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New linear lipopeptides produced by *Pseudomonas cichorii* SF1-54 are involved in virulence, swarming motility, and biofilm formation

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ABSTRACT

Pseudomonas cichorii is the causal agent of lettuce midrib rot, characterized by a dark-brown to green-black discoloration of the midrib. Formation of necrotic lesions by several plant pathogenic pseudomonads is associated with production of phytotoxic lipopeptides, which contribute to virulence. Therefore, the ability of *P. cichorii* strain SF1-54 to produce lipopeptides was investigated. A cell-free culture filtrate of SF1-54 showed surfactant, antimicrobial, and phytotoxic activities which are typical for lipopeptides. High-performance liquid chromatography analysis of *P. cichorii* SF1-54 culture filtrate revealed the presence of seven compounds with lipopeptide characteristics. Two related lipopeptides, named cichofactin A and B, were studied in more detail: they are linear lipopeptides with a decanoic and dodecanoic lipid chain, respectively, connected to the N-terminus of a 8-amino acid peptide moiety. Both cichofactins are new members of the syringafactin lipopeptide family. Furthermore, two non-ribosomal peptide synthethases encoding genes, *cifA* and *cifB*, were identified as responsible for cichofactin biosynthesis. A *cifAB* deletion mutant no longer produced cichofactins, was impaired in swarming motility, but showed enhanced biofilm formation. Upon spray-inoculation on lettuce, the cichofactin-deficient mutant caused significantly less rotten midribs than the wildtype, indicating that cichofactins are involved in pathogenicity of *P. cichorii* SF1-54. Further analysis revealed that *P. cichorii* isolates vary greatly in swarming motility and cichofactin production.

INTRODUCTION

Many plant pathogenic bacteria are known to produce non-host specific phytotoxins that contribute to virulence (Kunkel and Zhongying 2006). Well-characterized phytotoxins are those produced by *Pseudomonas syringae* and include chlorosis-inducing toxins (e.g., coronatine, phaseolotoxin, and tabtoxin) and necrosis-inducing toxins (e.g. syringomycin and syringopeptin) (Bender et al. 1999). The latter are cyclic lipopeptides (CLPs), which are amphipathic molecules composed of a fatty acid tail linked to a cyclic oligopeptide head. Due to this amphipatic nature they can insert into the plasma membrane of their host cells, which is their primary target, and form pores. This results in transmembrane fluxes of H^+, Ca^{2+} and K^+, and leads to a collapse of the pH-gradient across the plasma membrane, consequently resulting in cell death. This process is highly efficient, as only a few nanomoles of toxin suffice to induce pore formation (reviewed in Bender et al. 1999 and Bender and Scholz-Schroeder 2004). Besides phytotoxicity, these CLP toxins also exhibit hemolytic and antimicrobial activity (Hutchison et al. 1995; Hutchison and Gross 1997; Lavermicocca et al. 1997). This latter feature of lipopeptides has been exploited by researchers to develop bioassays that have expedited detection of lipopeptide production, purification and genetic studies on these cyclic lipopeptides.

But lipopeptides are not always phytotoxic. Depending on their structure, they may have diverse other roles and functions and can be involved in swarming motility, biofilm development and colonization, or display zoosporicidal and/or antimicrobial activity (reviewed in Raaijmakers et al. 2006, 2010). These functions are extensively described for antagonistic *Pseudomonas* spp., but to a lesser extent for plant pathogenic pseudomonads. The only available examples to date are the syringafactins, which are essential for swarming motility of *P. syringae* pv. *tomato* DC3000 and *P. syringae* pv. *syringae* B728a (Berti et al. 2007; Burch et al. 2010) and viscosin,

which is required for *P. fluorescens* strain 5064 to colonize the surface of broccoli florets (Hildebrand et al. 1998).

Pseudomonas cichorii is an important plant pathogenic bacterium, with a wide host range and a worldwide distribution. *P. cichorii* causes necrotic leaf and stem lesions on vegetables, ornamentals, cereals and woody plants (Bradbury 1986). Economically important hosts include lettuce, celery, chrysanthemum, tomato, coffee and soybean (Jagger 1914; Wilk and Dye 1974; Jones et al. 1984; Bradbury 1986; Yu and Lee 2012). In Belgian greenhouse-grown butterhead lettuces, *P. cichorii* causes a dark-brown to green-black discoloration of the midrib (Cottyn et al. 2009; Cottyn et al. 2011; Pauwelyn et al. 2011). Symptom development on lettuce leaves is strongly associated with apoptosis-like programmed cell death (Kiba et al. 2006; Kiba et al. 2009). Intriguingly, the type III secretion system is essential for pathogenicity of *P. cichorii* on eggplant, but not on lettuce (Hojo et al. 2008; Kajihara et al. 2012). Symptom development may be associated with the production of necrosis-inducing cyclic lipopeptide toxins by this bacterium. The production of antimicrobial and phytotoxic substances by *P. cichorii* has already been described (Hikichi et al. 1998; Hu et al. 1998; Lazzaroni et al. 2003), but no bioactive compound has been characterized up to date.

Therefore, the aim of this study was to examine the ability of *P. cichorii* to produce phytotoxic and antimicrobial compounds using bioassays. Seven bioactive compounds produced in culture by the highly virulent *P. cichorii* strain SF1-54 were isolated and partly characterized by reverse-phase high-performance liquid-chromatography (RP-HPLC) and liquid chromatography electrospray ionisation mass spectrometry (LC-ESI-MS). The structures of two of these lipopeptides produced by *P. cichorii* strain SF1-54 were elucidated using LC-ESI-MS and HR-ESI-MS-MS. They appear to be linear lipopeptides, consisting of 8 amino acids, and are

novel members of the syringafactin family of lipopeptides. In addition, genome mining of *P. cichorii* SF1-54 revealed two non-ribosomal peptide synthetase (NRPS) genes, *cifA* and *cifB*, that encode the lipopeptide synthetases responsible for biosynthesis of cichofactins. Based on the *cifA* and *cifB* gene sequences, a cichofactin-deficient mutant was constructed to investigate the function of these compounds for *P. cichorii* SF1-54.

RESULTS

Biological activity of strain SF1-54 and its secreted metabolites.

Injection of a *P. cichorii* SF1-54 bacterial suspension (1×10^8 CFU/ml) into lettuce or chicory leaves resulted in brown lesions in the treated area within 24 h post inoculation (Fig. 1A). Injection of a crude culture filtrate of *P. cichorii* SF1-54 caused identical symptoms (Fig. 1B), suggesting that a phytotoxic compound is produced and secreted into the SRM_{AF} medium. No symptom was visible by injection of non-inoculated SRM_{AF} medium in chicory. Although minor rotting symptoms were often observed on the lettuce leaves of the non-inoculated treatments, they looked clearly different from the symptoms caused by *P. cichorii* SF1-54 and its crude culture filtrate, indicating that they are possibly due to wounding, contamination or senescence (Fig. 1C).

Since symptom development is similar in lettuce and chicory leaves, only chicory leaves were used for further phytotoxicity tests, because they are less prone to contamination and easier to handle for reproducible infection.

P. cichorii SF1-54 inhibited growth of *Bacillus megaterium* (a bacterium), *Geotrichum candidum* (a filamentous fungus) and *Rhodotorula mucilaginosa* (a yeast). The indicator

microorganisms were selected according to the spectrum of antimicrobial activities of *P. syringae* pv. *syringae* toxins (Lavermicocca et al. 1997). In direct antagonistic assays against these microorganisms, a *P. cichorii* SF1-54 colony produced an inhibition zone of 25 to 41 mm against *B. megaterium*, of 38 to 53 mm against *G. candidum*, and of 50 to 56 mm against *R. mucilaginosa* under our experimental conditions. The crude culture filtrate and semi-purified culture extract (solid phase extraction on C18 cartridge) retained these inhibitory capacities against *B. megaterium*, *G. candidum*, and *R. mucilaginosa* (Fig. 2B).

The active molecules produced by strain SF1-54 in SRM_{AF} medium were isolated from the semi-purified culture extracts by repeated injections in semi-preparative reversed-phase HPLC. Repeated injections were performed on a protein and peptide C-18 column. The eluates were monitored spectrophotometrically at 220 nm. All eluates absorbing at 220 nm were independently collected in 23 fractions, corresponding to the observed peaks in the reverse phase HPLC chromatogram (Fig. 2A). All fractions were first tested for surfactant activity and seven compounds, indicated with a letter (A-G) on Fig. 2A, caused drop collapse, which is characteristic of biosurfactant metabolites.

Antimicrobial activity and phytotoxicity of these purified molecules were then assayed (Fig. 2B). Compound A inhibited the growth of *B. megaterium*. Compound B had antimicrobial activity against *G. candidum* and *R. mucilaginosa*, while compound C only inhibited *R. mucilaginosa*. Compounds D and E were both inhibitory to *R. mucilaginosa* and *B. megaterium*. Compounds F and G were both phytotoxic and inhibited *B. megaterium*. Thus, the culture filtrate of *P. cichorii* SF1-54 is a complex mixture of several bioactive metabolites.

Chemical characterization of compounds D and E (cichofactins).

The concentration of compounds D and E in the culture filtrate obtained from SRM_{AF} medium was 29 ± 15 mg/l based on calculated data from 5 different cultures. Several media other than SRM_{AF} and various incubation periods and temperatures were tested for production of all the bioactive compounds by *P. cichorii* SF1-54 but only compounds D and E were consistently produced in high amounts (data not shown), indicating that compounds D and E could be the main compounds produced by *P. cichorii* SF1-54. In addition to consistent production, compounds D and E exhibited not only surfactant but also inhibitory activities to *R. mucilaginosa* and *B. megaterium*. Thus, we first focused on identification and characterization of compound D and E.

HPLC-purified samples of compounds D and E were submitted to acid hydrolysis and further analysis of the released amino acids revealed that both molecules contained Leu, Val and Glx (representing either Gln or Glu) residues in similar molar ratios of 1.91:1.05:5.12 and 2.02:1.03:5.01, respectively. These results support the peptidic nature of compounds D and E and they were further characterized by high performance liquid chromatography-electrospray-mass spectrometry (HPLC-ESI-MS) using parameters to optimize in-source fragmentation. As shown in Figure S1 and Table 1, identification of b- and y"-ions revealed very similar fragmentation patterns of the $[M+H]^+$ ions (*m/z* 1109 and 1137, respectively) corresponding to compounds D and E. However, *m/z* values of b-ion fragments were 28 Da larger for compound E (Table 1), suggesting that its fatty acid chain contains two extra methylene groups. The presence of a 3-hydroxydecanoyl fatty acid in compound D and a 3-hydroxydodecanoyl fatty acid in compound E is fully supported by the detection of ions at *m/z* 284 and 312, respectively, in accordance to the [FA-Leu]-fragments.

Based on the fragmentation pattern, both lipopeptides could have the peptide sequence Leu-Leu-Gln-Leu-Gln-Val-Leu-Leu.

HR-ESI-MS-MS analyses

In depth structural characterization was obtained using High Resolution-ESI-MS-MS performed on the $[M+Na]^+$ form of compounds D and E. The exact m/z of $[M+Na]^+$ of compound D is 1131.732 and of compound E 1159.762 (calculated masses are 1131.737 and 1159.768 for $C_{55}H_{100}N_{10}O_{13}Na$ and $C_{57}H_{104}N_{10}O_{13}Na$, respectively). These masses suggest that the compounds are linear since their mass would be 18 Da smaller (loss of one water molecule for cyclization) if they were cyclic. Moreover, the first loss of Leu-COOH (*m/z* 1018 and 1046, respectively) is an additional evidence for a linear structure.

All pertinent fragments from the HR-ESI-MS-MS spectra of the $[M+Na]^+$ form of compounds D and E are shown in Table 2. The b-type ions are formed upon collision activation (CA) of the $[M+Na]^+$ species by rearrangement processes (Fuchs and Budzikiewicz 2001) depicted in Fig. S2. The degradation of the peptide chain by elimination of the C-terminal amino acid with back transfer of the hydroxyl group can be repeated several times (sequential loss of NH=CHR units). Thus, the HR-ESI-MS-MS fragmentation pattern supported the LC-ESI-MS data completely: a 3-hydroxydecanoyl fatty acid in compound D and a 3-hydroxydodecanoyl fatty acid in compound E coupled to a peptide moiety containing Leu-Leu-Gln-Leu-Gln-Val-Leu-Leu for both compounds.

Comparison of compounds D and E with previously described lipopeptides produced by *Pseudomonas* spp. revealed that these molecules are new members of the syringafactin family . The only difference is the replacement of Thr present at position 5 in syringafactins A and D by

Gln in the compounds produced by *P. cichorii* SF1-54. Because of the high structural resemblance of these compounds to the syringafactins A and D, we renamed compounds D and E cichofactins A and B, respectively.

Identification and bioinformatic analysis of putative cichofactin synthetase-encoding genes.

As cichofactins show high structural similarities with the syringafactins of *P. syringae* pv. *tomato* DC3000, the genome of *P. cichorii* SF1-54 (unpublished) was compared with the genome sequence of *P. syringae* pv. *tomato* DC3000 using MAUVE to identify the gene cluster which encodes the NRPS for cichofactins synthesis.

In the genome of *P. cichorii* SF1-54, a gene cluster was identified containing two large genes, *cifA* and *cifB*, that are 9,498 bp and 17,734 bp in size and encode for three and five NRPS modules with specific condensation (C), adenylation (A) and thiolation (T) domains, respectively (Fig. 3). Both genes exhibit two typical features of NRPS genes involved in lipopeptide synthesis: the presence of an N-terminal C-domain in the initiating module and two tandem thioesterase (TE) domains at the end of the NRPS cluster. Furthermore, *cifA* and *cifB* genes of *P. cichorii* SF1-54 showed respectively 76% and 75 % identity at the amino acid level to syringafactin-encoding genes *syfA* and *syfB* of *P. syringae* pv. *tomato* DC3000.

To predict the peptide sequence of cichofactins from genome sequencing data, bioinformatic analysis of the A-domain from each NRPS module was performed. The putative peptide sequence of cichofactins is Leu-Leu-Gln/Asp-Leu-Gln/Asp-Val-Leu-Leu (Table S1). The prediction with NRPSpredictor2 for the amino acids at third and fifth position is based on the specificity codes, which show in both cases 70% identity with the specificity code of Gln and Asp. According to the results of structural analysis of cichofactins, both the third and fifth amino

acids are Gln and the prediction is in full agreement with the peptide sequence of cichofactins determined by mass spectrometry.

Furthermore, the D/L configuration of amino acid residues of cichofactins was investigated by phylogenetic analysis of the peptide sequences of C-domains. In *Pseudomonas*, dual condensation/epimerization (C/E) domains are responsible for generating D-residues into lipopeptides (Balibar et al. 2005; Rausch et al. 2007). According to the phylogenetic analysis, C-domains of modules 2, 3, 4, 6 and 8 could be dual C/E domains; therefore, the peptidic structure of cichofactins could be D-Leu-D-Leu-D-Gln-L-Leu-D-Gln-L-Val-D-Leu-L-Leu. The locations of dual C/E domains of cichofactins are identical to those of syringafactins (Berti et al. 2007).

LuxR-type regulators and transporter genes are often positioned up- and downstream of the lipopeptide biosynthesis genes in various pseudomonads (Quigley et al. 1993; Roongsawang et al. 2003; Kang and Gross 2005; Berti et al. 2007; de Bruijn et al. 2008; Dubern et al. 2008; de Bruijn and Raaijmakers 2009; Vallet-Gely et al. 2010). Therefore, we examined the flanking regions of the cichofactin biosynthesis cluster in the *P. cichorii* SF1-54 genome. As shown in Fig. 3, two open reading frames downstream of the cichofactin biosynthesis cluster show sequence similarities to subunits of a multidrug efflux pump, MacA (77% protein identity with *P. entomophila* L48) and MacB (89% protein identity with *P. syringae* pv. *tomato* DC3000). On each side of the cichofactin biosynthesis cluster, two open reading frames were identified showing both sequence similarity with the LuxR family of transcriptional regulators (66% and 69% protein identity with *P. syringae* pv. *tabaci* ATCC 11528 and *P. syringae* pv. *maculicola* ES4326).

Biological role of cichofactins produced by *P. cichorii* SF1-54.

To investigate the biological role of the cichofactins and their importance in pathogenicity for *P. cichorii* SF1-54, we constructed a cichofactin-deficient mutant SF1-54-Δ*cifAB* by deleting a 9715 bp fragment within the cichofactin-encoding genes *cifA* and *cifB* (Figure 3). LC-ESI-MS analysis confirmed that this mutant was completely abolished in production of both cichofactins (Fig. 4). In addition, the production of the phytotoxic compounds F and G was about 10 times higher in the mutant in comparison with the wildtype. Deletion of the cichofactin biosynthesis genes did not influence the production of compound A, while the amounts of compounds B and C were too low to be detected in the semi-purified culture filtrate of the wildtype and the cichofactin mutant by LC-ESI-MS analysis.

When *P. cichorii* SF1-54 cells were suspended into a droplet of water on parafilm, the water droplet collapsed immediately, but a cell suspension of *P. cichorii* SF1-54-Δ*cifAB* did not cause spreading of the water on the parafilm surface (data not shown). Because of the biological activity of the remaining compounds, the cichofactin-deficient mutant retained the ability to inhibit growth of *B. megaterium*, *G. candidum*, and *R. mucilaginosa* in direct antagonistic assays. However, *P. cichorii* SF1-54-Δ*cifAB* produced remarkably larger inhibition zones than wild type against *B. megaterium*, probably due to the overproduction of compounds F and G. The mean inhibition zone of *P. cichorii* SF1-54-Δ*cifAB* was 47 mm compared to 35 mm for *P. cichorii* SF1-54.

The cichofactin-deficient mutant still induced discoloration and necrosis of chicory leaves, which is not surprising since the mutant overproduces the phytotoxic compounds F and G (Fig. 4).

Syringafactins produced by *P. syringae* pv. *tomato* DC3000 and *P. syringae* pv. *syringae* B748a function as biosurfactants and are involved in swarming motility (Berti et al. 2007; Burch et al. 2010). *P. cichorii* SF1-54 swarmed outwards from the inoculation point to cover the entire plate containing soft LB medium (0.5% agar) within 48 h, while swarming motility was completely abolished in the cichofactin-deficient mutant (Fig. 5A).

Because lipopeptides produced by plant-associated *Pseudomonas* spp. are often described to influence biofilm formation (Roongsawang et al. 2003; Kuiper et al. 2004; de Bruijn et al. 2007; de Bruijn et al. 2008), we tested the influence of cichofactins on biofilm production of *P. cichorii* SF1-54. *P. cichorii* SF1-54 formed biofilms in polystyrene 96-well microtiter plates, and the biofilm formation of the cichofactin-deficient mutant was significantly improved compared to the wildtype (Fig. 5B).

To determine the effect of cichofactins on the virulence of *P. cichorii* SF1-54, *P. cichorii* SF1-54 or its cichofactin-deficient mutant were spray-inoculated on butterhead lettuces in greenhouse experiments. Lettuce plants inoculated with the cichofactin-deficient mutant exhibited significantly less rotten midribs than lettuce plants inoculated with the wild type, which is also reflected in the significantly lower disease severity score obtained with the mutant ($P < 0.05$) (Fig. 5C). However, when the wildtype or the *cifAB*-deletion mutant were injected in lettuce midribs, both strains grew vigorously and reached similar population densities in the inoculated section at 1, 2 and 3 days post inoculation (Fig. S3). Furthermore, the effect of cichofactins on spread of *P. cichorii* in lettuce midribs was investigated (Table S2). Both the wildtype strain and the cichofactin-deficient mutant moved to the adjacent midrib section (Fig. S4) 3 days post inoculation but the mutant moved at a significant slower rate than the wild type.

A successful attachment is the first step in colonization and pathogenesis (Turnbull et al. 2001; Danhorn and Fuqua 2007). Hence, we wanted to understand whether cichofactins are involved in attachment of *P. cichorii* on three different regions of leaf surface (as indicated in Fig. S4). The wildtype strain and the cichofactin-deficient mutant attached equally well on the leaf surface, but for the petiole and center regions very small but significant differences in bacterial attachment were observed (Table S4).

Lettuce tissue inoculated with *P. cichorii* SF1-54 was analysed using LC-ESI-MS to determine lipopeptide production of *P. cichorii* SF1-54 *in planta*. Cichofactins were not detected the first day after strain injection but the concentration of cichofactins gradually increased over the next three days to reach amounts ranging from 1.2 to 8.1 µg/g leaf fresh weight in two different assays. No peaks corresponding to possible lipopeptide compounds were observed in uninfected lettuce tissue. In addition, no trace of the other lipopeptides could be detected (Figure 6).

P. cichorii strains and isolates differ in swarming motility and cichofactin production

Belgian *P. cichorii* isolates from lettuce can be categorized into three subgroups based on colony morphology and BOX-PCR patterns (Cottyn et al. 2009). Isolates belonging to the C3 group are significantly less virulent on lettuce than isolates of the C1 and C2 groups (Pauwelyn et al. 2011). The swarming ability of two additional *P. cichorii* isolates of each subgroup, next to SF1-54 (C1 group), the Japanese tar spot strain SPC9018 (C2 group), the Californian varnish spot strain 9D42 (related to the C3 group), the chrysanthemum strain NCPPB 907 (not related with any of the three groups), and the type strain NCPPB 943T (not related with any of the three groups) was investigated. As shown in Fig. 7A, *P. cichorii* isolates belonging to different groups

differ in their swarming motility, with isolates from group C1 and C2 swarming more rapidly than isolates of the C3 group. Surprisingly, the varnish spot strain 9D42, the type strain NCPPB 943T and chrysanthemum strain NCPPB 907 did not show swarming motility. Interestingly, these three strains are weakly or non-virulent on lettuce (Pauwelyn et al. 2011 and Table S5).

In addition, the level of cichofactin production in the various *P. cichorii* isolates was measured by HPLC-ESI-MS (Fig. 7B). Isolates of the C2 group secreted much more cichofactins than isolates of the C1 and C3 group. Very low amounts of cichofactins were produced by strains 9D42 and NCPPB 907, while no cichofactins could be detected in the type strain NCPPB 943T. However, the presence of the *cif* genes could be detected in all strains with the primers (Table 3) used to construct the deletion plasmid pMQ30- Δ*cifAB* (data not shown). Our results show a correlation between cichofactin production and swarming, and suggest indirectly an impact on virulence in lettuce.

DISCUSSION

Several plant pathogenic *Pseudomonas* spp., such as *P. syringae* pv. *syringae*, *P. fuscovaginae, P. corrugata* and *P. fluorescens* strain 5064, are known to produce (cyclic) lipopeptides (Flamand et al. 1996; Hildebrand et al. 1998; Bender et al. 1999; Catara, 2007). Since these lipopeptides are surfactants, exhibiting often antimicrobial and/or phytotoxic activity, bioactivity assays are commonly used to assess their presence (Bender et al. 1999; Raaijmakers et al. 2010).

In this study, we showed by means of bioassays that *P. cichorii* strain SF1-54 has surfactant, antimicrobial, and phytotoxic activity. These results confirm and extend the findings

of Hu *et al*. (1998), who showed the production of an antibiotic compound by *P. cichorii*, and of Lazzaroni *et al*. (2003), mentioning the production of both phytotoxic and antibiotic compounds by *P. cichorii.* However, these compounds were not further characterized. Since the culture filtrate of *P. cichorii* SF1-54 showed similar characteristics as *P. cichorii* SF1-54 cells, bioactivity is due to compounds secreted in the culture filtrate. Because tar symptoms caused by *P. cichorii* on crisphead lettuce in Japan are partly due to apoptosis-like programmed cell death (Kiba et al. 2006; Kiba et al. 2009), we suggest that phytotoxic compounds or virulence factors in the culture filtrate may be involved in the induction of apoptosis in lettuce.

HPLC chromatogram of the semi-purified extract from the culture filtrate of *P. cichorii* SF1-54 revealed that *P. cichorii* SF1-54 produces a complex mixture of several metabolites (Fig. 2). The HPLC elution pattern of the semi-purified extract was similar to the elution pattern of the lipopeptide metabolites of *P. syringae* pv. *syringae* (Ballio et al. 1991) and *P. fuscovaginae* (Flamand et al. 1996), which is characterized by the presence of two groups of compounds with different polarity, more specifically the lipodepsinonapeptides (such as syringomycins) and the more hydrophobic lipopeptides, such as syringopeptins and fuscopeptins. Preliminary data suggest that compounds B and C are pseudomycin-like (Ballio et al. 1994), while compounds F and G are structurally related to corpeptin, a phytotoxic lipopeptide produced by *Pseudomonas corrugata* (Emanuele et al. 1998), but this has to be confirmed by further chemical analysis.

HPLC fractionation of the culture filtrate of *P. cichorii* SF1-54 showed that the bioactivity of the strain could be ascribed to seven different compounds (Fig. 2). These compounds all showed surfactant activity, which is the primary characteristic of lipopeptide metabolites. However, bioactivities of these compounds are clearly different. These tests showed that two of them (D and E) were particularly interesting and their chemical structure was further

investigated. Because of the close resemblance of these compounds to syringafactins (Berti et al. 2007), they were renamed cichofactins A and B. Their molecular masses are 1109 Da and 1137 Da, respectively. Their similar bioactivity and their molecular weight difference of 28 Da that could correspond to two methylene groups, suggest these compounds are homologs, only differing in the length of their fatty acid moieties. This relationship has been found with several other *Pseudomonas* lipopeptides homologs such as several syringopeptins (Ballio et al. 1991; Isogai et al. 1995; Grgurina et al. 2002; Grgurina et al. 2005). Up till now cichofactins and syringafactins (Berti et al. 2007) are the only members of the syringafactin family of linear *Pseudomonas*-produced lipopeptides. It has been suggested that the syringafactin NRPS system in *P. syringae* evolved from the arthrofactin (a cyclic lipoundecapeptide produced by *Pseudomonas* sp. MIS38) system, after which three modules of the arthrofactin NRPS were deleted, including the module that incorporates the threonyl residue that forms the ester linkage involved in cyclization. This may explain why syringafactins (and cichofactins) are linear (Berti et al. 2007; Roongsawang et al. 2011).

The genes encoding two non-ribosomal peptide synthethases, *cifA* and *cifB*, were identified in the genome of *P. cichorii* SF1-54 as responsible for cichofactin biosynthesis. The putative peptide sequence of cichofactins predicted by *in silico* analysis of A-domain correlates perfectly with the peptide sequence determined by mass spectrometry. In the flanking regions of cichofactin biosynthesis clusters, genes coding for ABC transporter systems and LuxR-type regulators have been found (Fig. 3) as previously identified in the lipopeptide biosynthesis genes of many *Pseudomonas* strains (Quigley et al. 1993; Roongsawang et al. 2003; Kang and Gross, 2005; Berti et al. 2007; de Bruijn et al. 2008; Dubern et al. 2008; de Bruijn and Raaijmakers, 2009; Vallet-Gely et al. 2010). In *P. syringae pv. tomato* DC3000, only the LuxR-type

transcriptional regulator upstream of the syringafactin biosynthesis genes appeared to be essential, whereas a mutation of the LuxR-type regulator located downstream did not affect the production of that compound (Berti et al. 2007).

Several biological roles for lipopeptides have been proposed, including their function in motility, antimicrobial activity, biofilm formation, and pathogenicity (Raaijmakers et al. 2006; D'aes et al. 2010; Raaijmakers et al. 2010). Little is known about functions of the lipopeptides belonging to the syringafactin family except for the involvement in swarming motility (Berti et al. 2007; Burch et al. 2010; Burch et al. 2012). To clarify the functions of cichofactins in *P. cichorii* SF1-54, a cichofactin-deficient mutant was constructed. This mutant does not produce cichofactins anymore, but surprisingly, an enhanced production of the phytotoxic compounds G and F was observed (Fig. 4). A similar phenomenon has been observed in *Bacillus subtilis* 168 derivative strains which are able to synthesize lipopeptides after integration of a functional *sfp* gene (Coutte et al. 2010). Disruption of the plipastatin operon enhanced surfactin production. The redirection of common precursors, which needs ATP and cofactors from the disrupted biosynthetic pathway to the other intact biosynthetic pathway of a lipopeptide, may explain this phenomenon (Coutte et al. 2010).

Cichofactins were shown to be essential for surface motility in all *P. cichorii* isolates tested. This role has been established for many surfactants produced by pseudomonads such as syringafactin, entolysin, massetolide, viscosin, putisolvin, and arthrofactin (Roongsawang et al. 2003; Kuiper et al. 2004; Berti et al. 2007; de Bruijn et al. 2007; de Bruijn et al. 2008; Kruijt et al. 2009; Vallet-Gely et al. 2010). Furthermore, microtiter plate assays showed that the cichofactin-deficient mutant formed significantly more biofilm than the wildtype strain *P. cichorii* SF1-54 (Fig. 5B), indicating that cichofactins were involved in surface motility of *P.*

cichorii SF1-54. Several lipopeptides of *Pseudomonas* have been described to influence biofilm formation, but their role in biofilm formation differs considerably. For example, viscosin, massetolide and CLP1 (sessilin) produced by *P. fluorescens* strain SBW25, SS101, and *Pseudomonas* CMR12a, respectively, (de Bruijn et al. 2007; de Bruijn et al. 2008; D'aes et al. 2010) influence biofilm formation positively, while putisolvins, arthrofactin and CLP2 (orfamide) produced by *P. putida*, *Pseudomonas* sp. MIS38 and *Pseudomonas* CMR12a, respectively, (Roongsawang et al. 2003; Kuiper et al. 2004; D'aes et al. 2010) negatively affect biofilm formation.

Cichofactins are also involved in pathogenicity of *P. cichorii* SF1-54 on lettuce but are not phytotoxic per se. Lettuce plants spray-inoculated with the cichofactin-deficient mutant of P. cichorii SF1-54 showed significantly less rotten midribs compared to lettuces infected with the wildtype strain (Fig. 5C). However, upon injection, the wildtype and the cichofactin-deficient mutant displayed similar symptoms and reached similar population densities in the injected area (Fig. S3). It is suggested that this resulted from the production of the phytotoxic compounds F and G, which was highly enhanced in the cichofactin-deficient mutant (Fig. 4). Interestingly, when cichofactin production of *P. cichorii* was abolished, the mutant strain spread in lettuce midribs at a significantly slower rate than the wildtype (Table S2). These data suggest that cichofactins are important for the *in planta* spread of *P. cichorii*. Both cichofactins A and B were detected in infected plant tissue (Fig. 6), indicating their importance in infection/pathogenesis of lettuce leaves by *P. cichorii*. In contrast, the production of the other compounds in infected leaf tissues by *P. cichorii* SF1-54 remains unclear. They may be produced in very low amounts compared to cichofactins and thus in insufficient amounts for adequate detection using HPLC-ESI-MS. This latter hypothesis should not be surprising since syringomycins were detected in

infected leaf tissue only in minute amounts by repeated fractionations using HPLC (Grgurina et al. 2002). Another possibility is that they are produced at earlier time points and degrade to low amounts or interact with the plasma membrane or other components of the plant cell (Fogliano et al. 1999; Henry et al. 2011) which may thwart chemical detection.

Interestingly, *P. cichorii* isolates that have low virulence on lettuce produce less cichofactin than more pathogenic isolates (Fig. 7B). It remains to be investigated whether these isolates also differ in their production of the phytotoxic compounds F and G.

In conclusion, this study showed that *P. cichorii* SF1-54 produces seven bioactive compounds with lipopeptide characteristics, and two new lipopeptides which we focused on were identified as isomers and named cichofactins. Our results provide evidence that cichofactins are responsible for swarming motility, and are involved in virulence of *P. cichorii* SF1-54 on lettuce. We are currently investigating the role of compounds F and G in virulence and bioactivity of *P. cichorii*.

MATERIALS AND METHODS

Microorganisms and culture conditions.

Microorganisms used in this study are listed in Table 3. *P. cichorii* isolates and mutants, and *Escherichia coli* strains were kept at −80°C in Luria-Bertani 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. *Bacillus* and *Rhodotorula* strains were maintained at −80°C in LB broth with 20% glycerol, and *G. candidum* on potato dextrose agar (PDA, Difco, Erembodegem, Belgium) slants. *Pseudomonas* strains were routinely grown on Pseudomonas Agar F (PAF,

Difco) or SRM_{AF} medium (Gross, 1985). *B. megaterium* and *E. coli* strains were grown on LB medium at 28°C and 37°C, respectively. *R. mucilaginosa* and *G. candidum* were cultured on PDA at 28°C. *Saccharomyces cerivisiae* was grown on yeast-extract-peptone-dextrose medium at 30°C (Shanks et al. 2006). Antibiotics were added to the growth media (if required) at the following concentrations: 100 µg/ml gentamycin and 10 µg/ml nalidixic acid.

Biological activity assays.

Biosurfactant production was tested using the drop collapse assay as described by Kuiper *et al*. (2004). Briefly, cells from a single colony, previously grown on PAF-medium for 24 h, were transferred by a toothpick and suspended into a droplet of 50 µl sterile water on a hydrophobic background, more specifically parafilm 'M' laboratory film. When crude/semi-purified culture filtrate or purified compound was tested, 5 µl of the material was added to a droplet of 50 µl H_2O and placed onto parafilm. The reduction in surface tension was visualized by observation of the spread of the droplet over the parafilm surface.

Biological activity was assayed with *P. cichorii* SF1-54 suspension [1.0 x 10^8 CFU/ml in 50 mM phosphate buffer (PB, pH 7.0)], crude culture filtrate, semi-purified culture filtrate and purified compounds.

Phytotoxic activity was tested on chicory or lettuce leaves by injection of 300 µl bacterial suspension or culture filtrate with a syringe through the abaxial surface. The infiltrated leaves were incubated at 100% humidity and 25°C for 24 h and appearance of necrotic lesions was scored at 24 h after injection. PB, SRM_{AF} or methanol were used as negative control treatment.

Antimicrobial activity was tested against *B. megaterium, G. candidum* and *R. pilimanae* using the protocol described by Schaad et al. (2001) with minor modifications. Briefly, a

bacterial suspension of *P. cichorii* SF1-54 (1×10^8 CFU/ml) was spotted on PDA plates in triplicate, allowing growth at 25°C for 5 days. The areas of colony growth were marked and the colonies 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. When culture filtrate or purified compound was tested, 50 µl or 10 µl respectively, was spotted onto PDA plates (for *G. candidum* and *R. pilimanae*) or LB plates (for *B. megaterium*) and followed by drying of the drops. The plate was then sprayed with a spore/cell suspension containing about 10^6 cells or spores/ml of the indicator organism. After 24-48 h incubation at 28°C, inhibition of the indicator organism was scored.

Lipopeptide production, extraction and purification.

P. cichorii strain SF1-54 was grown in 100 ml Erlenmeyer flasks containing 30 ml of SRM_{AF} medium. After incubation for 6 days at 25°C without shaking, the *P. cichorii* SF1-54 culture was centrifuged (10 000g, 10 min, Sigma 4K15, Sartorius, Göttingen, Germany) and the supernatant was filter sterilized through a 0.2 µm filter (Millipore, Sartorius, Göttingen, Germany) to obtain the crude culture filtrate. Before use, 100 µl of this crude culture filtrate was plated on PAF-medium in three replications to confirm its sterility. The crude culture filtrate was loaded on C_{18} solid-phase extraction cartridges (C18 SPE Maxi-Clean™ cartriges, Alltech, Grace, Lokeren, Belgium), washed with 5% methanol and lipopeptides were desorbed with 100 ml 100% methanol to obtain the semi-purified culture filtrate.

To purify the *P. cichorii* SF1-54 metabolites, 200 ml semi–purified culture filtrate was further concentrated using rotavaporation (Büchi Rotavapor R-2000, Flawil, Switzerland) and vacuum speed (Savant Speed-Vac Plus SC110A, Thermo Fisher Scientific, Bremen, Germany)

and separated with an Agilent 1100 series HPLC (Hewlett Packard, Diegem, Belgium) on a protein and peptide C18 Column (5 µm, 4.6 mm×250 mm, VYDAC). Metabolites 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 1 ml min^{-1} and at room temperature. UV signal was monitored at 220 nm. All different fractions were collected, concentrated using rotavaporation and vacuum speed, and used for bioactivity assays.

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

Amino acid analysis

For the amino acid analysis, HPLC-purified compounds were collected in glass tubes and dried using vacuum speed (Savant Speed-Vac Plus SC110A). The samples were hydrolysed at 115°C for 6 h with 1.5 ml of 6 N HCl. After drying in a speedvac (Savant Speed-Vac Plus SC110A) and dissolving in 100 µl 0.1 N HCl, the samples were analyzed on a ZORBAX Eclipse AAA column (Agilent, Diegem, Belgium) using a Agilent 1100 series HPLC (Hewlett Packard) by strictly following the instructions, provided by the Agilent manufacturer. Briefly, the hydrolysed samples and amino acid standard solutions 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 2 ml/min and detected at 338 nm (OPA) and 262nm (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.

LC-ESI-MS

Analysis of lipopeptides produced by *P. cichorii* was carried out on a LC-ESI-MS (reverse phase HPLC Waters Alliance 2695/diode array detector, in tandem with a Single Quad Waters SQD Mass spectrometer, Waters, Zellik, Belgium) on a 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 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 l/h; cone voltage: 100V. A similar setting was used for lipopeptide fragmentation, except the cone voltage which was 75V instead of 100V. Both instruments were controlled with the MassLynx software (Waters).

HR-ESI-MS-MS

All measurements were conducted at the mass spectrometry lab of the Institute of Organic Chemistry at the University of Cologne on a LTQ-Orbitrap XL instrument (Thermo Fisher Scientific, Bremen), with a conventional ESI ion source (ESI-voltage: 3.5 kV, capillary temperature: 275°C, flow rate: 5 µl/min). Acidified methanol/water solutions were used in micromolar concentrations. MS^n-product ion spectra are produced in a linear ion trap (LTQ part of the Orbitrap) or in an octopole collision cell. The precursor ions were selected and activated until extensive fragmentation occurred.

Identification and bioinformatic analysis of the NRPS genes.

Whole genome sequencing of *P. cichorii* SF1-54 was performed at Genoscreen, France, with a Roche 455 Gs-FLX sequencer. The draft genome was automatically annotated using RAST server (Aziz et al. 2008). Putative NRPS encoding regions were found by comparison of the genome sequence of *P. cichorii* SF1-54 with the genomes of *P. syringae* pv. *tomato* DC3000 and *P. syringae* pv. *syringae* B748a (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 PCR and Sanger sequencing of the PCR-product (Agowa sequencing, Germany). Open reading frames (ORF) were identified using the program Glimmer (Delcher et al. 1999), available on the NCBI website. The catalytic domains present in the NRPS genes were identified using the NRPS/PKS analysis website (Bachmann and Ravel, 2009) (http://nrps.igs.umaryland.edu/nrps) and the NRPS-PKS website (Ansari et al. 2004) (http://www.nii.res.in/nrps-pks.html). The specificity prediction of the A-domain was conducted by using the web-based software NRPSpredictor2 (Rausch et al. 2005; Rottig et al. 2011) (http://nrps.informatik.uni-tuebingen.de/). The D/L configurations of amino acid residues of nonribosomal peptide synthetases were investigated by phylogenetic analysis of the C-domains via Phylogeny.fr website (Dereeper et al. 2008). 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 EBI site (www.ebi.ac.uk).

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

A cichofactin-deficient mutant of *P. cichorii* SF1-54Nal^R was constructed by means of 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*cifAB*, primers NRPS14mut-F1 and NRPS14mut-R1 were used to amplify the upstream fragment, and primers NRPS14mut-F2 and NRPS14mut-R2 for the downstream fragment (Table 3). The cichofactin-deficient mutant contained a deletion of 9715 bp in the cichofactin encoding genes, *cifA* and *cifB*. Deletion of the NRPS-fragment was confirmed by PCR and the mutant was characterized phenotypically.

Biological roles of cichofactin in *P. cichorii*

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

Biofilm formation was tested in polystyrene 96-well plates based on protocols described by Merrit *et al*. (2005) and Maddula *et al*. (2006) with minor modifications. Briefly, strains were grown overnight in LB broth. The 96-well flat bottom polystyrene microtiter plates (Greiner bio-one, Wemmel, Belgium) containing 120 µl King's B broth (King et al. 1954) were inoculated using 100-fold dilutions of these culture, previously adjusted to OD_{620} 0.8. Plates were incubated at 21°C for 24 h without shaking and final cell densities were determined (OD_{620}). Subsequently, the medium was removed from the microtiter plates, rinsed with water, filled with 150 µl 0.1% crystal violet and incubated for 10 min at room temperature. Excess of stain was removed by inversion of the plate followed by two washings with tap water. When the plates were air-dried, adherent cells were decolorized with 95% ethanol solution for 10 min and transferred to new microtiter plates in order to measure the absorbance at 595 nm. The experiment was carried out three times with 7 repetitions per treatment. Statistical analysis was performed in SPSS 17.0

(SPSS Inc., Chicago, Illinois, USA) for Windows using non-parametric Mann-Whitney U tests (P = 0.05).

Pathogenicity of the cichofactin-deficient mutant and of the *P. cichorii* strain NCPBB907 was tested at Inagro, Rumbeke, Belgium, according to the methods described in a previous study (Pauwelyn et al. 2011). The strains were inoculated on butterhead lettuce plants cv. Flandria (Rijk Zwaan, the Netherlands) at head formation. Tap water without bacteria was used as the non-inoculated treatment. Each treatment was assayed in three replicates with 24 plants per replicate and the experiment was carried out twice. At harvest, disease severity was assessed by giving each plant a score ranging from 0 to 4, where 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. In addition, the number of rotten midribs on each plant was scored. Data were analyzed using non-parametric Kruskal-Wallis and Mann-Whitney comparisons ($P = 0.05$).

To test the ability of *P. cichorii* SF1-54-Δ*cifAB* to grow in lettuce midribs, the midrib of the inner lettuce leaves was injected with 0.2 ml bacterial suspension (1×10^5 CFU/ml). The injected leaves were incubated at 100% humidity at 25°C. The inoculated sections (2 cm^2, indicated as section A in Fig. S4) were excised from the midribs of three lettuce leaves at 0, 1, 2, and 3 days post inoculation and macerated in 2 ml sterile 0.05M potassium phosphate buffer (pH 7.0). Tenfold serial dilutions from the macerate were prepared with PB and 100 µl aliquots of each dilution were spread on three PAF plates with 50 µg/ml naladixic acid. Colonies were counted 2 days after incubation at 28°C to estimate population densities. For assessment of bacterial spread in midribs, the adjacent sections (2 cm^2, indicated as section B in Fig. S4) were also excised from the midribs 3 days post inoculation to estimate population densities as

described above. The experiment was repeated twice using three leaves from three different plants per treatment.

Assays for bacterial attachment on lettuce leaves were performed according to the method of Kroupitski et al. (2011) with slight modification. Five leaf discs from different regions (near petiole, center, and near blade as shown in Fig. S4) from five different butterhead lettuce leaves were cut using an aseptic cork borer (1.4-cm diameter) and the entire experiment was repeated once. The leaf discs were immersed in 10-ml of a bacterial suspension in a 50-ml falcon tube and incubated at 25°C for 2 h without shaking. The leaf discs were washed twice for 1 min in 10-ml sterile water and macerated in 1 ml sterile 0.05M potassium phosphate buffer (pH 7.0) for estimation of bacterial population sizes as described before.

All data were statistically analysed using the software package SPSS 17.0 for Windows. As the data did not meet the conditions of normality and homogeneity of variance, non-parametric Kruskal-Wallis and Mann-Whitney comparisons ($P = 0.05$) were performed.

In planta detection of lipopeptides produced by _P. cichorii_ SF1-54.

A bacterial suspension of *P. cichorii* strain SF1-54 (1×10^6 CFU/ml) was inoculated in the midrib of lettuce leaves. 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 incubated at room temperature for 5 days. At 1, 2, 3, 4 and 5 days post inoculation, symptomatic tissue was excised from the lettuce leaves and ground in liquid nitrogen. After thawing, 10 ml of grinded symptomatic leaf tissue was suspended in 40 ml of 95% acetonitrile acidified with 0.1% formic acid, shaken for 1 h at room temperature, and then centrifuged (5 min at 10 000 rpm, Beckman avanti J-25i). The pellet was dissolved again in

10 ml 95% of acetonitrile acidified with 0.1% formic acid and centrifuged. Supernatants of both extractions were mixed, concentrated five times using rotavaporation, and analysed with LC-ESI-MS.

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AUTHOR CONTRIBUTION

E.P. and C.H.J. carried out most of the experiments and wrote the manuscript. M.O., H.B., and M.S. did the chemical characterizations; V.L. and P.J. helped with the bioinformatics analysis; P.B. coordinated the greenhouse experiments; M.H. and M.O. were involved in the design of the experiments and data analysis and revised the manuscript critically. All authors have approved the final version.

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Table 1. Listing of b-and y"-ions obtained from the $[M+H]^+$ species of compounds D and E formed in the LC-ESI-MS analysis. Numbers in parentheses are calculated values not observed in the mass spectra.

	b ions		y" ions	
	Compound D	Compound E	Compound D	Compound E
FA	(171)	(199)		
Leu	284	312	(939)	(939)
Leu	397	425	(826)	(826)
Gln	525	553	713	713
Leu	638	666	585	585
Gln	766	794	472	472
Val	865	893	344	344
Leu	978	1006	245	245
Leu	1091	1119	(132)	(132)
$[M+H]^+$	1109	1137		

Table 2. Listing of fragment ions (Figure S2) and of y"-ions obtained from the $[M+Na]^+$ species of compounds D and E formed in the HR-ESI-MS-MS analysis. Numbers in parentheses are calculated values not observed in the mass spectra.

	Fragment ions				y" ions	
	Compound D		Compound E		Compound D	Compound E
FA	(211)[a]	(165)	(239)	(193)		
Leu	324	(278)	(352)	(306)	(961)	(961)
Leu	437	391	465	419	848	848
Gln	565	519	593	547	735	735
Leu	678	632	706	660	607	607
Gln	806	760	834	788	494	494
Val	905	859	933	887	(366)	(366)
Leu	1018	972	1046	1000	(267)	(267)
Leu ($[M+H]^+$)	1131		1159		(154)	(154)

Table 3. Strains, plasmids and primers used in this study

Strain, plasmid or primer	Genotype or description [a]	Source/Reference
Strains		
Pseudomonas cichorii		
SF1-54, SF0068-02, SF0075-01, SF0057-3A1, AF0089-01, LMG8401, SF0125-01	Natural isolates from infected greenhouse butterhead lettuce in Belgium	Cottyn et al. 2011
9D42	Isolate from lettuce with varnish spot disease in California	Grogan et al. 1977
SPC9018	Isolate from lettuce with tar disease in Japan	Kiba et al. 2006
NCPPB 943^{T}	Type strain isolated from *Cichorium endivia* in Germany (=LMG2162^{T})	Cottyn et al. 2009
NCPPB 907	Isolate from *Chrysanthmum manifolium* in USA	Yamamoto et al. 2000
SF1-54NalR	Spontaneous nalidixic acid-resistant mutant of SF1-54	This study
SF1-54-ΔcifAB	Cichofactin deficient-mutant of SF1-54 NalR	This study
Escherichia coli S17-1	*thi pro hsd*R *rec*A::RP4-2-Tc^{R}::Mu Km^{R}::Tn7(λ*pir*), Sm^{R}	Simon et al. 1983
Bacillus megaterium LMG 7127	An indicator strain for lipopeptide	
Rhodolotula mucilaginosa MUCL 30397	An indicator strain for lipopeptide	
Geotrichum candidum MUCL 28959	An indicator strain for lipopeptide	
Saccharomyces cerevisiae	*MAT*a/*MAT*α *leu*2/*leu*2 *trp*1-289/*trp*1-289 *ura*3-52/*ura*3-52 *his*3-Δ1/*his*3-Δ1	Invitrogen

InvSc1		
Plasmids		
pMQ30	7.6 kb mobilizable suicide vector used for gene replacements in *Pseudomonas* spp.; *Sac*B, URA3, Gm^R	(Shanks et al., 2006)
pMQ30-*cifAB*	pMQ30 containing two ca. 1 kb fragments of the cichofactin biosynthesis genes to obtain a 9.7 kb deletion in these genes	This study
Primers (5'→3')		
NRPS14mut-F1	*GGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTG*AGGTGCTG GTGGACTTCAAT	This study
NRPS14mut-R1	*ATCGAAACTGACAGGCGTCT* GTACCTGACCCGAGGTAGCA	This study
NRPS14mut-F2	*TGCTACCTCGGGTCAGGTAC* AGACGCCTGTCAGTTTCGAT	This study
NRPS14mut-R2	*CCAGGCAAATTCTGTTTTATCAGACCGCTTCTGCGTTCTGAT*TGTGTTTCT GTCTCGCCTTG	This study

[a] Primer extensions are in italic font.

FIGURE LEGENDS

Figure 1. Necrosis induction in chicory and lettuce leaves at 24 hours after inoculation with **A**, *P. cichorii* SF1-54 (1×10^8 CFU/ml), **B,** crude culture filtrate of *P. cichorii* SF1-54, and **C,** non-inoculated SRM_{AF} medium (the un-inoculated control treatment). Each treatment was repeated 3 times, and a picture representative of each of the treatments is shown.

Figure 2. **A,** Reverse-phase-HPLC chromatogram of the semi-purified extract of *P. cichorii* SF1-54. All (bio)active peaks are indicated with a letter. **B,** Antimicrobial and phytotoxic activity of each bioactive fraction separated by reverse-phase-HPLC. Antimicrobial activity against *R. mucilaginosa* (Rm), *G. candidum* (Gc), and *B. megaterium* (Bm) and phytotoxicity to chicory (PT) of each fraction was assayed.

Figure 3. Schematic representation of the cichofactin biosynthetic gene clusters and the flanking open reading frames in the *P. cichorii* SF1-54 genome. The module and domain organization are indicated below *cifA* and *cifB* genes. Predicted amino acids specificity is represented below each module and all amino acids are identified by standard three-letter biochemical notation. The region between arrowheads was deleted in the cichofactin-deficient mutant.

Figure 4. LC-ESI-MS analysis of culture supernatants of *P. cichorii* SF1-54 and its cichofactin-deficient mutant.

Figure 5. Biological roles of cichofactins in *P. cichorii* SF1-54. **A,** Swarming behavior of *P. cichorii* SF1-54 and its cichofactin-deficient mutant on LB soft agar (0.5% w/v) plates. *P.*

cichorii SF1-54 covered the entire plate within 48 hours after inoculation (left panel), while its cichofactin-deficient mutant was impaired in motility (right panel). **B,** Biofilm formation of *P. cichorii* SF 1-54Nal^{R} and its cichofactin-deficient mutant. Spectrophotometric quantification of the biofilm formed by *P. cichorii* SF1-54Nal^{R} and its cichofactin-deficient mutant (*P. cichorii* SF1-54-Δ*cifAB*) 24 h after inoculation; the quantity of crystal violet stained biofilm was measured at 595 nm. The experiment (7 repetitions per treatment) was carried out 3 times and similar results were obtained each time. Representative data from one experiments are presented. Bars indicated with the same letter are not statistically different based on non-parametric Kruskal-Wallis and Mann-Whitney comparisons ($P < 0.05$). **C,** Virulence of *P. cichorii* SF1-54NalR and its cichofactin-deficient mutant (SF1-54-Δ*cifAB*) on greenhouse grown butterhead lettuce. Plants were inoculated with suspensions of *P. cichorii* strains (1×10^{6} CFU/ml) after head formation of lettuce. Virulence is represented as disease severity (left panel) and mean number of diseased midribs (right panel). Tap water was used as the un-inoculated control. The experiment was carried out twice with similar results. Data from one experiment are shown. Bars indicated with the same letter are not statistically different based on non-parametric Kruskal-Wallis and Mann-Whitney comparisons ($P < 0.05$).

Figure 6. Detection of *P. cichorii* SF1-54 lipopeptides in **A**, semi-purified culture filtrate of *P. cichorii* SF1-54; **B,** extract of lettuce tissue infected with *P. cichorii* SF1-54, and **C,** extract of non-inoculated lettuce tissue. Lipopeptides were extracted and analysed 5 days post inoculation.

Figure 7. **A,** Swarming behavior of *P. cichorii* isolates of different groups as defined by Cottyn et al. (2009) on LB soft agar (0.5% w/v) plates. Results were photographed 24 hours after

incubation. See Table 3 for strain details and Table S5 for pathogenicity data. **B,** Quantification of cichofactin production by *P. cichorii* isolates of different groups on LB soft agar (0.5% w/v) plates. After 24-h incubation, the bacterial cells on swarm plates were scraped and suspended in 100% acetonitrile, and the OD_{620} nm of each sample was measured as the indicator of biomass. Cichofactin production of each sample was measured by HPLC-ESI-MS by using single ion recording integration of peaks corresponding to the exact mass of A and B homologues. Data are expressed as relative production based on the peak area per OD_{620}. Vertical bars indicate standard deviations. For each isolate, two plates were analyzed. No production of cichofactins was detected in the type strain of *P. cichorii* NCPPB 943T.

Figure 1. Necrosis induction in chicory and lettuce leaves at 24 hours after inoculation with A, P. cichorii SF1-54 (1×108 CFU/ml), B, crude culture filtrate of P. cichorii SF1-54, and C, non-inoculated SRMAF medium (the un-inoculated control treatment). Each treatment was repeated three times, and a picture representative of each of the treatments is shown.

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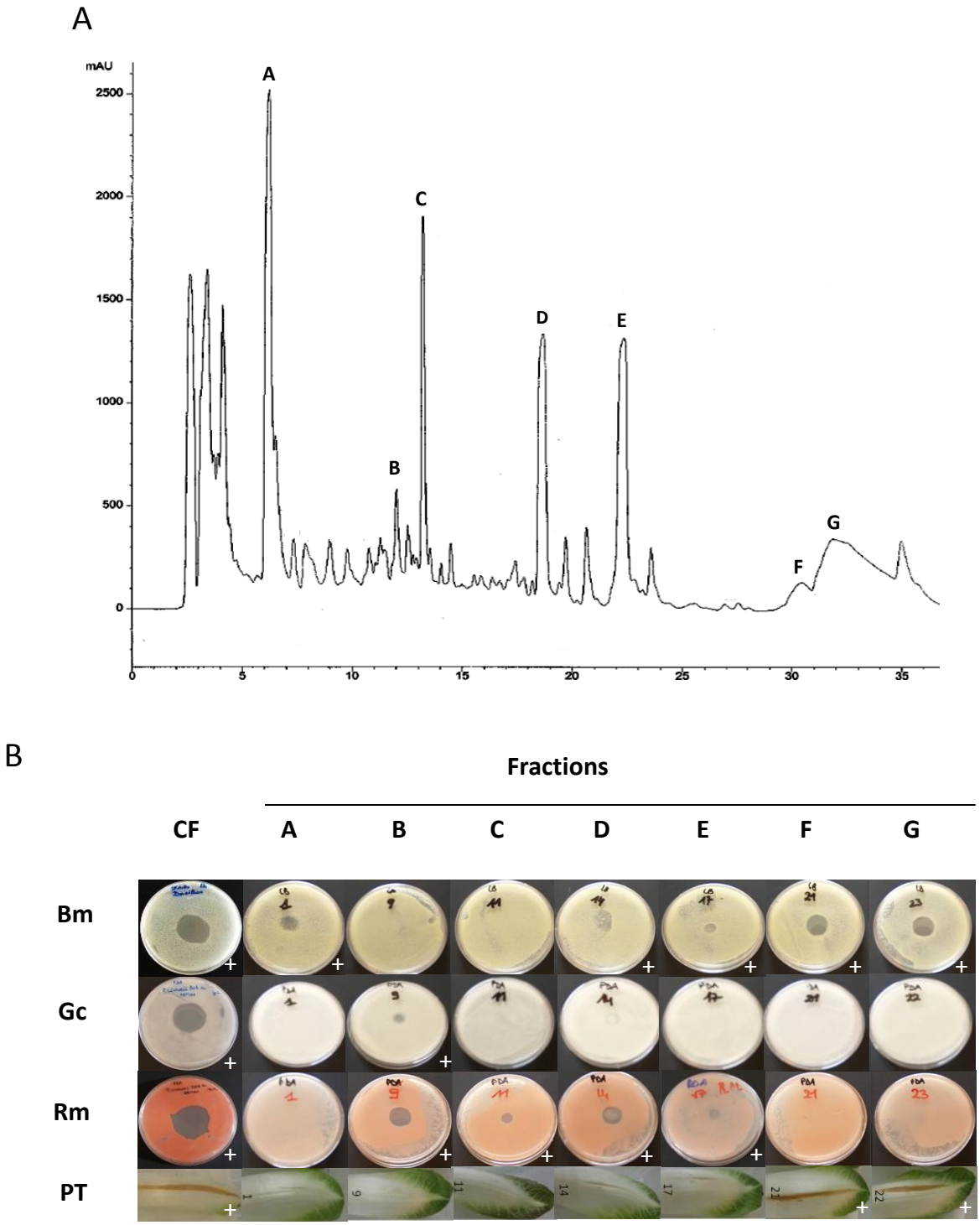

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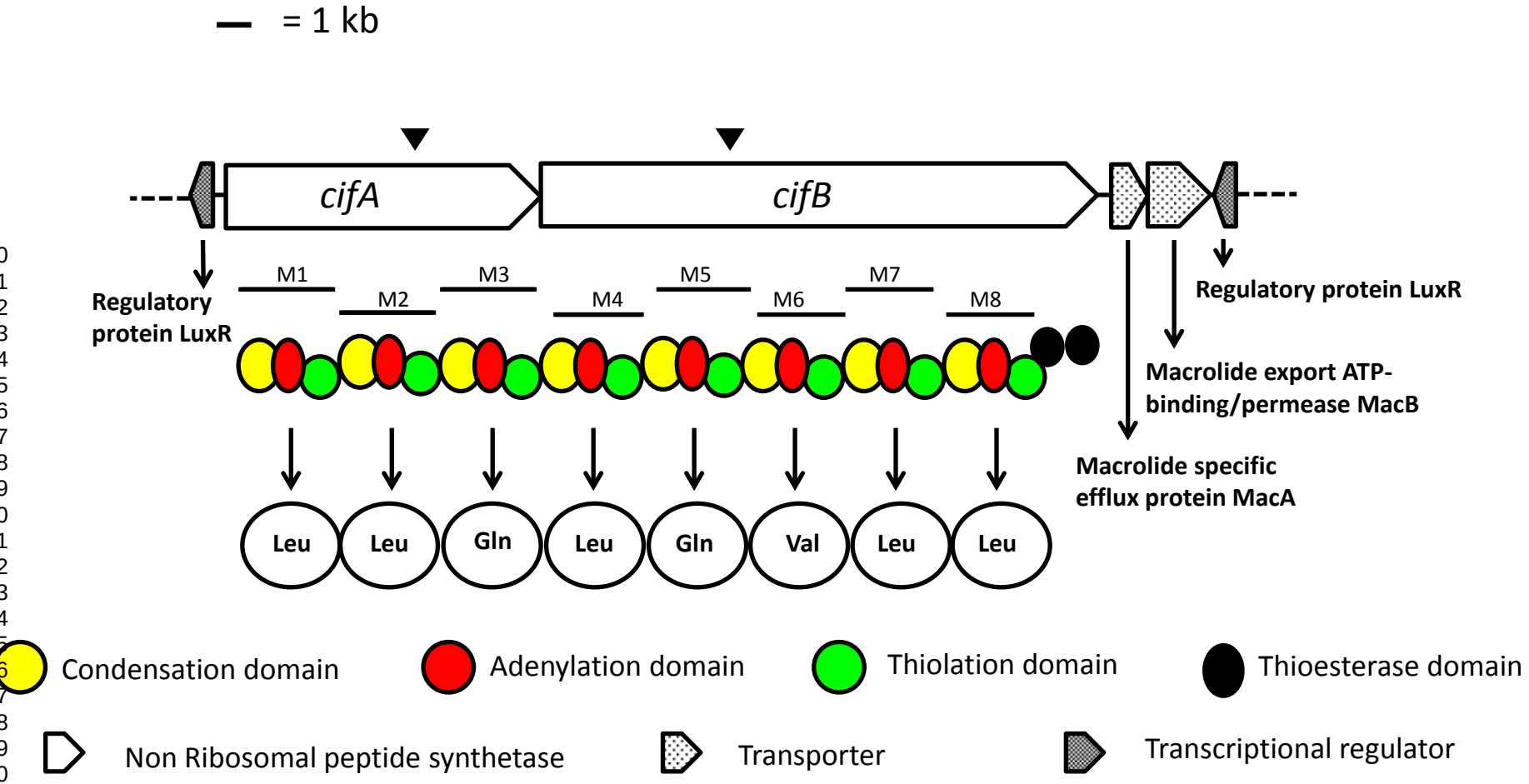

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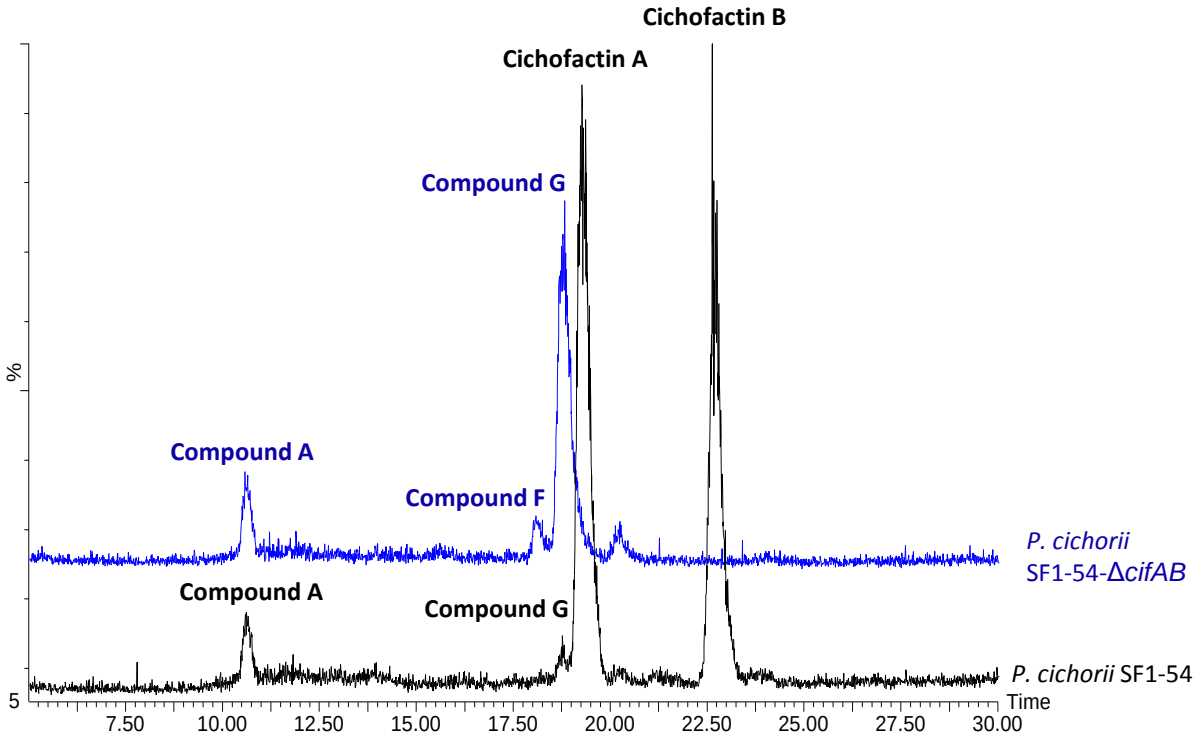

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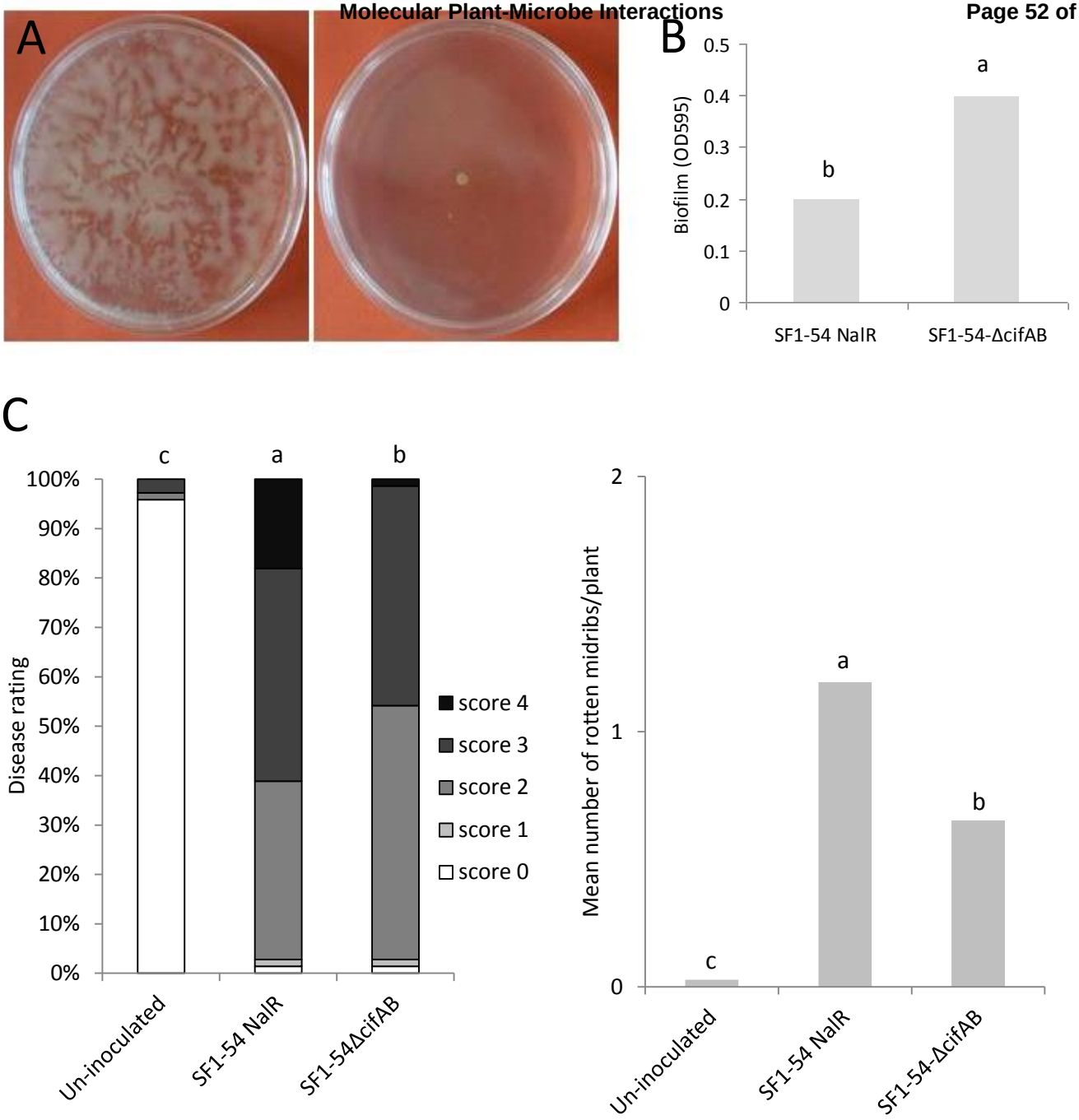

Figure 5. Biological roles of cichofactins in *P. cichorii* SF1-54. **A,** Swarming behavior of *P. cichorii* SF1-54 and its cichofactin-deficient mutant on LB soft agar (0.5% w/v) plates. *P. cichorii* SF1-54 covered the entire plate within 48 hours after inoculation (left panel), while its cichofactin-deficient mutant was impaired in motility (right panel). **B,** Biofilm formation of *P. cichorii* SF 1-54Nal^R and its cichofactin-deficient mutant. Spectrophotometric quantification of the biofilm formed by *P. cichorii* SF1-54Nal^R and its cichofactin-deficient mutant (*P. cichorii* SF1-54-Δ*cifAB*) 24 h after inoculation; the quantity of crystal violet stained biofilm was measured at 595 nm. The experiment (7 repetitions per treatment) was carried out 3 times and similar results were obtained each time. Representative data from one experiments are presented. Bars indicated with the same letter are not statistically different based on non-parametric Kruskal-Wallis and Mann-Whitney comparisons ($P < 0.05$). **C,** Virulence of *P. cichorii* SF1-54NalR and its cichofactin-deficient mutant (SF1-54-Δ*cifAB*) on greenhouse grown butterhead lettuce. Plants were inoculated with suspensions of *P. cichorii* strains (1×10^6 CFU/ml) after head formation of lettuce. Virulence is represented as disease severity (left panel) and mean number of diseased midribs (right panel). Tap water was used as the un-inoculated control. The experiment was carried out twice with similar results. Data from one experiment are shown. Bars indicated with the same letter are not statistically different based on non-parametric Kruskal-Wallis and Mann-Whitney comparisons ($P < 0.05$).

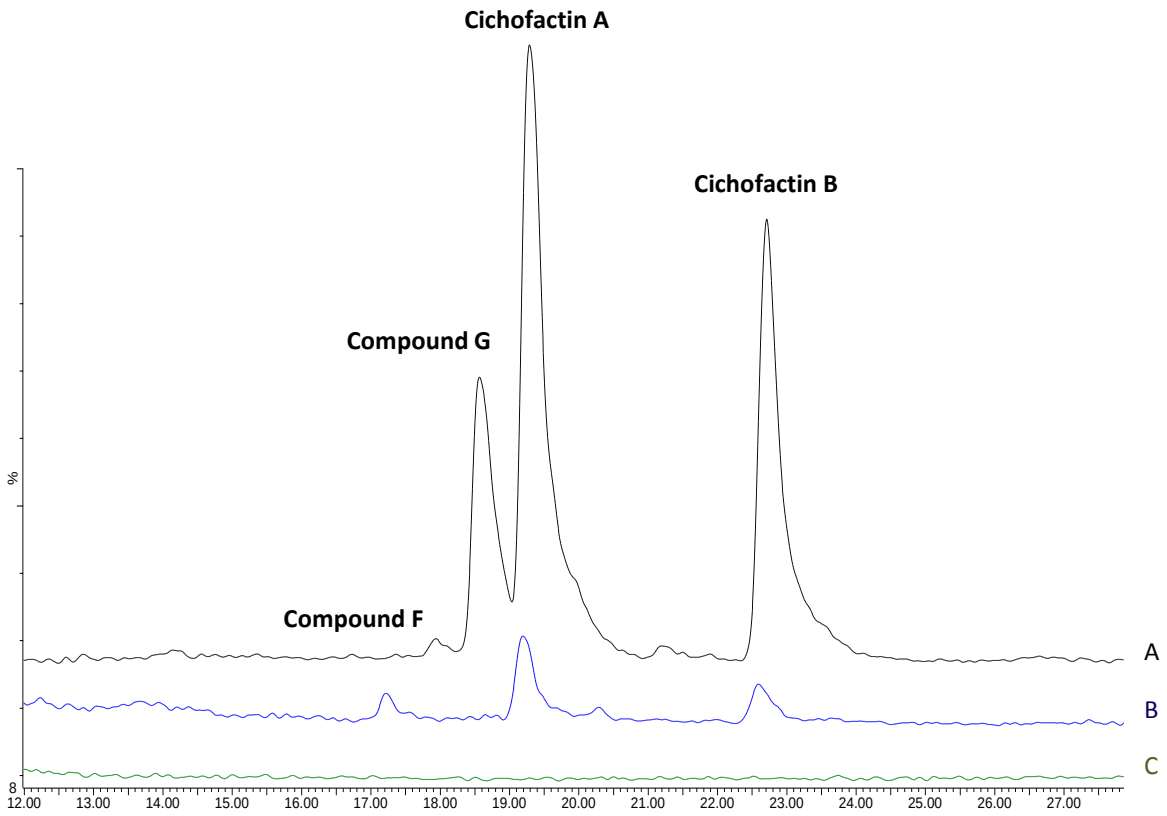

Figure 6. Detection of *P. cichorii* SF1-54 lipopeptides in **A**, semi-purified culture filtrate of *P. cichorii* SF1-54; **B,** extract of lettuce tissue infected with *P. cichorii* SF1-54, and **C,** extract of non-inoculated lettuce tissue. Lipopeptides were extracted and analysed 5 days post inoculation.

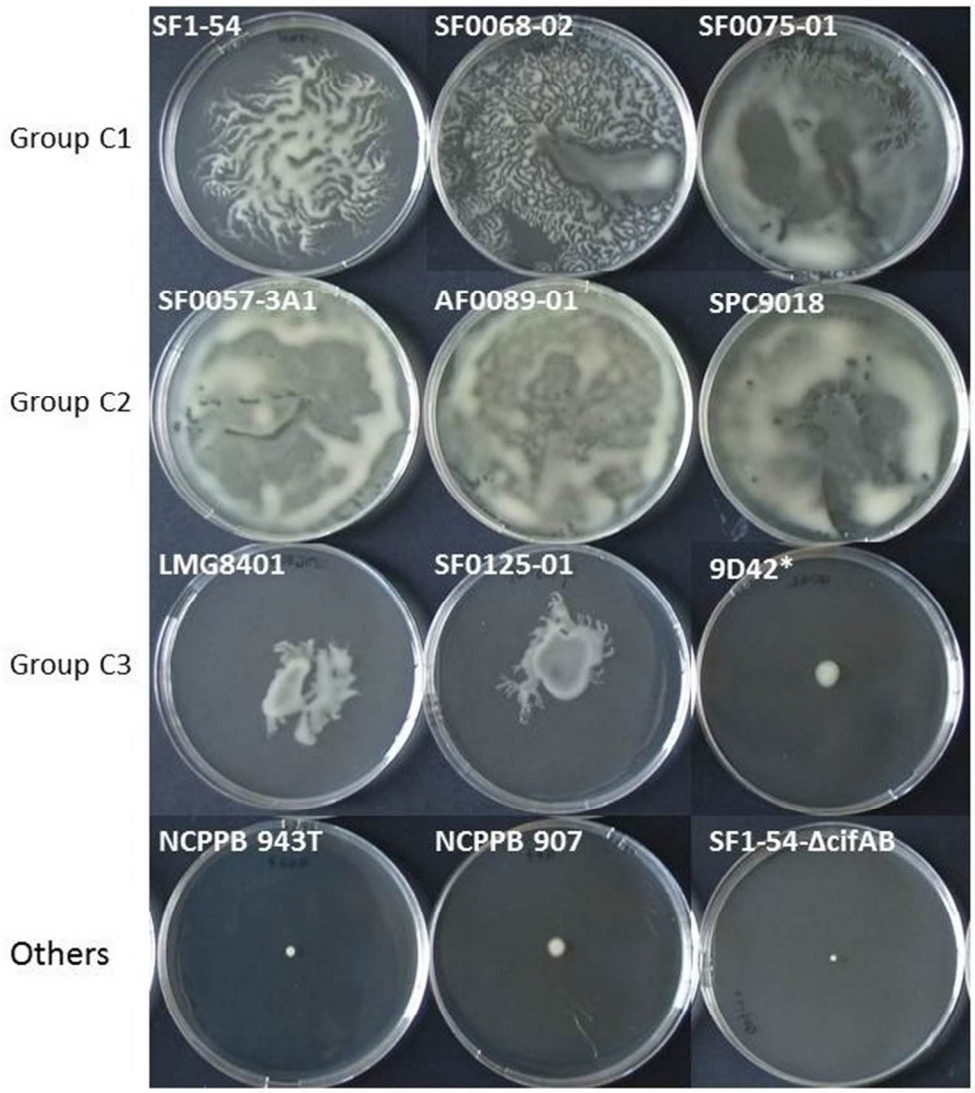

Figure 7. A, Swarming behavior of P. cichorii isolates of different groups as defined by Cottyn et al. (2009) on LB soft agar (0.5% w/v) plates. Results were photographed 24 hours after incubation. See Table 3 for strain details and Table S5 for pathogenicity data.
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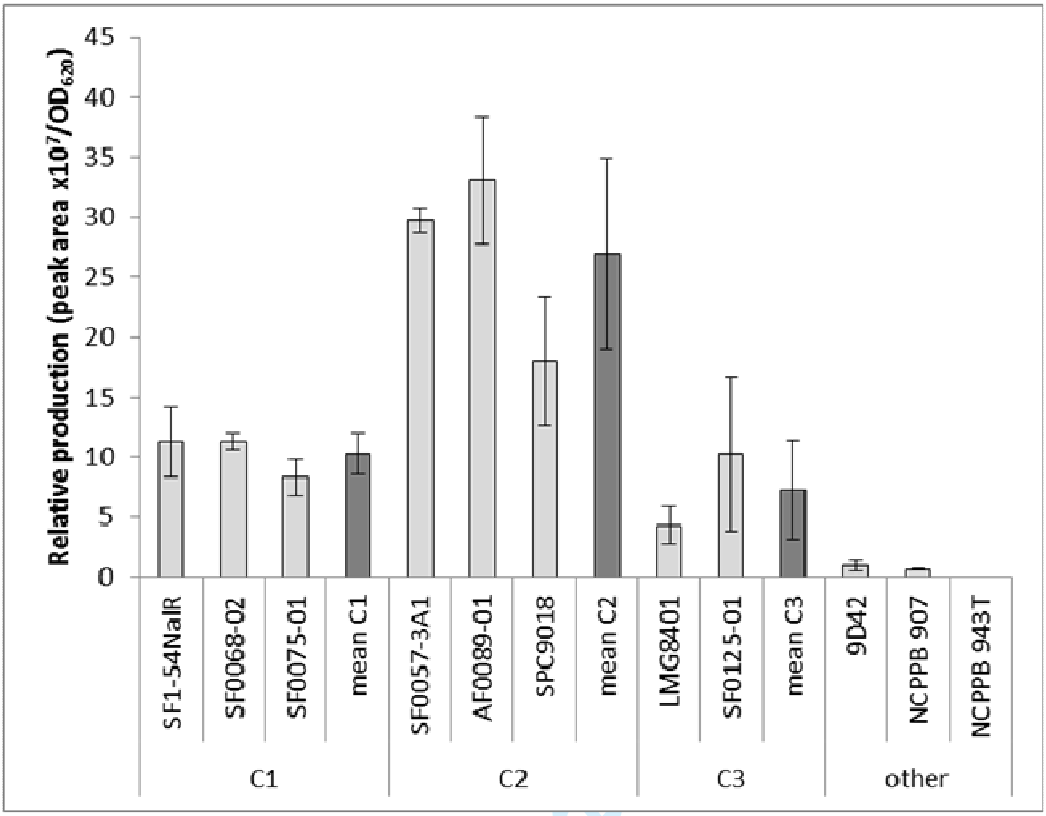


Figure 7. B, Quantification of cichofactin production by *P. cichorii* isolates of different groups on LB soft agar (0.5% w/v) plates. After 24-h incubation, the bacterial cells on swarm plates were scraped and suspended in 100% acetonitrile, and the $OD_{620\ nm}$ of each sample was measured as the indicator of biomass. Cichofactin production of each sample was measured by HPLC-ESI-MS by using single ion recording integration of peaks corresponding to the exact mass of A and B homologues. Data are expressed as relative production based on the peak area per OD_{620}. Vertical bars indicate standard deviations. For each isolate, two plates were analyzed. No production of cichofactins was detected in the type strain of *P. cichorii* NCPPB 943^{T}.

Table S1. Analysis of amino acid specificity code of each A-domain of cichofactin NRPS

Module of cichofactin NRPS [a]	Specificity code [b]	Highest homology with[c]	Predicted amino acid
M1	DAWFLGNVVK DAWFLGNVVK	SRF2_BACSU_m3	Leu
M2	DAWFLGNVVK DAWFLGNVVK	SRF2_BACSU_m3	Leu
M3	DAWQVGVVDK DAWQFGLIDK DSWKLGVVDK	Q9K5M2_m1 Q84BQ6_m2	Gln Asp
M4	DAWFLGNVVK DAWFLGNVVK	SRF2_BACSU_m3	Leu
M5	DAWQVGVVDK DAWQFGLIDK DSWKLGVVDK	Q9K5M2_m1 Q84BQ6_m2	Gln Asp
M6	DAMFMGGTFK DALWIGGTFK	Q9FDB3_m4	Val
M7	DAWFLGNVVK DAWFLGNVVK	SRF2_BACSU_m3	Leu
M8	DAWFLGNVVK DAWFLGNVVK	SRF2_BACSU_m3	Leu

[a] Module number of cichofactin NRPS of *P. cichorii* SF1-54

[b]Specificity code of the adenylation domain of the NRPS modules constitute the amino acid binding pocket (Stachelhaus et al., 1999; Challis et al., 2000). The most homologues specificity codes are grouped together. The specificity code was identified using the web-based software NRPSpredictor2 (Rausch et al., 2005; Rottig et al., 2011).

[c] UniprotKB query_module number

Table S2. Spread of *P. cichorii* SF1-54Nal^R and its cichofactin-deficient mutant SF1-54Δ*cifAB* in adjacent midrib sections or attachment on leaf discs of butterhead lettuce

	Population size (log CFU/cm^2)				
	2-cm midrib sections [a]		leaf discs of different regions [b]		
Strain	0 - 2	2 - 4	Petiole	Center	Blade
SF1-54 Nal^R	7.72 (0.67)	4.85 (0.48) a	6.96 (0.06) a	7.06 (0.05) a	6.84 (0.13)
SF1-54Δ*cifAB*	7.72 (0.44)	3.34 (0.66) b	6.82 (0.08) b	6.96 (0.04) b	6.85 (0.12)

[a]The bacterial suspensions were injected into midrib of butterhead lettuce near petiole. The first 2 cm section (0 - 2) was the inoculation point (also shown in Fig. S4). Bacterial population sizes were estimated at 3 days post inoculation. Values represent log transformed means and standard deviation (in parentheses) from 9 leaves in three experiments.

[b]Leaf discs of three different regions (near petiole, center, and near blade, see Fig. S4) were used for attachment assay. Values represent log transformed means and standard deviation (in parentheses) from ten leaves in two experiments.

Different letters indicate statistically significant differences between treatments by non-parametric Kruskal-Wallis and Mann-Whitney comparisons ($P < 0.05$).

Table S3. Virulence of different *Pseudomonas cichorii* isolates on butterhead lettuce

Strain	Subgroup[a]	Disease severity on lettuce[b]
SF1-54	C1	3.67
SF0068-02	C1	4.00
SF0075-01	C1	4.00
SF0057-3A1	C2	3.25
AF0089-01	C2	4.00
SPC9018	C2	4.00
LMG8401	C3	2.50
SF0125-01	C3	2.50
9D42	Related to C3	1.00
NCPPB907	other	0.00
NCPPB943^T	other	1.00

[a]Subgroup based on morphology and BOX-PCR according to Cottyn et al. (2011) and Pauwelyn et al. (2011)

[b]Disease severity expressed as mean disease score; data from Pauwelyn et al. (2011) except for NCPPB907 and NCPPB943^T that were tested in this study.

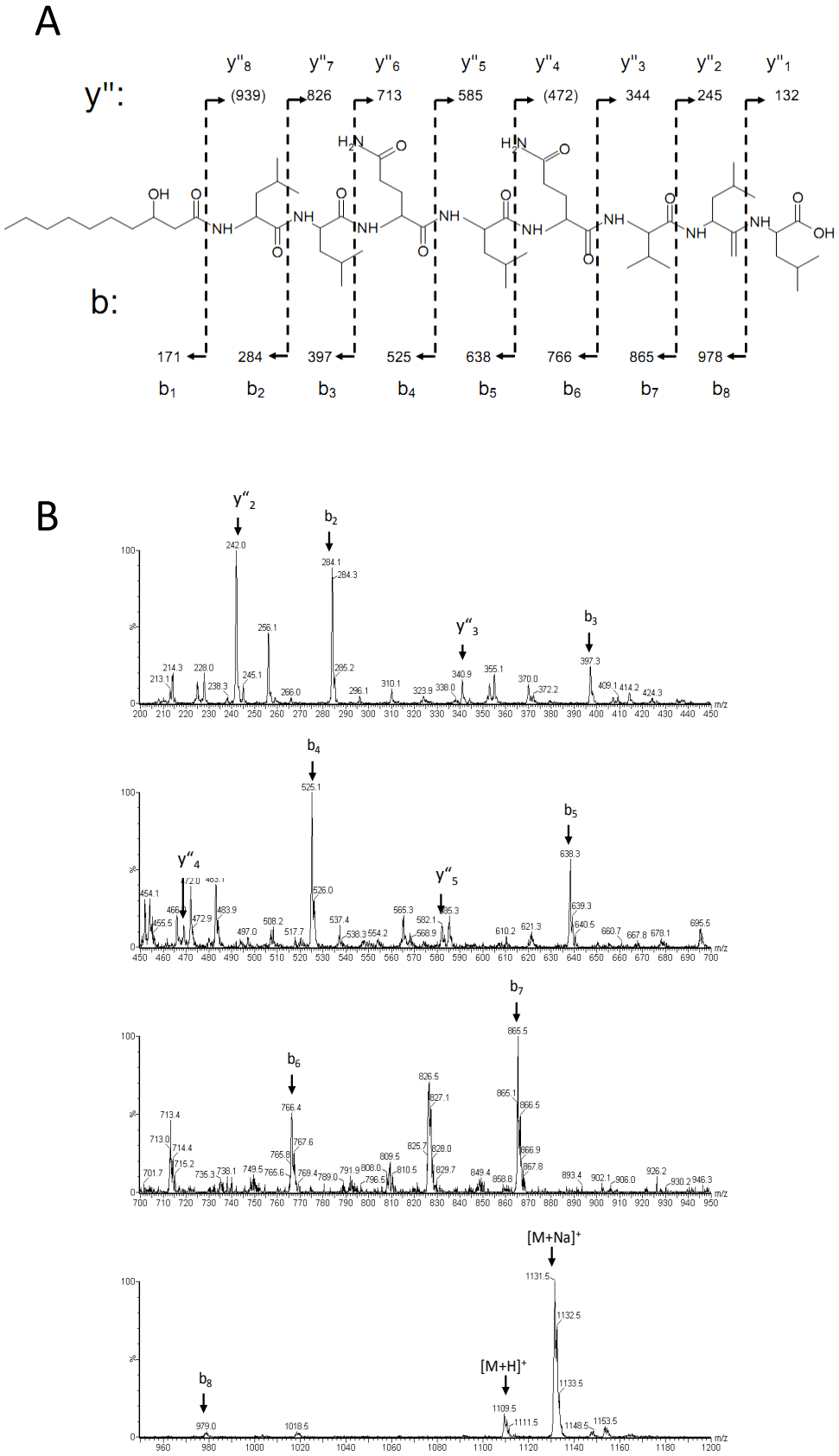
Supplemental figure legends

Figure S1. A, Proposed structure for compound D based on LC-ESI-MS in-source fragmentation spectrum of its $[M+H]^+$ ion. The sites of cleavage, yielding b- and y-ions are represented and the masses of b- and y-ion fragments are indicated. The masses between brackets are expected upon calculation. **B,** The ion-masses obtained from LC-ESI-MS-MS fragmentation spectrum of the $[M+H]^+$ form of compound D.

Figure S2. Scheme of the b-ions formed upon collision activation of the $[M+Na]^+$ species by rearrangement processes.

Figure S3. Population sizes of *P. cichorii* SF1-54Nal^R and its cichofactin-deficient mutant SF1-54-Δ*cifAB* in lettuce midribs. Values represent the mean ± SD of three independent experiments.

Figure S4. Illustration of a butterhead lettuce leaf used for assays of bacterial growth *in planta* or attachment to leaf surface. The populations of *P. cichorii* strains in the midrib sections were estimated. The midrib was injected with a bacterial suspension in section A. For attachment assays, leaf discs were taken from three regions: (I) near the edge of the blade; (II) at the center of the leaf; (III) near the petiole.



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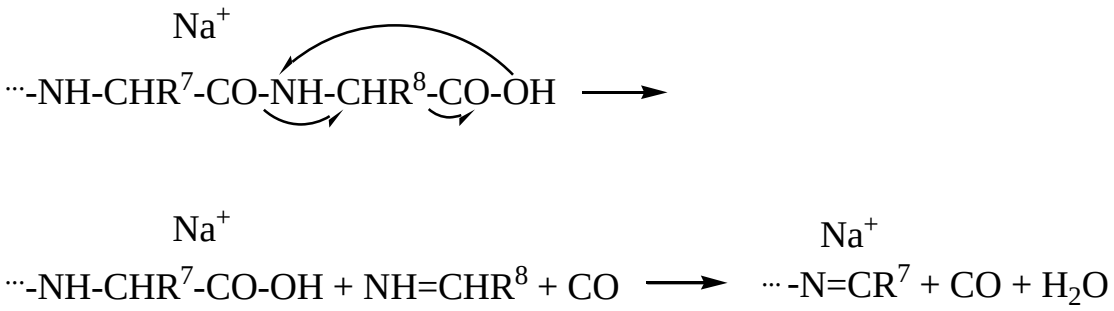

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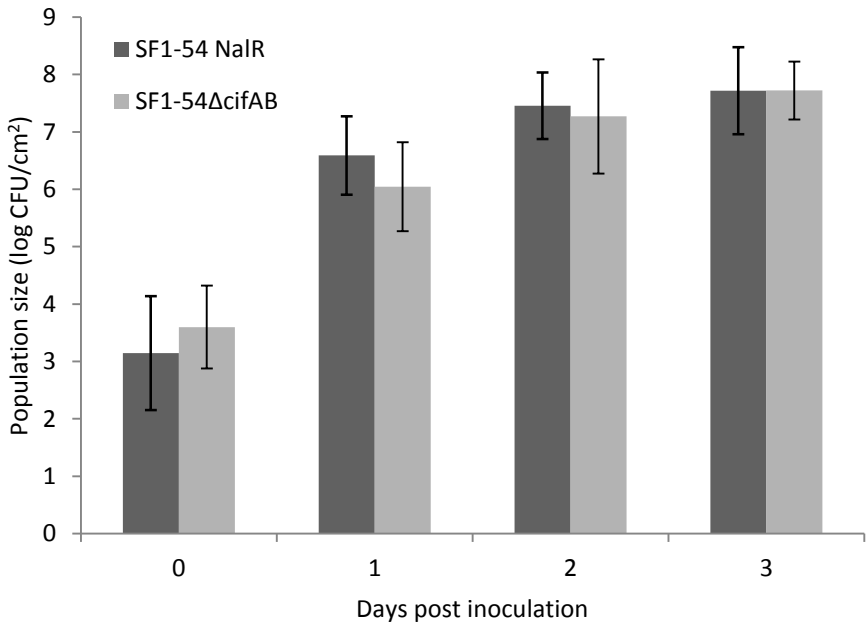

Figure S3. Population sizes of *P. cichorii* SF1-54Nal^R and its cichofactin-deficient mutant SF1-54-Δ*cifAB* in lettuce midribs. Values represent the mean ± SD of three independent experiments.

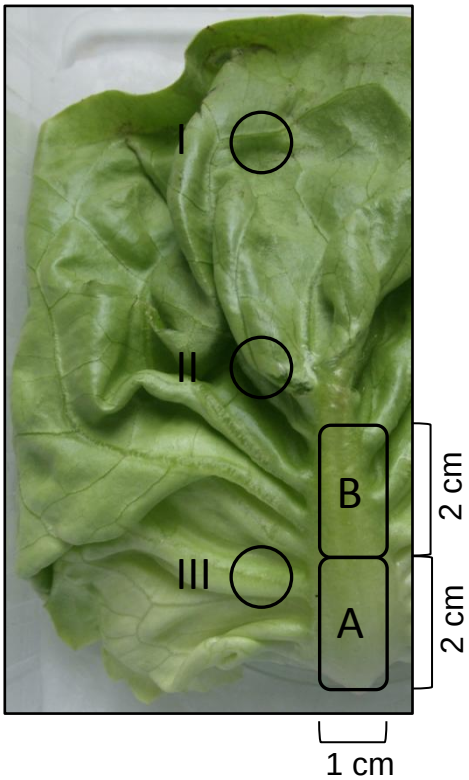

Figure S4. Illustration of a butterhead lettuce leaf used for assays of bacterial growth *in planta* or attachment to leaf surface. The populations of *P. cichorii* strains in the midrib sections were estimated. The midrib was injected with a bacterial suspension in section A. For attachment assays, leaf discs were taken from three regions: (I) near the edge of the blade; (II) at the center of the leaf; (III) near the petiole.