J. Microbiol. Biotechnol. (2013), 23(10), 1454–1459
http://dx.doi.org/10.4014/jmb.1307.07067

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Different Effects of Acidic pH Shock on the Prodiginine Production in *Streptomyces coelicolor* M511 and SJM1 Mutants

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Received: July 22, 2013
Revised: August 9, 2013
Accepted: August 10, 2013

First published online
August 14, 2013

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pISSN 1017-7825, eISSN 1738-8872

The changes in prodiginines productions caused by pH shock culture of *Streptomyces coelicolor* strains were estimated. In *Streptomyces coelicolor* M511, undecylprodiginine and streptorubin B productions increased 1.8-fold (37.22 mg/g) and 2.5-fold (18.61 mg/g), respectively, by pH shock (from 7.2 to 4.0). In contrast, this resulted in the significantly decreased prodigignines production in the *redP* deletion mutant SJM1; 3.7-fold for undecylprodiginine, 4.4-fold for streptorubin B, 5.2-fold for methylundecylprodiginine, and 6.4-fold for methyldodecylundecylprodiginine, respectively. RT-PCR analyses showed that, during pH shock, expression of *redD*, the transcription activator gene, was increased while the expression of *fabH*, the decarboxylative condensation enzyme gene in fatty acid biosynthesis, was decreased in both strains. The enhanced *redD* expression was in good accordance with the increased total prodiginines production of M511. However, for SJM1 mutant, the decrease of *fabH* expression occurred more strikingly, such that it became almost completely turned off during acidic pH shock culture. Therefore, a down-regulation of *fabH* was considered to be the cause of decreased amount of total prodiginines produced, although *redD* expression was high in SJM1 mutant.

Keywords: Fatty acid synthase, β-ketoacyl-ACP synthase III, prodiginine, branched-chain alkylprodiginine, acidic pH shock, *Streptomyces coelicolor* A3(2)

Prodiginines are a family of linear and cyclic oligopyrrole red-pigmented antibiotics that are produced by actinomycetes and other eubacteria [2]. Members of this class of antibiotics have been known for some time, as have their broad antifungal and antibacterial activities [17]. Recently, there has been a dramatic increase in interest in these natural products, as they have been reported to exhibit pronounced antimalarial, anticancer, and immunosuppressant activities [1, 4, 5]. A promising linear analog, PNU-156804, with improved immunosuppressant activity has been prepared [14], while another analog, GX15-070 (http://www.geminx.com), is currently undergoing phase II oncology trials [3]. *Streptomyces coelicolor* A3(2) has been known to generate the prodiginine antibiotics undecylprodiginine and a cyclized derivative, streptorubin B [2, 12, 13]. Over the last few years, research groups under the direction of Dr. Kevin Reynolds and Dr. Greg Challis have been studying prodiginine biosynthesis in *S. coelicolor* A3(2) [2, 12, 13]. This work has revealed that the 23 *red* genes in *S. coelicolor* A3(2) encode a fascinating and unusual pathway leading to the formation of undecylprodiginine and streptorubin B [2, 12]. Two genes (*redD* and *redZ*) are known to encode the pathway-specific regulators in the cluster [18]. Prior to the initiation of this study, six genes (*redX*, *redW*, *redU*, *redO*, *redN*, and *redM*) were known to be required for the biosynthesis of 4-methoxy-2,2′-bipyrrole-5-carboxaldehyde (MBC) [16], and four genes (*redP*, *redQ*, *redR*, and *redL*) were known to be involved in the biosynthesis of 2-undecylpyrrole (2-UP) [12, 13].

The biosynthesis of undecylprodiginine and streptorubin B is proposed as a bifurcated process involving the condensation of 2-UP and MBC (Fig. 1). Precursor incorporation experiments suggest that the 2-UP is generated by a decarboxylative casein-like condensation of glycine, with an acetate-

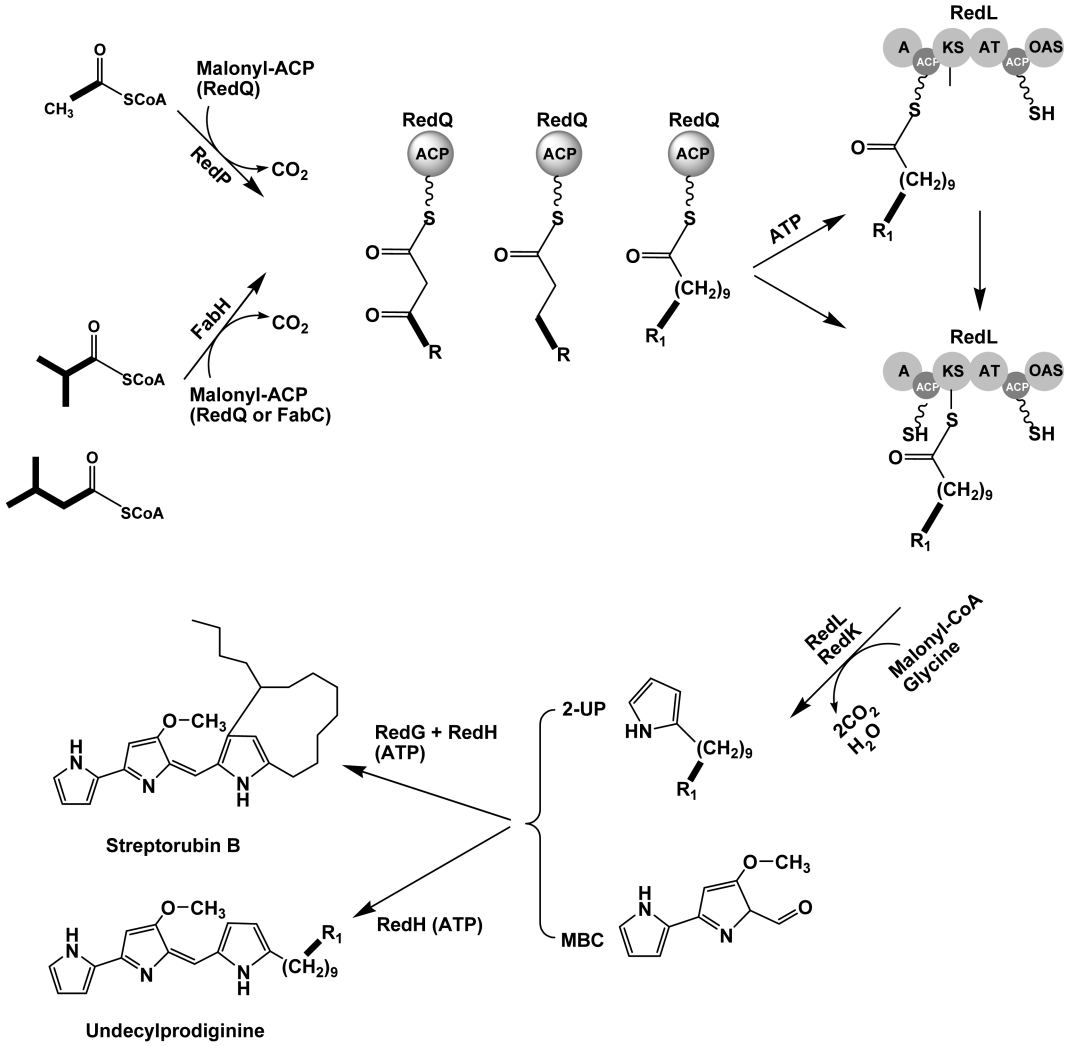

Fig. 1. Proposed role of RedP, RedR, RedQ, RedL, RedK, RedG, RedH, and FAS FabH in generating a long-chain acyl-ACP (RedQ) substrate for alkylprodiginine biosynthesis.

Undecylprodiginine, streptorubin B [$R_1 = CH_2CH_3$], methylundecylprodiginine [$R_1 = CH(CH_3)_2$], and methyldodecylprodiginine [$R_1 = CH_2CH(CH_3)_2$]. RedP and RedR are proposed to selectively elongate an acetyl CoA primer (R = CH_3) to a C_{12} acyl-ACP (RedQ) substrate, leading ultimately to undecylprodiginine biosynthesis. Replacement of either RedP and/or RedR with the FAS homologs FabH and FabF allows production of a range of acyl-ACP (RedQ) substrates from 3-methylbutyryl CoA [R = $CH_2CH(CH_3)_2$] and isobutyryl CoA [R = $CH(CH_3)_2$] and production of branched-chain alkylprodiginine analogs. RedL is a PKS-NRPS hybrid and consists of six domains containing two acyl carrier protein (ACP) domains, an adenylation (A) domain, a ketosynthase (KS) domain, an acyltransferase (AT) domain, and an α-oxoamine synthase (OAS) domain.

derived 3-ketomyristic acid derivative (Fig. 1). Initial analysis of the *red* gene cluster revealed *redQ* encoding an acyl carrier protein (ACP) and two genes, *redP* and *redR*, which encode homologs of the fatty acid biosynthetic enzymes FabH (3-ketoacyl-ACP synthase III) and FabF (3-ketoacyl-ACP synthase II), respectively [2, 12, 13]. It was proposed that RedP and RedR, in tandem with fatty acid biosynthetic enzymes that catalyze the conversion of 3-ketoacyl-ACP to acyl-ACP, were responsible for the formation of a RedQ-activated dodecanoic acid (Fig. 1) [12, 13]. The subsequent elaboration of dodecanoic acid to 2-UP was catalyzed by the RedK and RedL gene products [16]. The *redH* gene encoded a protein that was hypothesized to be responsible for the condensation of MBC and 2-UP to give undecylprodiginine [16], and the *redG* gene encoded a protein that was predicted to contain two domains, and has been shown to catalyze the oxidative carbocyclization of undecylprodiginine to streptorubin B (Fig. 1) [2]. In

particular, Reynolds' group have reported that two novel branched-chain alkylprodiginines, methylundecylprodiginine and methyldodecylprodiginine, have been obtained by substituting a FabH ketosynthase for RedP ketosynthase in the undecylprodiginine biosynthetic gene cluster [12]. Precursor incorporation experiments with perdeuterated valine indicate that these new analogs are produced from 3-methylbutyryl-CoA and isobutyryl-CoA starter units, respectively. The 2-methylundecylprodiginine produced in the SJM1 mutant results from the extension of the leucine catabolite 3-methylbutyryl-CoA with five malonyl units, whereas the 2-methyldodecylprodiginine is obtained by extension of the valine metabolite isobutyryl-CoA with six malonyl units (Fig. 1).

It has been reported that the regulation of secondary metabolite production for *Streptomyces* spp. in coordinated at several layers of control. This involves a complex regulatory network in response to environmental stimulus or stress, such as nutrient depletion, salt shock, oxidative stress, osmotic stress, acidic shock, and alkaline shock. There have been a number of studies on the application of an environmental stimulus or stress for the improvement and enhanced production of secondary metabolites [6–9, 10, 15]. Among the external stimulus or stress, although the acidic shock is simple and easy method, it should be an extremely effective approach to enhance the productivity of secondary metabolites [6, 10, 11]. Recently, we also found out that acidic pH shock dramatically increased the production of prodiginine in *S. coelicolor* M511 [11].

In this study, we examined this phenomenon in more detail compared with primary metabolites-derived prodiginines. The results showed that production of both prodiginines (the origin of primary metabolism and the origin of secondary metabolism) is differentially affected by acidic pH shock in *S. coelicolor* strains. This effect is likely mediated by the low-level expression, at acidic pH shock culture condition, of the gene encoding the homolog of the fatty acid biosynthetic enzyme FabH.

S. coelicolor A3(2) strain M511 (SCP1$^-$, SCP2$^-$ act$^-$ (Δ*actII-orf4*)) was kindly provided by Dr. Greg Challis (Warwick University, UK). *S. coelicolor* SJM1 (Δ*redP*::*oriT-aac(3)IV*) has been described previously and was generated by replacement of *redP* with the *oriT-aac(3)IV* cassette [12]. For an acidic pH shock experiment, *S. coelicolor* M511 and the SJM1 mutant were applied to two pH-shocked (PS) culture and pH-controlled (PC) culture, as previously described [9]. In the PS culture system, cells were grown on a cellophane film placed on R2YE solid medium, with an initial pH of 7.2 for 2 days, before being transferred to a new plate with a pH of 4, and then incubated for a further 5 days. R2YE with no TES buffer was used to eliminate the buffering effect. In the PC culture system, cells were grown on a cellophane film placed on R2YE with TES buffer to suppress spontaneous pH change, for 7 days without transfer to a new plate. The pH of the solid media was measured using pH-test strips (Sigma-Aldrich, St. Louis, USA) [9, 10]. Cell concentrations were measured as dry cell weight according to the modified methods of Kim *et al.* [9]. Before extraction of total prodiginine, the weight of the cell was adjusted to equalize the amount of cell. The overall prodiginine concentrations were determined by absorbance at 530 nm (ε_{530} = 100,500 M^{-1} cm^{-1}) of a metabolic mycelium extract [12]. Determination of the relative abundances of the various prodiginines was carried out by high-performance liquid chromatography (HPLC). The mycelia were extracted with methanol, and the prodiginines in a 10 µl sample were resolved using a Supelco Discovery HS C18 column (4.6 × 250 mm; Bellefonte, PA, USA) with a linear elution gradient ranging from CH_3OH:CH_3CN:H_2O (40%:10%:50%) to CH_3OH (100%) in 0.15% trifluoroacetic acid, at a flow rate of 0.5 ml/min and with detection at 530 nm [12]. For the quantitative analysis of each prodiginine, cyanidin chloride (1 mg/ml; Fluka, Germany) was used as an internal standard. This has a λ_{max} = 530 nm in methanolic HCl and a retention time of 10 min under the same HPLC conditions. Total RNA was isolated from *S. coelicolor* M511 and the SJM1 mutant grown on a cellophane film placed on R2YE solid medium, and was then quantified and assessed following the method of Kim *et al.* [9]. The RNA preparations were treated with DNase I (Qiagen, Valencia, CA, USA) to eliminate possible chromosomal DNA contamination. The concentration of RNA was determined by measuring the absorbance at 260 nm (A_{260}) using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, USA) with absorbance ratio at 260 and 280 nm (A260/A280). The purity of the RNA was estimated by the ratios of A260/A280. The reverse transcriptase PCR (RT-PCR) was performed with the Qiagen OneStep RT-PCR kit (Qiagen, Valencia, USA), using 100 ng of total RNAs as template. Conditions were as follows: cDNA synthesis, 50°C for 30 min followed by 95°C for 15 min; amplification, 35 cycles of 94°C for 1 min, 54°C for 1 min, and 72°C for 40 sec. The cycle number for each gene was optimized in order to obtain enough visibility of the RT-PCR band, and to ensure that amplification was in the linear range and that the results were semi-quantitative. Primers were designed to generate PCR products of approximately 500 bp using the following primers pairs: RedD_F 5'-ACATATTGGGAC

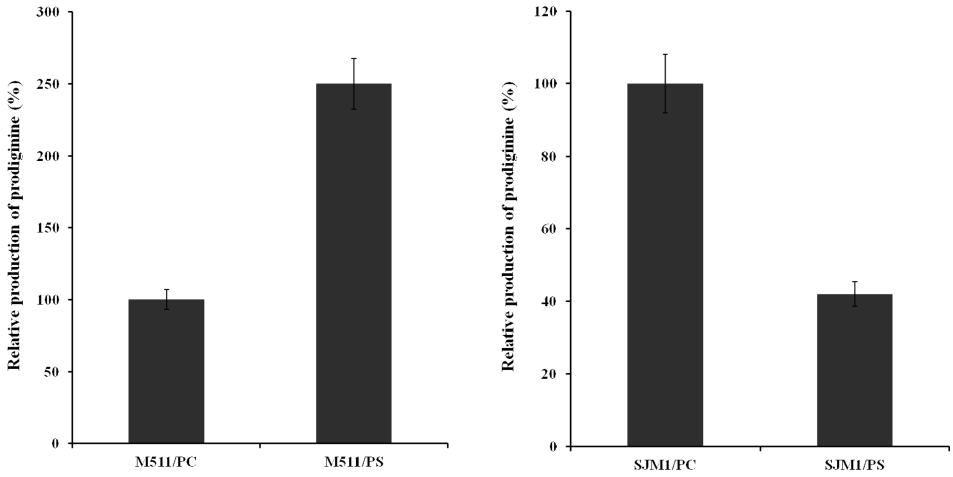

Fig. 2. Overall titers of total prodiginine in pH-shocked *S. coelicolor* M511 and the SJM1 mutants.
Overall titers of total prodiginine from *S. coelicolor* M511 (left) and *S. coelicolor* SJM1 mutant (right). The values are averages of two series of duplicate experiments. pH-controlled culture (PC); pH-shocked culture (PS).

CCGTATCG-3′ and RedD_R 5′-GAACATCGCGCTGAT GAGAT-3′; FabH_F 5′-TGATCCTCGAGAAGATCGAC-3′ and FabH_R 5′-AGACCGTCTGCTTGATCGTT-3′. With each pair of primers, the negative controls were carried out with *Taq* DNA polymerase (Stratagene, La Jolla, CA, USA) in the absence of reverse transcriptase, to confirm that the amplified products were not derived from chromosomal DNA. The RT-PCR experiments were carried out in duplicate using RNA samples from two independent cultures. The relative level of amplified mRNA was normalized to the mRNA expression level of the housekeeping gene *S. coelicolor hrdB*, which was amplified as an internal control using the primer pair HrdB_F 5′-GCGGTGGAGAAGTTCGACTA-3′ and HrdB_R 5′-TTGATGACCTCGACCATGTG-3′.

We previously observed that acidic pH shock elicited a significant enhancement of prodiginine production in *S. coelicolor* M511 grown on solid medium [11]. On the basis of this result, we hypothesized that artificial acidic pH shock might promote branched-chain alkylprodiginine production in the *S. coelicolor* SJM1 mutant. To test the hypothesis, we investigated the effect of acidic pH shock on branched-chain alkylprodiginines. When the pH was artificially changed to 4, the overall total prodiginine titer per cell weight was increased by approximately 2.5-fold compared with that under PC-cultured conditions of the *S. coelicolor* M511. However, the artificial pH 4.0 shock in *S. coelicolor* SJM1 mutant conversely gave rise to a 2-fold decrease in the overall total prodiginine titers per cell weight, compared with that under PC-cultured conditions of the *S. coelicolor* SJM1 mutant (Fig. 2).

To quantitate the effect of pH shock, M511 and the SJM1 mutant were grown for 5 days in PS-cultured condition and were analyzed throughout this period for cell growth and overall quantity of prodiginine analogs. There was no observable difference in the growth of either strain in either culture condition. Overall yields of prodiginines in pH-

Table 1. Production of prodiginines by *S. coelicolor* M511 and the SJM1 mutant strains by acidic pH shock stress.

Strain	Prodiginine titers (mg/g)[a] ± SD for strain			
	Undecylprodiginine	Streptorubin B	Methylundecylprodiginine	Methyldodecylprodiginine
S. coelicolor M511-PC	37.22 ± 0.08	18.61 ± 0.02	ND[b]	ND[b]
S. coelicolor M511-PS	67.96 ± 0.05	35.95 ± 0.04	ND[b]	ND[b]
S. coelicolor SJM1-PC	5.91 ± 0.01	3.51 ± 0.03	2.14 ± 0.04	2.53 ± 0.07
S. coelicolor SJM1-PS	1.56 ± 0.07	0.79 ± 0.08	0.41 ± 0.06	0.39 ± 0.05

[a]Cultures were grown for 7 days. Cell concentration was measured as dry cell weight. Cells collected off the cellophane film were washed with phosphate buffered saline. The washed cells were dried at 60°C for 24 h and were then weighed at room temperature. Standard error values are the results of two series of duplicate data acquisition.

[b]ND, not detected.

shocked experiments with the M511 strain were determined. There was a 1.8-fold (37.22 mg/g) increase in the level of undecylprodiginine production, as well as a dramatic 2.5-fold (18.61 mg/g) increase in the level of streptorubin B production, as compared with the levels produced under PC-cultured conditions (Table 1). However, when the SJM1 mutant was shocked with pH 4, there were 3.7-fold, 4.4-fold, 5.2-fold, and 6.4-fold decreases in the levels of undecylprodiginine, streptorubin B, methylundecylprodiginine, and methyldodecylprodiginine produced, respectively, as compared with the levels present in PC-cultured condition (Table 1). Markedly differing results were determined using the acidic pH shock treatment, where the overall prodiginine yield decreased.

To investigate the effects of pH shock on the expression of regulators related to prodiginine production, and genes involved in fatty acid synthesis and prodiginine biosynthesis, transcriptional analyses were performed by RT-PCR. The expression of genes encoding pathway-specific transcriptional activators for prodiginine, *redD*, and 3-ketoacyl-ACP synthase III for the initiation of fatty acid synthesis, *fabH*, was analyzed. Total RNA was extracted from the M511 strain and SJM1 mutant strain after culture for 60 h and was employed as a template for gene expression analysis by RT-PCR experiments. The results of these experiments are shown in Fig. 3. Under PS culture conditions, the transcription level of *redD* in the M511 strain was determined to be much higher than that under PC culture conditions (control). This result corresponds with the above-mentioned result that acidic pH shock enhanced overall prodiginine production in the M511 strain (Fig. 3). However, in the case of *fabH* expression, it was completely turned off at 60 h under the PS culture conditions, unlike that in the SJM1 mutant strain under PC culture conditions (control) (Fig. 3). This result proves that acidic pH shock is the cause of decreased overall prodiginine production in the SJM1 mutant strain. Moreover, this experiment demonstrates that acidic pH shock induces the overexpression of regulatory genes, whereas fatty acid biosynthesis is suppressed [9].

In conclusion, it is true that acidic pH shock is an extremely effective approach to enhance the production of secondary metabolites, including prodiginine. This dramatic increase in the transcription level of the regulators, as like *redD* under acidic shock conditions, may account for the observed production of prodiginine. However, the level of overall prodiginine derived from primary metabolism has shown the opposite result. We also showed the same result when using the artificial pH 4.0 shock in the *S. coelicolor* SJM2 mutant [13] in which overall total prodiginine led to a 2.5-fold decrease, compared with that under PC-cultured conditions of the *S. coelicolor* SJM2 mutant (data not shown). As a result of these two experiments, secondary metabolites assembled from a fatty acid synthase-derived starting material are circumspect in using the acidic pH-shock method for their overproduction, although this remains to be proven.

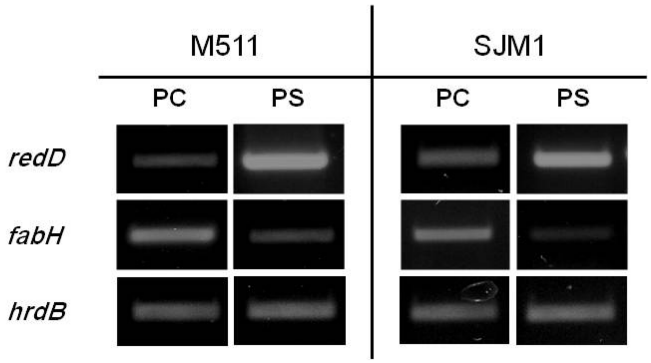

Fig. 3. Transcriptional analysis of *redD* and *fabH* in *S. coelicolor* SJM1 by pH-controlled culture (PC) and pH-shocked culture (PS). Total RNAs were extracted from mycelia harvested after 60 h of cultivation.

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