Characterization of Cicho-peptins, New Phytotoxic Cyclic Lipodepsipeptides Produced by *Pseudomonas cichorii* SF1-54 and Their Role in Bacterial Midrib Rot Disease of Lettuce

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The lettuce midrib rot pathogen *Pseudomonas cichorii* SF1-54 produces seven bioactive compounds with biosurfactant properties. Two compounds exhibited necrosis-inducing activity on chicory leaves. The structure of the two phytotoxic compounds, named cicho-peptin A and B, was tentatively characterized. They are related cyclic lipopeptides composed of an unsaturated C12-fatty acid chain linked to the N-terminus of a 22-amino acid peptide moiety. Cicho-peptin B differs from cicho-peptin A only in the last C-terminal amino acid residue, which is probably Val instead of Leu/Ile. Based on peptide sequence similarity, cicho-peptins are new cyclic lipopeptides related to corpeptin, produced by the tomato pathogen *Pseudomonas corrugata*. Production of cicho-peptin is stimulated by glycine betaine but not by choline, an upstream precursor of glycine betaine. Furthermore, a gene cluster encoding cicho-peptin synthetases, *cipABCDEF*, is responsible for cicho-peptin biosynthesis. A *cipA*-deletion mutant exhibited significantly less virulence and rotten midribs than the parental strain upon spray inoculation on lettuce. However, the parental and mutant strains multiplied in lettuce leaves at a similar rate. These results demonstrate that cicho-peptins contribute to virulence of *P. cichorii* SF1-54 on lettuce.

Some plant pathogenic *Pseudomonas* spp., such as *P. syringae* pv. *syringae*, *P. fuscovaginae*, and *P. corrugata*, produce necrosis-inducing lipopeptide phytotoxins (Bender et al. 1999; Catara 2007; Flamand et al. 1996) that belong to either the syringomycin group or the phytotoxic lipodepsipeptide group, including tolaasin and syringopeptin-like compounds (Gross and Loper 2009; Raaijmakers et al.

2006). The members of the syringomycin group are cyclic depsinonapeptides attached to a β -hydroxy fatty acid tail. Tolaasin and syringopeptin-like compounds are cyclic lipodepsipeptides with a longer and more hydrophobic peptide chain and a higher molecular weight (MW) (Gross and Loper 2009). These two groups of cyclic lipopeptides (CLP) cause cellular lysis by formation of transmembrane pores in the plasma membranes of host cells that lead to disruption of the membrane electrical potential (Brodey et al. 1991; Coraiola et al. 2008; Hutchison et al. 1995). Although these CLP can induce necrosis on plants, phytotoxic CLP contribute to virulence but are not essential for pathogenicity of plant pathogenic pseudomonads (Bender et al. 1999; Scholz-Schroeder et al. 2001).

Biosynthesis of CLP is accomplished via nonribosomal biosynthetic pathways through large multienzyme complexes, nonribosomal peptide synthetases (NRPS), which are encoded by large gene clusters. NRPS have a modular structure in which each module is responsible for the incorporation of one amino acid in the peptide chain. A module consists of a minimal set of three domains: an adenylation domain, a thiolation domain or peptidyl carrier protein domain (PCP), and a condensation domain (Bender et al. 1999; Raaijmakers et al. 2010). Based on the specificity code in the sequence of the A domains of the NRPS modules, the amino acid sequence of the synthesized CLP can be predicted in silico (Challis et al. 2000; de Bruijn et al. 2007; Gross et al. 2007; Stachelhaus et al. 1999).

Pseudomonas cichorii is an important plant-pathogenic bacterium, with a broad host range and a worldwide distribution. *P. cichorii* causes necrotic leaf and stem lesions on many economically important hosts, including lettuce, celery, chrysanthemum, tomato, coffee, and soybean (Bradbury 1986; Jagger 1914; Jones et al. 1984; Wilkie and Dye 1974; Yu and Lee 2012). In Belgium, *P. cichorii* causes midrib rot disease of greenhouse-grown butterhead lettuces (Cottyn et al. 2009, 2011; Pauwelyn et al. 2011). Symptom development on lettuce leaves is strongly associated with apoptosis-like programmed cell death (Kiba et al. 2006, 2009). Intriguingly, the type three secretion system is essential for pathogenicity of *P. cichorii* on eggplant but not on lettuce (Hojo et al. 2008; Kajihara et al. 2012). Production of phytotoxic substances by *P. cichorii* has been described (Hu et al. 1998; Lazzaroni et al. 2003; Pauwelyn

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et al. 2013; Shirakawa and Ozaki 1993), but their role in pathogenicity of *P. cichorii* on lettuce is not clear.

P. cichorii SF1-54, which was isolated from diseased butthead lettuce in Belgium, can produce seven bioactive compounds (named A to G) with biosurfactant properties (Pauwelyn et al. 2013). These bioactive compounds differ in inhibition spectra against different microorganisms and in the ability to cause necrosis on chicory leaves (Supplementary Table S1). Compounds D and E, which have been well-identified, characterized, and renamed cichofactins A and B, are new linear lipopeptides of the syringafactin family and are involved in virulence and surface motility, while compounds B and C appear to be related to pseudomycins (Pauwelyn et al. 2013). Compounds F and G are phytotoxic molecules and induce necrosis on chicory leaves. Moreover, they have antimicrobial activity against *Bacillus megaterium* but not against *Rhodotorula mucilaginosa*. In this study, we attempted to elucidate the structure of the phytotoxic compounds F and G produced by *P. cichorii* SF1-54. A dual approach, which combined chemical characterization by high performance liquid chromatography-electrospray ionization-mass spectrometry (HPLC-ESI-MS) and mining the NRPS-encoding genes in the SF1-54 genome, was used. Furthermore, a mutant deficient in production of cicho-peptides was constructed to study the involvement of the phytotoxins in virulence of *P. cichorii* SF1-54. Our results demonstrate that the phytotoxic CLP are important virulence factors for *P. cichorii* SF1-54 on lettuce. Interestingly, we also found that production of phytotoxic CLP by *P. cichorii* SF1-54 is linked to glycine betaine metabolism.

RESULTS

Chemical analysis of phytotoxic lipopeptides produced by *P. cichorii* SF1-54 (cicho-peptides).

Compounds F and G were purified by reverse phase (RP)-HPLC. Based on their phytotoxicity and antimicrobial activity against *B. megaterium* but not against *R. mucilaginosa*, compounds F and G were expected to be new phytotoxic lipopeptides because corpeptin and syringopeptin have similar properties (Emanuele et al. 1998; Lavermicocca et al. 1997).

To structurally characterize the phytotoxic metabolites, HPLC-purified compounds F and G were first submitted to total acid hydrolysis and subsequent HPLC separation of derivatized amino acids. With this methodology, it was not possible to establish a precise quantification of the various amino acids, but 10 different residues with high relative amounts of Ala and Val, more than one Gly, one residue of Ser, Thr, Leu, and Ile, as well as three unidentified amino acids were detected in the purified sample of the molecule. These first results confirm the peptidic nature of compounds F and G, which were further purified by HPLC-ESI-MS and characterized by ESI-tandem MS (MS/MS). The mass spectrum of purified extract of compounds F and G showed two major peaks at m/z 1,026.1 and 1,033.1, both doubly charged protonated forms ($[M+2H]^{2+}$), like syringopeptins (Grgurina et al. 2005; Monti et al. 2001). Based on the m/z of the doubly charged protonated ions, the MW of compounds F and G were calculated as 2,052.4 and 2,066.2 (exact mass measurements), respectively. Further characterization of the two molecules was obtained by selecting each ion and fragmenting it with argon. Identification of b and y⁺ ions reveals very similar fragmentation patterns of the $[M+H]^+$ ions corresponding to compounds F and G (Supplementary Fig. S1; Supplementary Table S2).

The first b ion fragments of compounds F and G were identical and compatible with 2,3-dehydroaminobutyric acid (dhAbu) and a fatty acid moiety that is expected to be a C_{12:1}-OH residue, upon calculation. Thus, the MW difference of 14

between compounds F (MW 2,052.4) and G (MW 2,066.2) can be attributed to a difference in the peptide chain rather than in the fatty acid chain. The first 12 b ions were the same in the fragmentation spectra of compounds F and G, indicating a structural identity of the N-terminal part of both molecules, which includes the fatty acid moiety linked to at least 12 amino acid residues. Moreover, the masses of the y⁺ ion fragments obtained from compound G are always 14 Da higher than those of compound F. This difference was maintained until the smallest y⁺ ions that could be detected, at m/z 459.26 for compound F and at m/z 473.27 for compound G. This indicated that the amino acid substitution explaining the 14-Da difference between compounds F and G should occur within the first five residues (discussed below) at the C-terminal end. Further fragmentation of this part of the molecules was not obtained, most probably due to the fact that those residues are involved in the formation of an internal cycle, as occurs in many CLP (Raaijmakers et al. 2006; 2010). Nevertheless, based on those ESI-MS fragmentation data, the putative amino acid sequence of the first 17 amino acids for both compounds F and G could be determined as dhAbu-Pro-Ala-Ala-Ala-Ala-Val-dhAbu-Gly-Val-Ile-Gly-Ala-Val-Ala-Val-dhAbu. Additionally, according to the spectrophotometric characteristics of both compounds (UV-visible spectrum obtained in diode array-coupled HPLC analysis [data not shown]), no aromatic amino acids are present in the peptide backbone. The proposed peptide structure of the fatty acid chain and the first 17 amino acids of the peptide chain, with their site of cleavage yielding b- and y⁺-type ions, are presented in Supplementary Fig. S2.

The complete peptide sequences of the two lipopeptides F and G could not be fully elucidated from these MS/MS analyses due to the high stability of the cyclic substructure involving the last five amino-acids. Multiple attempts to open the ring by chemical breakdown of the lactone bond (KOH 2M at 37°C for up to 4 h, triethylamine 14.28%, pH = 9, 37°C, 4 h or room temperature overnight) were not successful.

However, based on molecular weights, on amino acid composition, and on previously reported structures of *Pseudomonas* lipopeptides, the remaining part at the C-terminal region of compound G could be composed of five amino acid residues, which could be Ala, Leu (or Ile), Ser, αThr, and an unknown amino acid. This suggests compounds F and G are structural relatives of corpeptins (Emanuele et al. 1998). Therefore, we renamed compounds G and F cicho-peptides A and B, respectively, and next, used an in silico approach to determine full peptide sequences.

Sequence analysis of cicho-peptin synthetase-encoding genes.

Since genes responsible for biosynthesis of phytotoxic CLP in *P. cichorii* have not been reported, we performed genome mining based on the genes of syringopeptin synthetases (Feil et al. 2005; Scholz-Schroeder et al. 2003). First, the genome sequence of *P. cichorii* SF1-54 (Pauwelyn et al. 2013) was compared with the genomes of *P. syringae* pv. *syringae* B728a and *P. syringae* pv. *tomato* DC3000 (Buell et al. 2003; Feil et al. 2005), using the MAUVE program (Darling et al. 2010). Then, BlastN searches using the syringopeptin synthetase gene cluster of *P. syringae* pv. *syringae* B301D (*sypA*, *sypB*, and *sypC*: AF286216.2) (Scholz-Schroeder et al. 2003) as a query against the SF1-54 genome were performed. This resulted in the identification of a large NRPS gene cluster, spanning a region of 73,057 bp in the *P. cichorii* SF1-54 genome. The sequence, including the NRPS gene cluster and flanking regions, has been submitted to the GenBank database under accession number KJ513094. The structural organization of this NRPS gene cluster and its flanking genes is represented in Figure 1.

Six NRPS-encoding open reading frames (ORF) were identified in this gene cluster by the program Glimmer (Delcher et al. 1999). They are referred to as *cipA*, *cipB*, *cipC*, *cipD*, *cipE*, and *cipF*. These six genes, which are 14.71, 9.64, 4.62, 13.01, 9.42, and 21.53 kb in size, respectively, encode NRPS modules with specific condensation (C), adenylation (A), and thiolation (T) domains (Fig. 1). These genes constitute, together, 22 NRPS modules, which confirms that the lipopeptide produced by *P. cichorii* SF1-54 is composed of 22 amino acids. Hence, the results of sequence analysis strongly suggest that the *cip* gene cluster is responsible for cichoheptin biosynthesis. The *cip* gene cluster exhibits two typical features of NRPS genes responsible for biosynthesis of lipopeptides: the presence of an N-terminal C domain in the first module (C starter) and a tandem of thioesterase domains at the end of the *cip* cluster. Moreover, Blast analysis of cichoheptin synthetases revealed that the recently released genome sequence of *P. cichorii* JBC1 has a very similar gene cluster (PCH70_25300, PCH70_25310, and PCH70_25320, accession number CP007039.1) encoding hypothetical peptide synthetases. At the amino acid sequence level, cichoheptin synthetases showed 99% protein identity with these hypothetical peptide synthetases of *P. cichorii* JBC1.

Analysis of cichoheptin synthetases.

To predict the peptide sequence of cichoheptins from genome sequencing data, bioinformatics analysis of the A domain of each module of cichoheptin synthetases was performed. The putative peptide sequence of cichoheptins is Thr/dhAbu-Pro-Ala-Ala-Ala-Ala-Val-Thr/dhAbu-Gly-Val-Ile-Gly-Ala-Val-Ala-Val-Thr/dhAbu-Thr/dhAbu-Ala-Dab-Ser-Leu/Ile/Val (Supplementary Table S3). According to the biochemically determined peptide sequence of cichoheptins, the A domains of the first, eighth, and 17th modules are responsible for specific recognition of dhAbu, and the 18th module recognizes α Thr. The prediction is in full agreement with the peptide sequence determined by MS. Furthermore, it confirms that the unidentified C-terminal region of cichoheptins consists of five amino acid residues. Interestingly, the last module, CipF, is predicted to recognize three amino acids, Leu, Ile, and Val. The

difference in molecular weight between Leu/Ile and Val is 14, and that is exactly in agreement with the molecular weight difference between cichoheptin A and B. The peptide sequences of the last five amino acid residues are predicted to be α Thr-Ala-Dab-Ser-Leu/Ile (cichoheptin A) and α Thr-Ala-Dab-Ser-Val (cichoheptin B). The calculated masses of the C-terminal fragments of cichoheptins A and B are 18 Da higher than the masses determined by HPLC-ESI-MS, indicating that one water molecule is lost for cyclization, confirming that cichoheptins A and B are CLP with a C-terminal pentapeptide ring.

In *Pseudomonas* spp., dual condensation and epimerization (C/E) domains are responsible for generating D-amino acids into lipopeptides (Balibar et al. 2005; Rausch et al. 2007). As Gly and dhAbu are nonchiral monomers, the D/L configuration of other amino acid residues of cichoheptins was investigated by detection of specific signatures via weblogs within DownSeq of each C domain (Caradec et al. 2014). According to the signatures, the C domains of modules 3-8, 11-12, 14-17, and 22 are identified as dual C/E domains. Therefore, the following structure of cichoheptins A and B is proposed: dhAbu_D-Pro_D-Ala_D-Ala_D-Ala_D-Ala_D-Val_-dhAbu_Gly_D-Val_D-Ile_Gly_D-Ala_D-Val_D-Ala_D-Val_dhAbu_D- α Thr_L-Ala_L-Dab_D-Ser_L-Leu/Ile and dhAbu_D-Pro_D-Ala_D-Ala_D-Ala_D-Ala_D-Val_dhAbu_Gly_D-Val_D-Ile_Gly_D-Ala_D-Val_D-Ala_D-Val_dhAbu_D- α Thr_L-Ala_L-Dab_D-Ser_L-Val, respectively. The locations of dual C/E domains of cichoheptin synthetases are identical to those of syringopeptin synthetases, according to in silico analysis. Furthermore, the specific signature detection confirms that the first C domain is a C starter allowing the condensation with the lipidic moiety.

Analysis of the *cip* flanking sequences.

Immediately downstream of the *cip* gene cluster encoding the cichoheptin synthetases, an ABC transporter system (99% protein identity with *P. cichorii* JBC) as well as a LuxR transcriptional regulator (100 and 57% protein identity with *P. cichorii* JBC1 and *P. syringae* pv. *japonica* M301072PT, respectively) have been identified in the genome of *P. cichorii* SF1-54 (Fig. 1). The peptide sequences of the two ABC

Fig. 1. Schematic representation of the gene cluster involved in biosynthesis of cichoheptin and the surrounding open reading frames in the *Pseudomonas cichorii* SF1-54 genome. The module and domain organization is indicated below the *cipA*, *cipB*, *cipC*, *cipD*, *cipE*, and *cipF* genes. Predicted amino acid specificity is presented below each module and all amino acids are identified by standard three-letter biochemical notation. Dab = 2,4-diaminobutyric acid; dhAbu = 2,3-dehydroaminobutyric acid. The region between arrowheads was deleted in the cichoheptin-deficient mutant.

transporter components showed 72% protein identity with the macrolide efflux protein encoded by *macA* of the orfamide gene cluster of *Pseudomonas fluorescens* Pf-5 (PFL_2148) and 81% protein identity with the macrolide efflux protein encoded by *macB* of the syringopeptin gene cluster of *P. syringae* pv. *syringae* B728a (Pysr_2618), respectively. Upstream of the cicho-peptin synthetase genes, three genes are present that encode proteins exhibiting amino acid homology of 100% to proteins encoded by PCH70_25250 and PCH70_25260 and of 99% to PCH70_25270-encoded protein of *P. cichorii* JBC1. These three proteins also showed homology of 65% to PseA, 60% to PseB, and 72% to PseC. The PseABC efflux system is a tripartite resistance-nodulation-cell division (RND) transporter system, identified at the left border of the *sy-syp* genomic island of *P. syringae* pv. *syringae* B301D, with an important role in secretion of syringomycin and syringopeptin (Kang and Gross 2005). The gene located between the cicho-peptin biosynthesis genes and the genes encoding the PseABC efflux system shows 56% amino acid homology with a putative membrane protein of *P. corrugata*.

Cicho-peptin production is stimulated by glycine betaine.

Culture filtrate obtained from still SRM_{AF} medium (Mo and Gross 1991) showed clear phytotoxicity and contained cicho-peptin concentrations of 21 ± 16 µg/liter based on calculated data from nine different cultures. Culture filtrates obtained from shaken cultures did not show phytotoxicity. Production of cicho-peptins was much lower than that of cichofactins (Pauwelyn et al. 2013) and was easily influenced by culture conditions, revealing that SRM_{AF} medium is not optimal for *P. cichorii* to produce cicho-peptins. Moreover, we were intrigued by the fact that cicho-peptins contain two molecules of glycine and that such high content in glycine is not common in *Pseudomonas* lipopeptides (Gross and Loper 2009; Raaijmakers et al. 2006).

Glycine is a downstream product of choline metabolism (Supplementary Fig. S3). Choline and glycine betaine are potentially abundant in plant hosts including lettuce (Chen et al. 2013; de Zwart et al. 2003; Zeisel et al. 2003). Genes involved in choline uptake and metabolism are present in the genome of *P. cichorii* SF1-54 (Supplementary Table S4). Further analysis revealed that *P. cichorii* SF1-54NaI^R can utilize choline and its breakdown product glycine betaine but not glycine as the sole carbon source supplied to minimal medium under well-aerated conditions (Supplementary Fig. S4A). We further tested the effect of choline and glycine betaine on *P. cichorii* SF1-54NaI^R growth in SRM_{AF} medium under static culture conditions, which were used for lipopeptide production. Various concentrations of choline and glycine betaine were tested, but only glycine betaine could be utilized by *P. cichorii* under static culture conditions in SRM_{AF} medium. Among all conditions tested, *P. cichorii* exhibited the best growth in SRM_{AF} medium containing 10 mM glycine betaine until 72 h of incubation. In contrast, addition of choline caused an inhibitory effect on *P. cichorii* growth under static culture conditions. Thus, we decided to examine the effect of glycine betaine on cicho-peptin production by *P. cichorii* SF1-54NaI^R. Supplementation of glycine betaine at a final concentration of 10 mM in SRM_{AF} medium greatly enhanced cicho-peptin production by *P. cichorii* SF1-54NaI^R (1.3 and 3.0 fold increases for cicho-peptins A and B, respectively) (Fig. 2A to C). Production of those lipopeptides also reached similar levels by supplementation of glycine betaine at a higher (1% or 85 mM) concentration (Supplementary Fig. S5). Addition of 1 mM glycine betaine could not stimulate cicho-peptin production (Fig. 2A to C). In addition, supplementation of glycine betaine also enhanced production of cichofactins and compound A but not the

production of the pseudomycin-like compounds B and C (Fig. 2A).

The filtrate obtained from *P. cichorii* SF1-54NaI^R culture grown in SRM_{AF} medium in the presence of 10 mM glycine betaine induced the strongest necrosis on chicory leaves, among all treatments (Figs. 2D to I). The results are in accordance with the results of cicho-peptin production determined by HPLC-ESI-MS analysis (Figs. 2A to C).

In planta fate of cicho-peptins produced by *P. cichorii* SF1-54.

Lettuce tissue inoculated with *P. cichorii* SF1-54 was analyzed using HPLC-ESI-MS to determine lipopeptide production of *P. cichorii* SF1-54 in planta. Cicho-peptins were detected at 24 h postinfiltration in amounts of 4.1 ± 1.5 ng per gram of leaf fresh weight, while at 48 h postinoculation, no cicho-peptin was detected in infected lettuce tissue. In addition to cicho-peptins, cichofactins, which are involved in swarming motility of *P. cichorii*, were detected at each time point. In addition, no trace of compounds A, B, and C corresponding to other lipopeptides could be detected in infected lettuce tissue (Fig. 3).

Subsequently, we infiltrated lettuce leaves with a culture filtrate of *P. cichorii* SF1-54 to further study the distribution and stability of lipopeptides in lettuce tissue. The concentration of cicho-peptins gradually decreased from 4.4 ± 1.9 to 3.5 ± 0.1 ng per gram of leaf fresh weight over 48 h after infiltration, while the concentration of cichofactins slightly reduced from $6.4 \times 10^7 \pm 0.4 \times 10^7$ to $5.7 \times 10^7 \pm 0.7 \times 10^7$ peak area per gram of leaf fresh weight during the same time course.

The biological role of cicho-peptins produced by *P. cichorii* SF1-54.

To investigate the biological role of cicho-peptins and their importance in pathogenicity for *P. cichorii* SF1-54, we constructed a cicho-peptin-deficient mutant, SF1-54- Δ *cipA*, by deleting an 8,071-bp fragment in the cicho-peptin synthetase A-encoding gene *cipA* (Fig. 1). Cells of both SF1-54NaI^R and mutant SF1-54- Δ *cipA*, suspended into a droplet of water on parafilm, caused immediate drop collapse (data not shown), indicating that the mutant still had biosurfactant activity. HPLC-ESI-MS analysis confirmed that SF1-54- Δ *cipA* no longer produced cicho-peptins (compounds F and G in the chromatogram) but that deletion of the cicho-peptin biosynthesis gene did not influence the production of cichofactins A and B (i.e., compounds D and E in the chromatogram, which are no longer produced by the cichofactin mutant SF1-54- Δ *cifAB*), pseudomycin-like compounds B and C, and uncharacterized compound A (Fig. 4A).

As shown in Figure 4B, the culture filtrate of SF1-54- Δ *cipA* still caused discoloration of chicory tissue, but the symptoms were somewhat different from the necrosis induced by the culture filtrates of SF1-54NaI^R and SF1-54- Δ *cifAB*. Furthermore, induction of discoloration and necrosis on chicory leaves by injection with a cell suspension of SF1-54- Δ *cipA* was reduced in comparison with the leaves inoculated with SF1-54NaI^R and the cichofactin-deficient mutant SF1-54- Δ *cifAB* (Fig. 4C).

Moreover, SF1-54- Δ *cipA* exhibited significantly smaller inhibition zones against *B. megaterium* than the parental strain and SF1-54- Δ *cifAB* (Fig. 5A). Compared with the parental strain, growth inhibition of *B. megaterium* by SF1-54- Δ *cipA* was reduced by 45%. The parental strain and mutant did not differ in inhibitory activity against *Geotrichum candidum* and *R. mucilaginosa*.

P. cichorii SF1-54NaI^R swarmed outwards from the inoculation point to cover almost the entire plate containing soft Luria Bertani (LB) medium (0.5% agar) within 24 h. Swarming

behavior of the cichopectin-deficient mutant was identical to the parental strain, while the cichofactin mutant completely lost this ability, indicating that cichofactins but not cichopectins contribute to swarming activity of *P. cichorii* SF1-54 (Fig. 5B and C).

When lettuce leaves were infiltrated with strains SF1-54NaI^R or SF1-54Δ*cifAB*, cell collapse was observed in almost all of the infiltrated areas at 1 day postinoculation (Supplementary Fig. S6). When lettuce leaves were infiltrated with SF1-54Δ*cipA*,

Fig. 2. Effect of glycine betaine (betaine) on cichopectin production by *Pseudomonas cichorii*. *P. cichorii* SF1-54NaI^R was cultured in SRMAF medium with or without betaine (1 or 10 mM) and the culture filtrates were analyzed by reverse phase ultra-performance liquid chromatography coupled with electrospray ionization mass spectrometry (UPLC-ESI-MS) and by phytotoxicity assay. **A**, Chromatogram of semipurified extracts of SF1-54NaI^R. **B**, Extracted ion chromatogram of semipurified extracts of SF1-54NaI^R, using masses of the most abundant ions of compounds F and G (at *m/z* 1,027 and *m/z* 1,034, respectively). The y axes of liquid chromatography-mass spectrometry traces are linked at the same scale for comparison of lipopeptide production. Compounds F and G are cichopectins B and A, respectively. Two peaks labeled F at different retention times correspond to compounds with the same mass but which probably slightly differ in the position or stereoisomery of some amino acids, resulting in distinct chromatographical behaviors. **C**, Quantification of relative production of cichopectins by SF1-54NaI^R based on UPLC-ESI-MS data. Values represent the mean $\pm$ standard deviation of three independent cultures. Bars indicated with the asterisk are significantly different, based on Fisher's least significant difference test ($P < 0.05$). **D** to **I**, Assays of phytotoxicity of SF1-54NaI^R culture filtrates on witloof chicory leaves. Chicory leaves were inoculated with **D**, SRMAF medium; **E**, crude culture filtrate of SF1-54NaI^R grown in SRMAF medium; **F**, SRMAF medium + 10 mM betaine; **G**, crude culture filtrate of SF1-54NaI^R grown in SRMAF medium supplemented with 10 mM betaine; **H**, SRMAF medium + 1 mM betaine; **I**, crude culture filtrate of SF1-54NaI^R grown in SRMAF medium supplemented with 1 mM betaine. Photographs were taken at 2 days posttreatment.

Fig. 3. Liquid chromatography electrospray ionization mass spectrometry detection of *Pseudomonas cichorii* SF1-54 lipopeptides in **A**, semipurified extract of *P. cichorii* SF1-54NaI^R culture filtrate, **B**, extract of lettuce tissue infected with *P. cichorii* SF1-54NaI^R, and **C**, extract of mock-inoculated lettuce tissue. Lipopeptides were extracted at 24 h postinoculation and were analyzed. The y axes of liquid chromatography-mass spectrometry traces are linked at the same scale. Detected lipopeptides in the chromatograms are indicated with a letter. D and E, cichofactins. F and G, cichopeptins. Peaks detected in panel C do not correspond to lipopeptides produced by *P. cichorii*.

Fig. 4. **A**, Chromatograms of the semipurified extracts of *Pseudomonas cichorii* SF1-54NaI^R and its lipopeptide-deficient mutants. Seven bioactive compounds in the semipurified culture extracts were identified by electrospray ionization mass spectrometry analysis. Seven bioactive compounds are indicated with a letter. D and E, cichofactins; F and G, cichopeptins. In addition to the various forms of cichopeptin B, two cichopeptin A forms (peaks labeled G) were also detected at different retention times. They correspond to compounds differing by +1 mu for the doubly charged molecular ion (+2 mu for b fragment ions) (data not shown) which is most probably explained by a saturated form of the acyl chain in the minor form. The y axes of liquid chromatography-mass spectrometry traces are linked at the same scale for comparison of lipopeptide production. **B and C**, Symptoms caused by *Pseudomonas cichorii* SF1-54NaI^R and its lipopeptide-deficient mutants on chicory. **B**, Chicory leaves were inoculated with bacterial culture filtrates or **C**, bacterial suspensions. Photographs were taken at 1 (**B**) and 2 days postinoculation (**C**). Each treatment has been repeated three times and a picture representative of each of the treatments is shown.

symptom development was significantly slower than in the other two treatments ($P < 0.05$). The cichoheptin-deficient strains mainly caused brown necrosis on lettuce leaves 1 day postinoculation, while cell collapse was clearly observed in the infiltrated areas 2 days postinoculation.

To investigate the effect of cichoheptins on the virulence of *P. cichorii* SF1-54, greenhouse-grown butterhead lettuces were spray-inoculated with *P. cichorii* SF1-54Nal^R and its cichoheptin-deficient mutant in greenhouse experiments. The cichofactin mutant strain SF1-54- Δ cifAB was also included. Lettuce plants inoculated with the cichoheptin-deficient mutant SF1-54- Δ cipA exhibited a significantly lower number of rotten midribs than lettuce plants inoculated with the parental strain (Fig. 6A). However, plants inoculated with the cichoheptin mutant SF1-54- Δ cipA or the cichofactin mutant SF1-54- Δ cifAB did not differ in number of rotten midribs. When lettuce midribs were inoculated with SF1-54Nal^R and its lipopeptide-deficient mutants, all strains grew vigorously and reached similar population densities (approximately 1×10^8 CFU/cm²) in the inoculated midribs at 1, 2, and 3 days postinoculation (Fig. 6B).

DISCUSSION

Structure and bioactivity of cichoheptins.

Cichoheptin A and B are two structurally related cyclic lipopeptides composed of an unsaturated C12-fatty acid chain

linked to the N-terminus of a 22-amino acid peptide moiety. The difference between cichoheptins A and B occurs in the last amino acid residue, which is predicted to be Leu/Ile and Val for cichoheptins A and B, respectively. Because the production of cichoheptin B was lower than that of cichoheptin A when *P. cichorii* SF1-54 was cultured in SRM_{AF} medium, it is suggested that the A domain of the last module of CipF protein recognizes Leu/Ile more specifically or frequently than Val during the synthesis of cichoheptins. Like other phytotoxic CLP (Gross and Loper 2009; Raaijmakers et al. 2006), cichoheptin contains several noncanonical amino acid residues, Dab and dhAbu, one α Thr, and several Ala and Val (Fig. 7). Cichoheptins are structurally related to corpeptin B (Emanuele et al. 1998). The C-terminal pentapeptide rings of cichoheptins and corpeptin B are almost identical, but cichoheptins contain Ala₆, Gly₉, Gly₁₂, and Val₁₄ instead of Val₆, Hse₉, dhAbu₁₂, and Ala₁₄ in corpeptin B (Fig. 7). The fatty acid chain of corpeptin B is a *cis*-3-hydroxy-5-dodecenoate (Emanuele et al. 1998). The configuration of the fatty acid chain in cichoheptins is not clear, since we could not elucidate the exact position of the double bond.

Although structurally related, the bioactivities of cichoheptins and corpeptins are slightly different. Cichoheptins and corpeptins show antimicrobial activity against *B. megaterium* but not against *R. mucilaginosa* (Emanuele et al. 1998; Pauwelyn et al. 2013). Syringopeptins also have the same properties

Fig. 5. Phenotypic characterization of lipopeptide-deficient mutants of *Pseudomonas cichorii* SF1-54Nal^R. **A**, Antimicrobial activities of *P. cichorii* SF1-54Nal^R and its mutants against three indicator microorganisms. Values represent the mean $\pm$ standard deviation (SD) of five repetitions. **B**, Swarming activity of *P. cichorii* strains. Values represent the mean $\pm$ SD of two experiments with four repetitions per experiment. **C**, Swarming behavior of *P. cichorii* SF1-54 and its mutants on Luria Bertani soft agar (0.5% [wt/vol]) plates. Bars indicated with asterisks are statistically different based on nonparametric Kruskal-Wallis and Mann-Whitney comparisons ($P < 0.05$).

(Lavermicocca et al. 1997). However, phytotoxic lipopeptides showed different levels of phytotoxicity. Chicory leaves treated with purified cichoheptins A or B exhibited clear necrotic symptoms but no chlorosis was observed (Pauwelyn et al. 2013). Infiltration of crude culture filtrate or semipurified extract of *P. cichorii* SF1-54 in tobacco leaves also caused necrosis without symptoms of chlorosis (data not shown). In contrast, both corpeptins A and B are chlorosis-inducing cyclic lipopeptides and only corpeptin A can cause necrosis of tobacco leaves (Emanuele et al. 1998). Moreover, fuscopeptin and syringopeptin, two other long lipopeptides with internal cyclization at the C-terminal end, can induce necrosis of plant leaves (Ballio et al. 1996; Iacobellis et al. 1992). Thus, it is suggested that necrosis or chlorosis caused by phytotoxic lipopeptides could be related to these structural traits.

Cichoheptin production by *P. cichorii* is stimulated by glycine betaine.

We hypothesized that the choline metabolic pathway could be involved in biosynthesis of cichoheptins, because the peptide backbone of cichoheptin contains two residues of glycine, a downstream product of choline metabolism. The choline

catabolic pathway, which is conserved in three model *Pseudomonas syringae* strains, *P. syringae* pv. *phaseolicola* 1448a, *P. syringae* pv. *syringae* B728a, and *P. syringae* pv. *tomato* DC3000 (Chen and Beattie 2007, 2008; Chen et al. 2013), is also present in *P. cichorii* SF1-54. *P. syringae* can sensitively detect and scavenge choline (the detection limit is 0.1 μ M), which is presumably utilized for nutrition or osmoprotection (Chen and Beattie 2007, 2008; Chen et al. 2013). In *P. syringae* pv. *syringae* B728a, it has been shown that glycine betaine functions both in nutrition and as an intracellular signal modulating compatible solute synthesis under hyperosmotic stress conditions (Li et al. 2013). Although *P. cichorii* can utilize either choline chloride or glycine betaine as the sole carbon source under well-aerated culture conditions, supplementation of choline chloride and glycine betaine showed opposite effects on *P. cichorii* growth under static culture conditions for lipopeptide production. Addition of choline chloride in SRM_{AF} medium showed an inhibitory effect on *P. cichorii* growth when grown statically. In *Escherichia coli* and *Pseudomonas* spp., choline is oxidized to glycine betaine via two enzymes, choline dehydrogenase (BetA) and betaine aldehyde dehydrogenase (BetB). The corresponding *bet* genes require aerobic conditions for their expression (Eshoo 1988; Landfald and Strøm 1986; Wargo 2013) and the growth pattern of static *P. cichorii* cultures in choline-containing SRM_{AF} medium is similar to that of *E. coli* cultured in choline-containing medium under either fermentative or anaerobic conditions (Landfald and Strøm 1986). Furthermore, betaine aldehyde dehydrogenase is an NAD⁺-dependent dehydrogenase (Landfald and Strøm 1986; Velasco-García et al. 2000), suggesting that choline catabolism competes for available NAD⁺ with glycolysis and the tri-carboxylic acid (TCA) cycle. A pronounced competition for NAD⁺ may occur under static culture conditions, which were used for lipopeptide production by *P. cichorii* in this study. A possible competition for NAD⁺ may also explain the repression of *P. cichorii* growth when statically grown in choline-containing SRM_{AF} medium compared with *P. cichorii* growth in SRM_{AF} medium. On the other hand, inability of choline utilization due to catabolite repression seems less likely, because succinate rather than glucose affects choline utilization by *P. aeruginosa* and *P. syringae* via catabolite repression (Diab et al. 2006; Li et al. 2013). Succinate is not a component of SRM_{AF} medium.

Compared with glucose and choline, glycine betaine is a relatively poor carbon source for *P. cichorii* when glycine betaine is the sole carbon source. Surprisingly, however, supplementation of 10 mM glycine betaine significantly enhanced bacterial growth under culture conditions for lipopeptide production and greatly stimulated cichoheptin production by *P. cichorii* SF1-54 (Figs. 2A to C). Thus, cichoheptin formation appears to be linked to the uptake and catabolism of glycine betaine from the environment. Furthermore, the higher production observed for both cichoheptins upon addition of glycine betaine in the culture medium may result from an increased rate of the TCA cycle resulting from glycine betaine metabolism. The influence of the TCA cycle and amino acid production on lipopeptide production has already been suggested by de Bruijn and Raaijmakers (2009). They demonstrated that the addition of some amino acid residues to the medium partially rescued massetolide production and swarming motility in a *clpP* (caseinolytic protease) mutant of strain *P. fluorescens* SS101. It was also suggested that some amino acids may induce NRPS gene transcription (de Bruijn and Raaijmakers 2009), which is also possible for the expression of NRPS-encoding genes in *P. cichorii*.

Cichoheptins were detected in infected lettuce tissue at 24 h postinoculation but not at 48 h postinoculation. In the model

Fig. 6. Effect of cichoheptins on virulence of *Pseudomonas cichorii* SF1-54. **A**, Greenhouse-grown butterhead lettuce plants were inoculated with suspensions of the wild-type or cichoheptin-deficient strains (1×10^6 CFU/ml) after head formation of lettuce. The cichofactin-deficient mutant of *P. cichorii* SF1-54 was also included in the pathogenicity assay. Tap water was used as the uninoculated control. Virulence is represented as mean number of rotten midribs. Bars indicated with the same letter are not statistically different based on nonparametric Kruskal-Wallis and Mann-Whitney comparisons ($P < 0.05$). **B**, Population sizes of *P. cichorii* strains in lettuce. Values represent the mean standard deviation of three independent experiments. No significant difference was observed among population sizes of three *P. cichorii* strains in lettuce.

pathosystem *Arabidopsis thaliana* and *P. syringae* pv. *tomato* DC3000, the level of glycine betaine but not choline was significantly elevated in DC3000-challenged *Arabidopsis* at 12 h postinoculation (Ward et al. 2010), but the biological impact of this accumulation was not clear. We speculate that *P. cichorii* may trigger accumulation of glycine betaine in lettuce leaves during an early stage of infection. Initially produced cicho-peptins (at a relatively low level) may cause membrane disruption by interacting with phospholipid molecules as described for other cyclic lipodepsipeptides (Brodey et al. 1991; Coraiola et al. 2008), resulting in an increased glycine betaine release. The released glycine betaine may be sensed and metabolized by *P. cichorii* for further growth and cicho-peptin production.

Another hypothesis is that glycine betaine may play a regulatory role in *P. cichorii* SF1-54. According to a study about the interaction between *P. syringae* pv. *syringae* B728a and its native host bean, water was more limited in the leaf interior (apoplast) than on the leaf surface (Yu et al. 2013). Water limitation in the apoplastic sites may result from one or both low water content (e.g., rapid evapotranspiration) or from a high concentration of solutes that is caused by nutrient leakage and secretion in the mesophyll (Yu et al. 2013). Moreover, genes involved in uptake and catabolism of glycine betaine and in biosynthesis and transport of phytotoxic CLP syringomycin and syringopeptin in *P. syringae* pv. *syringae* B728a were more strongly induced in apoplastic sites than in epiphytic sites (Yu et al. 2013). Addition of 10 mM glycine betaine to SRM_{AF} medium may result in a relatively high-osmolality environment, mimicking apoplastic conditions. Further study is necessary to verify whether cicho-peptin production is stimulated by increasing osmolality.

In situ persistence of *P. cichorii*-produced lipopeptides.

Our data show the difference in persistence between cichofactin and cicho-peptin in infected leaves. When lettuce leaves were infiltrated with *P. cichorii*, severe cell collapse was observed in almost all of the infiltrated areas at 24 h postinoculation. At the same time point, cicho-peptins were clearly detected in infected lettuce tissue (Fig. 3). However, no cicho-peptin was detected at 48 h postinoculation, indicating the

importance of cicho-peptins at a relatively early stage during pathogenesis of lettuce midrib rot caused by *P. cichorii*. In situ cicho-peptin production by *P. cichorii* SF1-54 may be associated with viable lettuce cells because cicho-peptin could not be detected in completely necrotic lettuce cells. Similar phenomena have been observed in CLP-producing *P. fluorescens* strains, which exhibit an early phase of CLP production on germinating seeds rather than in bulk soil (Koch et al. 2002; Nielsen and Sørensen 2003; Nielsen et al. 2000). Additionally, it has been shown that germinating sugar beet seeds release unknown compounds that stimulate an early phase production of amphisin and tensin by *P. fluorescens* strains (Koch et al. 2002; Nielsen and Sørensen 2003; Nielsen et al. 2000). Thus, we suggest that unknown stimulants associated with viable lettuce cells may trigger in situ cicho-peptin production by *P. cichorii* SF1-54. On the other hand, cichofactins can be detected up to 5 days postinoculation, suggesting that cichofactin production is not related to viability of lettuce cells. The results are in agreement with our previous study demonstrating that *P. cichorii* SF1-54 can consistently produce cichofactins under various culture conditions (Pauwelyn et al. 2013). The phenomenon of in situ cichofactin production is also reflected in their involvement in the in planta spread of *P. cichorii* (Pauwelyn et al. 2013).

Furthermore, it was reported that syringomycin produced in plant tissue would be rapidly metabolized into other substances or irreversibly bound (Gross and DeVay 1977). When lettuce leaves were infiltrated with the culture filtrate of *P. cichorii* SF1-54, our data showed that cichofactin was quite stable in lettuce tissue. The concentration of cicho-peptin in treated lettuce tissue decreased gradually but not dramatically, indicating that cicho-peptin is not prone to degradation or metabolism by lettuce cells. However, we cannot exclude the possibility that cicho-peptins might be more susceptible to metabolic degradation in necrotic lettuce tissues than cichofactins during plant pathogenesis. It has been shown that *P. cichorii* triggers apoptosis-like programmed cell death in lettuce that is highly associated with generation of reactive oxygen species and de novo protein synthesis (Kiba et al. 2006, 2009). Reactive oxygen species are able to cause oxidative damage to biomolecules (Halliwell 2006). Alternatively, cicho-peptin could be degraded by *P. cichorii* SF1-54 or other microorganisms in

Fig. 7. Proposed structure of cicho-peptin and comparison with structures of corpeptin, fuscopeptin, and syringopeptin SP22. The fatty acid chain of each cyclic lipopeptide is indicated. For cicho-peptins, the general formula of the fatty acid chain is presented because the exact location of the double bond was not elucidated. Dab = 2,4-diaminobutyric acid, dhAbu = 2,3-dehydroaminobutyric acid, Hse = homoserine, *α*Thr = allothreonine. The four amino acids that differ between cicho-peptin and corpeptin are indicated in bold.

infected lettuce leaves. It has been suggested that lipopeptides produced by *Pseudomonas* and *Bacillus* species are degraded by other microorganisms or the residual producers in nonsterile soil (Asaka and Shoda 1996; Nielsen and Sørensen 2003; Raaijmakers et al. 2010). Apparently, *P. cichorii*-infected lettuce tissue is a complex environment like nonsterile soil. Further investigations are needed to study the in situ persistence of *P. cichorii*-produced lipopeptides, especially in complex environments.

Cichozeptins are important virulence factors of *P. cichorii*.

It proved to be very difficult to construct a *cipA*-deletion mutant of *P. cichorii* SF1-54. We tried three different deletion constructs, two constructs from different locations of *cipA* and one construct from *cipF*, and only the deletion construct described in this study was successfully maintained in *E. coli*. The other two deletion constructs had lethal effects on *E. coli*, a phenomenon that did not occur when we constructed a cichofactin biosynthesis mutant in our previous study (Pauwelyn et al. 2013). In addition, we obtained *cipA* mutants showing unexpected phenotypes (e.g., loss of cichofactin production) and several attempts were needed to obtain a cichozeptin-deficient mutant that was not affected in the production of cichofactins and the other lipopeptides.

Our data demonstrated that cichozeptins are important for virulence of *P. cichorii* SF1-54 on lettuce. Compared with the parental strain, discolored but not necrotic symptoms were observed on chicory leaves inoculated with the culture filtrate of a *cipA*-deleted mutant (Fig. 4C). Thus, cichozeptin is an important necrosis-inducing factor in culture filtrate. Infiltration with the *cipA*-deletion mutant, however, still caused necrotic symptoms on chicory and lettuce leaves that cannot visually be differentiated from necrosis induced by the parental strain (Fig. 4). The type three secretion system may be one of the candidate virulence factors for the cichozeptin mutant of *P. cichorii* SF1-54 to cause symptoms on chicory and lettuce. It has been shown that the type three secretion system is not essential for virulence of *P. cichorii* on lettuce (Hojo et al. 2008; Kajihara et al. 2012; Pauwelyn 2012). Thus, it is possible that both cichozeptin and the type three secretion system contribute to *P. cichorii*-induced necrosis in lettuce. We are currently investigating the roles of lipopeptides and type three secretion system in interactions between host plants and *P. cichorii* SF1-54.

The cichozeptin-deficient mutant of *P. cichorii* SF1-54 exhibited significantly weaker virulence to butterhead lettuce than the parental strain, but SF1-54- Δ *cipA* was as virulent as the cichofactin-deficient mutant (Fig. 6A), indicating that both phytotoxic cichozeptins and motility-related cichofactins affect virulence of *P. cichorii* SF1-54 on lettuce. It should be noticed that production of cichozeptin was not related to bacterial growth in planta (Fig. 6B). Collectively, cichozeptin contributes to induction of necrotic symptoms on lettuces and is a major virulence factor of *P. cichorii* SF1-54, like corpeptin and syringopeptin in the case of *P. corrugata* and *P. mediterranea* (Emanuele et al. 1998; Licciardello et al. 2009, 2012) and *P. syringae* pv. *syringae* B301D (Scholz-Schroeder et al. 2001).

In conclusion, we tentatively characterized cichozeptins, the phytotoxic CLP produced by *P. cichorii* SF1-54. Our results demonstrated that cichozeptins contribute to virulence of *P. cichorii* SF1-54 on lettuce and play an important role at the early stages of lettuce infection. We also found that production of cichozeptins by *P. cichorii* SF1-54 is stimulated in the presence of glycine betaine, a compound that can be used for nutrition or functions in signaling osmotic stress.

MATERIALS AND METHODS

Microorganisms and culture conditions.

Microorganisms used in this study are listed in Table 1. *P. cichorii* SF1-54, its mutants, and *E. coli* strains were stored at -80°C in LB broth (Sambrook and Russel 2001) with 20% glycerol for long-term storage. *R. mucilaginosa* MUCL 30397, *G. candidum* MUCL 28959, and *B. megaterium* LMG 7127 were used as indicator organisms for bioassays of antimicrobial activities. *B. megaterium* and *R. mucilaginosa* were maintained at -80°C in LB broth and in potato dextrose broth (Difco, Erembodegem, Belgium) with 20% glycerol, respectively. *G. candidum* was maintained on potato dextrose agar (PDA) (Difco) slants. *Pseudomonas* strains were routinely grown on *Pseudomonas* agar F (PAF) (Difco) or SRM_{AF} (Mo and Gross 1991) medium for production of lipopeptides. *B. megaterium* and *E. coli* strains were grown on LB medium at 28 and 37°C , respectively. *R. mucilaginosa* and *G. candidum* were cultured on PDA at 28°C . *Saccharomyces cerevisiae* was grown on yeast

Table 1. Strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Genotype or description ^a	Source/Reference
Strains		
<i>Pseudomonas cichorii</i>		
SF1-54	Natural isolate from infected greenhouse butterhead lettuce in Belgium	Cottyn et al. 2011
SF1-54NaI ^R	A spontaneous nalidixic acid resistant mutant of <i>P. cichorii</i> SF1-54	Pauwelyn et al. 2013
SF1-54 Δ <i>cifAB</i>	A cichofactin-deficient mutant of SF1-54NaI ^r	Pauwelyn et al. 2013
SF1-54 Δ <i>cipA</i>	A <i>cipA</i> -deletion mutant	This study
<i>Escherichia coli</i> S17-1	<i>thi pro hsdR recA::RP4-2-Tc^r::Mu Km^r::Tn7(λpir), Sm^r</i>	Simon et al., 1983
<i>Bacillus megaterium</i> LMG 7127	An indicator strain for lipopeptides	
<i>Rhodolotula mucilaginosa</i> MUCL 30397	An indicator strain for lipopeptides	
<i>Geotrichum candidum</i> MUCL 28959	An indicator strain for lipopeptides	
<i>Saccharomyces cerevisiae</i> InvSc1	<i>MATa/MATα leu2/leu2 trp1-289/trp1-289 ura3-52/ura3-52 his3-Δ1/his3-Δ1</i>	Invitrogen
Plasmids		
pMQ30	7.6-kb mobilizable suicide vector used for gene replacements in <i>Pseudomonas</i> spp.; <i>SacB</i> , <i>URA3</i> , Gm ^r	Shanks et al. 2006
pMQ30- <i>cipA</i>	pMQ30 containing two approximately 1-kb fragments of the cichozeptin biosynthesis <i>cipA</i> gene to obtain a 8.1-kb deletion in the gene	This study
Primers (5'→3')		
CipA-F1	<i>ccaggcaaatctgtttatcagaccgcttcgctgtatGTTGAATCGTCAGGCCAATCG</i>	This study
CipA-R1	<i>GCACCTTCGCCACCAITTCAGGTAGATCTCACCCGCAACACCC</i>	This study
CipA-F2	<i>GGGTGTTGCGGTGAGATCTACCTGAATGGTGGCGAAGGTGC</i>	This study
CipA-R2	<i>ggaattgtgagcggatacaatttcacaggaacagctgCCGACACCAACTCCAACGTCG</i>	This study

^a Primer extensions are in italic font. Tc^r, Km^r, Sm^r, and Gm^r indicate resistant to tetracycline, kanamycin, streptomycin, and gentamycin, respectively.

extract-peptone-dextrose medium at 30°C (Shanks et al. 2006). Antibiotics were added to the growth media (if required) at the following concentrations (µg/ml): gentamycin, 100 and nalidixic acid, 10.

Chemical analysis of *P. cichorii* SF1-54 lipopeptides.

Phytotoxic compounds F and G were purified from the culture filtrate of *P. cichorii* SF1-54 by RP-HPLC according to the method of Pauwelyn et al. (2013). Purified compounds were used for chemical characterization and structural determination.

Amino acid analysis. For amino acid analysis, HPLC-purified compounds were collected in glass tubes and were dried using vacuum speed (Speed-Vac Plus SC110A; Savant; Thermo Fisher Scientific, Bremen, Germany). The samples were hydrolyzed at 115°C for 6 h in 1.5 ml of 6 N HCl. After drying in a speedvac (Savant Speed-Vac Plus SC110A) and dissolving in 100 µl of 0.1 N HCl, the samples were analyzed on a ZORBAX Eclipse AAA column (Agilent, Hewlett Packard, Diegem, Belgium), using an Agilent 1100 series HPLC, by strictly following the instructions of the manufacturer. Briefly, the hydrolyzed samples and amino acid standard solutions (purchased from Sigma-Aldrich, Diegem, Belgium) were automatically derivatized in the autosampler with o-phthalaldehyde (OPA) for most amino acids and 9-fluorenylmethyl-chloroformate (FMOC) for lysine, hydroxyproline, and proline residues. After derivatization, 0.5 µl of each sample was injected with a flow rate of 2 ml/min and was detected at 338 (OPA) and 262 nm (FMOC). Amino acids in the lipopeptide hydrolysis products were identified and their relative amounts in the samples were estimated based on the amino acid standards analyzed simultaneously.

Mass spectrometry. Analysis of lipopeptides produced by *P. cichorii* SF1-54 was carried out on a LC-ESI-MS (RP-HPLC Waters Alliance 2695/diode array detector in tandem with a Single Quad Waters SQD Mass spectrometer; Waters, Zellik, Belgium) on an XTerra MS C18 Column (3.5 µm, 4.6 × 150 mm; Waters). Putative lipopeptides of *P. cichorii* SF1-54 were eluted with a gradient of acetonitrile acidified with 0.1% formic acid in water acidified with 0.1% formic acid at a constant flow rate of 0.5 ml/min at 40°C. Compounds were monitored in the positive ion mode and in-source settings in the SQD were as follows: source temp, 130°C; desolvation temp, 280°C; nitrogen flow, 600 liters/h; cone voltage, 100 V. Similar settings were used for lipopeptide fragmentation, except the cone voltage, which was 75 V instead of 100 V. Both instruments were controlled with the MassLynx software (Waters). Exact mass measurements and MS/MS experiments were performed with a FT-ICR mass spectrometer (9.4T Solarix; Bruker Daltonics, Bremen, Germany) equipped with an ESI– matrix-assisted laser desorption ionization dual ion source including the SmartbeamII laser (wavelength 355 nm). Identification of lipopeptides was performed based on mass accuracy, isotopic pattern simulation, and MS/MS. For MS/MS experiments, the selection window in the quadrupole was set to 5 Da and fragmentation was performed in the hexapole, using argon as collision gas and an optimized collision energy of 50 V.

Identification and bioinformatics analysis of the NRPS genes.

Putative NRPS-encoding regions were found by comparison of the genome sequence of *P. cichorii* SF1-54 (Pauwelyn et al. 2013) with the genomes of *P. syringae* pv. *tomato* DC3000 and *P. syringae* pv. *syringae* B728a (Buell et al. 2003; Feil et al. 2005), using the program MAUVE (Darling et al. 2010). Gap-filling in the putative NRPS-encoding gene regions was performed by way of polymerase chain reaction (PCR) and Sanger sequencing of PCR products (Agowa Sequencing, Berlin, Germany). ORF were identified using the program Glimmer

(Delcher et al. 1999), available on the National Center for Biotechnology Information website. The catalytic domains present in the NRPS genes were identified using the NRPS/PKS analysis website (Bachmann and Ravel 2009) and the National Institute of Immunology NRPS-PKS website (Ansari et al. 2004). The specificity prediction of the A domain was conducted by using the web-based software NRSPredictor2 (Rausch et al. 2005; Röttig et al. 2011). The D/L configurations of amino acid residues of nonribosomal peptide synthetases were investigated by phylogenetic analysis of the C domains via the Phylogeny.fr website (Dereeper et al. 2008) and detection of specific signatures occurring in the DownSeq regions of each C domain (Caradec et al. 2014). Genes adjacent to the putative NRPS genes were compared with the sequences available in GenBank, using the program BlastX. Multiple alignments and phylogeny tree design were performed using ClustalW2 tool available at the European Bioinformatics Institute website.

Growth conditions for enhanced production of cichozeptins.

Choline chloride, glycine, or glycine betaine (all purchased from Sigma-Aldrich) as the sole carbon source for *P. cichorii* growth were tested. *P. cichorii* SF1-54Nal^R was cultured in 5 ml of the standard minimal medium (Meyer and Abdallah 1978) containing 1% of each compound at 28°C with shaking at 150 rpm. Bacterial growth was measured at 620 nm.

SRM_{AF} medium supplemented with various concentrations of choline chloride or glycine betaine was tested for *P. cichorii* growth under static conditions. In a sterile 24-well plate, 1 ml of SRM_{AF} medium in the absence or in the presence of choline chloride or glycine betaine was added in a well and a bacterial suspension of *P. cichorii* SF1-54Nal^R was added in culture media to a final concentration of 1 × 10⁸ CFU/ml. The plate was sealed with parafilm and was incubated at room temperature as the conditions for lipopeptide production. Bacterial growth was measured at 620 nm.

SRM_{AF} medium supplemented with various concentrations of glycine betaine was tested for production of cichozeptin. A bacterial suspension of *P. cichorii* SF1-54Nal^R was added in culture media (25 ml in a 100-ml flask) to a final concentration of 1 × 10⁸ CFU/ml. Bacterial cultures were incubated at room temperature for 6 days without shaking. After 6 days of incubation, the culture supernatants were collected and filtrated through 0.22-µm filters. The cell-free culture filtrates were used for assays of antimicrobial activity and phytotoxicity and HPLC-ESI-MS analysis. Three independent cultures of each treatment were analyzed and each culture was assayed in triplicate.

In planta detection of lipopeptides produced by *P. cichorii* SF1-54.

Lettuce leaves were infiltrated with a bacterial suspension of *P. cichorii* SF1-54Nal^R (1 × 10⁶ CFU/ml) or a filter-sterilized culture filtrate obtained from *P. cichorii* SF1-54Nal^R culture grown in SRM_{AF} medium. The lettuce leaves were placed on upside down petri dishes into plastic infection trays filled with wetted sterile cotton. The infection trays were covered with a plastic lid to obtain high relative humidity and were incubated at 25°C. At 24 and 48 h postinoculation, symptomatic tissue was excised from the lettuce leaves and was ground in liquid nitrogen. After thawing, 1 g of ground symptomatic leaf tissue was suspended in 3 ml of 50% acetonitrile, was shaken for 1 h at room temperature, and was then centrifuged (5 min at 10,000 rpm). Lipopeptides in the supernatants of extractions were concentrated with C₁₈ solid-phase extraction cartridges (C₁₈ SPE Maxi-Clean cartridges; Alltech, Grace, Lokeren, Belgium) and were analyzed with LC-ESI-MS.

Construction of the cichopectin-deficient *P. cichorii* SF1-54 mutant.

A cichopectin-deficient mutant of *P. cichorii* SF1-54NaI^R was constructed by an in vivo cloning technique with the yeast *S. cerevisiae* InvSc1 (Hoang et al. 1998; Shanks et al. 2006). To construct deletion plasmid pMQ30- Δ cipA, primers CipA-F1 and CipA-R1 were used to amplify the upstream fragment, and primers CipA-F2 and CipA-R2 for the downstream fragment (Table 1). The cichopectin-deficient mutant contained a deletion of 8,071 bp in the cichopectin encoding gene *cipA*. Deletion of the *cipA* fragment was confirmed by PCR, and the mutant was characterized phenotypically.

Biological roles of cichopectin in *P. cichorii* SF1-54.

Phytotoxic activity was tested on chicory or lettuce leaves by injection of 300 μ l of bacterial suspension (1.0×10^8 CFU/ml in 50 mM phosphate buffer [PB], pH 7.0) or culture filtrate, with a needleless syringe used through the abaxial surface. The infiltrated leaves were incubated at 100% humidity and 25°C for 24 h and the appearance of necrotic lesions was scored at 24 h after injection. PB, noninoculated SRM_{AF} medium, or SRM_{AF} medium supplemented with different carbon sources were used as negative control treatment.

Antimicrobial activity was tested against *B. megaterium*, *G. candidum*, and *Rhodotorula pilimanae*, using the protocol described by Pauwelyn et al. (2013). Briefly, bacterial suspensions of *P. cichorii* strains (1×10^8 CFU/ml) were spotted on PDA plates in five replicates, allowing growth at 25°C for 5 days. The areas of colony growth were marked and the colonies were removed with a sterile swab. The plates were exposed to chloroform vapors for 20 min to kill the remaining bacterial cells, followed by dissipation of the vapors for an additional 40 min. The plate was then sprayed with a spore/cell suspension at a concentration of about 10^6 cells or spores per milliliter of the indicator microorganism. After 24 to 48 h of incubation at 28°C, inhibition of the indicator microorganism was scored.

Swarming motility of *P. cichorii* strains was assayed on soft LB plates (0.5% agar). Bacteria of a fully grown colony on PAF medium were applied in the center of the soft LB agar plates with a sterile toothpick, and the plates were subsequently incubated at 28°C up to 72 h after inoculation.

To test virulence in detached butterhead leaves, the leaves were inoculated by infiltration with 100 μ l of a bacterial suspension of each *P. cichorii* strain (5×10^5 CFU/ml). On one leaf, four sites were inoculated with the same strain. Infiltrated leaves were incubated in humid boxes at 28°C. Each strain was assayed in triplicate and the experiment was carried out twice. Disease symptoms were evaluated on the inoculated leaves 1 and 2 days postinoculation on a 0 to 3 scale: 0 = no disease symptoms, 1 = discoloration or small spot of the infiltrated tissue, 2 = brown necrosis of the infiltrated tissue, and 3 = collapse of the infiltrated tissue. Data were analyzed by the Kruskal-Wallis and Mann-Whitney nonparametric tests ($P = 0.05$) in SPSS 21.0 for Windows.

Pathogenicity of the lipopeptide-deficient mutants was tested at Inagro, Rumbeke, Belgium, according to the methods described in our previous studies (Pauwelyn et al. 2011, 2013). Butterhead lettuce cv. Hofnar plants (R. Zwaan, De Lier, The Netherlands) at head-formation stage were inoculated with *P. cichorii* strains. Tap water without bacteria was used as the uninoculated treatment. Each treatment was assayed in three replicates, with 24 plants per replicate. At harvest, disease severity was assessed by giving each plant a score ranging from 0 to 4, in which 0 = healthy plant, 1 = little black spots on the leaf periphery of the inner crop leaves, 2 = infection of small side ribs or black spots or stripes on the midrib, 3 = one, two or

three rotten midribs, and 4 = four or more rotten midribs. Disease score of all plants was calculated as previously described (Pauwelyn et al. 2011, 2013). All data were statistically analyzed using the software package SPSS 21.0 for Windows. As the data did not meet the conditions of normality and homogeneity of variance, nonparametric Kruskal-Wallis and Mann-Whitney comparisons ($P = 0.05$) were performed.

To test the ability of *P. cichorii* SF1-54 and its mutants to grow in lettuce midribs, the midrib of the inner lettuce leaves was inoculated with 1.0 ml of bacterial suspension (1×10^5 CFU/ml). The inoculated leaves were incubated at 100% humidity at 25°C. Nine leaf discs (1 cm diameter) were excised from the midribs of three lettuce leaves at 0, 1, 2, and 3 days postinoculation and were macerated in 7.0 ml of sterile 50 mM potassium PB (pH 7.0). Tenfold serial dilutions from the macerate were prepared with PB, and 100- μ l aliquots of each dilution were spread on three PAF plates with 50 μ g of nalidixic acid per milliliter. Colonies were counted 2 days after incubation at 28°C, to estimate population densities. The experiment was repeated twice. All data were statistically analyzed, using the software package SPSS 21.0 for Windows. As the data did not meet the conditions of normality and homogeneity of variance, nonparametric Kruskal-Wallis and Mann-Whitney comparisons ($P = 0.05$) were performed.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

The European Bioinformatics Institute website: www.ebi.ac.uk
 National Institute of Immunology NRPS-PKS website:
<http://www.nii.res.in/nrps-pks.html>
 The NRPS/PKS analysis website: <http://nrps.igs.umd.edu/nrps>
 NRPSpredictor2: <http://nrps.informatik.uni-tuebingen.de>