Modulation of multiple gene clusters expression by a single PAS-LuxR transcriptional regulator.

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ABSTRACT

PAS-LuxR transcriptional regulators are highly conserved enzymes governing polyene macrolide antifungal biosynthesis. PteF is one of such regulators, situated in the polyene macrolide filipin gene cluster from *Streptomyces avermitilis*. Its mutation leads to a drastic filipin production decline, but also to a severe loss of oligomycin production, an ATP-synthase inhibitor of macrolide structure, and a delay in sporulation, thus it has been considered as a transcriptional activator. Transcriptomic analyses were carried out in *S. avermitilis* Δ*pteF* and its parental strain *S. avermitilis* NRRL 8165 as control. Both strains were grown in YEME medium without sucrose, and samples were taken in the exponential and stationary growth phases. 257 genes showed altered expression in the PteF-deleted mutant, most of them in the exponential phase of growth. Surprisingly, despite PteF being an activator of filipin biosynthesis, a majority of the genes affected upon mutation showed overexpression thus suggesting a negative modulation of those genes. Genes affected were related to various metabolic processes, including genetic information processing; DNA, energy, carbohydrate, and lipid metabolism; morphological differentiation; and transcriptional regulation; among others, but particularly to secondary metabolite biosynthesis. Notably, ten secondary metabolite gene clusters out of 38 encoded by the genome, some of them encoding cryptic compounds, showed altered expression profiles in the mutant, suggesting a regulatory role for PteF wider than expected. Transcriptomic results were validated by quantitative reverse transcription polymerase chain reaction. These findings provide important clues to understand the intertwined regulatory machinery that modulates antibiotic biosynthesis in *Streptomyces*.

KEYWORDS

Antifungal, Gene regulation, LuxR, PAS domain, polyene macrolide, *Streptomyces*

48 **INTRODUCTION**

49

50 PAS-LuxR regulators are transcriptional factors that combine an N-terminal PAS
51 sensory domain (Hefti et al., 2004) with a C-terminal helix-turn-helix (HTH) motif of the LuxR
52 type for DNA-binding (Santos et al., 2012). The sensor domain is thought to detect a physical
53 or chemical stimulus and regulate, in response, the activity of the effector domain (Möglich et
54 al., 2009). The archetype of this class of regulators, PimM, was first identified in the antifungal
55 pimaricin biosynthetic gene cluster from *Streptomyces natalensis* (Antón et al., 2007). It was
56 characterised as a transcriptional activator of pimaricin biosynthesis since antifungal production
57 was abolished upon gene deletion, and later its mode of action was characterised at the
58 molecular level (Santos-Aberturas et al., 2011a). Since its discovery, homologous regulatory
59 proteins have been found to be encoded in all known biosynthetic gene clusters of antifungal
60 polyketides, and they have been shown to be functionally equivalent, to the extent that the
61 production of pimaricin is restored in *S. natalensis ΔpimM* upon introduction of heterologous
62 regulators of the PAS-LuxR class, such as *nysRIV* (nystatin), *amphRIV* (amphotericin), or *pteF*
63 (filipin) into the strain (Santos-Aberturas et al. 2011b). Furthermore, introduction of a single
64 copy of *pimM* into the amphotericin-producing strain *S. nodosus*, into the filipin-producing
65 strain *S. avermitilis*, or into the rimocidin producing strain *S. rimosus*, boosted the production
66 of all polyenes, thus indicating that these regulators are fully exchangeable (Santos-Aberturas
67 et al. 2011b).

68 Although given their location in the chromosome, PAS-LuxR regulators were initially
69 considered pathway-specific transcriptional regulators, recent results have shown that they
70 should be considered as regulators with a wider range of implications. The canonical operator
71 of PimM was used to search for putative targets of orthologous protein PteF in the genome of
72 *S. avermitilis*, finding multiple binding sites located inside or upstream from genes involved in
73 different aspects of both primary and secondary metabolism (Vicente et al,. 2015), thus

74 suggesting that the regulator could govern those processes. Several of these operators were
75 selected, and their binding to PimM DNA-binding domain demonstrated by electrophoretic
76 mobility shift assays (EMSAs). As a proof of concept, the biosynthesis of the ATP-synthase
77 inhibitor oligomycin whose gene cluster included two operators was studied (Vicente et al,.
78 2015). PteF deleted mutants, which showed a severe loss of filipin production and delayed spore
79 formation in comparison to that of the wild-type strain (Vicente et al 2014), also showed a
80 severe loss of oligomycin production and reduced expression of *olm* genes. Gene
81 complementation of the mutant restored phenotype, thus demonstrating that PteF was able to
82 co-regulate the biosynthesis of two related secondary metabolites, the polyketide macrolides
83 filipin and oligomycin (Vicente et al., 2015). This cross-regulation could therefore be extended
84 to all the clusters where operators were found, which suggests that PAS-LuxR regulators may
85 affect a plethora of processes previously unforeseen. In this sense, the introduction of PAS-
86 LuxR regulatory genes into different *Streptomyces* hosts has already proven useful for the
87 awakening of dormant secondary metabolite biosynthetic genes (Olano et al., 2014; Martínez-
88 Burgo et al., 2019).

89 Here we have used microarrays to study the transcriptome of *S. avermitilis* Δ*pteF* mutant
90 in comparison with that of its parental strain in order to deepen our knowledge about the
91 processes in which PteF is involved.

92

93 **MATERIALS AND METHODS**

94

95 **Strains and cultivation**

96 *S. avermitilis* NRRL 8165 and its mutant *S. avermitilis* Δ*pteF* (Vicente et al., 2014) were
97 routinely grown in YEME medium (Kieser et al., 2000) without sucrose. Sporulation was
98 achieved in TBO medium (Higgens et al. 1974) at 30˚C.

99

100 **Nucleic acid extractions.**

101 RNA was extracted as described elsewhere (Vicente et al., 2014). Briefly, 2 ml from
102 liquid cultures in YEME medium without sucrose were harvested by centrifugation and
103 immediately frozen by immersion in liquid nitrogen. Cells were resuspended in lysis solution
104 [600 µl RLT buffer (RNeasy mini kit; Qiagen); 6 µl 2-mercaptoethanol] and disrupted using
105 sonicator (Ultrasonic processor XL; Misonix Inc.). RNeasy ® Mini kit (Qiagen) was used for
106 RNA isolation using RNase-Free DNase Set (Qiagen) as specified by manufacturer, followed
107 by two consecutive digestions with TURBO^{TM} DNase from Ambion® according to the
108 manufacturer's instructions. Total RNA concentration was determined with a NanoDrop ND-
109 1000 spectrophotometer (Thermo Scientific), and quality and integrity were checked in a
110 Bioanalyzer 2100 apparatus (Agilent Technologies). Total genomic DNA (gDNA) was isolated
111 from stationary phase cultures following the salting-out procedure (Kieser et al., 2000).

112

113 **Microarray hybridizations.**

114 The microarray experiment was performed using a common reference design (Gadgil et
115 al. 2005). The microarray chip Custom Gene Expression Microarray, 8x15K (Agilent) was
116 customized in order to include different sets of probes as indicated elsewhere (Beites et al.,
117 2014). For each microarray hybridization, 10 pmol of Cy3-labelled cDNA obtained from total
118 RNA were mixed with 80 pmol of Cy5-labelled genomic DNA as the common reference.
119 Labelling, hybridization, washing and scanning conditions were carried out as indicated
120 previously (Rodríguez-García et al. 2007; Guerra et al., 2012). Three biological replicates from
121 independent cultures were made for each experimental condition.

122

123 **Identification of differentially transcribed genes**.

124 Microarray data were normalized and analysed with the Bioconductor package LIMMA
125 (Linear Models for Microarray Analysis) (Smyth 2004; Smyth et al. 2005). Spot quality weights

126 were estimated as indicated in the Supplementary section (Tables S1 and S2). Both local and
127 global normalizations were used (Wu et al., 2005). Firstly, weighted medians of $\log_2$ Cy3/Cy5
128 intensities were calculated for print-tip correction and afterwards global Loess was applied
129 (Smyth and Speed 2003). The normalized $\log_2$ of Cy3/Cy5 intensities is referred in this work
130 as the Mg value, which is proportional to the abundance of transcripts for a particular gene
131 (Mehra et al., 2006). The information from within-array spot duplicates (Smyth et al., 2005)
132 and empirical array weights (Ritchie et al., 2006) were taken into account in the linear models
133 (Smyth 2004). The Mg transcription values of the four experimental conditions were compared
134 using two contrasts, mutant versus wild type, corresponding to the two studied growth phases
135 (exponential and stationary). For each gene, the Mc value is the binary log of the differential
136 transcription between the mutant and the wild strain. The Benjamini-Hochberg (BH) false-
137 discovery rate correction was applied to the *p*-values. A positive Mc value indicates
138 upregulation, and a negative one, downregulation. For each contrast a result was considered as
139 statistically significant if the BH-corrected *p*-value was <0.05. In certain occasions, however,
140 when the transcription profile of a gene matched that of genes statistically significant and
141 functionally related, or for comparison with previous published results obtained by RT-qPCR
142 or by EMSA assays (Vicente et al., 2014, 2015), we used an uncorrected *p*-value with a level
143 of significance <0.05.
144 The microarray data are deposited in the National Center for Biotechnology
145 Information-Gene Expression Omnibus under accession number GSE185887.
146
147 **Assessment of filipin and oligomycin production.**
148 Filipin production was quantified as described elsewhere (Barreales et al., 2020),
149 whereas oligomycin was measured following the procedure described by Vicente et al (2015).
150
151

152 **Reverse transcription-quantitative PCR.**

153 Reverse transcription of total RNA was performed on selected samples with 5 µg of
154 RNA and 12.5 ng/µl of random hexamer primer (Invitrogen) using SuperScript™ III reverse
155 transcriptase (Invitrogen) as described previously (Barreales et al., 2018). Reactions were
156 carried out on two biological replicates with three technical replicates each and appropriate
157 controls were included to verify the absence of gDNA contamination in RNA and primer-dimer
158 formation. Primers (see Table S3) were designed to generate PCR products between 97 and 153
159 bp, near the 5´ end of mRNA. The PCR reactions were initiated by incubating the sample at
160 95°C for 10 min followed by 40 cycles at 95°C for 15 s, 62-70°C (depending of the set of
161 primers used) for 34 s, and 72 ºC for 30 s. To check the specificity of real-time PCR reactions,
162 a DNA melting curve analysis was performed by holding the sample at 60°C for 60 s followed
163 by slow ramping of the temperature to 95°C. Baseline and threshold values were determined
164 by the StepOnePlus software. C_t values were normalized with respect to *rrnA1* mRNA
165 (encoding 16S rRNA). Relative changes in gene expression were quantified using the Pfaffl
166 method (2001) and the REST© software (Pfaffl et al., 2002). The corresponding real-time PCR
167 efficiency (E) of one cycle in the exponential phase was calculated according to the equation E
168 = $10^{[-1/slope]}$ (Rasmussen, 2000) using 5-fold dilutions of genomic DNA ranging from 0.013 to
169 40 ng (n=5 or 6 with three replicates for each dilution) with a coefficient of determination $R^2 >$
170 0.99 (Fig. S1).

171

172 **RESULTS AND DISCUSSION**

173

174 **Identification of genes with an altered expression profile in *S. avermitilis ΔpteF* mutant.**

175 *S. avermitilis* Δ*pteF* and its parental strain *S. avermitilis* NRRL 8165 were grown in
176 YEME medium without sucrose, and samples were taken at the end of the exponential and at
177 the middle of the stationary growth phases (Fig. 1). Transcriptomic analysis was performed by

178 microarray hybridization to assess the genes with an altered expression in the mutant when
179 compared with the parental strain. Genomic DNA was used as a universal reference for all
180 hybridizations. A result was considered as statistically significant if the BH-corrected *p*-value
181 was <0.05. It is worth noting that these conditions are quite stringent, given that genes that
182 constitute direct targets of PteF (e.g. the filipin polyketide synthases *pteA1* and *pteA2*; Vicente
183 et al., 2014) are not statistically significant. With this criterion, microarrays analysis showed
184 significant differences (with a fold change above or below +/-2) in the expression of 208 genes
185 of the *pteF*-negative mutant at the end of the exponential phase, and 99 at the stationary phase
186 of growth (Table 1; Fig. 2).

187 Surprisingly, the lack of PteF resulted in the overexpression of a majority of the
188 differentially transcribed genes, at both sampling times, thus indicating that this regulator acts
189 as a negative modulator for those genes expression. This was unexpected given that PteF is an
190 activator of both the antifungal filipin (Vicente et al., 2014) and the ATP-synthase inhibitor
191 oligomycin (Vicente et al., 2015) biosynthesis.

192 These genes were related to different cellular processes, including genetic information
193 processing; energy, carbohydrate, and lipid metabolism; DNA replication and repair;
194 morphological differentiation; and transcriptional regulation, among others, but particularly to
195 secondary metabolite biosynthesis (Table 1).

196

197 <u>Genes involved in genetic information- and protein-processing, and amino acid metabolism.</u>

198 This group includes 24 genes that showed differential transcription in at least one of the
199 sampling times (Table 1). These genes code for enzymes involved in amino acid metabolism
200 (7 genes), proteins involved in transcription (8 genes, including 5 sigma factors), the ribosomal
201 protein L28 (*SAV2675*), two putative acetyltransferases of ribosomal proteins (*SAV703* and
202 *SAV758*), and enzymes involved in protein processing (5 genes) (Table S4).

203 Interestingly, while sigma factors *sig10* (*SAV898*), *sig13* (*SAV997*), and *sig60*
204 (*SAV213*), and ribosomal proteins acetyltransferases *SAV703* and *SAV758* showed increased
205 transcription levels in the mutant, *sig32* (*SAV3888*), *sig40* (*SAV4561*), the L28 ribosomal
206 protein encoding gene *rpmB1*, and the *whiB*-like transcriptional factor *wblE* were clearly
207 underexpressed in the mutant. The Wbl family of transcriptional factors is exclusive of
208 actinobacteria and their members have been correlated with diverse roles in morphological
209 differentiation and secondary metabolism (Fowler-Goldsworthy et al., 2011; Bush 2018).
210 Noteworthy, genes *rocA* (*SAV2723*) and *putA* (*SAV2724*), that encode delta-1-pyrroline-
211 5-carboxylate dehydrogenase and proline dehydrogenase, respectively, and that have been
212 related to proline catabolism (Menzel and Roth, 1981), and *rocD2* (*SAV7112*) and *SAV4551*,
213 which encode putative ornithine aminotransferases, and are also involved in proline metabolism
214 were underexpressed in the mutant, while *leuB* (*SAV2718*) which has been involved in valine,
215 leucine, and isoleucine biosynthesis biosynthesis, *paaI* (*SAV1986*) that encodes a phenylacetic
216 acid thioesterase, and putative cysteine desulfurase *SAV1061* were overexpressed.
217
218 <u>Genes involved in nucleotide and vitamin metabolism, and DNA replication, recombination,</u>
219 <u>and repair</u>.
220 Eighteen genes falling into this category were found to be differentially transcribed in
221 the mutant (Table 1). Ten of them are involved in DNA replication, recombination and repair.
222 Of these, seven putative transposases belonging to different families showed enhanced
223 transcription in the mutant. Additionally, two genes involved in DNA repair, *ku2* (*SAV879*)
224 which is probably involved in non-homologous DNA end-joining (Zhang et al., 2012), and
225 *uvrD1* (*SAV3463*) that codes for a putative ATP-dependent helicase, were also upregulated.
226 Conversely, *int12* (*SAV4626*), which encodes a tyrosine-family recombinase/integrase, showed
227 reduced transcription levels at stationary phase.

228 The remaining genes were differentially transcribed only in the exponential phase. Four
229 genes are involved in vitamin metabolism, three of them with lower transcription in the mutant,
230 including cobalamin methylase *cobJ* (*SAV6407*) and adenosyltransferase *cobA* (*SAV6413*), and
231 alkaline phosphatase *phoA* (*SAV5915*), which besides being part of the PhoRP two-component
232 system (Sola-landa et al., 2008) is also involved in folate metabolism. The fourth gene, *thiC*
233 (*SAV4265*) is a thiamine biosynthesis protein (Table S4). The rest of the genes are involved in
234 purine metabolism, including *pgmA*, *purA*, and *purN*, all with enhanced transcription, and *cpdB*,
235 with lower transcription.
236
237 Carbohydrate metabolism genes.
238 Thirteen genes fall into this category, including four most likely belonging to the same
239 operon (*SAV1009*, *galE5*, *mpg2*, *SAV1014*) and putatively involved in galactose metabolism,
240 and showing enhanced transcription in the mutant. Other genes involved in the metabolism of
241 this sugar were the alpha-galactosidase *agaB1* (*SAV1082*), which was underexpressed in the
242 mutant, and the phosphoglucomutase *pgmA* (*SAV803*), which showed the opposite behavior.
243 Interestingly, three genes of the tricarboxylic acid/glyoxylate cycle (citrate synthase *citA2*,
244 citrate lyase *citE2*, and methylmalonyl-CoA mutase *meaA1*) were overexpressed in the mutant
245 (Table S4).
246
247 Lipid metabolism genes.
248 Nine genes related to lipid metabolism were differentially transcribed. These include
249 the putative 3-oxoacyl-ACP synthase II *fabB2* (*SAV2944*), the acyl carrier protein *fabC4*
250 (*SAV217*), the enoyl-CoA hydratase *echA1* (*SAV492*), and the acetyl/propionyl CoA
251 carboxylase alpha subunit *accA2* (*SAV3866*), which are all presumably involved in fatty acid
252 biosynthesis, and the 1-acylglycerol-3-phosphate O-acyltransferase *plsC1* (*SAV1485*)
253 putatively involved in glycerophospholipid biosynthesis, among others. Interestingly, all these

genes showed increased transcription in the mutant during the exponential phase except *fabB2* which was underexpressed (Table S4). However, during the stationary phase *fabB2* also showed enhanced transcription.

Noteworthy, the direct binding of the PteF orthologue PimM to the promoters of two of these genes was already demonstrated (Vicente et al., 2015), thus they have been included in Table S4 although they did not meet the statistical criteria. These were the acyltransferase *plsC1* (Yao and Rock 2013) whose transcription was increased in the mutant (Mc 0.88, uncorrected *p* value 0.0471), and *fabB2* whose transcription was reduced (Mc -0.84, uncorrected p-value 0.0410 in t1) or increased (Mc 1.12, *p*-value 0.0048 in t2) depending on the growth phase.

Energy production genes.

Only three genes belonging to this group were found to be differentially transcribed in the mutant. All of them involved in oxidative phosphorylation and with reduced transcription in the mutant, two belonging to the operon *nuo* (*nuoJ1* and *nuoK1*), and the ATP synthase *atpF* (Table S4). Interestingly, all the genes belonging to the *nuo* operon (*SAV4837-SAV4850*), although in several cases not meeting the statistical criteria, showed the same decreased transcription profile in the mutant.

Transport and external signals processing.

This group includes 25 genes that showed differential transcription in at least one of the sampling times (Table 1). Interestingly, twelve of them code or participate in the formation of ATP-binding cassette transporters (Table S4). Of these, four are putatively involved in sugar transport (*SAV1804*, *SAV2246*, *SAV2247*, and *SAV2609*) and showed reduced transcription in the mutant.

278 Four transporters belonging to the major facilitator superfamily showed differential
279 transcription in the mutant, *SAV2455* with reduced transcription, and *SAV610*, the sulfate
280 transporter *SAV4600, and SAV6941* with enhanced transcription.

281 Noteworthy, in agreement with the enhanced transcription of *SAV610*, the genes *fecC1*
282 (*SAV600*) and *fecB* (*SAV602*) which constitute part of a putative ABC transporter
283 iron(III)/siderophore transport system were also overexpressed. Based on protein similarity,
284 SAV600-602 could constitute an ABC transport system homologous to the system FecBCD
285 from *E. coli* involved in iron dicitrate transport (Staudenmaier et al., 1989). *SAV602* and
286 *SAV610* genes flank a gene cluster involved in the biosynthesis of the siderophore nrp6 whose
287 expression is also upregulated in the mutant (see below and Tables 2 and S4). Altogether these
288 results suggest that the ABC system SAV600-602 and the transporter SAV610, would be
289 involved in iron transport using the siderophore nrp6. These transcriptomic results are further
290 supported by the direct binding of PimM to the promoters of *SAV602* and *SAV610* (Vicente et
291 al., 2015).

292

293 <u>Genes involved in cell envelope biosynthesis and morphological differentiation</u>.

294 This group includes eleven genes that showed differential transcription in at least one
295 of the sampling times. These genes code for enzymes involved in cell envelope biosynthesis
296 (the N-acetylmuramoyl-L-alanine amidase *ampD1*), and morphological differentiation (8
297 genes). The latter are particularly interesting because in *Streptomyces* morphological
298 differentiation is usually accompanied by physiological differentiation (McCormick and
299 Flärdh, 2012). The differential expression of genes involved in morphological differentiation
300 was somehow expected given that *S. avermitilis ΔpteF* mutants show a delay in spore formation
301 (Vicente et al., 2014).

302 Our results indicate that the transcriptional regulators *wlbE* and *bldC* that are associated
303 to deficient phenotypes in spore formation (*white*) and in aerial mycelium development (*bald*),

304 respectively, are underexpressed in the mutant. Similarly, the secreted subtilisin inhibitor *sit2*
305 involved in morphological differentiation via *sigU* in *S. coelicolor* (Gordon et al., 2008), and
306 *SAV2505* that encodes a DNA-binding protein orthologous to *S. lividans* transcriptional
307 regulator ClgR which controls the expression of ATP-dependent protease Clp involved in
308 morphological differentiation (Bellier et al., 2006), are also downregulated (Table S4).
309 Interestingly, *clpC1* gene had also been proposed as direct PteF molecular target given PimM
310 binding to its coding region (Vicente et al., 2015).

311 Conversely, the gene *ctpB* that encodes a cation-transporting P-type ATPase involved
312 in *Bacillus subtilis* sporulation activation (Campo and Rudner, 2007), the gene *mreC* needed
313 for spore cell-wall synthesis in *S. coelicolor* (Kleinschnitz et al., 2011), and both *kipI*, and its
314 antagonist *kipA*, which have been involved in sporulation control in *B. subtilis* (Wang et al.,
315 1997; Jacques et al., 2011), showed enhanced transcription in the mutant (Table S4).

316

317 Regulatory genes.

318 As described here, a large set of genes with diverse functions are under the control of
319 PteF, including several regulatory genes listed in the categories described above. This prompted
320 us to analyze other possible transcriptional regulators differentially expressed in the mutants,
321 as these could be mediators of the regulatory control. A complete list of regulatory genes whose
322 expression is affected in the mutant is presented in Table S4.

323 A total of 31 transcriptional regulators showed a significant differential transcription in
324 the mutant when compared with the parental strain. Such a large number reflects the pleiotropic
325 nature of PAS-LuxR regulators (Vicente et al., 2014, 2015; Aparicio et al., 2016), and probably
326 justifies all the biological processes affected by the mutation (see functional categories listed
327 above).

328 Among the regulators controlled by PteF, is interesting to highlight eight directly
329 involved in diverse secondary metabolites biosynthesis control, namely: *avaL2* (*SAV2268*) and

330 *avaL1* (*SAV2270*), both TetR-family regulators putatively involved in the biosynthesis of a γ-
331 butyrolactone (Ikeda et al., 2014); *avaR1* (*SAV3705*), which encodes the avenolide receptor
332 protein (Kitani et al., 2011; Wang et al., 2014; Zhu et al., 2016); *olmRII* (*SAV2901*) and *olmRI*
333 (*SAV2902*), both LuxR-family positive regulators of macrolide oligomycin biosynthesis (Yu et
334 al., 2012); *pteR* (*SAV410*), the SARP-LAL regulator of the polyene macrolide filipin
335 biosynthesis (Ikeda et al., 2014; Vicente et al., 2014; Payero et al., 2015); *aveR* (*SAV935*), a
336 LAL-family positive regulator of avermectin biosynthesis (Kitani et al., 2009); and *SAV2989*,
337 a MarR-family transcriptional regulator from the neopentalenolactone biosynthetic cluster
338 (Ikeda et al., 2014). All these regulatory genes showed decreased transcription in the mutant,
339 except for *pteR* and *aveR* that were overexpressed (Tables 2 and S4).

340 Interestingly, the expression of *olmRI* and *olmRII* genes had already been proven to be
341 negatively affected by the lack of PteF (Vicente et al., 2015). Furthermore, *pteF*-deletion
342 mutants showed a severe loss of oligomycin production, whereas gene complementation of the
343 mutant restored parental-strain phenotype, and gene duplication in the wild-type strain boosted
344 oligomycin production (Vicente et al., 2015). Similarly, *pteR* has also been reported as a PteF
345 molecular target, via the action of another hierarchical regulator which would be activated by
346 PteF (Vicente et al., 2014).

347 Besides the abovementioned regulators, it is also noteworthy the identification of
348 *SAV2301*, that codes for a RedD orthologue, the transcriptional activator of the
349 undecylprodigiosin pathway in *S. coelicolor* (Narva and Feitelson, 1990), *bldC* (*SAV4130*), a
350 MerR-family regulator involved in morphological differentiation and secondary metabolite
351 production in *S. coelicolor* (Hunt et al., 2005), and *cutS* (*SAV2404*), a sensor kinase involved
352 in actinorhodin biosynthesis in *S. lividans* (Chang et al., 1996), all of them being down-
353 regulated in the mutant (Table S4).

354

355

356 Secondary metabolite genes.

357 The functional group more clearly affected by *pteF* deletion was that of genes involved
358 in secondary metabolite biosynthesis (Table 1). In this category, when one or more genes
359 critical for metabolite biosynthesis were found statistically significant, the transcription of other
360 genes belonging to the same cluster with uncorrected p-values < 0.05 was also considered
361 significant. Following this broader criterion, sixty one genes belonging to this group, regardless
362 of regulatory genes mentioned above, showed a significant differential transcription in the
363 mutant when compared with the parental strain in at least one of the sampling times (Table S4).
364 Noteworthy, almost all genes were detected at the exponential growth-phase. In particular,
365 those related to secondary metabolism precursor biosynthesis were only detected at this
366 sampling time. These were: the ornithine aminotransferases *rocD3* (*SAV2285*) and *rocD2*
367 (*SAV7112*), and the proline dehydrogenase *putA* (*SAV2724*), which were underexpressed; and
368 the phosphoglucomutase *pgmA* (*SAV803*), the 3-isopropylmalate dehydrogenase *leuB*
369 (*SAV2718*), the phosphoribosylglycinamide formyltransferase *purN* (*SAV3445*), and the
370 putative citrate synthase *citA2* (*SAV3859*), which were overexpressed.

371 But the most striking results of microarray analyses were the identification of
372 differential transcription in 67 genes (including regulatory genes) belonging to 10 out of the 38
373 putative secondary metabolite gene clusters encoded by *S. avermitilis* genome (Ikeda et al.,
374 2014). Table 2 includes the transcriptional values of genes belonging to differentially expressed
375 secondary metabolite gene clusters. For gene cluster boundaries definition we used StrepDB
376 database (http://strepdb.streptomyces.org.uk) in conjunction with information described by
377 Ikeda et al. (2014).

378 The secondary metabolites whose biosynthesis would be affected by *pteF* deletion were
379 of different nature, and included: the polyketides filipin (*pte*), oligomycin (*olm*), avermectin
380 (*ave*), and the product of *pks3*; the non-ribosomal peptides nrp3 and the siderophore nrp6; the

381 vibrioferrin-like polyhydroxycarboxylate siderophore *avs*; the terpenoid
382 neopentalenoketolactone (*ptl*); the γ-butyrolactone (*gbl*); and melanin (*melC-1*).
383 In all these clusters, the differential transcription of at least one key biosynthetic gene
384 was observed. The number of genes affected were: eleven in the *nrp6* cluster (out of 12), ten
385 (out of 13 and 14 respectively) in the case of the filipin and oligomycin clusters, eight (out of
386 11) in the case of the *pk3* cluster, seven in the case of the avermectin (out of 19) cluster, six in
387 the *nrp3* cluster (out of 10), six in the *ptl* cluster (out of 14), five (out of 5) in the *gbl* cluster,
388 and two in the *avs* (out of 4) and melanin *melC-1* (out of 2) clusters (Table 2).
389 Furthermore, a closer look at the transcription of the remaining genes of each of these
390 clusters revealed that most of the genes of a given cluster, followed the same tendency. Figure
391 3 shows the transcription profiles of secondary metabolite gene clusters genes affected by the
392 mutation including regulatory genes, and Table 2 the transcription values observed for each of
393 the genes.
394 Seven of the secondary metabolite gene clusters showed an overall reduced
395 transcription, including filipin *pte*, oligomycin *olm*, neopentalenoketolactone *ptl*, and melanin
396 *melC*-1 clusters, the silent cluster for γ-butyrolactone *gbl*, and the cryptic gene clusters *pk3* and
397 *nrp3*. On the opposite, three gene clusters showed overall enhanced transcription, including the
398 macrolide avermectin *ave*, the siderophore *avs*, and the cryptic non-ribosomal peptide *nrp6*
399 (Fig. 3).
400 Interestingly, besides the genes mentioned above, all the genes belonging to the clusters
401 coding for the terpenoid albaflavenol/albaflavenone (*ezs*), and the cryptic polyketide *pk4* also
402 followed the same tendency. In these cases, transcription values did not meet the statistical
403 criteria, but their uncorrected *p*-values were < 0.05 in all instances (Table 2). In the case of the
404 *ezs* genes (SAV3031-3032) they showed an average of two fold more transcription in the
405 mutant, whereas *pk4* genes (*SAV7184-7186*) showed between 7 and 9 fold less transcription
406 than in the parental strain.

407

408 **Filipin and Oligomycin production are strongly reduced in *S. avermitilis ΔpteF*.**

409 Although many of the metabolites whose biosynthesis would be affected by *pteF*
410 deletion are of unknown structure (cryptic) and others are not produced under laboratory
411 conditions (silent) (Ikeda et al., 2014), the production of two of them could be readily monitored
412 in *S. avermitilis ΔpteF*. These were the antifungal pentaene filipin which is encoded by the *pte*
413 cluster where the regulator is situated, and the ATP-synthase inhibitor oligomycin which is
414 encoded by the *olm* cluster (Fig. 1). In both cases, production of the secondary metabolite was
415 strongly reduced upon inactivation of the regulatory gene *pteF*. This is in agreement with the
416 reduced transcription of most biosynthetic genes of both clusters (Fig. 3). The exceptions were
417 the discrete thioesterase *pteH*, the cholesterol oxidase *pteG*, and the SARP-LAL regulator *pteR*
418 of the filipin cluster, which were overexpressed. These results corroborate our previous
419 observations by RT-qPCR (Vicente et al., 2014; Vicente et al., 2015).

420

421 **Validation of microarray results by using quantitative RT-PCR.**

422 Quantitative RT-PCR was used on reversed transcribed RNA samples to confirm that
423 differential expression indicated by the microarray data was supported by an independent
424 method. The selected genes covered a wide range of expression, including up-regulation and
425 down-regulation. Twelve genes were validated including genes for the biosynthesis of filipin
426 (*pteC*, *pteB*, *pteR*, *pteG*), oligomycin (*olmRI, olmRII, olmB*), avermectin (*aveR*), the isomerase
427 of *pk3* cluster (*SAV2273*), one ABC transporter of the *nrp6* cluster (*fecB*), the alpha
428 galactosidase *agaB1*, and the heat shock internal membrane protease *htpX1* (*SAV4891*).

429 Overall, the RT-qPCR data and microarray data showed a good concordance (Fig. S2).
430 The range of dynamics for relative $\log_2$ fold change obtained from RT-qPCRs (-6.53 to +7.54)
431 was higher than that obtained from Mc values from microarrays (-7.24 to +2.94), indicating
432 that RT-qPCRs are more sensitive. This probably reflects on the Pearson's correlation

433 coefficient (R^2) for the plot, resulting in a lower value than what could be expected.
434 Nevertheless, the obtained value (R^2 = 0.892) still indicates a good correlation of results.
435
436 **Concluding remarks.**
437
438 Up to date, PAS-LuxR regulator-encoding genes have been found only in polyene
439 macrolide gene clusters, thus constituting a landmark of these type of clusters. In this context,
440 they are transcriptional activators essential for the biosynthesis of the polyene encoded within
441 the cluster. Their expression is a bottleneck in the biosynthesis of the antifungal and thus
442 polyene production is easily incremented upon gene dosage increase (Aparicio et al., 2016).
443 Additionally, heterologous gene complementation of mutants restores strain ability to produce
444 the antifungal compound, thus proving that these regulators are highly conserved (Santos-
445 Aberturas et al. 2011b). Recently, we have obtained evidence indicating that although these
446 regulators were initially thought to be pathway-specific, they actually are regulatory proteins
447 with a wider range of connotations in addition to polyene biosynthesis. Thus, PteF, the regulator
448 of filipin biosynthesis, was proven to control oligomycin production in *S. avermitilis* (Vicente
449 et al., 2015). This prompted us to propose that introduction of PAS-LuxR regulatory genes into
450 *Streptomyces* species could prove useful for the awakening of dormant secondary metabolite
451 biosynthetic genes (Vicente et al., 2014; 2015). This hypothesis was confirmed when PimM,
452 the archetype of PAS-LuxR regulators was introduced into *S. albus* J1074, and production of
453 the hybrid non ribosomal peptide-polyketide antimycin was activated (Olano et al., 2014).
454 Recently, a similar result has been described in *S. albus* S4, where a PimM orthologue (the
455 candicidin regulator FscRI) has been identified as required for antimycin production (McLean
456 et al., 2016).
457 Here we have studied the transcriptome of *S. avermitilis* Δ*pteF* mutant in comparison
458 with that of its parental strain. Our results corroborate our previous observations (Vicente et al.,

459 2014, 2015), reinforcing the idea that PAS-LuxR regulators control many different cellular
460 processes of bacterial metabolism at the transcriptional level, but in particular stress the
461 importance of PAS-LuxR involvement on secondary metabolite biosynthesis.

462 Notably, ten (or twelve if we include *ezs* and *pk4* gene clusters) out of the 38 putative
463 secondary metabolite gene clusters encoded by *S. avermitilis* genome (Ikeda et al., 2014)
464 showed altered expression in the mutant. In some instances, the modified expression of
465 biosynthetic genes of a given cluster could be explained by the effect of the mutation on the
466 expression of one or more cluster-situated regulators. This is the case of the *aveR* regulator of
467 the avermectin *ave* cluster, the regulators *avaL1* and *avaL2* of the γ-butyrolactone *gbl* cluster,
468 the oligomycin regulators *olmRI* and *olmRII*, and the MarR regulator (*SAV2989*) of the
469 pentalenolactone *ptl* cluster. AveR, the transcriptional activator of avermectin biosynthesis
470 (Kitani et al., 2009), is overexpressed four-fold in the mutant and concomitantly the remaining
471 genes of the *ave* cluster showed enhanced transcription. Conversely, OlmRI and OlmRI,
472 positive regulators of oligomycin biosynthesis (Yu et al., 2012), showed decreased transcription
473 in the mutant (Mc values -1.56 and -1.47 respectively), and so did the remaining genes of the
474 cluster. It is not known whether AvaL1 and AvaL2 are positive regulators, but it is conceivable
475 given that they show reduced transcription values upon mutation of the *pteF* gene (fold changes
476 of 6.4 and 8.9, respectively) together with the remaining genes of the *gbl* cluster, including the
477 γ-butyrolactone synthase *avaA*. Both AvaL1 and AvaL2 show convincing similarity to γ-
478 butyrolactone receptor proteins, and although these proteins normally act repressing
479 transcription of the synthase gene (Zou et al., 2014; Zhou et al., 2015; Barreales et al., 2020),
480 there are cases that display the opposite behavior, like FarA from *S. lavendulae* that activates
481 the transcription of the synthase *farX* (Kitani et al., 2010). The same occurs with the MarR
482 regulator of the *ptl* cluster (Ikeda et al., 2014) whose transcription is diminished (2-fold) in the
483 mutant as well as that of all *ptl* genes. In the remaining gene clusters there are no cluster-situated
484 regulatory genes, thus the effect of the mutation must be explained either by a direct action of

485 PteF on key biosynthetic genes or via the action of other regulatory genes. In this sense, 28
486 regulatory genes not situated in the clusters indicated above, most of them with unknown
487 function, were differentially expressed upon mutation of *pteF* (Table S4).

488 To our knowledge, this is the second time a genome-wide transcriptomic study is
489 conducted to describe the pleiotropic nature of a cluster-situated regulator, that of the regulator
490 of lincomycin biosynthesis LmbU from *S. linconensis* (Li et al., 2020). Cross-regulation of
491 disparate natural-product biosynthetic gene clusters by a cluster-situated regulator has already
492 been described by several groups although not in genome-wide studies (Santamarta et al., 2011;
493 Vicente et al., 2015; McLean et al., 2016). Moreover, the ability of some of these regulators to
494 modulate the effects of regulators that act more globally (Huang et al., 2005), as well as the
495 competition between global regulators (Santos-Beneit et al., 2009), have also been reported.
496 Our findings go beyond, and indicate that PAS-LuxR regulators should be considered wide
497 domain regulators. They affect the expression of multiple genes involved in both primary and
498 secondary metabolism.

499 The findings reported here should provide important clues to understand the intertwined
500 regulatory machinery that modulates antibiotic biosynthesis in *Streptomyces*, and suggest that
501 the heterologous expression of PAS-LuxR regulators is likely to represent a powerful general
502 strategy for novel bioactive natural product discovery.

503

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505

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511

512 **CONFLICT OF INTEREST:**

513

514 The authors declare that they have no conflict of interest.

515

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699

Modulation of multiple gene clusters expression by a single PAS-LuxR transcriptional regulator.

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Figures 1-3, Tables 1 and 3

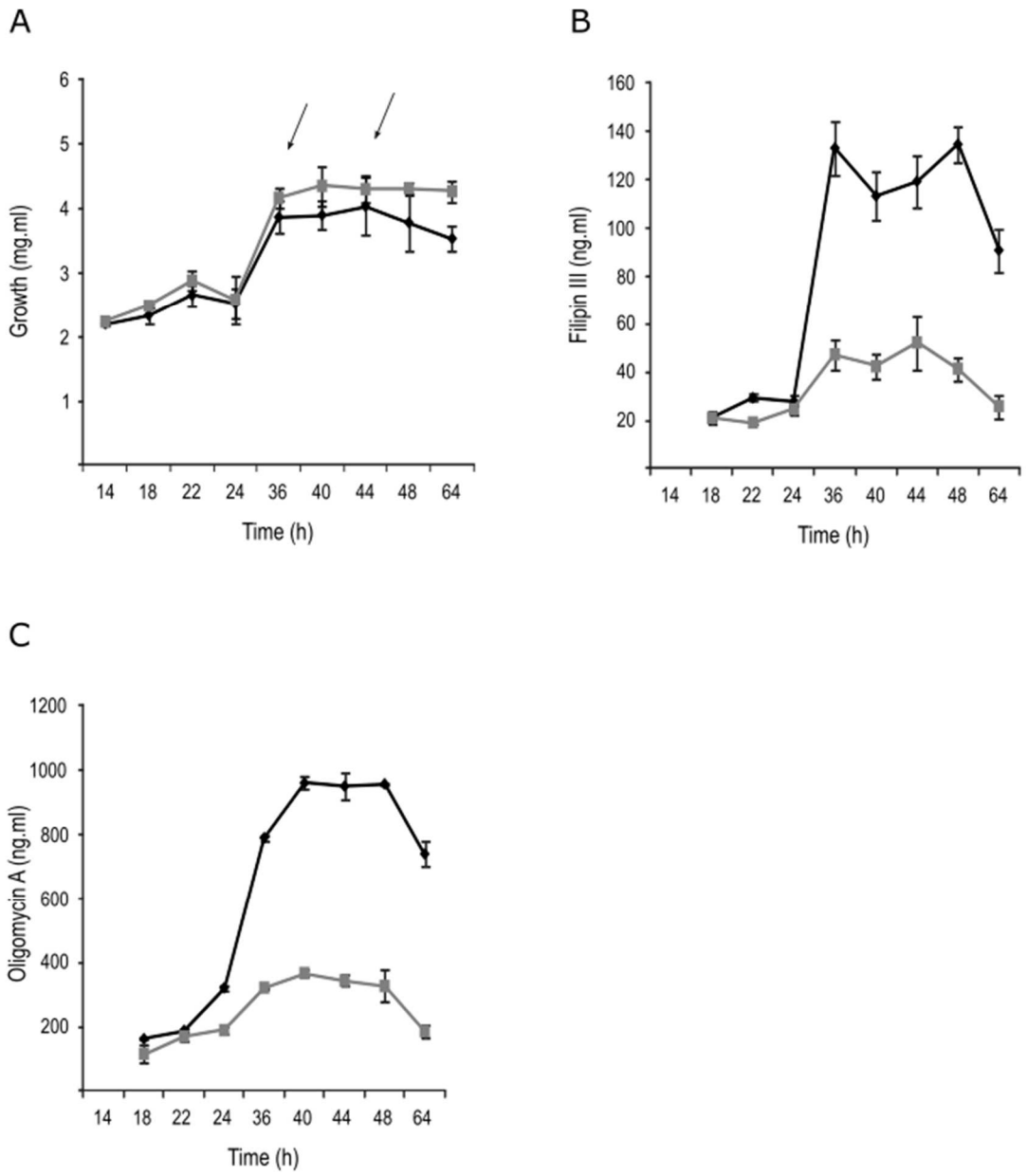

Fig. 1.- Growth and antibiotic production in YEME medium without sucrose. Strains *S. avermitilis* wt (black), and Δ*pteF* mutant (gray). A) Growth curves. B) Filipin production. (C) Oligomycin production. Arrows indicate RNA samples harvesting times.

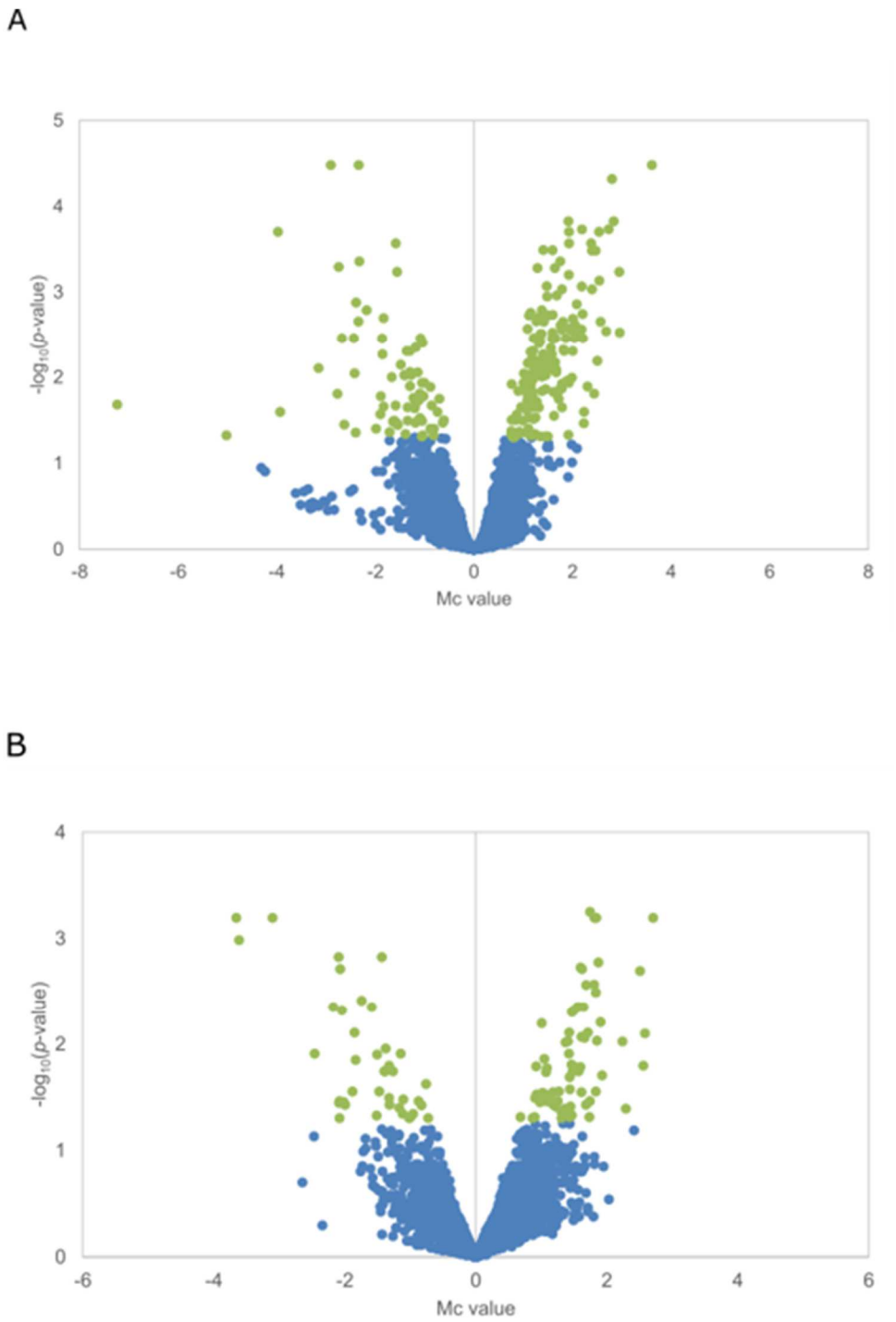

Fig. 2.- Differentially expressed genes in the mutant strain Δ*pteF*. Volcano plots show differential gene expression distribution during exponential phase (A) and stationary phase (B). Statistically significant genes are shown in green ($\log_{10}$ *p*-value ≥ 1.3).

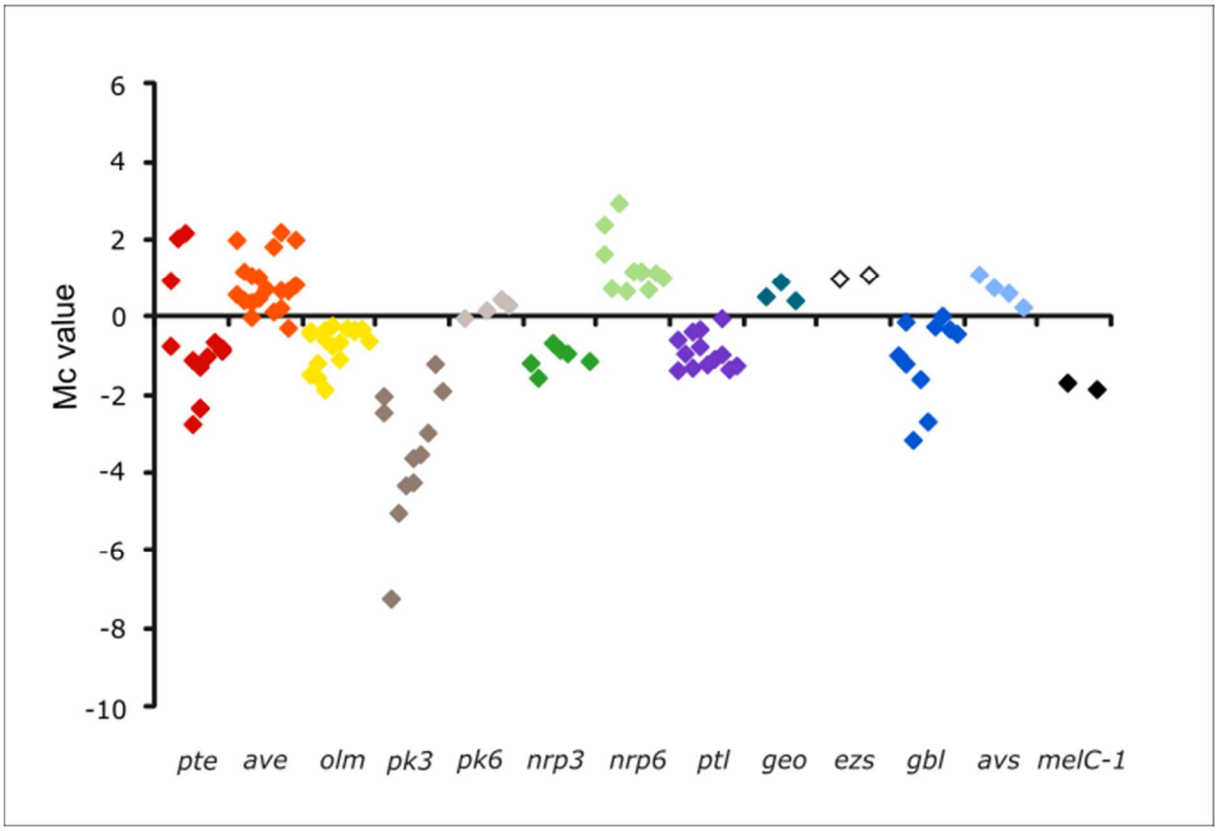

Fig. 3.- Transcription profiles of secondary metabolite gene clusters genes in *S. avermitilis* *ΔpteF*. Only clusters whose transcription was affected by the mutation are included. All the genes of a given cluster are shown in the plot, including regulatory genes. Coloured squares are the plots of differential transcription values for individual genes in the mutant. *pte*, filipin (red); *ave*, avermectin (orange); *olm*, oligomycin (yellow); *pk*, polyketide (gray); *nrp*, non-ribosomal peptide (green); *ptl*, neopentalenoketolactone (purple); *geo*, geosmin (teal); *ezs*, albaflavenol/albaflavenone (white); *gbl*, γ-butyrolactone (dark blue); *avs*, vibrioferrin-like siderophore (light blue); *melC*-1, melanin (black).

Table 1: Differential transcription and functional classification of genes affected by *pteF* deletion.

	Genes underexpressed[1]	Genes overexpressed[a]	Total
Exponential phase (t1)	63	145	**208**
Stationary phase (t2)	35	64	**99**

	Identified genes[2]	
Function	t1	**t2**
Genetic information- and protein-processing, and amino acid metabolism	20 (7 ↓; 13 ↑)	11 (5 ↓; 6 ↑)
Nucleotide and vitamin metabolism, DNA replication, recombination and repair	16 (4 ↓; 12 ↑)	4 (1 ↓; 3 ↑)
Carbohydrate metabolism	13 (3 ↓; 10 ↑)	1 (1 ↓)
Lipid metabolism	8 (1 ↓; 7 ↑)	4 (4 ↑)
Energy production	2 (2 ↓)	1 (1 ↓)
Transport and external signals processing	20 (12 ↓; 8 ↑)	8 (4 ↓; 4 ↑)
Cell envelope biosynthesis and morphological differentiation	9 (4 ↓; 5 ↑)	5 (4 ↓; 1 ↑)
Regulation	27 (12 ↓; 15 ↑)	12 (3 ↓; 9 ↑)
Secondary metabolism	60 (34 ↓; 26 ↑)	6 (2 ↓; 4 ↑)
Miscellaneous	38 (11 ↓; 27 ↑)	19 (1 ↓; 18 ↑)

[1] Only statistically significant genes with a fold-change value equal higher to +/-2 are included.
[2] All identified genes were accounted for

Table 2: Transcriptional values of genes belonging to differentially expressed secondary metabolite gene clusters in *S. avermitilis ΔpteF* when compared to its parental strain. Differential transcription values *Mc* were obtained by subtracting mutant *Mg* values from parental strain *Mg* values. Only clusters whose transcription was affected by the mutation are included. The *p*-values are indicated in bold type when found statistically significant (see Materials and methods). For simplicity, designations ‘‘putative’’ have been removed.

Gene		Description	Mc	Corrected p value	p value
		Filipin cluster (*pte*)			
SAV407	*pteH*	thioesterase	0.95	0.1395	**0.0076**
SAV408	*pteG*	cholesterol oxidase	**2.05**	**0.0025**	0.0000
SAV410	*pteR*	SARP-family transcriptional regulator	**2.18**	**0.0009**	0.0000
SAV411	*pteE*	ferredoxin	**-1.10**	0.2533	**0.0233**
SAV412	*pteD*	cytochrome P450 monooxygenase	**-2.74**	**0.0005**	0.0000
SAV413	*pteC*	cytochrome P450 monooxygenase	**-2.33**	**0.0004**	0.0000
SAV414	*pteB*	dehydrogenase	**-2.34**	**0.0000**	0.0000
SAV415	*pteA5*	modular polyketide synthase	**-1.01**	0.1136	**0.0054**
SAV416	*pteA4*	modular polyketide synthase	**-1.28**	**0.0095**	0.0002
SAV417	*pteA3*	modular polyketide synthase	-0.64	0.3514	**0.0457**
SAV418	*pteA2*	modular polyketide synthase	-0.87	0.3639	0.0506
SAV419	*pteA1*	modular polyketide synthase	-0.80	0.4388	0.0752
		Non-ribosomal peptide-6 (*nrp6*)			
SAV600	*fecC1*	ABC transporter iron(III)/siderophore transport system ATP-binding protein	**2.40**	**0.0003**	0.0000
SAV601	*fecD1*	ABC transporter iron(III)/siderophore permease	0.75	0.5625	0.1290
SAV602	*fecB*	ABC transporter iron(III)/siderophore-binding protein	**2.95**	**0.0006**	0.0000
SAV603	*nrps6*	non-ribosomal peptide synthetase	0.68	0.3119	**0.0342**
SAV604		hypothetical protein	**1.18**	**0.0224**	0.0005
SAV605	*fadD2*	acyl-CoA synthetase	**1.20**	**0.0049**	0.0001
SAV606		hypothetical protein	0.71	0.3525	**0.0464**
SAV607		taurine catabolism dioxygenase	**1.15**	**0.0017**	0.0000

SAV608	*fabC2*	acyl carrier protein	**1.02**	0.1136	**0.0054**
SAV609	*fabH4*	3-oxoacyl-ACP synthase III	**1.15**	0.1182	**0.0058**
SAV610		MFS transporter protein	**1.19**	**0.0166**	0.0004
SAV611		beta-hydroxylase	**1.64**	**0.0005**	0.0000
Avermectin cluster (*ave*)					
SAV935	*aveR*	LuxR-family transcriptional regulator	**2.00**	**0.0049**	0.0001
SAV936	*aveF*	C-5 ketoreductase	0.59	0.6016	0.1518
SAV937	*aveD*	C5-O-methyltransferase	0.43	0.6148	0.1603
SAV938	*aveA1*	type I polyketide synthase	**1.18**	0.1820	**0.0122**
SAV939	*aveA2*	type I polyketide synthase	0.39	0.7264	0.2537
SAV940	*aveC*		0.00	0.9993	0.9950
SAV941	*aveE*	cytochrome P450 monooxygenase	**1.04**	0.1376	**0.0074**
SAV942	*aveA3*	type I polyketide synthase	0.49	0.6328	0.1725
SAV943	*aveA4*	type I polyketide synthase	0.73	0.4435	0.0764
SAV944[3]	*orf-1*	reductase	0.14	0.9330	0.6990
SAV945	*aveBI*	dTDP-L-oleandrose transferase (glycosyltransferase)	0.70	0.6052	0.1543
SAV946	*aveBII*	dTDP-glucose 4.6-dehydratase	0.23	0.8027	0.3477
SAV947	*aveBIII*	glucose-1-phosphate thymidyltransferase	**1.08**	0.2027	**0.0154**
SAV948	*aveBIV*	dTDP-4-keto-6-deoxy-L-hexose 4-reductase	-0.28	0.8828	0.5032
SAV949	*aveBV*	dTDP-4-keto-6-deoxyhexose 3.5-epimerase	0.68	0.5387	0.1160
SAV950	*aveBVI*	dTDP-4-keto-6-deoxy-L-hexose2.3-dehydratase	0.84	0.4308	0.0711
SAV951	*aveBVII*	dTDP-6-deoxy-L-hexose 3-O-methyltransferase	**2.01**	**0.0020**	0.0000
SAV952	*aveBVIII*	dTDP-4-keto-6-deoxy-L-hexose 2.3-reductase	**1.83**	**0.0049**	0.0001
SAV953	*aveG*	thioesterase	**2.20**	**0.0018**	0.0000
Melanin cluster (*melC-1*)[4]					
SAV1136	*melC1*	tyrosinase co-factor protein	**-1.68**	0.0776	**0.0015**
SAV1137	*melC2*	tyrosinase	**-1.85**	**0.0078**	0.0000
γ-butyrolactone cluster (*gbl*)					

[3] Not involved in avermectin biosynthesis
[4] Values from stationary phase (t2) analysis

SAV2266	*avaC*	phosphatase	-0.98	0.0794	**0.0030**
SAV2267	*avaB*	oxidoreductase	**-1.20**	0.2777	**0.0279**
SAV2268	*avaL2*	TetR-family transcriptional regulator	**-3.15**	**0.0078**	0.0001
SAV2269	*avaA*	gamma-butyrolactone biosynthesis protein	**-1.59**	0.1456	**0.0083**
SAV2270	*avaL1*	TetR-family transcriptional regulator	**-2.68**	**0.0035**	0.0000
		Polyketide-3 cluster (*pk3*)			
SAV2272		hypothetical protein	**-2.45**	0.2000	**0.0150**
SAV2273		isomerase	**-7.24**	**0.0208**	0.0005
SAV2274		secreted protein	**-5.02**	**0.0473**	0.0014
SAV2275		transmembrane efflux protein	**-4.32**	0.1124	**0.0053**
SAV2276		3-oxoacyl-ACP synthase III	**-4.24**	0.1242	**0.0063**
SAV2277		thioesterase	**-3.62**	0.2222	**0.0183**
SAV2278		F420-dependent dehydrogenase	**-3.52**	0.3037	**0.0327**
SAV2279		acyl-CoA synthetase	**-2.97**	0.3522	**0.0462**
SAV2280	*pks3-1*	modular polyketide synthase	**-1.20**	0.6288	0.1702
SAV2281	*pks3-2*	modular polyketide synthase	**-1.90**	0.5893	0.1436
SAV2282	*pks3-3*	acyl carrier protein	**-2.03**	0.3974	0.0593
		Oligomycin cluster (*olm*)			
SAV2890	*ccrA1*	crotonyl-CoA reductase	-0.42	0.4368	0.0743
SAV2891		hypothetical protein	**-1.18**	0.2280	**0.0193**
SAV2892	*olmA4*	modular polyketide synthase	-0.33	0.4760	0.0913
SAV2893	*olmA5*	modular polyketide synthase	-0.22	0.6421	0.1789
SAV2894	*olmB*	cytochrome P450 monooxygenase	**-1.09**	0.1512	**0.0087**
SAV2895	*olmA7*	modular polyketide synthase	-0.64	0.0746	**0.0028**
SAV2896	*olmA6*	modular polyketide synthase	-0.74	0.1443	**0.0080**
SAV2897	*olmA3*	modular polyketide synthase	-0.32	0.3610	**0.0493**
SAV2898	*olmA2*	modular polyketide synthase	-0.31	0.5785	0.1377
SAV2899	*olmA1*	modular polyketide synthase	-0.61	0.1817	**0.0121**
SAV2900		P450-like protein	-0.57	0.2474	**0.0224**
SAV2901	*olmRII*	LuxR-family transcriptional regulator	**-1.47**	0.0712	**0.0026**

SAV2902	*olmRI*	LuxR-family transcriptional regulator	**-1.56**	**0.0006**	0.0000
SAV2903	*olmC*	Thioesterase	**-1.86**	0.1235	**0.0062**
Neopentalenolactone cluster (*ptl*)					
SAV2989		MarR-family transcriptional regulator	**-1.06**	**0.0487**	0.0015
SAV2990	*gap1*	glyceraldehyde-3-phosphate dehydrogenase	**-1.36**	0.2469	**0.0222**
SAV2991	*ptlH*	1-deoxypentalenic acid 11-beta hydroxylase	-0.93	0.1529	**0.0089**
SAV2992	*ptlG*	transmembrane efflux protein	-0.37	0.9068	0.5988
SAV2993	*ptlF*	1-deoxy-11beta-hydroxypentalenic acid dehydrogenase	-0.76	0.2639	**0.0251**
SAV2994	*ptlE*	Baeyer-Villiger monooxygenase	**-1.30**	**0.0087**	0.0001
SAV2995	*ptlD*	dioxygenase	**-1.21**	0.0962	**0.0041**
SAV2996	*ptlC*	hypothetical protein	**-1.07**	0.5367	0.1153
SAV2997	*ptlB*	farnesyl diphosphate synthase	-0.96	0.5690	0.1322
SAV2998	*ptlA*	pentalenene synthase	**-1.34**	0.5592	0.1269
SAV2999	*ptlI*	pentalenene C13 hydroxylase; cytochrome P450	**-1.24**	0.4943	0.0993
SAV3000	*ptlR*	AraC-family transcriptional regulator	-0.58	0.5379	0.1158
SAV3001	*ptlJ*	lyase	-0.31	0.6109	0.1579
SAV3002	*ptlL*	hypothetical protein	-0.03	0.9754	0.8867
Albaflavenol/albaflavenone cluster (*ezs*)					
SAV3031	*cyp14*	epi-isozizaene hydroxylase (cytochrome P450 monooxygenase)	**1.00**	0.2918	**0.0301**
SAV3032	*ezs*	epi-isozizaene synthase (sesquiterpene cyclase)	**1.10**	0.1621	**0.0097**
Non-ribosomal peptide-3 cluster (*nrp3*)					
SAV3155		MbtH-like protein	**-1.18**	0.2696	**0.0261**
SAV3156	*nrps3-1*	non-ribosomal peptide synthetase	**-1.56**	0.0867	**0.0035**
SAV3157		export protein	-0.66	0.6407	0.1773
SAV3158	*nrps3-2*	non-ribosomal peptide synthetase	-0.84	0.6149	0.1623
SAV3159	*nrps3-3*	non-ribosomal peptide synthetase	-0.93	0.5290	0.1123
SAV3160		aminotransferase	**-1.13**	0.4384	0.0749
SAV3161	*dapF2*	diaminopimelate epimerase	**-1.03**	0.5045	**0.1029**
SAV3162		hypothetical protein	**-1.52**	0.1668	**0.0101**

SAV3163		hypothetical protein	**-1.24**	0.3227	**0.0384**
SAV3164		hypothetical protein	**-1.59**	**0.0003**	0.0000
Polyketide-4 cluster (*pk4*)					
SAV7184	*pks4*	modular polyketide synthase	**-3.22**	0.2980	**0.0315**
SAV7185		UDP-glucose:sterol glucosyltransferase	**-3.05**	0.2753	**0.0271**
SAV7186	*cyp26*	cytochrome P450 hydroxylase	**-2.88**	0.2419	**0.0213**
Polyhydroxycarboxylate siderophore cluster (*avs*)					
SAV7320	*avsA*	siderophore synthetase component	**1.11**	**0.0295**	0.0007
SAV7321	*avsB*	siderophore synthetase component	0.77	0.2474	**0.0223**
SAV7322	*avsC*	siderophore synthetase component	0.63	0.4654	0.0862
SAV7323	*avsD*	diaminopimelate decarboxylase	0.25	0.8125	0.3606