on the activity of tailocins (*67-***
556 ***69*)**

557 Tailocins were incubated for 1 hour at 37 °C with the following enzymes: proteinase K
558 (Sigma-Aldrich, final concentration: 0.5 mg mL^{-1}), DNase (Sigma-Aldrich, final concentration:
559 10 U mL^{-1}), or lysozyme (Sigma-Aldrich, final concentration: 0.5 mg mL^{-1}), and at room
560 temperature (22 °C) with sodium dodecyl sulfate (SDS, Sigma Aldrich, final concentration:
561 1%), or chloroform (POCH, 50% v/v). The remaining activity of treated tailocins was then
562 assessed in a spot test as described above. The processed samples had a total volume of
563 200 µL, and a tailocin concentration of approximately 10^{10} particles mL^{-1}. The samples were
564 prepared by mixing 20 µl of PEG-purified samples (about 10^{11} particles mL^{-1}) with the
565 appropriate volume of the factor stock to a final volume of 200 µL with PBS. The exception
566 were the chloroform-treated samples where 200 µL of sample were mixed on a shaker with
567 200 µL of chloroform. Prior to testing, the chloroform-treated sample was centrifuged (5 min
568 at 4000 RCF), and the aqueous phase was removed for assay. The experiments were
569 repeated three times, each with three technical repetitions per tested condition.

570

571 **Binding of tailocins to nonviable bacterial cells (*68*)**

572 Overnight bacterial cultures of *M. paradisiaca* NCPPB 2511 and *D. dadantii* strain 3937
573 grown in TSB were washed twice with PBS buffer and then killed by incubation with 5 mg mL^{-}
574 ^{1} chloramphenicol (Sigma-Aldrich) for 60 min, with shaking (120 rpm, 28 ºC). The killing of
575 the cells after 1 h was confirmed by plating 4 aliquots (10 µL) of treated culture on TSA

576 plates, incubating at 28 ºC for 24 h, and verifying last of bacterial growth. After killing, the
577 nonviable bacterial cells were again washed three times with PBS to remove the remaining
578 antibiotic. The PEG-purified tailocin samples were then added to suspension of viable and
579 nonviable susceptible and nonsusceptible bacteria to a final concentration of approximately
580 10^{10} particles mL^{-1} and incubated for 40 min with shaking at 120 rpm at 28 ºC. After
581 incubation, samples were filtered through a 0.2 µm PES (polyether sulfone) membrane filter,
582 and the remaining tailocins was assessed by a spot test using *M. paradisiaca*. The
583 experiments were repeated three times, each with three technical replication per tested
584 condition.

585

586 **Testing the influence of pH on the bactericidal activity of tailocins**

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

592

593 ***Caenorhabditis elegans* toxicity assay**

594 Sensitivity of *Caenorhabditis elegans* to tailocins produced by *D. dadantii* may influence the
595 was tested as before (*70*) with slight modifications. Briefly, wild-type Bristol N2 strain of *C.*
596 *elegans* nematode obtained from the *Caenorhabditis* Genetic Center (CGC, University of
597 Minnesota, Minneapolis, USA) was maintained as described before (*71, 72*) on Nematode
598 Growth Medium (NGM) plates with *Escherichia coli* strain OP50 as a food source. The
599 toxicity of the tailocins was tested on nematode cultures synchronized as described earlier
600 (*73*). Briefly, nematode eggs were harvested from cultures treated with a mixture of 5M
601 NaOH and 5.25% NaOCl (1:3, v:v) – a treatment that eliminates adult worms. Recovered

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

612

613 ***Statistical analysis***

614 All statistical tests were conducted using either Past 4.13 software (*74*) or Microsoft Office
615 Excel (www.office.com). The Shapiro-Wilk (*75*) and F-tests (*76*) were used to test for
616 normality and variance equality of data, respectively. For pairwise testing, the t-test (*77*) was
617 applied for data having a normal distribution and equal variances, the Welch test (*78*) was
618 used for samples with a normal distribution but unequal variances, while the U Mann-
619 Whitney test (*79*) was applied for data that were not normally distributed. One-way analysis
620 of variance (ANOVA) (*80*) was used to compare more than two data groups. Levene's test
621 (*81*) was used to test the homogeneity of variance, and the normality of the residuals was
622 conducted using the Shapiro-Wilk test. Welch's one-way analysis of variance, followed by the
623 Games-Howell post hoc test (*82*), was used for the data groups with non-homogeneous
624 variance and normally distributed residuals. For groups with a non-homogeneous variance
625 and without normally distributed residuals Kruscal-Wallis's one-way analysis of variance (*83*)
626 followed by Dunn's post hoc test (*84*) was applied.

627

628 **Competing Interest Statement**

629 The authors declare no competing interests.

630

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645

646 **Author contributions**

647 Conceptualization: RC, MB, DMK

648 Methodology: MB, DMK, MN, MS, MR, PC, KW

649 Investigation: MB, MS, MN, MR, DMK, KW

650 Visualization: MB, DMK, MN, MR,

651 Supervision: RC, DMK

652 Writing—original draft: MB, DMK, RC

653 Writing—review & editing: RC, DMK, MB

654 Funding acquisition: RC

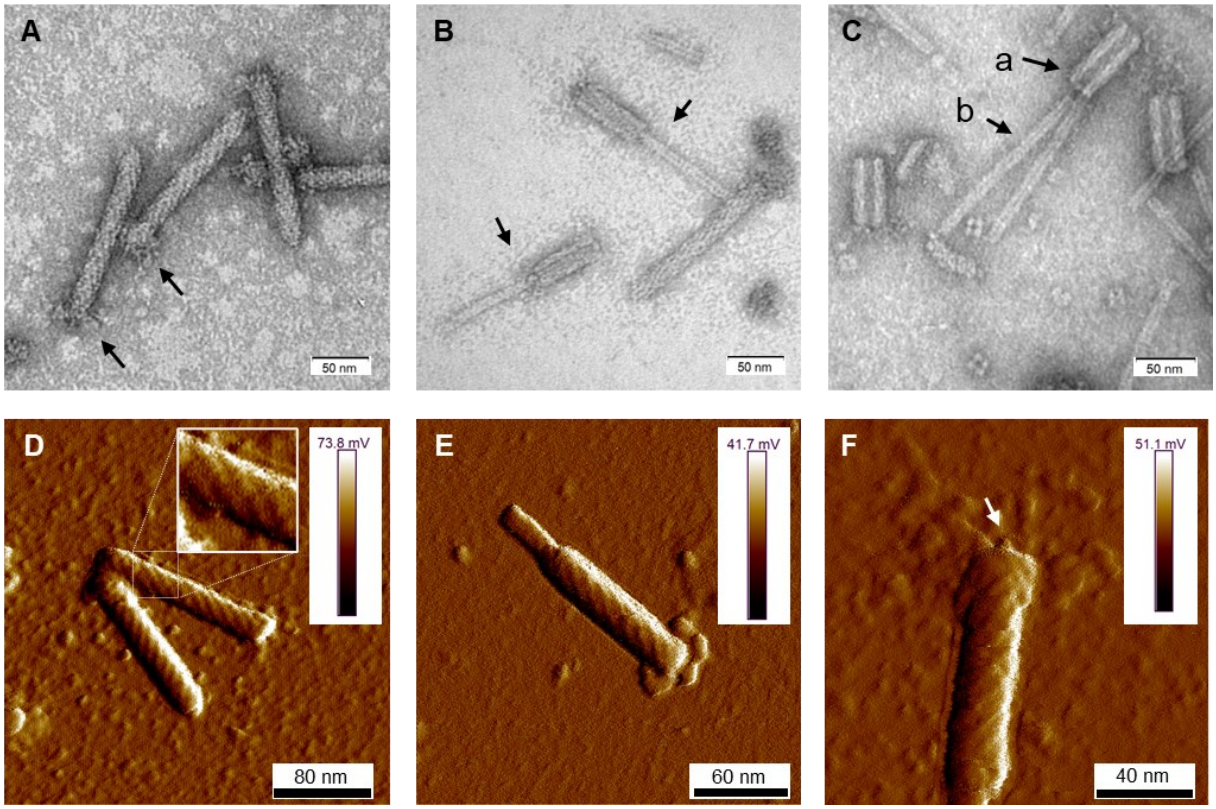
655

Data and materials availability:

All data are available in the main text or the supplementary materials.

Figures

Fig. 1 The morphology of tailocins from *D. 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 ºC); Arrows indicate: **a** – empty sheath with a hollow inner channel, **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; (**F**) an extended particle with an arrow pointing at the presumed tube-attached spike.



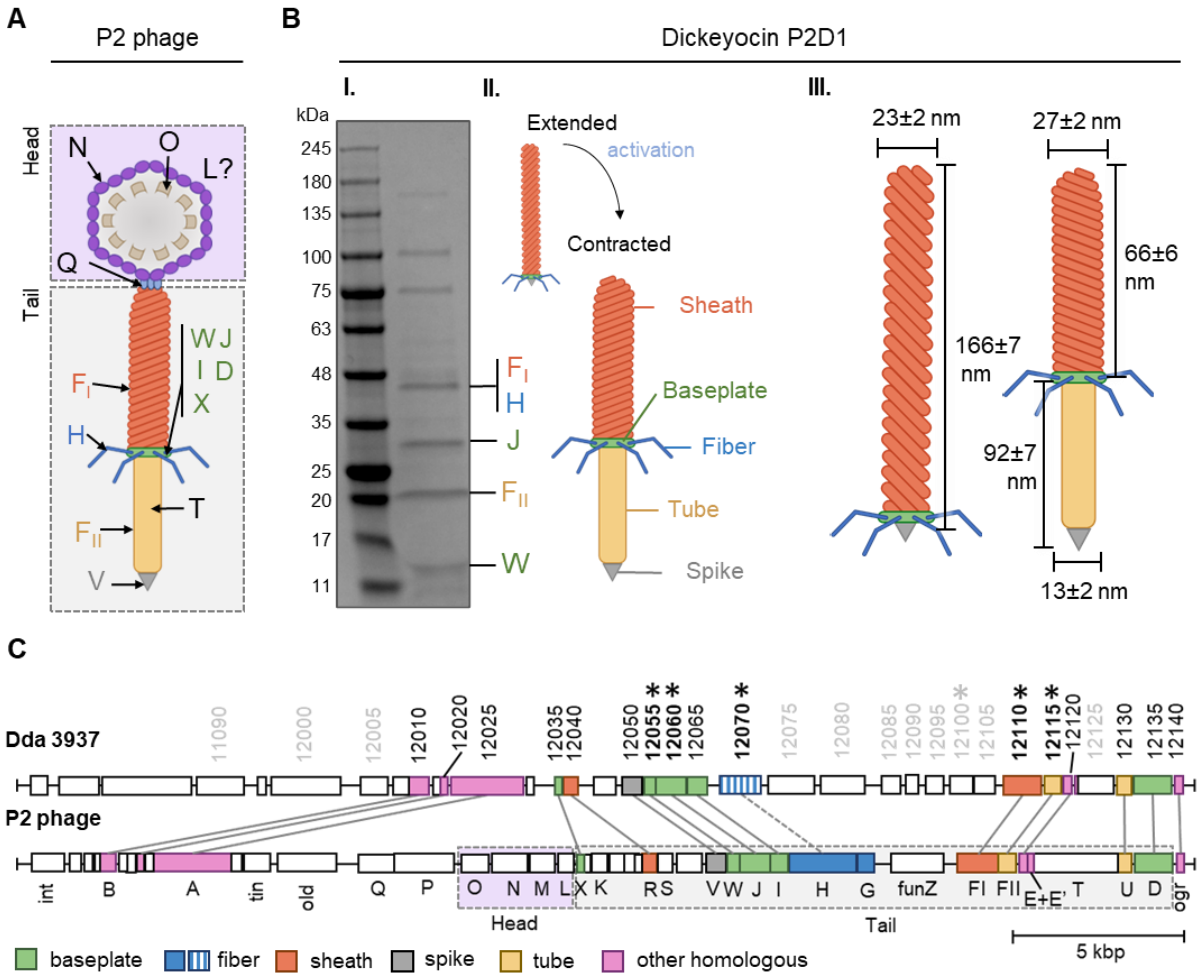
692 **Fig. 2 The building blocks of tailocins of *D. dadantii* strain 3937 and their encoding**
693 **genes in relation to those of the *Enterobacteria* P2 bacteriophage.**

694 **Panel A** shows a schematic representation of the structure of the P2 phage, together with
695 the nomenclature of the building proteins. The graph was prepared based on Cristi and
696 Calendar, 2016 (PMID: 27144088, https://pubmed.ncbi.nlm.nih.gov/27144088/). WJIDX –
697 baseplate proteins, F_I – sheet, H - fiber, F_{II} – tube, V – spike, T – tape measure protein, Q –
698 portal protein, N – major capsid protein, O – capsid scaffold, L – head completion protein.

699 **Panel B** shows: **I.** an SDS-PAGE separation of proteins from mitomycin-induced cultures of
700 *D. dadantii* 3937. Bands containing proteins homologous to those of the P2 phage are
701 provided with the respective protein designations; **II.** schematic representation of extended
702 and contracted forms of dickeyocin P2D1; **III.** dimentions of extended and contracted forms
703 of dickeyocin P2D1.

704 **Panel C** shows the alignment between the complete genome sequence of the P2 phage
705 (NC_001895; 33 593 bp) and a region of the same length in the genome of *D. dadantii* 3937
706 (NC_014500.1; range: 2723487 to 2757061). The numbering of genes in *D. dadantii*
707 corresponds to the numbering of loci in the genome (locus tag prefix DDA3937_RS).
708 Homologous proteins are marked with the same color.

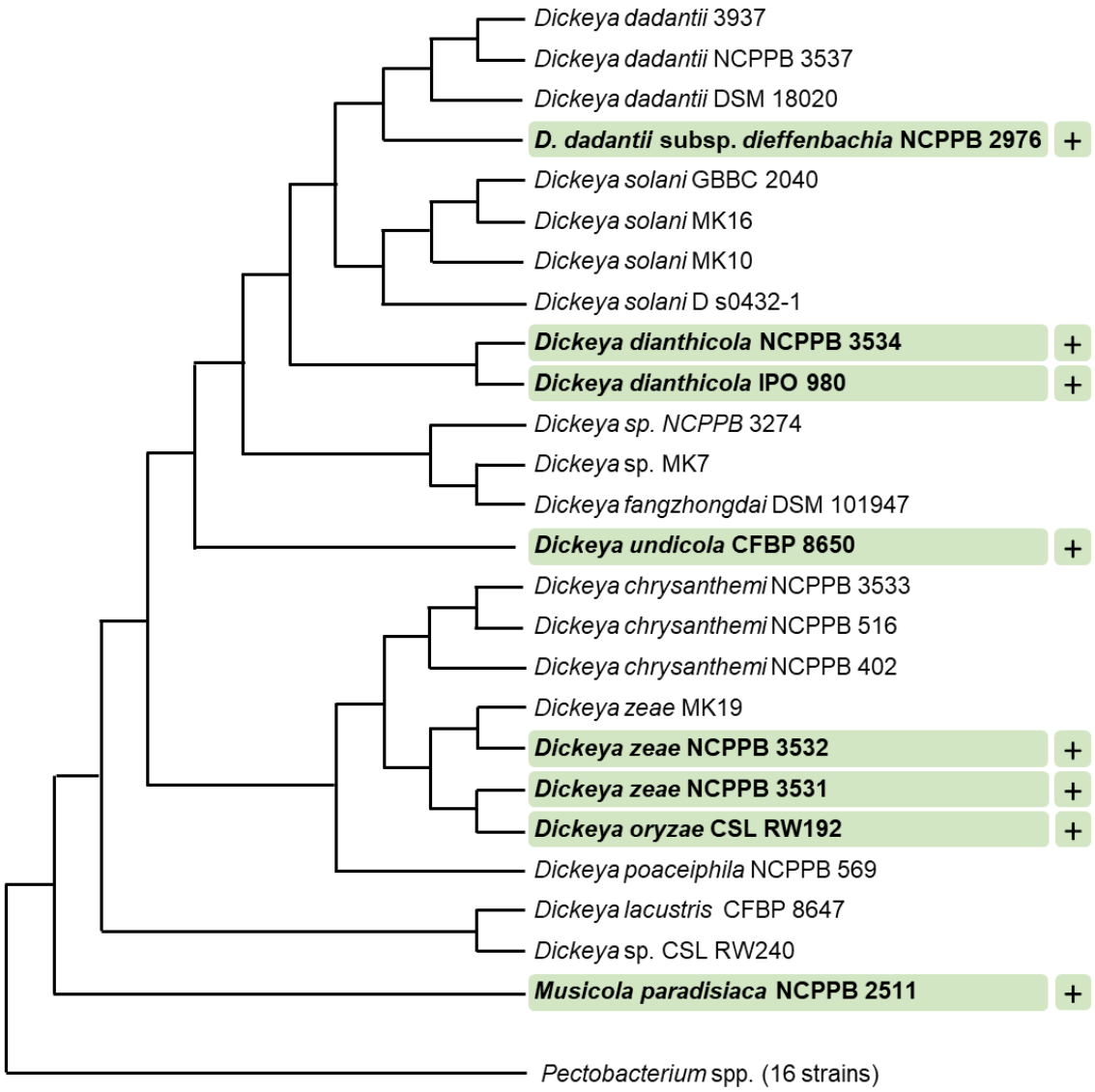
709



710

711

712 **Fig. 3 Target range of tailocins from *D. dadantii* 3937.** Strains susceptible to P2D1
713 dickeyocin were marked with a plus (+). A phylogenetic tree for 41 SRP strains was obtained
714 using EDGAR 3.2. It was built out of a core of 1616 genes per genome (nucleotide
715 sequences), 66256 genes in total. The core had 1560951 bp per genome, 63998991 in total.
716 The lengths of the branches do not reflect the phylogenetic distance. All analyzed
717 *Pectobacterium* spp. were collapsed. This included 16 strains: *P. cacticida* CFBP 3628, *P.*
718 *fontis* CFBP 8629, *P. betavasculorum* CFBP 2122, *P. peruviense* CFBP 5834, *P.*
719 *atrosepticum* SCRI1043, *P. atrosepticum* NCPPB 549, *P. parmentieri* CFBP 8475, *P.*
720 *parmentieri* SCC3193, *P. polonicum* DPMP 315, *P. punjabense* CFBP 8604, *P. actinidiae*
721 LMG 26003, *P. brasiliense* LMG21371, *P. polaris* NCPPB 4611, *P. versatile* CFBP 6051, *P.*
722 *carotovorum* CFBP 2046, *P. aroidearum* NCPPB 929.
723



724

Fig. 4 Interaction of P2D1 dickeyocins with the cells of a susceptible strain *M. 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.

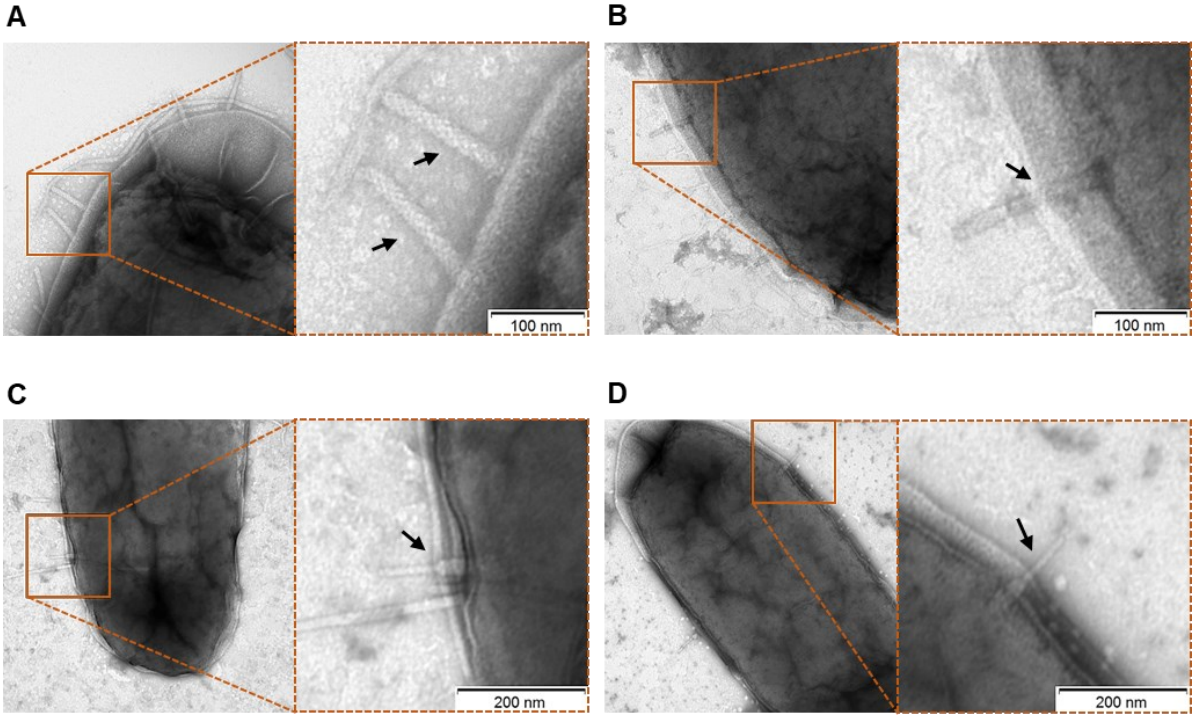
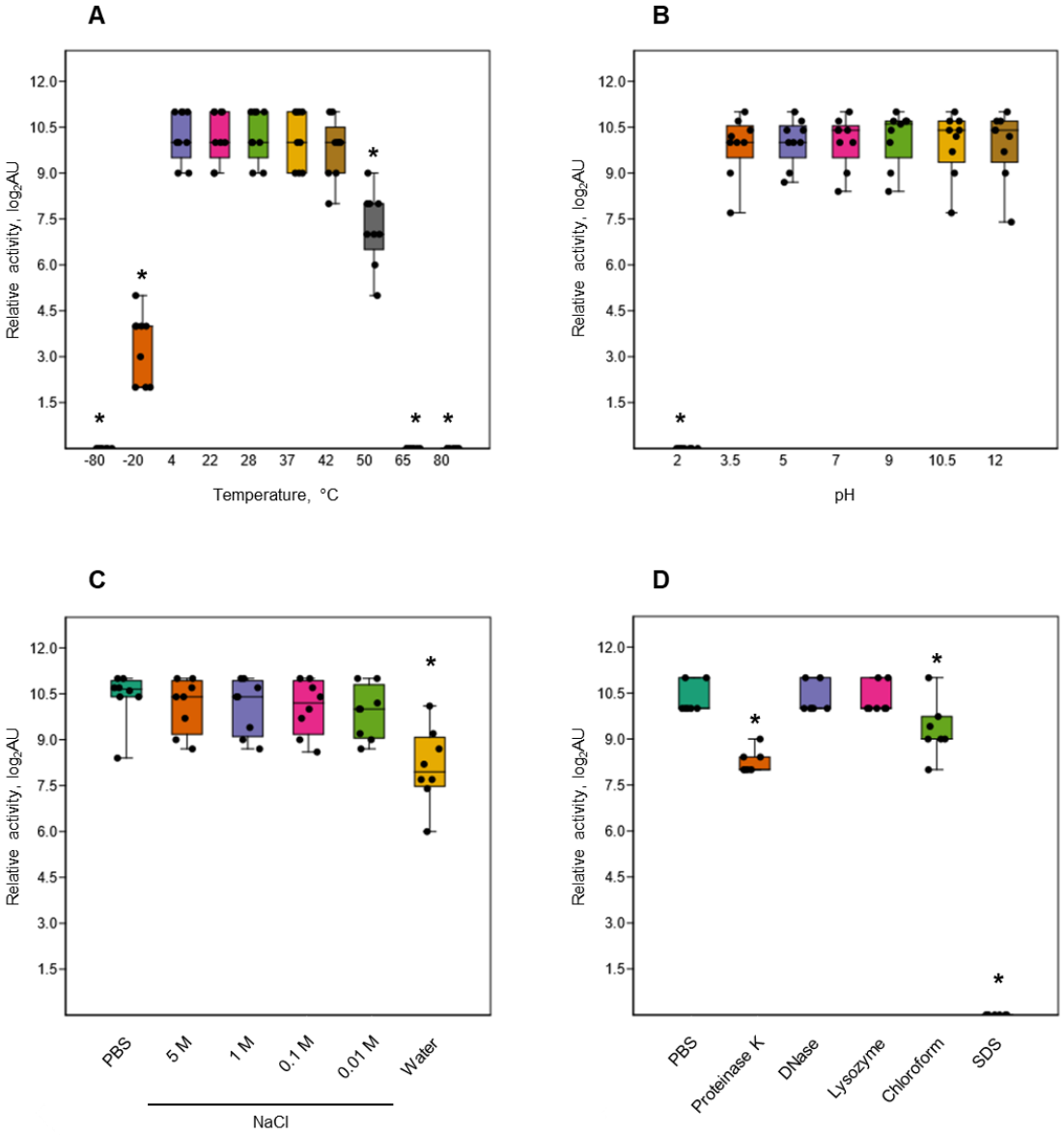


Fig. 5 Stability of P2D1 dickeyocins from *Dickeya dadantii* strain 3937 upon different treatments. The graphs show the influence of temperature (A), pH (B), salinity (C), as well as enzymatic digestion (proteinase K, DNase, and lysozyme), chloroform treatment, and SDS detergent (D) on bactericidal activity of P2D1 against *M. 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.



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