Fe$^{2+}$ chelator proferrorosamine A: a gene cluster of *Erwinia rhapontici* P45 involved in its synthesis and its impact on growth of *Erwinia amylovora* CFBP1430

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**INTRODUCTION**

Iron is a cofactor in key metabolic processes and is essential for almost all bacteria. Although iron is abundant in nature, it does not normally occur in its biologically relevant form Fe$^{2+}$, which requires anaerobic or microaerophilic conditions and low pH. In aqueous, pH-neutral, oxygenated environments, Fe$^{3+}$ is the dominant redox state of iron. Fe$^{3+}$ forms insoluble oxyhydroxide compounds, resulting in extremely low concentrations of free Fe$^{3+}$. Bacteria commonly overcome this problem by releasing siderophores – small molecules with high Fe$^{3+}$ affinity (Earphart, 1996).

Proferrorosamine A [proFRA; 1-L-2-(2-pyridyl)-1-pyrroline-5-carboxylic acid] belongs to a family of rare microbial Fe$^{2+}$ chelators (Vande Woestyne *et al.*, 1991). This Fe$^{2+}$-chelating compound was first described as a metabolite of *Pseudomonas roseus fluorescens* [formerly *Bacillus roseus fluorescens* (Pouteau-Thouvenot *et al.*, 1965)], and was later also described in *Erwinia rhapontici* [formerly *Pseudomonas GH* (Shiman & Neilands, 1965; De Vos *et al.*, 1993)] and *Erwinia persicina* (Feistner *et al.*, 1983). Binding of Fe$^{2+}$ to proFRA leads to the formation of the (pink) ferrosoamine complex. Bacteria synthesize an Fe$^{2+}$ chelator when iron is predominantly present as Fe$^{3+}$ in their environment and a hypothetical explanation for this unsuspected finding is that the Fe$^{2+}$ chelator endows a, yet to determined, competitive advantage for the producing bacteria in microbial communities (Liu *et al.*, 1981). Whilst proFRA has been physicochemically characterized (Vande Woestyne *et al.*, 1992), genes involved in its biosynthesis are currently not available or annotated in public databases.

The diagnosis protocol of the phytopathogen *Erwinia amylovora*, the causative agent of fire blight (Vanneste, 2000; Piqué *et al.*, 2015), takes into account the colony...
morphology of *E. amylovora* on the detection medium (EPPO, 2013). In mixed cultures, this morphological diagnosis is sometimes hindered due to an unusual pink coloration of the colonies on King’s B (KB) agar. This pink coloration of *E. amylovora* colonies is due to the presence of *E. rhapontici* or *E. persicina* and their production of proFRA. After the formation of the pink iron complex, ferrorosamine accumulates in *E. amylovora* colonies, leading to the pink color (Stockwell et al., 2008). Interestingly, pink *E. amylovora* colonies also exhibit attenuated growth on KB agar depending on the proximity of a proFRA producer. Thus, we hypothesize that the Fe$^{2+}$ chelator is capable of inhibiting the growth of *E. amylovora*.

To better understand proFRA biosynthesis and its effect on bacterial growth, proFRA-negative *E. rhapontici* P45 mutants were generated and thereby, for the first time to our knowledge, a gene cluster involved in proFRA synthesis was determined. Using *E. amylovora* CFBP1430 as a model strain, we demonstrate that proFRA exerts an inhibitory effect on its growth that can be neutralized upon the saturation of proFRA with Fe$^{2+}$. These findings lay the foundation for further studies and potential applications of proFRA in biocontrol applications.

**METHODS**

**Cultivation of bacteria.** Strains (Table 1) were routinely grown overnight at 28°C in LB medium (Roth), tryptic soy broth (TSB; Sigma Aldrich) or KB medium (1.5 g MgSO$_4 \times 7$H$_2$O 1$^{-1}$, 1.5 g K$_2$HPO$_4$ 1$^{-1}$, 16 g Bacto-Peptone 1$^{-1}$, 10.6 g glycerol 1$^{-1}$), *E. rhapontici* strain P45 was identified by MALDI-TOF-MS (using the Mabritec database), and sequencing of the *atpD* (GenBank accession number GU225728.1) and *gyrB* (GenBank accession number JF311456) genes (Brady et al., 2008; Rezzonico et al., 2010).

**Transposon mutagenesis.** Transposon mutagenesis was performed using pJA1, an oriK$^-$-based suicide vector. The plasmid contains a Tn10 transposase and confers kanamycin resistance (Badarinarayana et al., 2001). *Escherichia coli* SM10 zipir (pJA1) and *E. rhapontici* P45 were individually grown overnight in LB medium, plated together in the centre of an IPTG-containing LB plate (10 μl 100 mM IPTG solution, spotted onto the centre of the plate) and incubated at 37°C for 5 h. A dilution series was plated onto MM2 agar plates (4 g l$^{-1}$ asparagine 1$^{-1}$, 2 g K$_2$HPO$_4$ 1$^{-1}$, 0.2 g MgSO$_4 \times 7$H$_2$O 1$^{-1}$, 3 g NaCl 1$^{-1}$, 10 g sorbitol 1$^{-1}$, 15 g agar 1$^{-1}$). After 4–5 days of growth at 28°C, single colonies were picked, inoculated in 96-well plates containing KB medium (40 μg kanamycin ml$^{-1}$) and grown for 4 days at 28°C. Then, 40 μl 10 mM FeSO$_4$ was added to each well. A pink coloration was indicative of proFRA production. Mutants lacking the pink coloration were streaked onto KB plates and the coloration test was repeated by adding 10 mM FeSO$_4$ solution. This evaluation procedure was repeated three times. Mutants lacking a pink coloration in all three experiments were classified as proFRA-negative transposon mutants. To identify the transposon insertion sites of proFRA-negative mutants, an arbitrary PCR (Holenstein, 2011) was performed. Transposon-insertion specific primers were generated as forward primers in the T7 promoter region of the transposon: arPCR-T7 (5’-GCACCTTAAACGCTAGCAGTATACGACTC-3’) and the nested primer arPCR-T7 inner (5’-TGACGGTACCATCTGACGAC-3’). The arbitrary PCR amplified only the T7 promoter flanking region of the chromosomal insertion site. A crude DNA extract of selected transposon mutants was prepared by suspending 24–48 h bacterial colonies in 300 μl double-distilled H$_2$O, boiling at

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95 °C for 30 min and centrifuging at 12 000 g for 1 min. The supernatant was diluted 1 : 10 with double-distilled H₂O and 1 μl used as PCR template. Amplifications were performed using AccuStar Taq polymerase (Quanta Biosciences). PCR products were sequenced using an ABI Prism BigDye Terminator version 1.1 cycle sequencing kit (Applied Biosystems).

**Site-directed mutagenesis of *E. rhapontici* P45.** A proFRA-negative mutant of *E. rhapontici* P45 (ΔrosFE:: Kan<sup>R</sup>) was generated using an ABI Prism BigDye Terminator version 1.1 cycle sequencing kit (Applied Biosystems).

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**Fig. 1.** (a) Physical map of the gene cluster involved in proFRA production in *E. rhapontici* P45. Coding sequences are indicated by large horizontal arrows which show the direction of transcription. Black vertical arrows show the integration site of transposons. Small grey arrows show the position of the primers used in PCR and DNA cloning (i.e. rosXhoF, rosXhoR, SacIrosR and AparosF). The predicted transcriptional terminator is indicated. (b) Proposed biosynthetic pathway for proFRA from lysine, O-acetylserine and malonyl-CoA. Alternative routes A and B differ in the timing of dehydroalanine incorporation (after versus before release from the polyketide synthase). PLP, Pyridoxal phosphate.
by site-directed mutagenesis. To that end, the designated ros gene cluster was amplified in two PCRs using PfuUltra II Fusion HS DNA polymerase (Agilent Technologies). The resulting two fragments of 3.8 kb (primers AparosF 5'-TTTGTCCGGCCACGTTGACGAGTTTAA-3' and SacIrosF 5'-CGACCATCAAGCATTTTATC-3', annealing temperature 58 °C) and 6.0 kb (primers rosXhoF 5'-CATTTCCTCAGGGTGCGCAG-3' and SacIrosR 5'-GGGGGGAGGTACCATCCTGGTCGAGGTAGGTTACCTC-3', annealing temperature 58 °C) included a putative transcriptional terminator located downstream of rosG and the potential promoter region of the ros cluster. Primers AparosF and SacIrosF introduced restriction sites for Apal andSacI, respectively, whilst rosXhoF and rosXhoR annealed up- and downstream of a XhoI restriction site within the operon.

The Apal/Xhol-digested 3.8 kb product was ligated into the similarly digested pKS vector [pBluescriptKS(-): +; Stratagene] followed by the XhoI/Sacl-digested 6.0 kb fragment, which was ligated into the relevant restriction sites of the vector. Resulting plasmid pKSros harbour- pounding the 9.8 kb ros operon, which carries two EcoRV restriction sites: one located within the 3' terminal sequence of rosE and the second within rosF. pKSros was EcoRV-digested and the excised 2.7 kb fragment replaced by a HindIII-digested kanamycin cassette from pSB315 (Galán et al., 1992). pKSros (ArosEF:: KanR) was then KpnI/ Slacl-digested and the insert ligated into the similarly digested suicide vector pKAS32, resulting in pVSros. When using pKAS32 derivatives for the positive selection of an allelic exchange, a streptomycin-resistant parental strain is a prerequisite (Skorupski & Taylor, 1996).

Spontaneous streptomycin-resistant colonies of E. rhapontici P45, E. rhapontici P45S, were isolated by increasing streptomycin concentrations in TSB. pVSros was conjugated into E. rhapontici P45SIT using two-parental mating employing E. coli S17-1 λpir (Miller & Mekalanos, 1988; Simon et al., 1988). Kanamycin- and streptomycin-resistant mutants were selected on MM2 medium containing 10 µg ml−1 and 100 µg ml−1 streptomycin, respectively. Strain E. rhapontici ArosE:: KanR (SpF6) was tested via PCR for the presence of the kanamycin resistance gene (aph157 5'-GTCCACGGAGGATTTGACGCAATC-3'/aph606 5'-CGACCATCAAGCATTTTATC-3', annealing temperature 58 °C) and absence of the 2.7 kb EcoRV fragment (proofE 5'-ACTCTCAATAAAAGGGGTTTGGC-3'/proofF 5'-GAAATGGCTTGTATCAGTATT-3', annealing temperature 58 °C).

**Complementation and restoration of the proFRA-positive phenotype.** AparosF/XosXhoF and rosXhoF/SacIrosR vector products were ligated into vector pMP7605 (Lagendijk et al., 2010) analogous to the ligation into pKS described earlier. The resulting plasmid pMPros (GenBank under accession number KT210364) was conjugated into E. rhapontici P45SIT using two-parental mating employing E. coli S17-1 λpir (Miller & Mekalanos, 1988; Simon et al., 1988). Kanamycin- and streptomycin-resistant mutants were selected on MM2 medium containing 40 µg ml−1 kanamycin and 25 µg gentamicin ml−1.

**Preparation of proFRA extract and purification.** To prepare proFRA-containing crude extract, KB plates (containing 0.75 % agar and a 1.5 % agar overlay) were inoculated with E. rhapontici P45 or transposon mutants. After 2 weeks of growth, the agar around bacterial growth was cut into pieces and 1 ml of 50 ml double-distilled H2O was added for each 1 g agar. After overnight incubation at room temperature the supernatant was filter-sterilized (0.2 µm). To quantify proFRA, 5 µl of FeSO4 solution was added to 1 ml of proFRA-containing extract. Pink coloration of ferrosemamine was measured as A600 (Thermo Scientific Genesys 10 UV and 1.6 ml single-use cells, polystyrene; Carl Roth).

ProFRA was purified following the protocol of Feistner et al. (1997). In brief, complemented mutant strain E. rhapontici ArosE:: KanR (pMPros) was cultivated in TSBS overnight. An aliquot of 1 ml culture was centrifuged, and the cell pellet washed twice with PBS (2.5 g K2HPO4 1 l−1, 1.2 g KH2PO4 1 l−1) and resuspended in 120 ml MM2 medium. After 2 weeks of cultivation, cells were removed by centrifugation and the supernatant filtered through Nalgene 0.2 µm filters. The filtrate was passed over a cation exchange column (Dowex AG 50WX8; Bio-Rad), washed with 100 ml water and proFRA eluted with 10 % aqueous NH4OH (100 ml). The proFRA eluate was concentrated to 15 ml via rotary evaporation.

**Growth curves.** The effect of proFRA on E. amylovora CFBP1430 growth was monitored using a Bioscreen C (Oy Growth Curves) growth curve analysis system. proFRA crude extracts from E. rhapontici P45 or transposon mutants were diluted 1 : 2 with fresh KB medium. To study the effect of proFRA on E. amylovora CFBP1430 growth, proFRA eluate was mixed with KB medium in a twofold serial dilution starting from 1 : 2 up to 1 : 32. Deionized water replaced the proFRA eluate in the control serial dilution. To study the effect of iron addition to proFRA on E. amylovora CFBP1430 growth, 25 or 50 µl 1 mM FeSO4 solution was added to 1 ml proFRA eluate. The ferrioxamine-mixtures were diluted 1 : 4 with KB medium.

Samples of 200 µl mixture were used as a negative control; 700 µl was inoculated with 3 µl E. amylovora CFBP1430 overnight culture and loaded in three replicates (200 µl per well) on a Bioscreen C honeycomb plate. Measurement of OD600 was performed every 1.5 or 3 h, for at least 18 h at 26 °C with 10 s of shaking prior to each measurement. Two to three independent growth experiments were performed.

**Evaluation of growth inhibition on plate.** KB plates were overlaid with 0.75 % soft agar containing 5 × 104 cfu E. amylovora CFBP1430 ml−1 from an overnight culture (double-layer technique). Filter papers soaked with 20 µl bacterial cultures (OD600 1) of WT or mutant E. rhapontici P45 strains were placed on top of the soft agar layer and incubated at 28 °C for 48 h. Alternatively, an undiluted culture of the parental E. rhapontici strain P45 or mutant strains was transferred to a KB plate as a single streak using sterile cotton buds. A 10−6 dilution (in PBS) of an E. amylovora CFBP1430 overnight culture was streaked on the same plate, from 2 and 4 cm from the bottom or top edge to the centre. Plates were incubated for 4 days at 28 °C.

**Sequences.** Predicted ORFs were analysed using BLASTN and BLASTX (Altschul et al., 1990) against the National Center for Biotechnology Information database. The ros gene cluster sequence was deposited in GenBank under the accession number KT210364.

**RESULTS**

**A 9.3 kb gene cluster, comprising seven genes, is involved in proFRA biosynthesis.**

Although the chemical structure of proFRA has been known for decades, little is known about genes involved in biosynthesis of this iron chelator and sequence information is still not available to the public. Strain ‘P45’, isolated from a fire-blight-infected apple tree sample in 2004 (canton Lucerne, Switzerland), induces a pink coloration of E. amylovora colonies when co-cultured on KB plates. ‘P45’ was identified as E. rhapontici and therefore designated E. rhapontici P45. The strain was subjected to random insertion transposon mutagenesis, aiming to identify the proFRA synthesis genes. In total, 3600 E. rhapontici P45 transposon mutants were screened for defective proFRA synthesis. The chromosomal flanking regions of the integrated transposons of proFRA-negative mutants were sequenced. To characterize
the genetic environment of the transposon insertion sites, *E. rhapontici* P45 was sequenced (unpublished data) and the transposon insertion flanking sequences aligned to the draft genome. Insertion sites of four proFRA-negative mutants (TM1-2, TM18, TM31h and TM31c) could be pinpointed to one contig of the draft genome. The sequence matching and flanking the insertion sequence sites of these four transposon mutants was manually annotated. The transposons inserted within a 9.3 kb gene cluster composed of seven genes (Fig. 1). Given their putative involvement in proFRA biosynthesis, the genes were designated *rosA*–*rosG*. A putative 30 bp transcriptional terminator was located downstream of *rosG*. The transposons of proFRA-negative mutants *E. rhapontici* TM1-2 and TM31h integrated in the 5’ end of *rosE*, transposon TM18 in the 3’ end of *rosD*, and transposon TM31c in the 5’ end of *rosC*.

The deduced amino acid sequences of the proteins encoded by the gene cluster shared no significant similarities to proteins in other sequenced *Erwinia* species. The deduced amino acid sequence of *rosA* (1608 bp) showed the highest similarity to a 2,3-dihydroxybenzoate-AMP ligase of *Providencia burhodogranariea* (71 %; GenBank accession number WP_008913818.1) and to a protein of *Serratia marcescens* (57 %; GenBank accession number WP_033650395.1) predicted to be involved in siderophore biosynthesis. The *rosB* (735 bp)-encoded protein has similarity to the pyochelin biosynthetic protein PchC of *Providencia burhodogranariea* (70 %; GenBank accession number WP_008913819.1). Gene *rosC* (1029 bp) encoded a protein with similarity to the cysteine synthase of *Serratia marcescens* (79 %; GenBank accession number WP_033643090.1), whereas *rosD* (555 bp) encoded a protein that exhibited highest similarities to hypothetical proteins of *Pantoea* sp. A4 (78 %; GenBank accession number WP_017346023.1) and *Serratia marcescens* BIDMC 50 (73 %; GenBank accession number ETX45718.1). The protein encoded by *rosE* (1176 bp) had similarities to a hypothetical protein of *Pantoea* sp. A4 (81 %; GenBank accession number WP_017346024.1), to the FAD-dependent oxidoreductase of *Streptomyces rimosus* (49 %; GenBank accession number WP_030643530.1) and to the NikD protein of *Streptomyces tendae* (48 %; GenBank accession number CAB46534.1). *rosF* (2892 bp) encoded a protein similar to a polyketide synthase of *Providencia burhodogranariea* DSM 19968 (80 %; GenBank accession number WP_008913820.1). *RosG* (635 bp) exhibited high similarities to a potential leucine dehydrogenase of *Serratia marcescens* (75 %; GenBank accession number WP_033643093.1).

To verify its involvement in proFRA synthesis, site-directed mutagenesis was performed, which targeted a region of the *ros* cluster that had not been affected by transposon insertion. A 2.7 kb fragment composed of the 3’-terminal sequence of *rosE* and *rosF* was replaced by a kanamycin resistance cassette (Fig. 1a). The resulting strain *E. rhapontici* Δ*rosFE*::KanR did not synthesize proFRA. Mutants *E. rhapontici* Δ*rosFE*::KanR and *E. rhapontici* TM1-2 were used for in trans complementation using plasmid pMPros. Whilst *E. coli* S17-1 λ-*pir* (pMPros) did not produce detectable amounts of proFRA when cultivated on KB agar, complementing *E. rhapontici* Δ*rosFE*::KanR (pMPros) and *E. rhapontici* TM1-2 (pMPros) restored proFRA synthesis. proFRA was even overproduced in the complemented strains, most likely due to a plasmid copy effect (San Millan et al., 2015).

**proFRA biosynthesis pathway**

Based on the gene homologies, we propose the biosynthetic model shown in Fig. 1(b). RosE is similar to NikD from nikkomycin biosynthesis, which catalyses picolinate formation in the same way as shown here (Carrell et al., 2007). After activation by the adenylating enzyme RosA, the polyketide synthase RosF elongates the picolinyl starter. The next characterized homologue of RosF is CrmA, which has the same function in caerulomycin biosynthesis (Zhu et al., 2012). RosC, with similarity to cysteine synthases, is then proposed to generate a pyridoxal phosphate-bound dehydroalanine residue, which can be attacked by the polyketide intermediate either after (Fig. 1b, route A) or during polyketide elongation (route B). Spontaneous imine formation would then yield the pyrroline moiety of proFRA.

**proFRA inhibits growth of *E. amylovora* CFBP1430**

To test the growth-inhibitory effect of proFRA on the model strain *E. amylovora* CFBP1430, the double-layer
technique was used. Filter discs soaked with *E. rhapontici* P45 or corresponding transposon mutants were placed onto *E. amylovora* CFBP1430 lawns. After 2 days, a halo indicative of growth inhibition was detected around *E. rhapontici* P45. The haloes were smaller when the filter was soaked with one of the transposon mutants (Fig. 2), indicating that proFRA inhibited growth of *E. amylovora* CFBP1430. The proFRA effect was also investigated on single colonies of *E. amylovora* CFBP1430. After 4 days of cultivation, growth inhibition and colouring could be detected when *E. amylovora* CFBP1430 colonies were in close vicinity (2 cm) to strain *E. rhapontici* P45. *E. amylovora* CFBP1430 colonies further away (4 cm) showed no coloration or growth deficiency (Fig. 3a, b). proFRA-negative mutants caused neither coloration nor growth inhibition in *E. amylovora* CFBP1430. When the complemented strain *E. rhapontici* TM1-2 (pMPros) was used in competition against *E. amylovora* CFBP1430, both effects could be restored (Fig. 3b). Thus, colour and reduced growth of *E. amylovora* CFBP1430 colonies were dependent on production of proFRA.

Fig. 3. Growth inhibition and pink coloration of *E. amylovora* CFBP1430 colonies following exposure to proFRA. (a) KB plates with parental strain *E. rhapontici* P45, proFRA-negative transposon mutant TM31c (strains streaked along the plate, undiluted) and *E. amylovora* CFBP1430 (from bottom or top edge to centre, diluted). (b) proFRA-negative transposon mutant TM1-2, complemented mutant strain TM1-2 (pMPros) (strains streaked along the plate, undiluted) and *E. amylovora* CFBP1430 (from bottom or top edge to centre, diluted).

Fig. 4. Effect of proFRA on growth of *E. amylovora* CFBP1430. Bioscreen growth curve of *E. amylovora* CFBP1430 inoculated in KB medium containing extracts of parental strain *E. rhapontici* P45 or proFRA-negative mutants. Data are presented as mean ± SD.
For further growth studies, crude extracts from parental strain *E. rhapontici* P45 and proFRA-negative transposon mutants TM1-2, TM18 and TM31c were obtained. As culturing of these strains in liquid KB medium did not lead to any visible pink coloration of the medium (after addition of FeSO₄) even after 1 week of incubation, strains were cultivated on KB plates and a crude extract was prepared. Presence or absence of proFRA was tested by adding a 1 mM FeSO₄ solution to 1 ml extract. Only *E. rhapontici* P45 WT extracts yielded a visible pink coloration (*A₅₅₆* 0.296 compared with 0.03 as maximal *A₅₅₆* value of proFRA-negative mutants, i.e. background activity). The extracts were inoculated with *E. amylovora* CFBP1430 and bacterial growth was measured by determining OD₆₀₀. *E. rhapontici* P45 WT extracts significantly reduced growth of *E. amylovora* CFBP1430 when compared with extracts of proFRA-negative *E. rhapontici* P45 transposon mutants (Fig. 4).

**Growth reduction of *E. amylovora* CFBP1430 is due to Fe²⁺ complexation**

To verify a dose-dependent effect of proFRA on the growth of *E. amylovora* CFBP1430, growth assays with variable concentrations of proFRA eluate were performed. The maximal proFRA eluate/KB dilution decreasing the growth of *E. amylovora* CFBP1430 was determined to be 1:16 (Fig. 5a). Titration of the 1:16 proFRA eluate/KB dilution with FeSO₄ and measurement of ferroasamine revealed the dilution to be saturated at *A₅₅₆* 0.1, which corresponds to ~30 μM proFRA (Vande Woestyne et al., 1991). Addition of 25 or 50 μl 1 mM FeSO₄ solution to the 1:4 proFRA eluate/KB dilution abolished the negative effect on the growth of *E. amylovora* CFBP1430 (Fig. 5b).

**DISCUSSION**

Observations in our diagnostic laboratory indicated that co-culture of the plant pathogens *E. rhapontici* P45 and *E. amylovora* on KB agar leads to pink coloration and growth inhibition of the latter *in vitro*. This observation is due to the production of proFRA by *E. rhapontici* P45. Whilst the colouring effect of proFRA on *E. amylovora* was reported previously (Stockwell et al., 2008), the influence on growth deficiency had not been noticed. This discrepancy with previous observations might be attributed to different levels of residual iron in the prepared KB media. Preliminary data from our laboratory indicate that *E. rhapontici* P45 causes coloration on KB agar not only in *E. amylovora*, but also in *Erwinia piriformis*, *Yersinia mollaretii* and *Dickeya dadantii*. Thus, proFRA affects additional members of the *Enterobacteriaceae*. One can assume that the chelator of a cofactor as common as iron targets more than one family of the bacterial community. However, the spectrum of activity of proFRA has yet to be determined.

To study and elucidate the molecular mechanism underlying this inhibition, proFRA-negative mutants of *E. rhapontici* P45 were generated, using random transposon and site-directed mutagenesis. Thereby, we identified a 9.3 kb cluster, comprising seven genes (designated *rosA–rosG*). Based on the homologies of the corresponding Ros proteins, a proFRA biosynthetic pathway model was proposed. Significant identities of the proteins cannot be found within *Erwinia* species currently available in public sequence databases.

In this study, *E. amylovora* CFBP1430 was used as a model strain for proFRA-induced effects. proFRA exerts a growth-reducing effect on *E. amylovora* CFBP1430 when cultivated on KB agar or liquid KB medium. In contrast to the

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**Fig. 5.** Growth of *E. amylovora* CFBP1430 in KB medium containing (a) proFRA eluate or (b) proFRA saturated with different amounts of 1 mM FeSO₄ (25 or 50 μl). *E. amylovora* CFBP1430 was inoculated into medium composed of proFRA eluate mixed with KB medium in a twofold serial dilution. Dilution steps of proFRA from 1:2 up to 1:32 are indicated in (a). Addition of FeSO₄ to a 1:4 proFRA eluate/KB dilution abolished the growth-reducing effect on *E. amylovora* CFBP1430 (b). Data are presented as mean ± SD.
growth curve assays, KB plate tests were performed with proFRA-synthesizing bacteria, not with proFRA eluate. Thus, we cannot exclude that colony size of E. amylovora CFBP1430 on plates was affected by additional synthesis products of E. rhapontici P45, e.g. siderophores. Saturating proFRA with Fe$^{2+}$ before addition to the KB medium abolishes the negative effect on the growth of E. amylovora CFBP1430. Consequently, proFRA-negative transposon mutants or extracts fail to decrease growth of E. amylovora CFBP1430. In addition, proFRA leads to the pink coloration of E. amylovora CFBP1430 colonies, i.e. the accumulation of ferrorosamine in E. amylovora CFBP1430 on KB plates. Whether this accumulation leads to an additional reduction in bacterial growth remains to be analysed.

proFRA-negative Tn5 mutants of Pseudomonas rosaeus fluorescens formed siderophores only after the addition of proFRA to the medium (Vande Woestyne et al., 1991), implying proFRA production to be essential for siderophore synthesis. Here, the siderophores produced were not determined, but the parental strain E. rhapontici P45 and proFRA-negative mutants were placed on siderophore chrome azurol S agar and formed virtually indistinguishable haloes (data not shown). However, this finding is no confirmation for the siderophore synthesis not being altered in the proFRA mutants, as redundant systems might cover the loss of one system on the detection agar (Schwyn & Neilands, 1987).

The model strain used in this study, i.e. E. amylovora CFBP1430, is a causative agent of fire blight. This plant disease is a major threat to pome fruit production worldwide. Despite being one of the most intensively studied bacterial plant diseases, its control is still problematic, and E. amylovora continues to spread across Europe and the Middle East. Currently, the most effective control of the pathogen is the treatment of flowers with the antibiotic streptomycin. However, antibiotic application in the environment is controversial (McManus 2014) and resistant strains of E. amylovora emerged where streptomycin was applied. e.g. in the USA, Canada and Israel (Loper et al., 1991; Chiu & Jones, 1995; Sholberg et al., 2001; McManus et al., 2002; Manulis et al., 2003; McGehee & Sundin, 2011). Alternatives to the control of E. amylovora, such as biological control measures, are therefore required (Johnson & Temple, 2013).

E. amylovora harbours only one siderophore system – the hydroxamate siderophore desferrioxamine E (Feistner et al., 1993; Kachadourian et al., 1996; Smits & Duffy, 2011). Thus, the causative agent of fire blight has a limited capability for Fe$^{2+}$ competition in bacterial communities. The importance of this system for the pathogenicity of E. amylovora has been demonstrated using mutants deficient in siderophore synthesis or uptake which exhibit decreased pathogenicity on apple flowers (Dellagi et al., 1998).

The growth deficiency of bacteria that are exposed to siderochelins has been reported before. Recently, siderochelins with anti-mycobacterial activity have been isolated from an Amycolatopsis sp. (Lu et al., 2015). Siderochelin A was noted to be weakly active against anaerobic and aerobic bacteria. Addition of ferrous iron, but also Fe$^{3+}$, Co$^{2+}$ or Zn$^{2+}$, abolished the effect, implying the biological activity to be connected to the chelating activity of siderochelin A (Liu et al., 1981; Mitscher et al., 1984). Thus, iron limitation as an advantage in environmental competition seems to be also true for the Fe$^{2+}$ form, although it is still unknown why bacteria synthesize Fe$^{2+}$ chelators under aerobic conditions, when the ferrous form is scarce.

The predicted genome size of ~5 Mbp of E. rhapontici P45 indicates that the strain has 1 Mbp additional genetic information over the mean genome size of sequenced pathoadapted Erwinia spp., which is ~3.8–4 Mb (Kamber et al., 2012). Further analysis of the E. rhapontici P45 genome will help identifying additional factors with the potential of growth inhibition on competitors. It is reasonable to test such substances, e.g. proFRA, not only in vitro, but also in vivo for growth inhibition of E. amylovora, whilst ensuring concomitantly that these substances are not harmful to the host plant. Previous experiments showed that proFRA negatively affects the germination of cress and wheat seedlings (Feistner et al., 1997). However, when we sprayed crude proFRA extract on apple blossoms in a preliminary experiment, we did not detect any negative effect on these blossoms. Therefore, whilst E. rhapontici P45 is a plant pathogen and as such not suitable for plant protection, individual metabolic components produced by this strain might be an alternative for the control of bacterial plant pathogens such as E. amylovora. Additionally, now that we have identified the genes responsible for the production of proFRA we are able to screen bacteria for the presence of those genes and the production of proFRA. This opens new avenues in the identification of non-pathogenic biocontrol agents.

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REFERENCES


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