Different Ancestries of R Tailocins in Rhizospheric Pseudomonas Isolates

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Abstract

Bacterial genomes accommodate a variety of mobile genetic elements, including bacteriophage-related clusters that encode phage tail-like protein complexes playing a role in interactions with eukaryotic or prokaryotic cells. Such tailocins are unable to replicate inside target cells due to the lack of a phage head with associated DNA. A subset of tailocins mediate antagonistic activities with bacteriocin-like specificity. Functional characterization of bactericidal tailocins of two Pseudomonas putida rhizosphere isolates revealed not only extensive similarity with the tail assembly module of the Pseudomonas aeruginosa R-type pyocins but also differences in genomic integration site, regulatory genes, and lytic release modules. Conversely, these three features are quite similar between strains of the P. putida and Pseudomonas fluorescens clades, although phylogenetic analysis of tail genes suggests them to have evolved separately. Unlike P. aeruginosa R pyocin elements, the tailocin gene clusters of other pseudomonads frequently carry cargo genes, including bacteriocins. Compared with P. aeruginosa, the tailocin tail fiber sequences that act as specificity determinants have diverged much more extensively among the other pseudomonad species, mostly isolates from soil and plant environments. Activity of the P. putida antibacterial particles requires a functional lipopolysaccharide layer on target cells, but contrary to R pyocins from P. aeruginosa, strain susceptibilities surpass species boundaries.

Key words: bacteriocin, phage tail, antibacterial, pyocin.

Introduction

Bacteria are able to secrete very diverse secondary metabolites and ribosomally synthesized antimicrobials to antagonize competitors with similar niche preferences. Molecular sizes of these antibacterials vary largely, ranging from small peptides to multiprotein complexes (Hibbing et al. 2010). Historically, the (poly)peptides which exhibit high specificity and selectively kill close phylogenetic relatives of the producer are collectively referred to as bacteriocins. In recent years, it has become clear that bacteriocin-like activity of Gram-negative bacteria can also be mediated by type VI secretion substrates, proteins causing contact-dependent inhibition, or Rhs (rearrangement hot spot) proteins (Braun and Patzer 2013; Ruhe et al. 2013; Durand et al. 2014; Hayes et al. 2010, 2014; Russell et al. 2014).

The Escherichia coli colicins and the Pseudomonas aeruginosa pyocins are among the best-characterized “classical” bacteriocins of Gram-negative bacteria. In the latter species, five types have been described (Ghequire and De Mot 2014). S-type pyocins are bacteriocins with a modular organization similar to colicins. They consist of a receptor-binding domain typically targeting outer membrane receptors involved in iron uptake (Baysse et al. 1999; Denayer et al. 2007; Elfarash et al. 2012, 2014), a translocation domain, and a carboxy-terminal toxin domain with nuclease or pore-forming activity. Self-intoxication of a producer is prevented by tightly regulated coexpression of an immunity gene (Ghequire and De Mot 2014). Such accessory immunity partner appears not to be required for lipid II-degrading M-type pyocins that cause inhibition of peptidoglycan biosynthesis similarly to colicin...
M (Barreteau et al. 2012). L pyocins carrying a tandem of lectin domains have no counterpart among colicins (Ghequire et al. 2014; McCaughey et al. 2014). The carboxy-terminal lectin domain, recognizing α-rhamnose, allows anchoring onto the cellular surface (McCaughey et al. 2014), but target specificity is mainly conferred by the amino-terminal domain (Ghequire et al. 2013).

Two morphologically distinct types of bactericidal “tailocsins”, high-molecular-weight (HMW) bacteriocins showing structural similarities with bacteriophage tails, have been described in *P. aeruginosa* (Michel-Briand and Baysse 2002). The R-type pyocins are rigid and contractile, whereas the F-type pyocins are flexible but noncontractile. Based on gene cluster similarities, a common ancestry with tailed enterobacteriophage, P2 (P2like virus genus of the *Myoviridae* family) for the R-type and with phage λ (λ*Bactdeviridae* genus of the *Siphoviridae*) for the F-type, was proposed by Nakayama et al. (2000). To date, five R-type and three F-type pyocins have been described (Ghequire and De Mot 2014). Although the F type pyocin receptors remain unrevealed, it was demonstrated that an α-l-rhamnose and two α-glucose residues of the lipopolysaccharide (LPS) outer core account for the O-serotype-specific character of susceptibility for R1, R2, and R5 pyocins, respectively (Köhler et al. 2010). In the case of pyocin R3, a glucose residue in the core oligosaccharide is involved in bacteriocin susceptibility as well. A deletion in the *wapB* promoter prevents the addition of this terminal carbohydrate residue and accounts for bacteriocin resistance (Kocincova and Lam 2013). Atomic-resolution structures of pyocin R2 in extended configuration (sheath and tube) and in the contracted state (sheath) obtained by cryo-electron microscopy suggest a possible mechanism for release of stored energy to drive perforation of bacterial membranes by the inner tube (Ge et al. 2015). With its inner surface being lined by negative charges, the tube then functions as a cation-conducting channel that dissipates the proton potential of the target cell.

The expression of *P. aeruginosa* S-type, R-type, and F-type pyocin genes is controlled by the PrtN activator that binds to regulatory P boxes in their promoter regions. In noninducing conditions, expression of *ptrN* is repressed by PrtR. Upon exposure to stress conditions, such as DNA damage by chemicals or UV irradiation, activated RecA triggers auto-proteolytic cleavage of PrtR, which abrogates *ptrN* repression and leads to pyocin production (Ghequire and De Mot 2014). RecA-mediated pyocin production can also be triggered by the CRISPR/Cas phage immunity system in sessile *P. aeruginosa* cells (Heussler et al. 2015). Under denaturing conditions, pyocins are enclosed within *P. aeruginosa* membrane vesicles, equally dependent on the SOS response route (Toyofuku et al. 2014). The *P. aeruginosa* *ptrR* and *ptrN* genes are located upstream of the pyocin clusters, in opposite orientation (Ghequire and De Mot 2014). PrtR also binds its own promoter, pointing to autorepressive regulation and resulting in relatively stable PrtR expression levels (Sun et al. 2014). In addition, PrtR also controls expression of *ptrB*, encoding a Type III secretion system repressor, which is located in opposite direction of *ptrR* just ahead of the pyocin cluster (Wu and Jin 2005).

Also in soil-dwelling and plant-associated pseudomonads different types of bacteriocins, often subject to stress-induced production, have been characterized: Pyocin M homologs in *Pseudomonas fluorescens* and pathovars of *Pseudomonas syringae* (Barreteau et al. 2009; Grinter et al. 2012); L-type bacteriocins in *Pseudomonas protegens*, *Pseudomonas putida* and *P. syringae* (Parret et al. 2003, 2005; de los Santos et al. 2005; Ghequire et al. 2012); a gyrase-inhibiting B-type microcin in *P. syringae* (Metelev et al. 2013); and a phage tail-like bacteriocin in *P. fluorescens* SF4c (Fischer et al. 2012). Using hybridization of genomic DNA from 30 *P. fluorescens* strains with DNA probes derived from P2-like tail genes in *P. fluorescens* SBW25 and λ-like tail genes in *P. fluorescens* QBr1-96, the widespread occurrence, either individually or combined, of such phage-like genes was demonstrated (Mavrodi et al. 2009). Comparative genomics of the *P. fluorescens* clade revealed that in most strains the *mutS–cinA* intergenic region is occupied by mosaic P2- and/or λ-type phage-related clusters differing in size and gene composition, but their exact nature (intact prophage or remnant, HMW bacteriocin) remains elusive (Loper et al. 2012).

In this study we characterized HMW bacteriocins in two *P. putida* strains, isolated from the rhizosphere of banana (strain BW11M1) and rice (strain RW10S2), and identified the corresponding gene clusters. Phylogenetic analysis of regulatory, tail assembly and lysis genes of HMW bacteriocins and related (pro)phages was used to acquire insight in the diversity and ancestry of these mobile elements ubiquitously present in pseudomonad genomes. Based on this genomic perspective we propose a phylogeny-based classification of R tailocsins in pseudomonads.

**Materials and Methods**

**Strains, Media, and Growth Conditions**

Bacterial strains used in this study are listed in table 1. *Pseudomonas* strains were cultured in Tryptic Soy Broth (TSB 3%, BD Biosciences) at 30°C, on a rotary shaker at 200 rpm. *Escherichia coli* and *P. aeruginosa* strains were grown at 37°C in LB (2.5%; MP Biomedicals). Media were solidified with 1.5% agar (Invitrogen) and supplemented with kanamycin (50 μg/ml; Sigma-Aldrich) or spectinomycin (100 μg/ml; Sigma-Aldrich) when required.

Genomic DNA from *Pseudomonas* strains was extracted using the Puregen Yeast/Bact. Kit B (Qiagen). Plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (Qiagen). Bacterial stocks were stored in the appropriate medium at −80°C in 25% (v/v) glycerol (VWR International).
Large-Scale Production and Purification of R Tailocin

One-milliliter volumes of overnight test tube cultures of *P. putida* BW11M1 were transferred to 500 ml erlenmeyers. After the OD_{600} reached 0.5, filter-sterilized mitomycin C (Sigma-Aldrich) was added to a final concentration of 0.1 μg/mL. The culture was incubated further until cell lysis occurred. Subsequently, benzonase nuclease (2 U/ml; Sigma-Aldrich) was added to the lysed culture to reduce the viscosity, followed by a 30-min incubation at 37 °C. Debris and membrane fragments were removed by centrifugation at 8,000 × g for 30 min at 4 °C. Supernatants were filter sterilized (0.22 μm; Millipore) and supplemented with polyethylene glycol (PEG) 6000 (Sigma-Aldrich) and NaCl (VWR International) at final concentrations of 10% (w/v) and 0.5 M, respectively. This mixture was stirred overnight (200 rpm, 4 °C) and centrifuged the following day at 8,000 × g for 1 h at 4 °C. The precipitate was resuspended in 10 ml TN50 buffer (50 mM NaCl, Tris–HCl 10 mM, pH 7.5) and centrifuged at 4,000 × g for 15 min at 4 °C to remove small undissolved debris. Finally, bacteriocin particles were sedimented at 48,400 × g for 3 h at 4 °C, resuspended in 2 ml TN50 buffer and filtered (0.45 μm; Millipore) and further purified by gel filtration using a Sephacryl S-500 HR column (16/60, GE Healthcare Life Sciences; fractionation range for dextrans: 4×10^{6} – 2×10^{7} Da) at an elution rate of 0.7 ml/min. Collected fractions were tested for antibacterial activity and analyzed by transmission electron microscopy, SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), and mass spectrometry. Bacteriocin samples were kept at 4 °C until use.

Transmission Electron Microscopy

Purified tailocins from *P. putida* BW11M1 were transferred to 0.7% formvar-coated copper grids (Aurion, Wageningen, The Netherlands) by placing the grids in the tailocin emulsion for 30 s. Consequently, the tailocins were incubated in 0.25% phosphatungstic acid (pH 7) for 30 s at room temperature and washed in distilled water. After sample drying, analysis was performed with a Phillips EM208 S transmission electron microscope (80 kV), equipped with a Morada Soft Imaging System camera. Digital processing of the obtained images was performed with the iTEM-FEI software (Olympus SIS, Münster, Germany).

Bacteriocin Assay

Spot activity assays with purified tailocins from *P. putida* BW11M1 and RW10S2, and extracts of derived tailocin mutants, were performed as follows. First, cell lawns were prepared by overlaying TSA (Tryptic Soy Agar) plates with 5 ml of TSB soft agar (0.5 %), seeded with 25 μl of the indicator strain (~10^{8} CFU/ml). Next, 10-μl volumes of collected bacteriocin were spotted on top of these plates and allowed to air-dry. Heat sensitivity of the purified bacteriocins was tested by heating the tailocins in a heat block at 75 °C for 10 min. After cooling to room temperature, samples were spotted onto cell lawns as described above. Plates were incubated overnight at 30 °C (37 °C for *P. aeruginosa*). The following day, plates were evaluated for the presence of halos. TN50 buffer was used as a negative control.

Protein Electrophoresis and Amino-Terminal Amino Acid Sequencing

Samples with bacteriocin activity collected after gel filtration chromatography were analyzed through SDS-PAGE (Invitrogen) at 200 V for 0.7 h using the Precision Plus Protein Kaleidoscope ladder (Bio-Rad) as a size standard. After separation, the gels were electroblotted onto polyvinylidene difluoride (PVDF) membranes (Invitrogen) and stained with Coomassie blue R-250 (Bio-Rad) to visualize proteins. Protein bands were cut from the membrane, destained in methanol (VWR International) and amino-terminal sequencing was performed by automated Edman degradation, using a Procise 491 cLC protein sequencer (Applied Biosystems, Foster City, CA).

Recombinant DNA Methods

Standard methods were used for the preparation of competent *E. coli* and *P. putida* cells, heat shock transformation of *E. coli* and DNA electrophoresis (Green and Sambrook 2012). SMART ladder (Eurogentec) was used as a standard for DNA size estimation. Restriction enzymes were used according to the supplier’s specifications (Roche Diagnostics). DNA ligation was performed using T4 DNA ligase (Invitrogen). Sequencing of plasmid DNA was performed by GATC Biotech (Constance, Germany).

Construction of Tailocin Mutants

Terminal regions of *ptbI* (encoding phase tail sheath protein) and *ptbF* (encoding baseplate protein) from the R tailocin gene clusters of *P. putida* BW11M1 and RW10S2, respectively, were amplified by polymerase chain reaction (PCR) with *PfX* DNA polymerase (Invitrogen), using genomic DNA as a template. Primers used are listed in supplementary table S1, Supplementary Material online. Amplicons were purified with the QIAquick PCR Purification kit (Qiagen), digested with *HindIII* and *BamHI* for the upstream, and *BamHI* and *EcoRI* for the downstream fragments, and subsequently ligated in suicide plasmid pAKE604 (El-Sayed et al. 2001). Sequence-verified plasmids pCMGP6226 and pCMGP6227, carrying the fused upstream and downstream fragments of BW11M1 *ptbI* and RW10S2 *ptbF*, respectively, were transformed to *E. coli/S17-1 λpir* and transferred to the corresponding *P. putida* strains through biparental conjugation. Selection with kanamycin and spectinomycin yielded transformants that were individually grown in a test tube without antibiotic for 12 h. Serial dilutions were plated out on TSA containing...
### Table 1

Susceptibility of *Pseudomonas* Species Groups to R Tailocins of *Pseudomonas putida* Strains BW11M1 and RW10S2

| Species                  | Strain     | Genotype/Characteristics                          | Susceptibility to Tailocin $R_{BW11M1}$ | Susceptibility to Tailocin $R_{RW10S2}$ | Origin or Reference               |
|--------------------------|------------|--------------------------------------------------|-----------------------------------------|----------------------------------------|-----------------------------------|
| *Pseudomonas aeruginosa* | PA14       | Opportunistic pathogen, human isolate            | –                                       | –                                      | *Pseudomonas* genome DB           |
| *Pseudomonas aeruginosa* | PAO1       | Opportunistic pathogen, human isolate            | –                                       | –                                      | *Pseudomonas* genome DB           |
| *Pseudomonas agarici*    | LMG 2112   | Mushroom isolate, type strain                    | –                                       | –                                      | BCCM collection                   |
| *Pseudomonas aureofaciens* | LMG 1245  | River clay, type strain                          | –                                       | –                                      | BCCM collection                   |
| *Pseudomonas chlororaphis* | LMG 5004  | Contaminated plate, type strain                  | –                                       | –                                      | BCCM collection                   |
| *Pseudomonas cichorii*   | LMG 2162   | *Cichorium*, type strain                         | –                                       | –                                      | BCCM collection                   |
| *Pseudomonas entomophila* | L48        | Entomopathogenic strain that kills *Drosophila melanogaster* | –                                       | +                                      | *Pseudomonas* genome DB           |
| *Pseudomonas fluorescens* | 13-79      | Wheat rhizosphere                                | –                                       | –                                      | Weller and Cook (1983)            |
| *Pseudomonas fluorescens* | 2-79       | Wheat rhizosphere                                | –                                       | –                                      | Weller and Cook (1983)            |
| *Pseudomonas fluorescens* | A506       | Pear isolate                                     | –                                       | –                                      | *Pseudomonas* genome DB           |
| *Pseudomonas fluorescens* | BW11P2     | Banana rhizosphere                               | +                                       | –                                      | Vlassak et al. (1992)             |
| *Pseudomonas fluorescens* | DR54       | Viscosinamide producer, sugar beet rhizosphere isolate | +                                       | –                                      | Nielsen et al. (1999)             |
| *Pseudomonas fluorescens* | F113       | Sugar beet rhizosphere                            | –                                       | –                                      | *Pseudomonas* genome DB           |
| *Pseudomonas fluorescens* | LMG 1794   | Prefilter water-works tank, type strain          | –                                       | –                                      | BCCM collection                   |
| *Pseudomonas fluorescens* | OE 28.3    | Wheat rhizosphere                                | –                                       | –                                      | CMPG collection                   |
| *Pseudomonas fluorescens* | P0-1       | Soil isolate                                     | +                                       | –                                      | *Pseudomonas* genome DB           |
| *Pseudomonas fluorescens* | CMPG2247   | $R_{BW11M1}$ tailocin-resistant *P0-1* mutant     | –                                       | –                                      | This study                        |
| *Pseudomonas fluorescens* | CMPG2248   | $R_{BW11M1}$ tailocin-resistant *P0-1* mutant     | –                                       | –                                      | This study                        |
| *Pseudomonas fluorescens* | CMPG2249   | $R_{BW11M1}$ tailocin-resistant *P0-1* mutant     | –                                       | –                                      | This study                        |
| *Pseudomonas fluorescens* | CMPG2250   | $R_{BW11M1}$ tailocin-resistant *P0-1* mutant     | –                                       | –                                      | This study                        |
| *Pseudomonas fluorescens* | SBW25      | Sugar beet leaves                                | –                                       | –                                      | *Pseudomonas* genome DB           |
| *Pseudomonas fluorescens* | WCS365     | Potato rhizosphere                                | –                                       | –                                      | Geels and Schippers (1983)        |
| *Pseudomonas gingeri*    | LMG 5327t1 | Mushroom, type strain                            | –                                       | –                                      | BCCM collection                   |
| *Pseudomonas marginalis* | LMG 2210   | *Cichorium* isolate, pathovar reference strain   | –                                       | –                                      | BCCM collection                   |
| *Pseudomonas mendocina*  | LMG 1223   | Isolate from ethanol-enriched soil               | –                                       | –                                      | BCCM collection                   |
| *Pseudomonas protegens*  | Pf-5       | Soil isolate                                     | –                                       | –                                      | *Pseudomonas* genome DB           |
| *Pseudomonas putida*     | GB-1       | Fresh water isolate, manganese oxidizer          | –                                       | –                                      | *Pseudomonas* genome DB           |
| *Pseudomonas putida*     | OE 47.1    | Maize rhizosphere                                | –                                       | –                                      | CMPG collection                   |
| *Pseudomonas putida*     | OE 53.2    | Maize rhizosphere                                | –                                       | –                                      | CMPG collection                   |
| *Pseudomonas putida*     | OE 55.1    | Maize rhizosphere                                | –                                       | –                                      | CMPG collection                   |
| *Pseudomonas putida*     | OE 55.7    | Maize rhizosphere                                | –                                       | –                                      | CMPG collection                   |
| *Pseudomonas putida*     | WCS358     | Potato rhizosphere                                | –                                       | –                                      | *Pseudomonas* genome DB           |
| *Pseudomonas savastanoi* | LMG 6768   | Nerium oleander isolate                          | –                                       | +                                      | BCCM collection                   |
| *Pseudomonas syringae*   | DC3000     | Model pathogen, bacterial speck on tomato and *Arabidopsis* | –                                       | +                                      | *Pseudomonas* genome DB           |

(continued)
sucrose (7%; VWR International) to select for the loss of the cassette. Allelic exchanges of the recombinants were checked by PCR with Taq polymerase (BIOKE´), using primers PGPRB-10022 and PGPRB-10025 for strain BW11M1, and PGPRB-10120 and PGPRB-10123 for strain RW10S2. Putative mutants were sequence verified for in-frame deletions following PCR amplification with Pfx. Resulting deletion mutants were CMPG2245 (deletion of internal 780-bp fragment of ptbJ in BW11M1) and CMPG2246 (deletion of internal 681-bp fragment of ptbF in RW10S2).

Generation of Spontaneous Tailocin Resistant Mutants

A bacteriocin assay was performed on 24-h-old cultures of R tailocin-susceptible strains Pf0-1 (for the BW11M1 R tailocin) and LMG 5456 (for the RW10S2 R tailocin), using the spot-on-lawn assay. After 48-h incubation, tailocin-resistant mutants can be isolated as colonies growing inside the tailocin halos. One mutant (if present) per halo per cell culture was streaked to single colony and verified for resistance to the tailocin by spot assay (Pf0-1 mutants CMPG2247–CMPG2250; LMG 5456 mutants CMPG2251–CMPG2254). To exclude the isolation of bacteriocin-tolerant contaminant bacteria, genomic DNA of the presumed mutants was isolated and the boxA element was amplified using primer BOXA1R (PGPRB-10181; supplementary table S1, Supplementary Material online) as described (Koeuth et al. 1995). After gel electrophoresis, the banding patterns of the wild-type strains and resistant mutants were compared.

LPS Purification and SDS-PAGE Analysis

LPS was prepared by the method of Hitchcock and Brown (1983). Briefly, overnight bacterial cultures were diluted to OD600 0.5 and washed twice with phosphate-buffered saline (Sigma-Aldrich). Cell pellets of wild-type strains and resistant mutants were dissolved in 250 μl Hitchcock and Brown lysis buffer (SDS 20 mg/ml [Sigma-Aldrich], glycerol 10% v/v, bromophenol blue 0.02 mg/ml [Sigma-Aldrich], 1 M Tris–HCl [Invitrogen] pH 6.8), and heated at 100 °C for 30 min. After cooling, 1.5-μl volumes of proteinase K (20 mg/ml; Sigma-Aldrich) were added and the mixtures incubated overnight at 55 °C. The following day, the samples were heated for 5 min at 100 °C before loading 5-μl volumes on the gel for SDS-PAGE analysis. Gels were electrophoresed for 1 h at 150 V and LPS was visualized with the SilverQuest Silver Staining Kit (Invitrogen) using the basic staining protocol.

Liquid Chromatography-Tandem Mass Spectrometry Analysis

The identification of proteolytic peptides resulting from in-solution digestion of HMW fractions was performed on an UHPLC-HRMS (ultra-high-pressure liquid chromatography-high resolution mass spectrometry) platform combining a NanoLC-Ultra system (Eksigent) with a TripleTOF 5600 System (AB SCIEX). Tryptic peptides were separated in a 25-cm C18 reverse-phase column (Acclaim PepMap 100, 3 μm; Dionex) by a linear acetonitrile gradient (4–35% v/v, flow rate of 300 nL/min, 20 min) in water containing 0.1% (v/v) formic acid. The time-of-flight analyzer was regularly and

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### Table 1 (Continued)

| Species            | Strain | Genotype/Characteristics                          | Susceptibility to Tailocin BW11M1 | Susceptibility to Tailocin RW10S2 | Origin or Reference |
|--------------------|--------|---------------------------------------------------|----------------------------------|----------------------------------|---------------------|
| Pseudomonas syringae | GR12-2R3 | Grass rhizosphere                                 | −                                 | −                                 | Lifshitz et al. (1987) |
| Pseudomonas syringae | LMG 1247 | Isolate from Syringa vulgaris, pathovar reference strain | −                                 | +                                 | BCCM collection     |
| Pseudomonas syringae | LMG 5192 | Nicotiana tabacum isolate                          | −                                 | +                                 | BCCM collection     |
| Pseudomonas syringae | LMG 5295 | Raphanus sativus isolate                           | −                                 | −                                 | BCCM collection     |
| Pseudomonas syringae | LMG 5456 | Cucumis sativus isolate                            | −                                 | +                                 | BCCM collection     |
| CMPG2251            | R<sub>RW10S2</sub> tailocin-resistant              | −                                 | −                                 | This study            |
| CMPG2252            | R<sub>RW10S2</sub> tailocin-resistant              | −                                 | −                                 | This study            |
| CMPG2253            | R<sub>RW10S2</sub> tailocin-resistant              | −                                 | −                                 | This study            |
| CMPG2254            | R<sub>RW10S2</sub> tailocin-resistant              | −                                 | −                                 | This study            |
| CMPG2251            | R<sub>RW10S2</sub> tailocin-resistant              | −                                 | −                                 | This study            |
| CMPG2252            | R<sub>RW10S2</sub> tailocin-resistant              | −                                 | −                                 | This study            |
| CMPG2253            | R<sub>RW10S2</sub> tailocin-resistant              | −                                 | −                                 | This study            |
| CMPG2254            | R<sub>RW10S2</sub> tailocin-resistant              | −                                 | −                                 | This study            |
| Pseudomonas tolaasii | CH36    | Mushroom                                           | −                                 | −                                 | CMPG collection     |
| Pseudomonas tolaasii | LMG 2342 | Mushroom, type strain                              | −                                 | −                                 | BCCM collection     |
| Pseudomonas viridiflava | LMG 2352 | Phaseolus sp. isolate, type strain                 | −                                 | +                                 | BCCM collection     |

**Note.**—“+,” sensitive; “−,” insensitive. Pseudomonas genome DB, Pseudomonas genome database (Winsor et al. 2011).
automatically calibrated with trypptic peptides of β-galactosidase from E. coli, which maintains an average mass error below 10 ppm across all injections. Mass spectra (MS) were acquired across 400–1,500 m/z with 0.5-s accumulation time. A maximum number of 50 precursors per cycle was selected according to an intensity threshold of 200 counts per second. Each selected precursor was accumulated for 50 ms and submitted to fragmentation with N2 as the collision gas. Tandem mass spectrometry (MS/MS) spectra were acquired across 100–1,800 m/z and an exclusion time of 30 s was applied. The acquired data were analyzed using ProteinPilot Software v. 4.1 (AB SCIEX) and searched against the UniProt TrEMBL database or a local database covering the BW11M1 (Li et al. 2013) and RW10S2 (Rokni-Zadeh et al. 2012) genomes. The search parameters included amnoncarboxidomethyl cysteine, all biological modifications and amino acid (AA) substitutions, and one missed trypsin cleavage. Sequenced peptides (95%) represent the number of distinct peptides having at least 95% confidence. Multiple modified and cleaved states of the same underlying peptide sequence are considered distinct peptides due to different molecular formulas. Sequence coverage (95%) corresponds to the percentage of matching AAs from identified peptides having confidence greater than or equal to 95%, divided by the total number of AAs in the sequence.

**Phylogenetic Analysis**

The predicted AA sequences of the *P. putida* BW11M1 and RW10S2 tail proteins (PtbJ, PtbK, and PtbH), lysis proteins (PtbB and PtbR), and regulator protein (PtbA) were used as BLAST (Basic Local Alignment Search Tool) homology search queries against the nonredundant protein database and draft genomes of pseudomonads to identify and delineate related tailocin gene clusters and prophages. The same approach was used to screen bacteriophage genomes (subset *TcDB*). Of these, *P. putida* RW10S1 (Li et al. 2011) and RW10S2 (Rokni-Zadeh et al. 2012). Of these, *P. putida* RW10S1 was equally produced of the presumed bacteriocin activity by strain BW11M1 was obtained by exposing the culture to mitomycin C, which was accompanied by cell lysis. After cellular debris removal, HMW particles were collected by PEG precipitation and subsequent gel filtration. SDS-PAGE analysis of this HMW bacteriocin-active fraction of *P. putida* BW11M1 revealed the presence of several proteins (fig. 1A). After blotting to a PVDF membrane, amino-terminal sequences of the most prominent bands were determined through Edman degradation. Peptide sequences matched to phage-like structural proteins (predicted tail sheath, baseplate, and tail tube protein) encoded by a *ptb* (phage tail-like bacteriocin) gene cluster identified in the draft genome of *P. putida* BW11M1 (fig. 2). Genomic regions highly similar to this cluster were also retrieved in the draft genomes of *rice* rhizosphere isolates *P. putida* RW10S1 (Li et al. 2011) and RW10S2 (Rokni-Zadeh et al. 2012). Of these, *P. putida* RW10S2 was equally examined for HMW bacteriocin production, resulting in an active particulate fraction with a protein banding pattern similar to the one observed for the purified BW11M1 sample (data not shown).

Increased production of the presumed bacteriocin activity by strain BW11M1 was obtained by exposing the culture to mitomycin C, which was accompanied by cell lysis. After cellular debris removal, HMW particles were collected by PEG precipitation and subsequent gel filtration. SDS-PAGE analysis of this HMW bacteriocin-active fraction of *P. putida* BW11M1 revealed the presence of several proteins (fig. 1A). After blotting to a PVDF membrane, amino-terminal sequences of the most prominent bands were determined through Edman degradation. Peptide sequences matched to phage-like structural proteins (predicted tail sheath, baseplate, and tail tube protein) encoded by a *ptb* (phage tail-like bacteriocin) gene cluster identified in the draft genome of *P. putida* BW11M1 (fig. 2). Genomic regions highly similar to this cluster were also retrieved in the draft genomes of *rice* rhizosphere isolates *P. putida* RW10S1 (Li et al. 2011) and RW10S2 (Rokni-Zadeh et al. 2012). Of these, *P. putida* RW10S2 was equally examined for HMW bacteriocin production, resulting in an active particulate fraction with a protein banding pattern similar to the one observed for the purified BW11M1 sample (data not shown).

Activity of both bacteriocins was assayed against a diverse set of pseudomoad isolates by applying 10-μl spots of purified HMW complexes on trypticase soy agar plates, overlaid with soft agar seeded with cells of the respective indicator strains. After overnight incubation, plates were scored for the presence of growth inhibition halos (fig. 1B and C).

**Results**

**Strain-Specific Antibacterial Activities of Phage-Related Complexes Purified from Two *P. putida* Rhizosphere Isolates**

*Pseudomonas putida* strain BW11M1 produces a 30-kDa lectin (LlpA) with bacteriocin activity against a number of fluorescent pseudomonads (Ghequire et al. 2012), and the cyclic lipopeptide xantholysin that inhibits the growth of several xanthomonads and fungi (Li et al. 2013). The culture supernatant of this strain also shows antagonistic activity against some *Pseudomonas* isolates that are insensitive to LlpA and xantholysin, suggesting that BW11M1 is capable of producing at least one other, yet unidentified anti-*Pseudomonas* substance. This antagonistic effect was abolished upon heat treatment of the culture supernatant (see further) and, compared with LlpA, resulted in relatively small inhibition zones which are typically observed for large, slowly diffusing inhibitory protein complexes such as phage-like bacteriocins (Ghequire and De Mot 2014).

GenBank accession numbers of the nucleotide sequences of the R tailocin clusters from *P. putida* strain BW11M1, RW10S1 and RW10S2 are KP698091, KP698092 and KP698093, respectively.
The test panel consisted of a set of *Pseudomonas* culture collection strains, together with fluorescent *Pseudomonas* rhizosphere isolates from our own collection. Results are summarized in Table 1. For the phage-like complex from strain BW11M1, 3 of 38 tested isolates proved to be susceptible, whereas 7 of them were killed by the protein complex from strain RW10S2, together covering about 26% of the strains tested. Susceptible strains belong to different species, including *P. fluorescens* and *P. syringae*, whereas no *P. putida* indicator was found. None of the strains was susceptible to both particles. As initially no sensitive *P. aeruginosa* strain could be identified, this species panel was expanded to strains belonging to 15 different O serotypes, but again no susceptible strain could be retrieved (data not shown).

To verify that the observed antagonistic activities are not caused by other copurified bacteriocins or phages, defined nonmarked BW11M1 and RW10S2 mutants were constructed by deletions in *ptbJ* and *ptbF*, respectively, that encode predicted structural proteins of the phage-like complexes (putative tail sheath protein and baseplate protein, respectively). The PEG-precipitated HMW fractions derived from culture supernatants of mitomycin-induced BW11M1 and RW10S2 *ptb* mutants did not display the wild-type antibacterial activity (Fig. 1D and E), thereby confirming that the observed bacteriocin activities were solely attributable to the respective purified particles.

Biochemical Characterization Reveals LPS-Targeting R-Type Tailocins

Based on extensive gene synteny and significant AA sequence homologies of encoded proteins with the R-type pyocin
backbone of *P. aeruginosa* (fig. 2), the Ptb system likely represents an R-type of phage tail-like bacteriocin. This was further queried by analyzing the purified BW11M1 and RW10S2 particles through MS/MS. The samples were digested with trypsin and the resulting peptides were analyzed using an UHPLC-nanoESI-MS/MS approach. Multiple proteins were identified as *ptb* gene products encoding phage tail proteins: PtbC, PtbE, PtbF, PtbG, PtbH, PtbJ, PtbK, PtbN, PtbO, PtbP, and PtbQ (fig. 2, supplementary table S2, Supplementary Material online). In the RW10S2 sample, PtbL was detected as well. No indication was found for the possible presence of phage head compartment proteins.

Transmission electron microscopy on the purified HMW fraction of BW11M1 (fig. 1A) revealed structures consisting of a double cylinder, quite similar to previously obtained structures of R-type pyocins (Michel-Briand and Baysse 2002). Measured dimensions of the BW11M1 tail-like complexes or tailocins were 167 nm (length) and 13 nm (width) when sheaths are in extended form. Part of the complexes displaying higher electron density apparently represent contracted forms, resulting in thickened sheaths of 66 nm long and 20 nm wide. As compared with the *P. aeruginosa* pyocin R2 complexes studied by cryo-electron microscopy (Ge et al. 2015), slightly higher length and width ratios of uncontracted versus contracted sheaths are observed for the BW11M1 tailocin (2.53 vs. 2.4 and 1.48 vs. 1.3, respectively). As expected, bacteriocin activity of the BW11M1 and RW10S2 tailocins was lost after heat treatment, due to disassembly of the multiprotein complexes (data not shown).

To isolate spontaneous tailocin-resistant mutants, stationary phase cultures of *P. fluorescens* Pf0-1 and *P. syringae* LMG 5456 were subjected to spot-on-lawn assays with RBW11M1 and RRW10S2 particles, respectively. Colonies emerging inside the halos were picked, and bacteriocin resistance of these suspected mutants was confirmed by the absence of halos in a subsequent spot-on-agar test (table 1). Given the involvement of LPS in cellular killing by R pyocins, LPS banding profiles of the isolated clones were analyzed and compared with the wild-type indicator strains (fig. 1G). Major differences in the LPS patterns were observed, often in combination with altered constitution of the core regions, pointing toward the loss of (part of) the mutant’s LPS functionality.

**Fig. 2.**—Gene organization of the R tailocin clusters from *P. putida* strains BW11M1 and RW10S2. The genomic region of strain LESB58 is included to illustrate synteny with a canonic R pyocin cluster from *P. aeruginosa*. Arrows represent coding regions which are colored according to their predicted function (color legend in boxes). Flanking gene pairs *mutS–cinA* (black) and *trpE–trpG* (gray) are included. The *ptb* genes are denoted *ptbA* through *ptbT*. The cargo genes of strains BW11M1 (bacteriocin LlpA) and RW10S2 (unknown; marked with asterisk) are highlighted. The scale bar represents a DNA region of 2,000bp.

**Tailocin Gene Clusters of *P. putida* and *P. aeruginosa*: Located at Different Hot Spots, Carrying Similar Tail Cassettes but Equipped with Distinct Lysis and Regulatory Modules**

The overall organization of the *P. putida* BW11M1 and RW10S2 tailocin gene clusters is similar to those of *P. aeruginosa* for R-type pyocin production (fig. 2). However, although their tail assembly modules exhibit extensive synteny, the lysis cassettes differ substantially. Although both species share homologous genes encoding a cytoplasmic membrane-permeabilizing holin (PtbB; 64% AA identity) and a peptidoglycan hydrolase (PtbR; 50% AA identity), positioned proximal and distal to the tail region, respectively, the *P. putida* systems encode different accessory proteins predicted to destabilize the cell envelope. The gene products PtbS and PtbT belong...
to the same protein family as, respectively, Rz (an integral membrane protein; Pfam PF03245) and Rz1 (an outer membrane lipoprotein; Pfam PF06085), the spanins that are part of the phage λ lysis cassette (Catálão et al. 2013). The remarkable “Siamese twin” spanin gene organization, with the Rz1 coding region being embedded entirely within the Rz gene but transcribed in a different reading frame (+1), is conserved in both P. putida strains, as well as in P. fluorescens SF4c (Fischer et al. 2012). However, in the latter strain the “hidden” rz1 homolog was not annotated due to the overlap by the open reading frame specifying the Rz-like gene product (Fischer et al. 2012). Another deviation from the prototypical R-type pyocin gene organization is the absence of equivalents of the P. aeruginosa regulatory genes prtN and prtB, located downstream and upstream of prtR, respectively (fig. 2). A counterpart of the prtR gene (pttA4) is present but, unlike prtR, it is transcribed from the same DNA strand as the tail and lysis genes. Notably, PtbA shares rather low AA identity (38%) with the P. aeruginosa repressor protein. Yet another striking difference with the P. aeruginosa system concerns the genomic position of the P. putida BW11M1 and RW1052 tailocin gene clusters that are found between the stress response genes mutS and cinA–recA–recX. Partial sequence analysis of the genomic regions upstream of mutS and cinA in P. fluorescens SF4c revealed the same location (Fischer et al. 2012), whereas R-type pyocin genes are consistently positioned between the AA anabolic genes trpE and trpG.

Abundance of R-Type Tailocins in Rhizospheric Pseudomonads

To explore the occurrence of R-type tailocins, we searched Pseudomonas genomes for regions carrying phage tail-like genes homologous to those identified in P. putida BW11M1/RW1052 and exhibiting organizational similarity. This analysis revealed several tailocin-type gene clusters (~47), consistently sandwiched between mutS and cinA (supplementary table S3, Supplementary Material online). Their diversity is illustrated by the fact that only two of these have an identical nucleotide sequence: The strains Pseudomonas simiae WCS417 and Pseudomonas sp. R81 were both isolated from wheat roots but originate from remote agricultural areas (the Netherlands and India, respectively). In addition, near identity (99.5%) is displayed between the corresponding genomic regions in the biocontrol strains P. fluorescens A506, originating from pear phyllosphere (USA), and P. fluorescens WCS374, retrieved from potato rhizosphere (the Netherlands). Apparently, occurrence of strains with a particular tailocin element is not geographically restricted.

This search further revealed that the P. aeruginosa-type of pyocin R gene cluster is not only confined to this pathogenic species but also occurs in several environmental isolates degrading xenobiotics or plant-derived metabolites: For example, Pseudomonas knacmussii B13 (Miyazaki et al. 2014), Pseudomonas nitroreducens H8P1 (Garcia et al. 2014), P. nitroreducens TX1 (Huang et al. 2014), and Pseudomonas sp. M1 (Soares-Castro and Santos 2013). A pyocin cluster nearly identical to the latter, except for the tail fiber region, is present in two other strains, Pseudomonas sp. AAC and PI1.

Spanin-Like Proteins Are Part of the Lytic Cassettes of Most P. putida and P. fluorescens Tailocins

The BW11M1 and RW1052 PtbB proteins, together with the large majority of the pseudomonad tailocin holins, belong to the same family (http://www.tcdb.org) (Saier and Reddy 2015) as the P. aeruginosa pyocin R holins (TC—1.E.20) and are not related to the P2 type (TC—1.E.3) or ΦCTX type (TC—1.E.56). Apart from a short, highly charged carboxy-terminal stretch (<20 AA), the PtbB sequences are strongly conserved among pseudomonads (supplementary file S1A, Supplementary Material online). Induced expression of the P. fluorescens Q8r1-96 PtbB homolog in E. coli cells engendered rapid cell lysis (Mavrodi et al. 2009).

The predicted BW11M1 and RW1052 peptidoglycan hydrolases (PtbRs) lack a detectable secretory signal peptide and presumably act as canonical endolysins on peptidoglycan exposed by the action of the respective holins (Catálão et al. 2013). This type of endolysin appears to be common to the pseudomonad tailocins and the lytic capacity of the P. fluorescens Q8r1-96 PtbR homolog was demonstrated previously (Mavrodi et al. 2009). Belonging to the glycoside hydrolase family 19 (Pfam PF00182; CAZY-GH19; http://www.cazy.org), these enzymes are different from the lysozyme deployed by P2 (glycoside hydrolase family 24; PF00959) and the peptidoglycan-binding endolysin of ΦCTX (carrying domains PF01471 and PF11860). Remarkably, among characterized Caudovirales phages the most similar homologs are not found among Myoviridae members, with only less than 40% AA identity to the endolysin of enterobacteriophage 4MG (Kim et al. 2014). Significantly better matches are detected for a strain of the Podoviridae lysing P. aeruginosa (Jeon et al. 2012) and a strain of the Siphoviridae infecting P. syringae pv. actinidiae (Di Lallo et al. 2014) (supplementary file S2, Supplementary Material online).

The Rz/Rz1 module is widespread among phages infecting Gram-negative bacteria (Young 2014) and is also part of most of the pseudomonad tailocins. However, it is absent from the gene clusters of P. aeruginosa and a few other isolates (P. fluorescens strains A506 and FH5; Pseudomonas sp. strains CF150, CFT9, WCS347, and WCS417). The rz/rz1 gene pair is equally absent from ΦCTX that carries partially overlapping spanin genes (Summer et al. 2007). Close to their carboxy-terminal end, the Rz proteins of BW11M1 and RW1052 carry a cysteine residue that is conserved in the other inspected pseudomonad tailocins (supplementary file S1B, Supplementary Material online). The smaller Rz1 proteins have, in addition to the characteristic amino-terminal cysteine
required for lipoprotein formation, two other cysteines that are perfectly conserved among these tailocins (supplementary file S1C, Supplementary Material online). This suggests that disulphide bond formation could be important for the function of these spanins (Berry et al. 2012, 2013).

Dedicated Tailocin Regulatory Proteins Differ between *P. aeruginosa* and Other Pseudomonads

The regulatory proteins of pseudomonad tailocins, PrtR (*P. aeruginosa*) and PtbA (mainly *P. putida* and *P. fluorescens* groups) appear to be specific for these bacteriocins as no close homologs were identified in (pro)phages. The best matches to PtbAs (~55% AA identity) and PrtRs (~42% identity) are shown by, respectively, lambda prophage O6 of *P. protegens* PI-5 (Mavrodi et al. 2009) and *P. aeruginosa* temperate phage PAN70 (Rangel et al. 2013). The PrtR and PtbA sequences show strong sequence conservation within each subfamily (>80% AA sequence identity) but there is only low similarity between members from the respective subfamilies (<40% AA sequence identity). Although in *P. aeruginosa* PrtR represses *prtN* expression, no homolog of the latter is found in combination with *ptbA*.

A Bacteriocin Cargo Embedded in the R Tailocin Gene Cluster of *P. putida* BW11M1

Comparison of the tailocin gene clusters of *P. putida* BW11M1 and *P. putida* RW10S2 revealed the presence of the BW11M1 structural gene encoding the lectin-like bacteriocin LlpA (fig. 2). A second “cargo” gene located upstream of *llpA* encodes a homolog of bacteriophage Mu translational regulator Com (Hattman 1999). This gene organization is conserved in *Pseudomonas mosselii* SJ10. Also elsewhere in the tailocin clusters of *P. fluorescens* DSM 8569 and F113, *Pseudomonas parafulva* CR501-1, *P. putida* RW10S2, and *Pseudomonas* sp. GM67, a com homolog is apparently linked to a (different) cargo gene of unknown function (fig. 3). Rather unexpectedly, we detected LlpA in the MS-analyzed purified RW11M1 tailocin sample (supplementary table S2, Supplementary Material online). LlpA is readily detectable in the culture supernatant of BW11M1 stationary phase cells but its production can be significantly enhanced by UV-induced DNA damage (Parret et al. 2003). Apparently, when LlpA and the tailocin are coexpressed, (part of) the lectin remains associated with the particles. Also in the RW10S2 tailocin preparation, the com-linked cargo protein was detected (fig. 2).

Although the tail region of the tailocins in the various strains is well conserved, significant sequence variation is apparent near either end of it, but in particular in the regions flanking the repressor and holin genes (fig. 3). Most of the predicted gene products are hypothetical proteins of unknown functions, but to some a tentative function can be assigned: A peptidoglycan peptidase (Lee et al. 2013) and a YafO-like protein synthesis inhibitor (Zhang et al. 2009) in *P. fluorescens* SS101, a putative Zeta family toxin inhibiting peptidoglycan synthesis in *P. fluorescens* strains BBC6R8 and ATCC 17400 (Mutschler and Meinhart 2011), a putative LPS-modifying acyltransferase in *P. putida* UASWS0946, and a potential UV radiation-protective RuA–RuB module in *P. putida* BW11M1 (Sundin et al. 2000).

![Fig. 3.](http://gbe.oxfordjournals.org/)

**Fig. 3.**—General organization of *Pseudomonas* R tailocin clusters with insertion sites of cargo genes (teal). Arrows illustrate coding regions and are colored as in figure 2, except for the tail assembly module (in gray), and their respective functions are shown in dotted boxes. Cargo genes inserted at one of the four sites, together with their *Pseudomonas* strain of origin, are shown in separate boxes. The (predicted) function of the cargo gene products is specified by a color legend. Homologous genes are shown in the same color.
genes, encoding an oxygenase, an AA transporter, and a linked LysR family regulator. A larger cargo region with presumptive metabolic capacity (including carboxylase, 2-hydroxyacid dehydrogenase, and methyltransferase genes) is conserved in five strains.

**Distinct Tailocin Clades Pointing to Different (Pro)phage Ancestries**

Inspection of *Pseudomonas* genomes for regions bearing similarity to the *P. putida* BW11M1/RW1052 tailocin genetic backbones also revealed many putative prophage clusters typically located at sites other than the *mutS*-cinA intergenic region (supplementary table S3, Supplementary Material online). To scrutinize the evolutionary relationship of the pseudomonad tailocins with these prophages, as well as with related sequenced phages, we focused on their most conserved proteins: Ptb proteins equivalent to the phage P2 proteins building the tail spike (PtbD, GpV), tail sheath (PtbI, GpFII), tail tube (PtbK, GpFI), baseplate hub (PtbQ, GpD), and baseplate (PtbE–PtbF, GpW–GpJ). The Gp nomenclature refers to P2 proteins, as described by Sarris et al. (2014).

Inspection of *P. aeruginosa* sequences (Rp2) and the *P. putida* mutS pseudomonad (pro)phages were assigned. This implies that the structurally related tailocin/prophage pairs, as co-occurring in *P. fluorescens* strains A506, F113, Q2-87, and Q8r1-96 and in *Pseudomonas veronii* 1YdBTEX2, are probably of different origins. The phylogenetic distance of Rp3 from the Rp1/Rp2 clade can be illustrated by the marginal sequence identity between the main tail structural proteins: The *P. fluorescens* SF4c (Rp3) tube and sheath proteins share only between 19% and 24% AA identity with the respective protomers of *P. aeruginosa* PAO1 (Rp1) and *P. putida* BW11M1 (Rp2). The closest protein neighbors for the large group of Rp3 tailocins are not found among characterized pseudomonad phages but in the tail structural proteins of *Vibrio para-ahaemolyticus* phage VP882 (Lan et al. 2009) and *Halomonas aquamarina* phage ΦHAP-1 (Mobberley et al. 2008) (fig. 5). With the *P. fluorescens* SF4c tailocin sheath and tube proteins, these phages share 36–39% and 55–57% identity, respectively. VP882 and ΦHAP-1 are temperate phages with a linear plasmid-like genome, belonging to the *Hapunaleivirus* genus within the *Myoviridae* (http://viralzone.expasy.org). A few of the identified pseudomonad prophages exhibit structural protein homology and pronounced synteny (illustrated for *P. fluorescens* A506, fig. 5) with P2 and ΦCTX, both members of the genus *P2likevirus* of *Myoviridae* (Nakayama et al. 1999; Alber et al. 2002). Although photorhabdincin bears prominent phylogenetic relatedness with these phages (fig. 4), this is not the case for any of the pseudomonad tailocins. Maltocin, on the
other hand, appears to share a common ancestry with a *Pseudomonas plecoglossicida* phage, PPpW-3 (Kawato et al. 2015; fig. 5). The similarity between pseudomonad tailocins and related prophages is not confined to the conserved structural proteins but extends to the associated lytic modules of most of these prophages. These cassettes include holins belonging to the same TC/C0 family. However, the tailocin- and prophage-derived proteins tend to cluster on separate branches, even for representatives encoded by a single strain, as is the case for *P. putida* BW11M1. This indicates that the tailocin-type (PtbB) and prophage holins evolved separately (data not shown). A similar conclusion can be drawn for the tailocin-related prophages (supplementary file S2, Supplementary Material online). Likewise, although the spanin-encoding segment is typically poorly annotated, careful inspection reveals that the rz/rz1 spanin gene architecture appears to be common to the tailocin-related prophages.

**Tail Fiber Proteins: Highly Diversified Determinants of Target Strain Specificity**

The tail fiber protein (TfpH) of *R* pyocins is the major specificity determinant for killing *P. aeruginosa* strains (Williams et al. 2008). The equivalent PtbH proteins share an amino-terminal domain of unknown function (~150–160 AA, DUF3751; Pfam PF12571), followed by a polypeptide sequence that is quite variable in sequence and length. The PtbH proteins can be subdivided into three main groups. A first type of putative tail fiber protein is essentially composed of only the conserved DUF3751 domain ("a-type" tail fiber; supplementary table S3, Supplementary Material online). This "short" DUF3751 variant, present in a subset of tailocins, and prophages, is also found in the nonpseudomonad phages VP882 and HAP-1 (fig. 5). A similar architecture is also present in pseudomonad homologs from several other tailocin clusters, many
presumptive prophages, and phages such as /C8 CTX and PS17. In a third set of tail fibers (c-type), DUF3751 is accompanied by an additional domain of unknown function (Pfam PF07484), originally identified in the tail fiber protein Gp12 of bacteriophage T4 (Makhov et al. 1993). Although this fiber protein type is very abundant among pseudomonad R tailocins, including those of /P. fluorescens/ strains SF4c (998 AA) and /P. putida/ RW10S2 (748 AA), it is only occurring in a few of the retrieved prophages (supplementary table S3, Supplementary Material online).

Due to the huge sequence divergence and length differences of the carboxy-terminal regions in TfpH/PtbH proteins, only their amino-terminal part with the DUF3751 domain can be meaningfully aligned. The phylogenetic tree inferred from this alignment displays a dichotomous topology reminiscent of those of the much more conserved tail structural proteins, albeit with additional subclusters and more dispersed branches (fig. 6). One major cluster of a-type DUF3751 proteins encompasses those present in about half of Rp3-type tailocins (17 strains), being clearly related to the /Vibrio/ and /Halomonas/ phage-associated proteins. The b-type DUF3751-containing pseudomonad proteins are dispersed across the phylogenetic tree and represent members of the Rp1 and Rp2 tailocins, along with a second set of Rp3-type tailocins (16 strains). The DUF3751 domain of most /P. aeruginosa/ strains bears close similarity with the corresponding /ÇTX and PS17/ domains. However, a second P2-related type is present in the atypical strain /P. aeruginosa/ PA7 (Roy et al. 2010) and is shared by the /P. knackmussii/ /P. nitroreducens/ group of strains.

TfpH and its cognate chaperone (encoded by the gene downstream of tfpH), the tail tape measure protein (encoded by the ptm gene located further downstream) that controls the final length of the tail tube (Leiman and Shneider 2012), are specified by the least conserved DNA stretch (nucleotide sequence and coding length) in /P. aeruginosa/ R-type pyocin clusters. The equivalent tail tape measure protein PtbN of /P. putida/ BW11M1 and RW10S2 tailocins differs substantially in length (762 vs. 676 AA) and shares only marginal AA sequence identity (21%). This reflects the poor sequence conservation and broad size range (354–963 AA) within the TfpH/PtbN family. The equivalent pseudomonad prophage proteins are significantly longer (780–1,315 AA; average 980 AA, P value < 2.2 e-16; fig. 6). This would suggest that the tailocin tubes are generally
Phylogenetic analysis of tail fiber proteins in *Pseudomonas* R tailocins. The maximum-likelihood tree was derived from a multiple AA sequence alignment of the amino-terminal DUF3751 domains in PtbH proteins and in homologs from characterized phages (with P2 sequence as root), predicted pseudomonad prophages, and γ-proteobacterial R tailocin-like antibacterial particles. The same colors were used as for the PtbK tree (fig. 4). The scale bar represents 0.4 substitutions per site. Bootstrap values (percentages of 1,000 replicates) are shown at the major branches. The size distributions (in amino acids) of phage tail tape measure proteins of R tailocins (PtbN) and homologs from *Pseudomonas* prophages are shown in box plots.
shorter than those of structurally related pseudomonad (pro)phages.

Even between tailocin gene clusters that overall share a very high level of nucleotide sequence identity, the similarity in the DNA regions encoding tail fiber/chaperone/tape measure proteins can drop substantially. This can be illustrated by the extensive synteny between the gene clusters of *P. putida* BW11M1 and *P. mosselli* SJ10 that even spans their cargo genes (including a 99% identical copy of the encoded bacteriocin LpA), and yet the nucleotide sequence identity between their *ptbH–ptbl–ptbN* regions is only about 50% (compared with >90% for the remaining parts).

**General Discussion**

Phage tail-derived elements integrated in bacterial genomes mediate diverse interactions with other organisms. Afp (antifeeding prophage of *Serratia entomophila*) and PVC (*Photobacteridus* virulence cassette) are similar delivery vehicles that inject toxins into insect hosts (Hurst et al. 2004; Yang et al. 2006). Ordered arrays of MAC (metamorphosis-associated contractile structure) produced by biofilm-producing *Pseudomonas luteoviolacea* trigger metamorphosis in larvae of a marine invertebrate upon contact with the sessile bacteria (Shikuma et al. 2014). These systems share some features with the Type VI secretion machinery that delivers inhibitor or effector molecules to fellow prokaryotes or eukaryotic host cells (Durand et al. 2014; Russell et al. 2014) but their genetic make-up and contractile tail structure hint to an evolutionary relatedness to R-type pyocins (Heymann et al. 2013; Sarris et al. 2014). Most strains of the opportunistic human pathogen *P. aeruginosa* have the capacity to release HMW complexes (R- and/or F-type pyocins) with intraspecies bacteriocin activity (Ghequire and De Mot 2014). Early studies on the morphology of these killing particles revealed striking similarity with phage tails, which was further substantiated upon identification of the corresponding gene clusters (Nakayama et al. 2000). Similar phage tail-like bacteriocins have been described for several other γ-proteobacterial genera (*Coetzeae* et al. 1968; Thaler et al. 1995; Strauch et al. 2001; Jabrane et al. 2002; Gaudriault et al. 2004; Smarda and Benada 2005; Yamada et al. 2006) and some *Firmicutes* (Zink et al. 1995; Gebhart et al. 2012).

Following functional and genetic characterization of R-type tailocins in two *P. putida* strains, comparative analysis of pseudomonad genomes revealed that this type of tailocin and related prophage clusters are equally abundant in species other than *P. aeruginosa*. The phylogeny of the tailocin structural proteins in pseudomonads reveals an unexpected dichotomy with two main clades: One including the tailocins from the *P. putida* strains, related to those of *P. aeruginosa*, and a second distant clade represented mainly by *P. fluorescens* strains. For neither of these groups, particularly close similarity is apparent to the corresponding tail proteins of the HMW bacteriocins from other γ-proteobacteria. In addition, our comparative analysis advocates for a reappraisal of the current view, stemming from the work of Nakayama et al. (1999, 2000) about the shared ancestry of R-type pyocins with the P2-related pseudomonad phage *ΦCTX*. Although the P2/ΦCTX-relatedness is pronounced for photorhabdigin, the *P. aeruginosa* R pyocins appear more closely related to *P. aeruginosa* phage PS17. Production of this type of R pyocin is apparently not confined to the pathogen *P. aeruginosa* as quite similar gene clusters are found at the same genomic location (*trpE–trpG* intergenic region) in the genomes of other species such as *P. knackmussii* and *P. nitroreducens*. A large number of pseudomonad prophages encode structural proteins with homology to the tailocin sequences from the *P. aeruginosa/P. putida* mixed clade. For the *P. fluorescens* group of tailocins, on the other hand, no such close prophage relatives can be pinpointed. Their nearest structural protein neighbors are found in prophages that are maintained as linear plasmid-like entities in γ-proteobacterial hosts (*Halomonas, Vibrio*), which suggests a different evolutionary origin and route of acquisition compared with the tailocins of the *P. aeruginosa* and *P. putida* complexes. Based on these observations, we propose a new phylogeny-based classification of these particles in pseudomonads (fig. 4): Tailocins Rp1, Rp2, and Rp3, with “R” referring to the type, “p” referring to pseudomonads, and the number specifying the “species complex” of origin (with *P. aeruginosa*, *P. putida*, and *P. fluorescens*, respectively, as main representative species). Rp1 tailocins consistently differ from Rp2 and Rp3 tailocins with respect to genomic location, regulatory module, and lysis cassette. Possibly, adaptation to specific niches, for instance, by a pathogen such as *P. aeruginosa* to a human host environment or a saprophyte such as *P. fluorescens* or *P. putida* to a plant root environment, has driven such differences in tailocin domestication (Touchon et al. 2014).

Compared with the limited number of different tail fiber proteins found among *P. aeruginosa* R-type pyocins, the sequences of these tailocin proteins have diverged extensively among other pseudomonad species. Based on domain architecture, the subtypes a (short), b (“long” with only DUF3751), and c (long with DUF3751 and PF07484) are distinguished, which can be further differentiated by AA sequence and length (supplementary table S3, Supplementary Material online). This can be useful to subdivide the Rp types: For instance, *P. putida* BW11M1 and RW1052 produce Rp2b and Rp2c tailocins, respectively, whereas *Pseudomonas vanobens* DSM 16006 is designated a potential Rp2a producer; tailocins from *P. fluorescens* AS06, F113, and SF4c would be typed Rp3a, Rp3b, and Rp3c, respectively; *P. aeruginosa*, *P. knackmussii*, and *P. nitroreducens* share the same type of tailocin (Rp1b). The huge diversity among tail fiber proteins suggests that a particular tailocin will probably be able to kill only a confined subset of other pseudomonad strains and
hence typically display a narrow spectrum, as observed for the HMW bacteriocins from *P. putida* BW11M1 and RW1052.

Recently, a fourth type of R-type tailocin (Rp4), syringacin of the phytopathogen *P. syringae* pv. *syringae* B728a, was functionally characterized. Based on comparative analysis of baseplate and tail sheath proteins, this tailocin is only distantly related to the Rp1–Rt2–Rt3 clusters, in line with its striking genomic synteny to Stf (Hockett et al. 2015). Stf is a member of a group of temperate serotype-converting Mu-like Shigella flexneri phages within the Myoviridae (Grose and Casjens 2014). Hence, this study lends further support to our differential ancestry hypothesis for pseudomonad tailocins. Despite an apparently quite different route of tail gene domestication, the Rp4 tailocin has adopted a similar lysis cassette, including the “Russian doll” organization of spanin genes (although rzi1 is not yet annotated in strain B728a). *Pseudomonas syringae* houses the R-type syringacin cluster at the same genomic position as where *P. aeruginosa* harbors its tailocins (between *trpE* and *trpG*). It also carries an Rp1-type regulatory module (*rztE*–*rztA*, equivalent of *ptrR*–*ptrN*) but lacks the accessory *ptrB* gene that coordinates pyocin production and expression of Type III secretion in *P. aeruginosa* (Wu and Jin 2005). However, in strains of other species (such as *Pseudomonas brassicaeaeurn* NFM421, *P. fluorescens* Q8r1–96, *Pseudomonas mandelii* JR-1, and *P. protegens* Pf–5) only a PtBA-like regulatory gene is present in their Rp4-like cluster that is integrated between *mutS* and *cinA*, features which are typically associated with the Rp2 and Rp3 tailocins.

In several *P. aeruginosa* strains the R1 gene cluster is linked to a F-type tailocin, both relying for expression and release on a common set of regulatory and lysis genes (Ghequire and De Mot 2014). Inspection of genomic regions with one of the three other R-type tailocins has revealed that, in addition to clusters encoding a single tailocin, multiple combinations of them exist (some including a tailocin of the F-type) in which two or three tailocins are neatly fused between the regulatory and lytic genes, suggesting a capacity for concomitant production of two or three different tailocins (Ghequire and De Mot 2015). The presence in the *mutS–cinA* intergenic region of a prophage adjacent to a tailocin, each with their own regulatory and lytic modules, as observed in *P. putida* BW11M1, apparently also occurred in some other strains. In these combinations tail regions of prophage and tailocin(s) are not necessarily of the same type, which indicates that these mobile elements can be co-opted independently.

Several plant-associated pseudomonad strains have recruited additional genes to the external regions of the tailocin gene clusters, leaving the tail-assembly module intact. These acquired genes may contribute to the ecological fitness and competitiveness of their carrier bacteria. Such function in interference competition is likely for the Rp2-embedded lectin-like bacteriocin genes of *P. putida* BW11M1. Apparently, one of two lectin-like bacteriocins (LipA1; Parret et al. 2005) and a secreted chitinase (glycoside hydrolase family 18; PDB 4Q6T) are actually encoded by cargo genes of an Rp4-like tailocin cluster present in *P. protegens* Pf–5. The bacteriocin-within-tailocin configuration is equally present in the *P. fluorescens* Q8r1–96 genome, where this strain’s colicin M-like lipid II-degrading bacteriocin (Barreteau et al. 2012) is also encoded by an Rp4-type tailocin cargo gene. Such Rp4-associated colicin M-like genes are found in other isolates as well ( *P. syringae* 642 and *P. brassicaeaeurn* NFM421). The Rp4 cargo gene *rztE* of *P. syringae* B728a also encodes a putative glycoside hydrolase (family 5; Pfam PF00150). Our observation that some proteins encoded by cargo genes embedded within tailocin gene clusters may remain physically associated with mature tailocin particles requires further scrutiny to assess whether such hitchhiking is a more general phenomenon and is of biological relevance.
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