Regulation of secondary metabolites in plant-beneficial microorganisms: Strategies to activate a cryptic biosynthetic gene cluster in *Pseudomonas protegens*

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Abstract

Climate changes and overpopulation present hard challenges for global agriculture, threatening the food security of the world population. Some microbes have plant growth-promoting activities and can increase the yield of crops. Nevertheless, the complex microbial interactions alongside the unknown information hidden in the microbial genome make it harder to regulate plant-beneficial metabolites. This project aims to studystrategies for the activation of an unknown cryptic biosynthetic gene cluster (BGC) of the potential plant growth-promoting strain *Pseudomonas protegens* DTU9.1. BGCs encode proteins, here a polyketide synthase (PKS), that synthesise secondary metabolites. Comparisons in different media show that no significant differences were caused by PKS deletion regarding growth nor fluorescence. Intra- and extracellular metabolites from extracts of both the WT and ΔPKS were analysed by LC-MS and compared. Several metabolites showed different expression levels between the two strains.

Antibiotic rifamycin showed to induce the BGC expression, being influenced by the culture growth phaseon which is added. Cultures with D-arabitol as a carbon source resulted in higher expression levels of the BGC than cultures with glucose. With RNA polymerase engineering, there was an increase of 4.2-fold in the unknown BGC expression.

This study shows that the use of different carbon sources might be a key factor for BGC expression activation. The moment of supplementation of the inductive antibiotic to the culture should also beconsidered. RNA polymerase engineering demonstratesto be a promising technique for cryptic BGC expression activation.

Keywords: cryptic biosynthetic gene cluster, polyketide synthase, secondary metabolites, activation, plant-beneficial bacteria, *Pseudomonas protegens*

Introduction

Global population is expected to increase in approx. 2 billion people in the next 30 years[1]. The demand for food, fibres, and fuel, predicts the expansion of agricultural production to be approximately 70% by 2050[2]. Expansion of the cultivated area to increase crop production or improvement of the yields of the existing crops are possible solutions. The former implies the replacement of natural landscape and might result in increased water pollution, and greenhouse emissions, and many other problems[3]. The latter usually relies on the use of chemical fertilizers and pesticides, but the use of agrochemical products is linked to several animal and human diseases, and environmental issues like soil, air, and water contamination[4,5].

Fortunately, the awareness about global environmental issues changed the preferences of consumers and the focus of companies: global sustainable investment raised by 68% since 2014[6]. European Union (EU) has been restricting the use of hazardous substances[7].

Plant growth-promoting microorganisms (PGPM) have the potential for several applications in the field of agro-environmental sustainability, from disease suppression in plants to bioremediation of contaminated soils[8]. According to du Jardin[9], beneficial microorganisms can be divided into biocontrol agents and biostimulants, withbiofertilizers making part of the second group.

Using microorganisms to suppress phytopathogen populations and control plant diseases[10] has been explored to replace the use of agrochemicals. In 2017, Australia, Brazil, Canada, Europe, Japan, New Zealand, and the United States had registered a total of 101 microbial biological control agents for plant disease control[11].

Modes of action of PGPM differ and can be primarily divided into indirect or direct. Indirectly, microorganisms can act via plant metabolism: plants protect themselves from pathogens through chemical and physical mechanisms activated by stimuli that are recognized by specific recognition receptors. Some of the recognized stimuli have origin in pathogens, but beneficial microbes are also capable of inducing resistance in plants. Bacterial compounds that are plant-resistance inducers include lipopolysaccharides, flagella, iron-regulated compounds, and others[10,12]. This type of induced resistance is only activated in the presence of the stimuli.However, when the effect lasts longer in the absence of the stimuli, the phenomenon is called priming[10]. For example, *Bacillus subtilis* GB03 is a biological fungicide whose volatile organic compounds induce plant resistance on *Arabidopsis* by regulating auxin homeostasis and cell expansion, enhancing photosynthesis by decreasing glucose sensing and abscisic acid levels; conferring salt tolerance by regulating the tissue-specific expression of sodium transporter HKT1; and stimulating iron acquisition [13–17]. Another type of indirect action is competition with the pathogen for nutrients and space. It was successfully applied, for example, in fruit rot prevention, using fast colonizing yeasts to control pathogenic populations, such as fungi[10,18].

Directly, beneficial microorganisms can interact with pathogens by 1) hyperparasitism, occurring when a parasite infects another parasite, a phenomenon more often observed in fungi; or 2) antibiosis, by producing secondary antimicrobial metabolites with inhibiting effects[10]. Some classes of antibiotic compounds have strong evidence for their function as biocontrol agents, such as phenazines, pyoluteorin, pyrrolnitrin, cyclic lipopeptides, and hydrogen cyanide (HCN).

Microorganisms can promote plant growth by producing phytohormones (as auxins, gibberellins, and cytokines), modulating the levels of stress-related ethylene, contributing to nitrogen fixation, enhancing phosphorus availability, acting as biostimulants[19]. An experiment with *Bacillus subtilis* showed that the strain alleviates drought stress in potatoes which maintained a higher photosynthetic process, contents of chlorophyll, soluble

proteins, total soluble sugars, and enzymatic activities of superoxide dismutase, peroxidase, and catalase in the presence of the plant growth promoting bacteria than without it[20].

Despite the enormous potential of microorganisms for plant growth promotion, there are two main reasons for their low levels of application: (1) the technical difficulties, owing to a lack of fundamental information on them and their ecology, and (2) the costs of product development and regulatory approvals required for each strain, formulation, and use. Technical difficulties are mainly dueto the complexity of soil microbiomes. Together withabiotic factors, intra- and interspecific interactions of these microbes are responsible for microbiomes modulation, affecting "occurrence, abundance, diversity,distribution, communication, and functions"[21]. Hence, the study of PGPM when performed under different conditions, leads to inconsistentresults[19]. The social-economic constraints are mainly due to rentability analysis, reluctance in adopting new techniques by farmers, and the complex and time- consuming regulatory processes for biocontrol products registration, mainly in EU.

Secondary metabolites are small molecules, biologically active, not vital for normal growth, but naturally produced by organisms conferring physiological adaptative tools and improving fitness[22]. Secondary metabolism pathways are associated with low growth rate, stress response, and breakdown of cellular components. As primary metabolism is related to energy production and cellular synthesis, its intermediates are usually substrates or products of enzymes with a high rate of activity and consequently, have fast turnover rates within the cell, being present in low quantities. Contrarily, secondary metabolites are precursors of only a few reactions and have a slower turnover rate when inside the cell. This leads to their intracellular accumulation and, when on adequate concentrations, to their secretion to the extracellular medium[23]. Secondary metabolites can have antagonistic effects, but also act as signalling molecules, affecting several key biological processes of the microorganisms.

Secondary metabolites are produced by enzyme complexes encoded in biosynthetic gene clusters (BGCs). These clusters usually include a gene encoding a skeleton structure which is one of several key signature enzymes that catalyse the production of compounds. From these key enzyme, polyketide synthases (PKS) and nonribosomal peptide synthetases (NRPS) are usually targeted for natural product discovery due to the broad range of bioactive properties of the synthesized products (polyketides and non-ribosomal peptides, respectively)[24]. The remaining genes of the BGC generally encode for tailoring enzymes that modify the secondary metabolite skeleton, such as oxidoreductases, methyltransferases, and acyltransferases. In some cases, BGCs might include genes for pathway-specific regulators and/or for resistance to the pathway end-product[24,25].

Cryptic genes are silent/low-level expressed genes under normal laboratory conditions, that can be activated by genetic mechanisms or environmental stimuli[26]. The number of putative metabolites predicted to be encoded in cryptic BGCs is higher than the ones already reported to be produced in some species[27]. They represent an immense source of bioactive compounds with potential utility for human activities, explaining the importance of correspondent gene expression activation. Homologous expression of cryptic BGC can be achieved by several techniques such as changes in culture conditions, external chemical triggers, and ribosome and RNA polymerase engineering. The last technique makes use of spontaneous antibiotic-resistant mutants with alterations in genes related to ribosome or RNA polymerase (like *rpsL* and *rpoB* genes)[28]. These mutations are responsible for different gene expression patterns. Heterologous expression can be achieved by cloning, although some challenges might appear due to the usually large size of BGCs[29].

This study is focused on *Pseudomonas protegens* DTU9.1 strain with highly effective biocontrol activity against plant pathogens. An *in-silico* genome analysis (*AntiSmash*[30]) predicted the existence of a BGC encoding for a PKS not identifiedin the data base. The compound resultant from this PKSmight have relevant functions. Metabolites with biocontrol activities are desired for biocontrol agents. However, it is also important to confirm the absence of prejudicial effects caused by these microorganisms. Hence, this study aims to find out ways of activating the expression of the unknown BGC in *P. protegens* DTU9.1, contributing to a further identification of the secondary metabolite synthesized by the PKS.

Results and Discussion

The unkBGC has low expression levels

To seek changes in the expression of the unknown biosynthetic gene cluster (BGC) within different culture conditions, the *P. protegens* DTU9.1 wild type (WT), the unknown PKS knockout mutant (ΔPKS), and the WT with a transcription reporter gene for the promoter upstream the BGC region (Punk-GFP) were cultured in four different media: LB, KB, ABTG, and TSB. The variance between WT and ΔPKS growth rates was not consideredstatistically significant, Figure 1.A, meaning that the deletion of the gene did not influence the growth rate under the assayed condition.

The fluorescence levels (relative fluorescence units (RFU)) of the cultures after 24h of growth are displayed in Figure 1.B, as well as the negative control (only media). In all the media, the addition of the fluorescent reporter gene leads to an increase of the fluorescent signal of 988, 1097, 1320, and 1358 RFU, for ABTG, KB, TSB, and LB media, respectively. This means that the promoter is active in all the studied conditions. LB and KB media intrinsic levels of fluorescence are very high, making it difficult to analyse bacterial levels of fluorescence. The lower levels of fluorescence of the cultures in comparison to the media after 24h can be due to the consumption of the fluorescent compounds in the media.

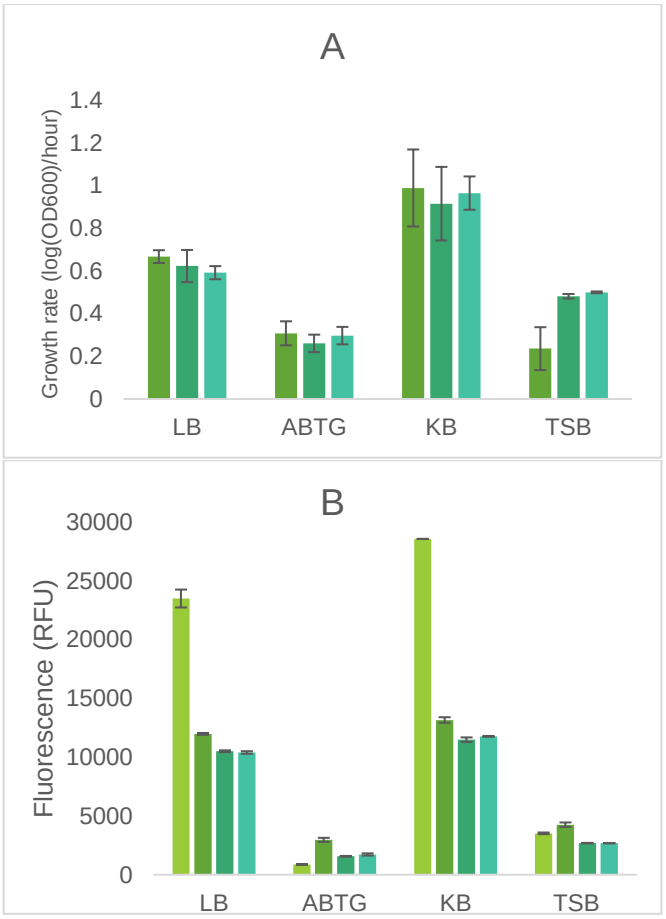


Figure 1. Bar chart – Growth rates and fluorescence of WT, ΔPKS, and Punk-GFP strains in LB, KB, ABTG and TSB media. The three strains, WT (■), ΔPKS (■), and Punk-GFP (■), were cultured in KB, LB, TSB, and ABTG media. **(A)** Growth rate. **(B)** Fluorescence levels measured after 24h of culture (not normalized to the medium fluorescence (■)). Standard deviation bars are displayed.

WT and ΔPKS were plated in four solid media and again, no significant difference was observed in the growth between each strain, evaluating the diameter of the spots, Figure 2.

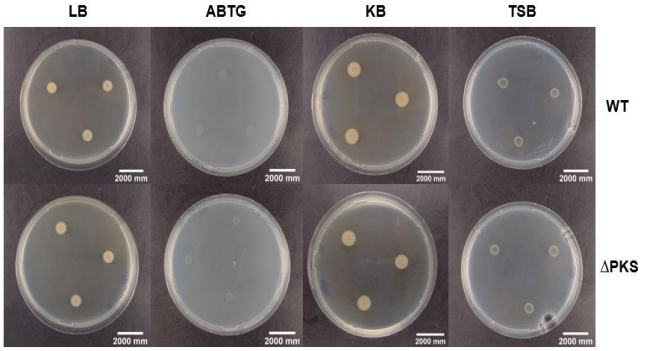


Figure 2. WT and ΔPKS spots in LB, KB, ABTG and TSB media after 2 days.

Together, the information leads to the conclusion that the unkBGC is expressed, but in low levels, not impacting the cellular growth.

WT and ΔPKS present different expression of intracellular metabolites

After confirmation of unkBGC expression, intra- and extracellular metabolites of the WT and ΔPKS in the late stationary phase were compared by LC-MS. The fold change of metabolites up/downregulated in ΔPKS extracts in comparison to WT extracts was analysed with *MetaboAnalyst 5.0*[31] based on peak areas. In the extracellular extract, LC-MS showed that only 8 metabolites were considered differently expressed comparing fold change between WT and ΔPKS strains and none of them was completely absent in the ΔPKS extract. In the intracellular extract, 118 metabolites were considered differently expressed with 2 of them having areas close to zero in the ΔPKS extract, Table 1.

Table 1. List of the metabolites with lowest fold change in ΔPKS in comparison with WT.

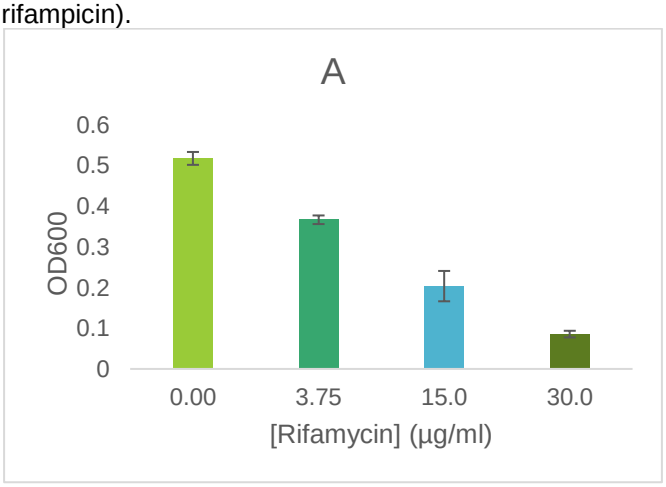
Average m/z	Average retention time (min)	Fold change
270.09402	2.226	0.011892
248.11168	2.240	0.016812

Analysing the chromatograms, (data not included), no evidence of a missing peak in ΔPKS extract in this retention times is visible.

Although several metabolites were differently expressed in the WT and the ΔPKS, respectively, the low expression level might not be enough to generate a product detectable by LC-MS. Hence, the identification of the produced compound is dependent on the overexpression of the unknown BGC.

Do antibiotics rifamycin and rifampicin activate the promoter?

The antibiotics rifamycin and its derivative rifampicin were tested on Punk-GFP strain to assess their effect in the activation of promoter Punk (promoter region upstream the unknown BGC) expression by measuring the fluorescence intensity. The presence of rifamycin and rifampicin increased the fluorescence signal, Figure 3.B and 4.B, with an exception for the concentration of 3.75 µg/ml of rifampicin, which strongly inhibited culture growth, Figure 3.A and 4.A. However, the normalized fluorescence/OD600 plot, Figures 3.C and 4.C, show higher values for higher concentrations (below MIC) in both cases (with the exception for 3.75 µg/ml of rifampicin).



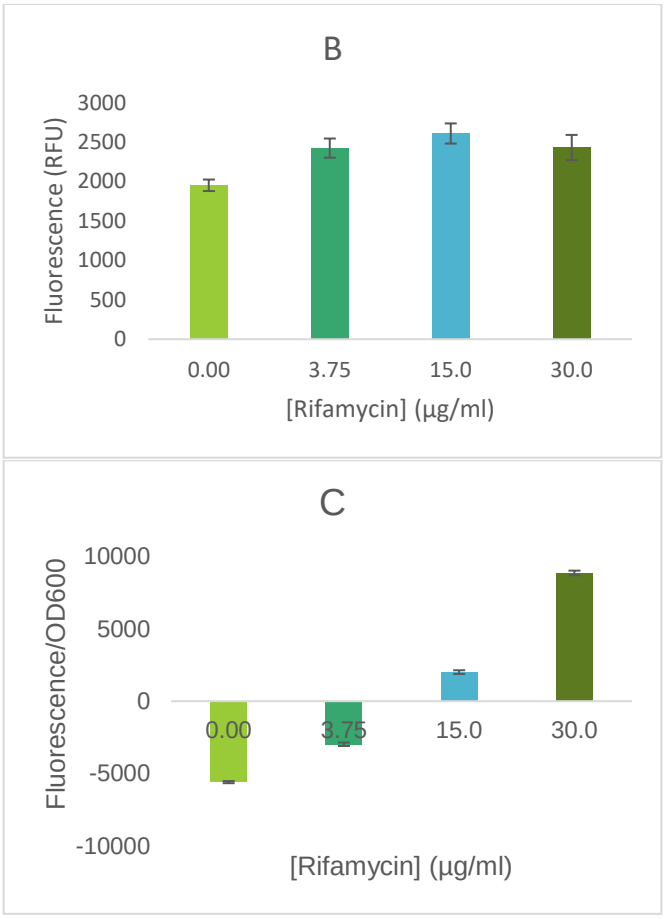
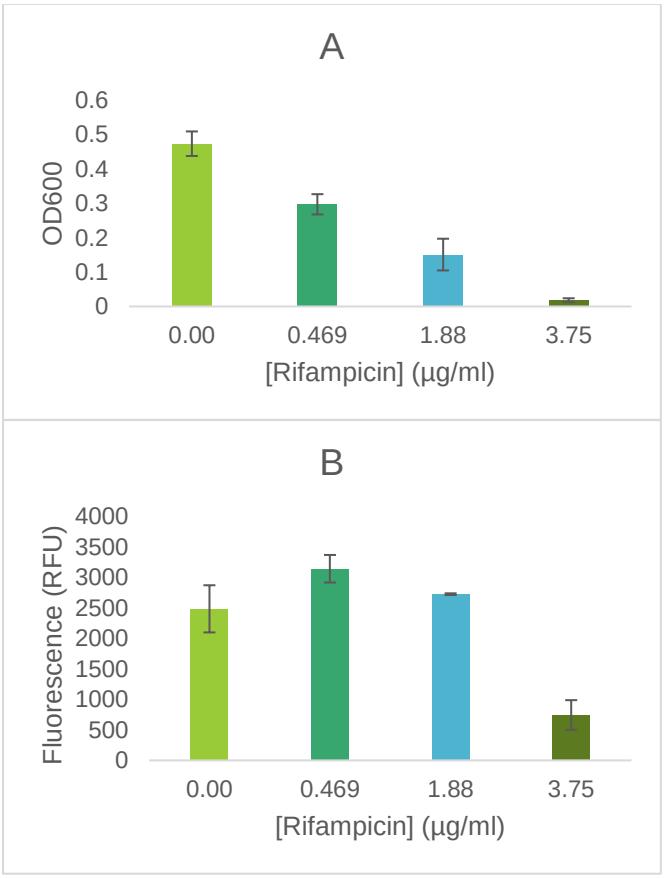


Figure 3. Rifamycin effect on promoter Punk activation in Punk-GFP after 24h of growth. Results of exposure to rifamycin concentrations of 0.00 µg/ml, 3.75 µg/ml, 15.0 µg/ml and 30.0 µg/ml. **(A)** growth (OD600), **(B)** fluorescence (RFU), **(C)** fluorescence normalised per OD600. Standard deviation bars are displayed.



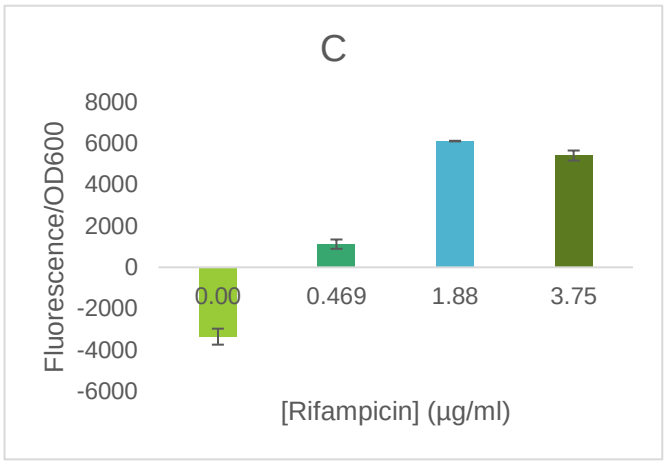
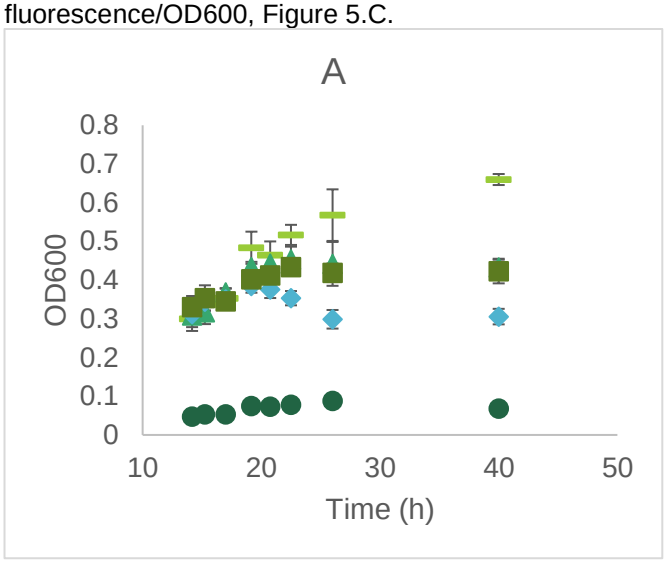
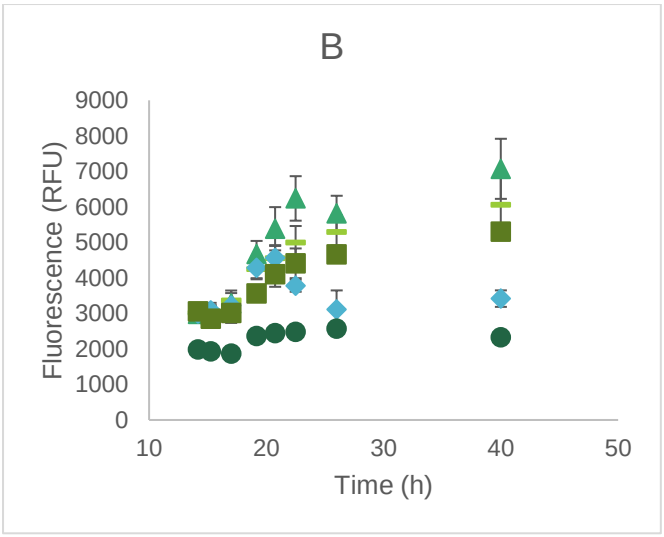


Figure 4. Rifampicin effect on promoter Punk activation in Punk-GFP after 24h of growth. Results of exposure to rifamycin concentrations of 0.00 µg/ml, 0.469 µg/ml, 1.88 µg/ml and 3.75 µg/ml. **(A)** growth (OD600), **(B)** fluorescence (RFU), **(C)** fluorescence normalised per OD600. Standard deviation bars are displayed.

Yim and colleagues showed that some promoters of *Salmonella enterica* were affected by the antibiotic rifampin (rifampicin) concentration and that their expression generally peaked at a concentration close to the MIC value[32]. For this, it would be of interest to narrow the interval of values tested for concentrations closer to the MIC value.

The inductive effect of rifamycin was confirmed by culturing Punk-GFP in shake flasks in two different modes: with antibiotic supplementation 1) from the beginning of the culture and 2) at the middle of the growth phase. Although both cultures with 3.75 µg/ml rifamycin had the same OD600 after 40h of growth, Figure 5.A, the culture with the late addition of 3.75 µg/ml rifamycin showed the highest fluorescence, Figure 5.B. This means that the addition of the compound in a later growth phase induced the promoter Punk differently. On the opposite, the late addition of 25 µg/ml of rifamycin had a stronger inhibitory effect, Figure 5.A, significantly reducing the fluorescence, Figure 5.B. As seen before, the concentration closer to the MIC value added at the beginning of growth resulted in higher fluorescence/OD600, Figure 5.C.





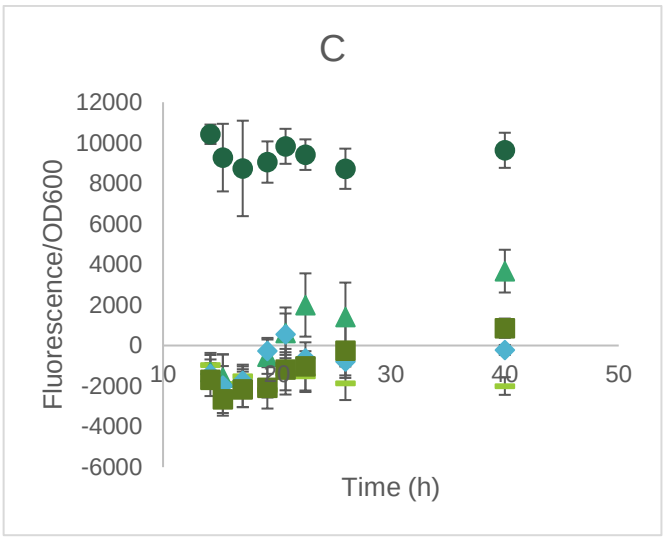


Figure 5. Scattering plot - Rifamycin effect on promoter Punk activation of Punk-GFP (shake flasks). Results of exposure to rifamycin concentrations of 0.00 µg/ml (-), 3.75 µg/ml (■),25.0 µg/ml (●), and 3.75 µg/ml (▲), and 25.0 µg/ml (♦), added only after 15h 30 min of growth. A) OD600, (B) fluorescence, (C) fluorescence normalised per OD600 was calculated. Standard deviation bars are displayed.

Again, the fluorescence/OD600 increased with the increase of antibiotic concentration. Additionally, this assay shows that the addition of rifamycin at the exponential phase can change this tendency: with a lower concentration of rifamycin, the fluorescence of the culture increased without reduction of the growth; with a higher concentration of rifamycin, the fluorescence of the culture decreased with reduction of the growth.

Intrinsic fluorescence levels of *P. protegens* must be considered for GFP reporter gene analysis

To evaluate the inhibitory effect caused by the antibiotic rifamycin, the promoter upstream of *phlA* gene (PphlA), the first gene of the 2,4-DAPG biosynthetic operon, was used as a control as it is already known not to be induced by rifamycin (data not shown). After 24h of growth, the fluorescence/OD600 values increased with the increase in antibiotic concentration.

The increased levels of fluorescence/OD600 with the increase of antibiotic concentration were also observed when the WT and ΔPKS were cultured with different concentrations of rifamycin, Fig. 6.B.

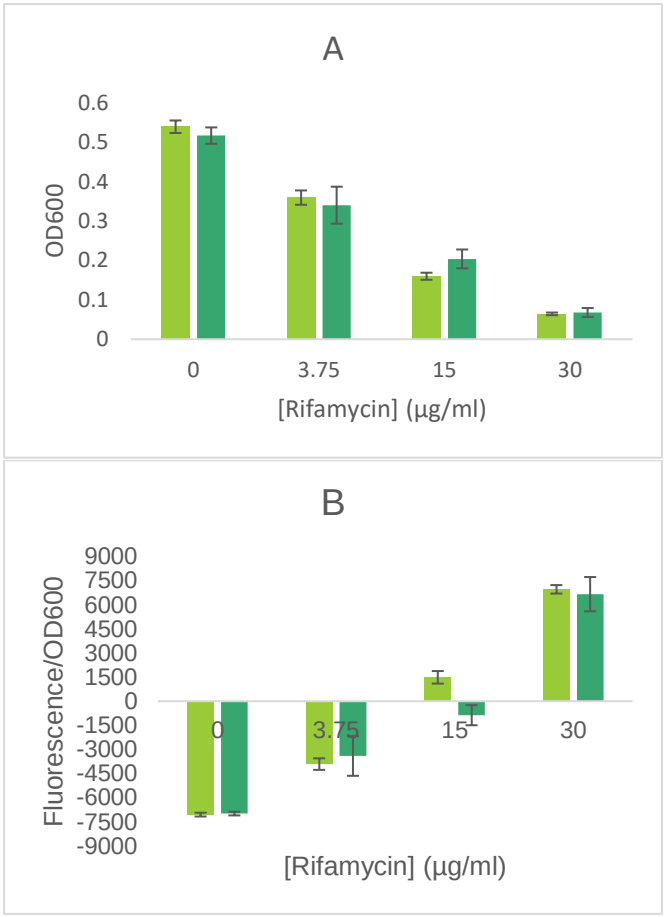


Figure 6. Bar graph – Rifamycin effect of WT (■) and ΔPKS (■) growth and intrinsic fluorescence curves after 24h of culture. Results of exposure to rifamycin concentrations of 0.00 µg/ml, 3.75 µg/ml and 15.0 µg/ml. (A) growth (OD600), (B) fluorescence (RFU), (C) fluorescence normalised per OD600. Standard deviation bars are displayed.

For this, assays using other type of reporter gene (e.g.: luciferase) or qPCR should be used to avoid the fluorescent background effects.

The final OD600 is significantly different ($p<0.05$) between WT and ΔPKS when cultured with 15.0 µg/ml of rifamycin, Figure 4.9.A, meaning that the absence of the unknown BGC made the cell react differently, adding a metabolic burden to the WT. Together with the information obtained from the shake flasks, it is possible to argue that rifamycin induces promoter activation.

Carbon source D-arabitol induces unkBGC expression

To test a condition that did not inhibit culture growth, the effect of carbon source D-arabitol on the unknown promoter was observed. WT and Punk-GFP were cultured with ABTG medium supplemented with D-arabitol (data not shown), but also with ABT medium supplemented with D-arabitol.

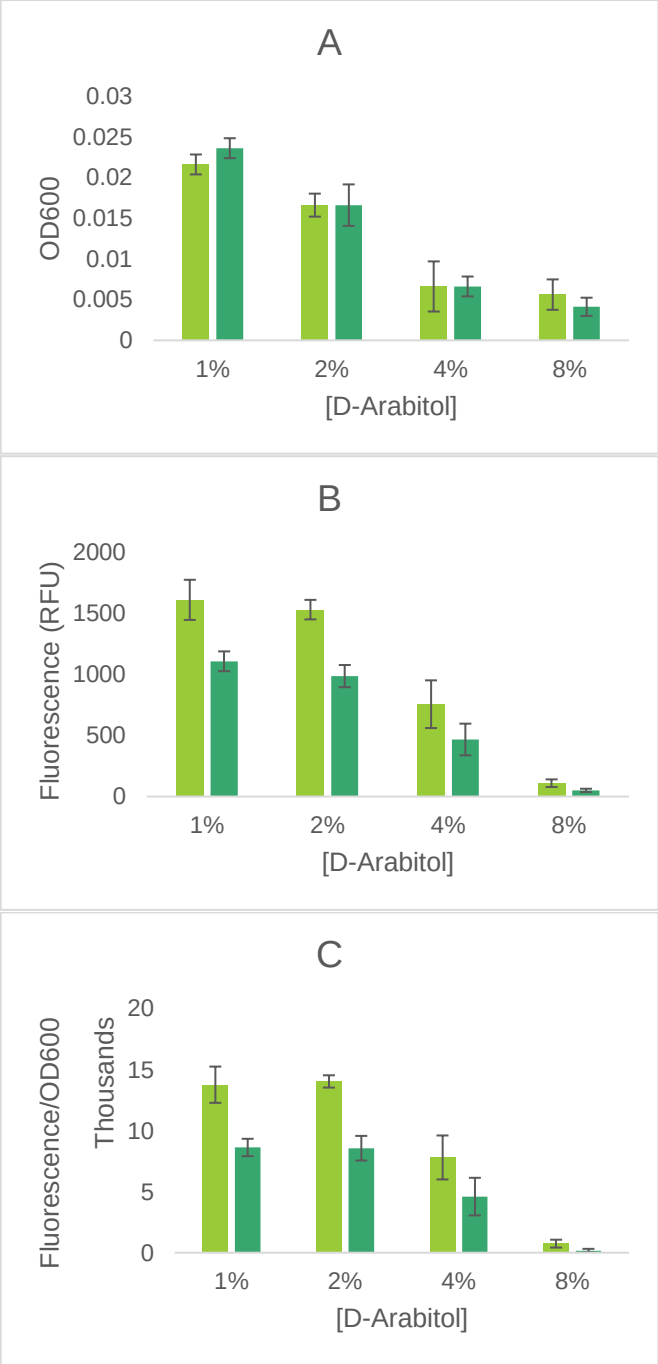


Figure 7. Bar graph – D-Arabitol effect on promoter Punk activation (Punk-GFP(■) compared to WT (■)) after 30h of growth. Results of cultivation with D-arabitol concentrations of 1%, 2%, 4% and 8%. (A) growth (OD600), (B) fluorescence (RFU), (C) fluorescence normalised per OD600.

Supplementation of D-arabitol to ABTG medium did not change growth nor fluorescence levels. Cultures grown in ABT with D-arabitol led to low final values of OD600, being the maximum value of 0.02 when cultured in 1% D-arabitol, Figure 7.A. However, there was a significant increase of fluorescence compared to the control (WT without reporter gene), being this difference larger when the cultures were grown with 1% and 2% D-arabitol, meaning that the promoter was more active in these two conditions, Figure 4.10.B and 4.10.C. Moreover, the difference in normalised fluorescence (5474 RFU/OD600 for 2% D-arabitol), Figure 4.10.C, is larger than the calculated for the conditions in Figure 4.1 (the highest value, 2971 RFU/OD600, was obtained for TSB media). The increased fluorescence levels in the Punk-GFP strain in comparison to the WT indicate that the use of D-arabitol as a carbon source might lead to a higher expression of the unkBGC in comparison to cultures with ABTG. Metabolite production is affected by the composition of carbon and nitrogen source, and the resultant products of the degradation of these molecules[33]. Glucose is a quickly metabolized carbon source, generating the effect of catabolite repression, impairing the production of some catabolic enzymes[34]. Production of secondary metabolites is commonly linked to lower growth rates[33].

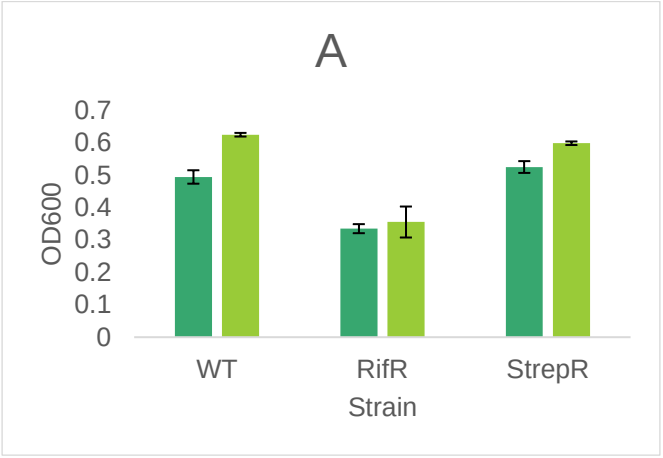
Rif^R mutant overexpresses the unkBGC

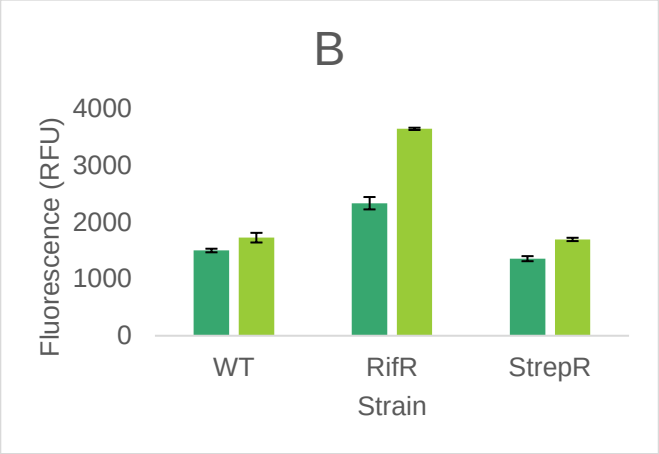
An attempt of RNA polymerase and ribosome engineering was performed by selecting rifamycin (Rif^R) and streptomycin (Strep^R) spontaneous resistant mutants, expecting point mutations in *rpoB*, and *rpsL* genes, respectively. The antibiotic resistance mutation rates were 8.54×10^{-13} and 6.53×10^{-11}, respectively.

The expression of the unknown BGC in the mutants and the WT at 14h, 24h, and 30h were analysed by qPCR (data not shown). Statistical analysis indicates that the difference between the values obtained for each hour is not significant. However, the qPCR still suggests that the production is higher in the late exponential phase.

The generated melting curve of reference gene *rpoD* amplicons for the 14h samples showed a good profile, as the curves peak in one single T_m (no extra amplicons were produced) with the same height (similar quantity of amplified product). Contrarily, the plot of reference gene *gyrB* amplicons for the 30h samples showed curves peaking at slightly different T_m and different heights, meaning that this set of samples included several outliers. The other samples showed similar curves.

The analysis of the ribosome and RNA polymerase engineering was pursued with a GFP expression assay. There is a clear difference in growth between the Rif^R mutant and the rest of the strains, Figure 8.A. The difference in normalised fluorescence with the reporter gene compared to without the reporter gene is higher for Rif^R strain. The ratio between the differences of normalised fluorescence of Rif^R strains and normalised fluorescence of WT strains, Figure 8.C estimates an increase in expression of the unkBGC of approximately 4.2-fold with RNA polymerase engineering.





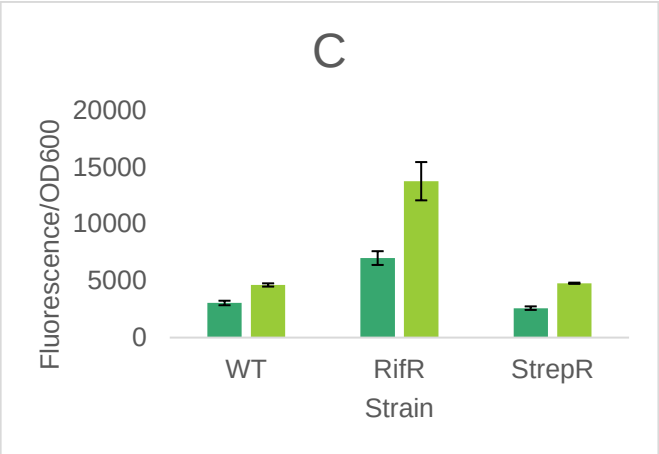


Figure 8. Bar graph – Promoter Punk expression in WT, Rif^{R,} and Strep^{R} strains after 30h. ■ - *without GFP reporter gene,* ■ - with GFP reporter gene. (A) growth (OD600), (B) fluorescence (RFU), (C) fluorescence normalised per OD600 was calculated. Standard deviation bars are displayed.

Surprisingly, there is no significant difference between WT and Strep^{R} growth and fluorescence, Figures 8.A and 8.B. Sequencing results for *rpsL* mutation check were revisited and the analysis was repeated with different primers, evaluating a broader region of the genome. The first results indicated an insertion that the second results contradicted, explaining why the ribosome engineering failed.

The point mutation in the *rpoB* gene resulted from the replacement of a thymine to a cytosine, leading to aspartate-521 to glycine substitution, a common mutation in the *rpoB* gene, induced by rifamycin antibiotics[35].

Although a more exact determination of relative expression was not obtained by qPCR, the expression assay clearly shows that RNA polymerase engineering led to an overexpression of the unknown BGC.

Insertion of a stronger promoter by allelic replacement – troubleshooting

To increase the gene expression and compound production for possible chemical identification of the compound and phenotype studies in the WT, the process of replacing the original promoter for a stronger one was initiated.

After the transformation of the competent cells, the false positive (FP) control, where very few or no colonies were expected, showed a similar number in comparison to the transformation plate (T) (FP – 16 CFU, T – 18 CFU). Several attempts to solve the problem were performed: by retrying the digestion steps for both fragments (including one trial with the addition of Fast AP and another with freshly bought enzymes), using a sample of digested plasmid previously used. The difference in the number of FP and transformant colonies increased, but when the transformants were analysed by colony PCR, by amplifying the region where the promoter should have been inserted, the resultant bands had several different sizes, as exemplified in Figure 9 (1-8). The eight colony PCR fragments had different sizes ranging from 500 base pairs (bp) to 1100 bp when the expected size was approximately 2000 bp, when using the primers 120/121. This primer pair results in fragments with 500 bp when applied in plasmid pNJ1.

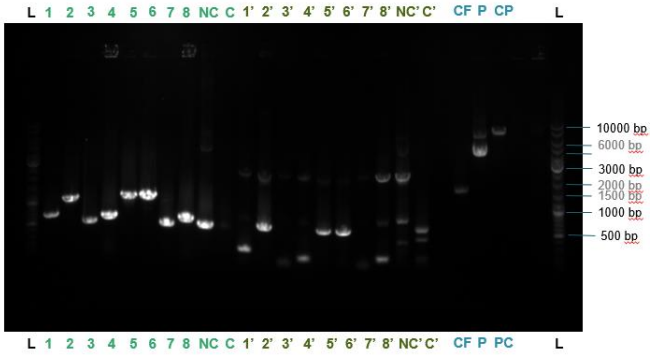


Figure 9 Agarose gel electrophoresis of colony PCR products of transformed *E. coli* CC118 λspir. Two sets of primers were used: 120/121 and 363/366. **Legend**: L – GeneRuler^{TM} DNA ladder mix, 1-8 – PCR products resultant from primers' 120/121 application in eight different transformant colonies, NC – control with the original plasmid pNJ1, C - control with the non-digested plasmid pNJ1, 1'-8' – PCR products resultant from primers' 363/366 application in eight different transformant colonies; CF – digested SOE fragment, P – original plasmid pNJ1, PC – digested plasmid pNJ1 loaded with DNA Gel Loading Dye (6X).

The colonies showing bands with sizes closer to what was expected were selected for plasmid purification and posterior sequencing of the local of insertion (primers 120/121). The sequencing results showed that the plasmid seemed to have been torn apart and fused again, as the different parts were aligning with different regions of the original plasmid.

The integrity of the gene fragment created with gene splicing by overlap extension (SOE) PCR was evaluated in an agarose gel, confirming the right size, Figure 9 (CF). In the PCR products generated with primers 120/121, the control with the original plasmid (NC) shows the correct band size (~500 bp). In the PCR products generated with primers 363/366, both the plasmid controls (NC' and C') show several bands, which means that the pair of primers were binding to multiple regions of the plasmid. All the PCR products include a band with approximately 2000 bp (1' to NC'), with an exception for the digested plasmid product (C'). The other bands have sizes inferior to 1000 bp, meaning that none of the transformants evaluated included the SOE PCR fragment (~1500 bp).

Due to the mentioned technical difficulties, no colonies with the desired plasmid were obtained. For this, the

process of insertion of a stronger promoter upstream of the unkBGC was halted.

Conclusion and future remarks

This project aimed to identify successful strategies to the activation of a cryptic BGC in *Pseudomonas protegens* DTU9.1, a plant-beneficial bacteria. The identification of the resultant secondary metabolites of cryptic BGCs can unravel mechanisms relevant for the use of microorganisms as plant growth-promoting agents.

Expression assays indicate that the unknown BGC is being expressed. The deletion of the unknown PKS, analysed by LC-MS analysis, showed to interfere with the concentration of several metabolites, mainly the intracellular metabolites. However, the low expression levels were not enough to analyse the structure and function of the putative produced compound. Hence, the expression activation is required to progress in this study.

Based on the expression assays performed with antibiotic media supplementation, it can be concluded that rifamycin is a strong candidate for the activation of the unknown BGC. The assay with delayed addition of the antibiotic indicates that the best moment for an elicitor addition to the media should also be a case of study. The cultivation of the strain with D-arabitol as an alternative carbon source led to lower growth levels but higher fluorescence/OD600 values, adding that the expression of the BGC might be carbon source composition and/or low growth dependent. In both cases, and as expression assays were subject to fluorescent background effects, the fold change in expression levels should be evaluated with qPCR, a more precise method. Based on these results, studies with other carbon sources should be addressed. The use of artificial root exudates containing different concentrations of organic (including carbon sources) and inorganic molecules could be particularly relevant. Another alternative is to use a *fed-batch* culture maintaining glucose at a very low concentration, reducing the effect of catabolite repression. This method would also facilitate the expression evaluation by addition of antibiotics in a later growth phase.

RNA polymerase engineering, by selecting a spontaneously rifamycin-resistant colony with a *rpoB* gene mutation, resulted in the highest expression increase observed in this project. The study of other mutations in the *rpoB* gene and ribosome-related genes might result in a more expressive BGC activation. The conjugation of some of these mutations can even result in more stable phenotypes, improving the growth rate usually impaired by these mutations.

Switching the endogenous promoter with a stronger promoter was a technique attempted in this report. Although technical limitations hampered the conclusion of the process, successful results could have opened the doors to the metabolite identification, by a retrial in LC-MS, or using nuclear magnetic resonance (NMR) which has more analytical precision, for the metabolite identification.

Co-cultivation, epigenetics modifications, or metabolic engineering are common strategies for cryptic BGC activation[36]. Additionally, the biofilm production capacity of *P. protegens* strains could be used to assess if the unknown BGC is activated by phenomena related to the populational density (quorum sensing).

After successful overexpression of the BGC, bioactivity assays should be conducted. For example, bactericidal and fungicidal activity, assessed by co-cultivation the strain with other microorganisms. The plant growth-promoting effect could be analysed by the cultivation of plants in presence of the strain in the roots space. The effect on stress toleration could be observed by culturing the cells under different stresses, like UV-light, oxidative stress, or osmotic stress.

Materials and Methods

Chemical, strains, and culture conditions. Chemical products used were acquired from *Sigma-Aldrich*®, *Milipore*® and *ACROS ORGANICS* and PCR reagents were obtained from Thermo-Fisher SCIENTIFIC (with indicated exceptions). Plasmids were ordered from IDT®. This study focused on *Pseudomonas protegens* DTU9.1 (WT), including PKS deletion mutant (ΔPKS) and strains spontaneously resistant to streptomycin ($Strep^R$) and rifamycin (Rif^R), selected with 135 µg/ml streptomycin and 65 µg/ml rifamycin, respectively. GFP-tagged derivative of WT, $Strep^R$, and Rif^R carrying the plasmid pSEVA237::P_{unk}-GFP, were used in the unknown BGC expression assays. *Escherichia coli* CC118λspir and *E. coli* HB101, containing the plasmid pRK600 were used as donor and helper in triparental mating technique, respectively. Plasmid pSEVA237::P_{phlA}-GFP was used in the WT as negative control. The media used were ABT, ABTG (4% glucose), LB, KB, TSB, PIA. Three or four biological replicates were used in each assay. DNA sequences were sequenced by *Eurofins*, previously purified with NucleoSpin® Gel and PCR Clean-up kit (*Macherey-Nagel*).

Unknown BGC expression assays. Growth (optical density at a wavelength of 600 nm (OD600)) and fluorescence (relative units of fluorescence (RFU)) were analysed in a 96-well microtiter plate at 30 °C and 282 rpm for 24h or 30h (with initial double agitation of 282 rpm for 10 secs). The initial OD600 was set to 0.01 (*Amersham Biosciences – Ultrospec 10*) and each well contained a volume of 200 µl. A negative control with only media was included, to be used as blank and contamination assessment. The plates were incubated in a multimode microplate reader (*Biotek Synergy H1 Hybrid Reader).* Excitation and emission wavelength for fluorescence reading were 485 and 513 nm, respectively.

Colonies comparison. 5 µl of WT and ΔPKS cultures were washed in NaCl 0.9% (OD600 = 1.0) and spotted on ABTG, TS, LB, and KB agar plates, with three biological replicates for each condition. Differences were observed after two days of growth on the plates and the diameter of the colonies was measured with *ImageJ* software.

Shake flasks assay. 100 ml Erlenmeyer flasks contained a total volume of 25 ml at 30 ºC and 200 rpm (*Infors HT Labotron*). The initial OD600 was 0.01. The volume of samples analysed each time was 150 µl, and the samples were incubated in a multimode microplate reader, at 282 rpm for 6 min (with initial double agitation of 282 rpm for 10 secs), reading OD600 and fluorescence values 4 times.

LC-MS. Extracts of cellular metabolites were physically separated by liquid chromatography (LC) and had their mass analysed by mass spectrometry (MS). The

resultant data was analysed by *MS-Dial* software and *MetaboAnalyst 5.0* online tool. The chromatograms were constructed with *Agilent MassHunter* software. Extracellular metabolites were extracted from 100 ml of cultures cultivated in 250 ml Erlenmayer flasks 30ºC and 200 rpm. The samples were centrifuged for 5 min at 8000 rpm and the supernatant was filter sterilized (pore diameter of 0.25 µm). 20 ml of the extraction solvent ethyl acetate were added to the 20 ml of filtered supernatant. The mixture was vortexed for 5 min and centrifuged for 5 min at 8000 rpm, resulting in two distinct phases: an upper organic phase and the lower aqueous phase. 14 ml of the upper phase were collected, dried with a nitrogen pump, and dissolved in 200 µl of methanol. Intracellular metabolites were extracted from 100ml cultures cultivated in 250 ml Erlenmeyer flasks at 30ºC and 200 rpm. The entire volume of cultures was centrifuged for 5 min at 8000 rpm, washed with 25 ml of 0.9% NaCl, and centrifuged again. The supernatant was completely removed, and the pellets were resuspended in 10 ml ethyl acetate. The suspension was sonicated for 20 mins and then centrifuged. 8 ml of the ethyl acetate layer was transferred to a falcon tube, dried with a nitrogen pump, and reconstituted with 200 µl of methanol. The final methanol solutions were transferred to LC-MS vials and sent to the Natural Products Chemistry group (DTU Bioengineering) for LC-MS analysis.

Isolation of RNA and synthesis of cDNA. WT, $Rif^{R,}$ and $Strep^{R}$ were incubated in 100ml Erlenmeyer flasks containing 50 ml of ABTG medium (OD600 = 0.01) at 30 ºC with orbital agitation at 200 rpm for 30h. Samples were collected at 14h, 24h, and 30h, and centrifuged. The supernatant was discarded, and the pallets were stored at -23 ºC. RNA was extracted from the pellets with RNeasy® Mini kit (*QUIAGEN*) according to the protocol supplied by the manufacturer. 1 µg of the obtained sample was converted into complementary DNA (cDNA) using the iScript™ Reverse Transcription Supermix for RT-qPCR (*Bio-Rad*) kit, according to the protocol supplied by the manufacturer. RNA and cDNA concentrations were measured by spectrophotometry with DS-11+Spectrophotometer (*DeNovix*®).

qPCR. Samples were prepared with PowerUp™ SYBR™ Green Master Mix following manufacturer instructions to a final volume of 10 µl per sample. The samples were analysed in technical and biological triplicates and a no-template control was included, using water instead of cDNA. The reactions were performed and analyzed by the instrument *Stratagene Mx3005P qPCR*. Primer efficiency was validated using a 5-point 5-fold serial dilution of templates. The thermal cycle applied was 50 °C for 2 min, 95 °C for 2 min, 40 cycles of 95 °C for 15 sec, 55 for 15 sec and 72 for 1 min, with a final melting curve between 60 °C and 95 °C.

Splicing Overlap Extension (SOE) PCR. A fragment of DNA containing the promoter p14g (amplified from the plasmid pBG42A) surrounded by elements homologous upstream and downstream of the target genomic region (amplified from the bacterial genome) was constructed.

Restriction digestion. Both SOE product and the plasmid pNJ1 went through restriction digestion using the enzymes SacI and XbaI, and 10X FastDigest Green Buffer (ThermoFisher Scientific). The thermal conditions for restriction digest were 37 ºC for 20 and 60 min (plasmid and fragment, respectively), and 65 ºC for 20 min.

Ligation. The concentrations used for vector/insert ligation were such to obtain a base pair ratio (insert/plasmid) of approximately 1:5 (plasmid: 6692 bp; insert: 1489 bp). Ligation reaction was performed using thermocycler program with ligation at 22 °C for 2h and protein degradation at 65 °C for 10 min steps.

Transformation. The constructed plasmid was transformed into *E. coli* CC118 λpir by heat shock transformation. The cells were mixed with 2.5 µl DNA and placed in ice for 30 mins, incubated at 42°C for 1 min, and placed again in ice for 2 mins. 450 µl of LB were added to each tube and the mixed sample was incubated at 37°C for 1 hour for recovery. The cells were spread on selective LB agar plates.

Statistical Analysis. In MetaboAnalyst 5.0, data filtering was based on interquartile range, samples were normalized by sum and the data scaling chosen was Pareto scaling. Differences were considered statistically significant for p-values lower than 0.05.

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