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High-Yielding Lovastatin Producer *Aspergillus terreus* Shows Increased Resistance to Inhibitors of Polyamine Biosynthesis

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Abstract: The biosynthesis of pharmaceutically significant secondary metabolites in filamentous fungi is a multistep process that depends on a wide range of various factors, one of which is the intracellular content of polyamines. We have previously shown that in *Aspergillus terreus* lovastatin high-yielding strain (HY) exogenous introduction of polyamines during fermentation can lead to an increase in the production of lovastatin by 20–45%. However, the molecular mechanisms of this phenomenon have not been elucidated. In this regard, we carried out an inhibitory analysis at the key stage of polyamine biosynthesis, the conversion of L-ornithine to putrescine by the enzyme ornithine decarboxylase (ODC). *A. terreus* HY strain showed upregulation of genes for biosynthesis of polyamines, 3–10-fold, and increased resistance compared to the original wild-type strain upon inhibition of ODC on synthetic medium with 5 mM α-difluoromethylornithine (DFMO), by 20–25%, and 5 mM 1-aminooxy-3-aminopropane (APA), by 40–45%. The data obtained indicate changes in the metabolism of polyamines in *A. terreus* HY strain. The observed phenomenon may have a universal character among fungal producers of secondary metabolites improved by classical methods, since previously the increased resistance to ODC inhibitors was also shown for *Acremonium chrysogenum*, a high-yielding producer of cephalosporin C.

Keywords: polyamines; *Aspergillus terreus*; ornithine decarboxylase; filamentous fungi; secondary metabolism

1. Introduction

Filamentous fungus *Aspergillus terreus* is the main industrial producer of cholesterol-lowering drug lovastatin (LOV) and simvastatin obtained on its basis [1]. These drugs are competitive inhibitors of hydroxymethylglutaryl-CoA reductase (HMG-CoA; EC 1.1.1.88), which catalyzes the rate-limiting step of the isoprenoid biosynthesis pathway associated with the production of cholesterol in humans and ergosterol in fungi [2]. In nature, LOV is produced by fungi from various taxonomic groups, for example, *Ascomycetes*, *Aspergillus*, *Doratomyces*, *Gymnoascus*, *Hypomyces*, *Monascus*, *Paecilomyces*, *Penicillium*, *Phoma*, *Trichderma* or basidiomycetes *Lenzites*, *Omphalotus*, *Phanerochaete*, *Pleurotus*, *Trametes* and many others [3–5]. Since, in fungi, this secondary metabolite (SM) inhibits the biosynthesis of ergosterol, which is necessary for building the cytoplasmic membrane, the fungal LOV producers should be resistant themselves to it [6,7]. This is realized by placing into the LOV biosynthetic gene cluster (BGC, *lov* genes) not only genes for biosynthesis but also for LOV resistance [7,8]. Since *lov* genes for biosynthesis are linked to genes for LOV resistance, the horizontal transfer of *lov* BGC to one or another representative of fungi kingdom gives them the ability to not only attack competitors with

LOV weapons, but also to defend themselves from exogenous LOV [8]. This may explain such a wide distribution of *lov* BGC in fungi [3].

The most important industrial LOV-producing strains are derived from *A. terreus* [1]. In the current study, we investigated *A. terreus* lovastatin high-yielding (HY) strain obtained after classical strain improvement (CSI) program after multi-round mutagenesis and screening [9]. In *A. terreus* HY strain, the LOV production is increased 200–300-fold compared to the original *A. terreus* wild-type (WT) strain [9,10]; *lov* genes and *laeA* gene (for global regulator of a fungal secondary metabolism) are upregulated [10,11]; the number of *lov* BGC copies does not increase [12]. The *A. terreus* HY strain also demonstrates a significant decrease in viability, expressed in a decrease in the growth rate on agar and liquid nutrient media, a partial reduction in the formation of conidia and the size of mycelium [10,12]. This may be due to the fact that CSI programs operate with random mutations and, during strain improvement, concomitant changes can be accumulated that negatively affect strain viability [13–15]. As a result, in the process of multi-round mutagenesis, a stage begins when the next mutagenic effect no longer leads to a further increase in the production of the target SM [16]. This final stage usually occurs after 20–50 rounds of mutagenesis, and determines the technological limit of the method, in relation to a particular strain [9,16–18].

Previously, it was shown that exogenously introduced polyamines (PAs), such as 1,3-diaminopropane (DAP) or spermidine (Spd), are able to increase the production of target SM in strains that have reached their technological limit [19,20]. In our previous work, we showed that the addition of PAs to complete agar medium leads to an increase in the viability of *A. terreus* HY, which is expressed in an increase in the number of germinating colonies [12]. Furthermore, the addition of 5 mM Spd during the fermentation of *A. terreus* HY increased the LOV yield by 20–45% and was accompanied by upregulation of the *lov* genes and *laeA*; however, the molecular mechanisms involved in this process have not been studied [12]. Recently, *Acremonium chrysogenum* cephalosporin C (CPC) high-yielding strain showed an increase in the content of PAs during the fermentation process, about 5-fold [21]. The improved strain *A. chrysogenum* strain also demonstrated the increased resistance to inhibitors of ornithine decarboxylase (ODC; EC 4.1.1.17), an enzyme of the key stage of PAs biosynthesis [21]. The question of whether this effect is unique or reflects universal trend arising from CSI of filamentous fungi has recently been discussed [16].

In this regard, the aim of our work was to perform the comparative inhibitory assay of ODC, the key and rate-limiting enzyme of PAs biosynthesis in filamentous fungi, for *A. terreus* WT and HY strains, amid the expression analysis of genes for lovastatin and polyamines biosynthesis.

2. Materials and Methods

2.1. Materials

1-aminooxy-3-aminopropane (APA) was a kind gift from Prof. A.R. Khomutov (Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow, Russia) and was synthesized as described previously [22]. Spermidine (Spd) was obtained from MP Biomedicals. α-difluoromethylornithine (DFMO, eflornithine, ornidyl) was obtained from Merck.

2.2. Microorganism Strains Used in the Work

The *A. terreus* strains used: WT (ATCC 20542, wild type) and HY (No. 43–16, highly active producer of LOV, obtained on the basis of the ATCC 20542 strain by UV mutagenesis [9]) were kindly provided by Dr. V.G. Dzhavakhiya, the head of the Department of Molecular Biology of the All-Russian Scientific Research Institute of Phytopathology (Moscow region, Bolshie Vyazemy, Russia).

2.3. Cultivation of A. terreus Strains

The filamentous fungi were cultivated on agarized ATA medium (100 g/L glucose, 20 g/L soy flour, 5 g/L malt extract, 5 g/L meat peptone, 5 g/L NaCl, 0.5 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \times 7H_2O$, 20 g/L agar,

pH 6.0–6.2) or agarized Czapek-Dox (CDA) medium (30 g/L sucrose, 2 g/L $NaNO_3$, 1 g/L K_2HPO_4, 0.5 g/L $MgSO_4 \times 7\ H_2O$, 0.5 g/L KCl, 0.01 g/L $FeSO_4 \times 7\ H_2O$, 20 g/L agar, pH 7.0–7.4). CDA medium was supplemented with DFMO, in the concentration range 0.1–5 mM, or APA, in the concentration range 0.1–5 mM, or Spd, in the amount of 0.5 mM, or used without additions (control). To the CDA medium also 1 or 5 mM DFMO and 0.5 mM Spd were added together; or 1 or 5 mM APA and 0.5 mM Spd were added together.

2.4. Quantification of Fungal Growth Inhibition

To determine the effect of ODC inhibitors, as well as the specificity of this inhibition on the growth of *A. terreus* strains, the drop and dilution method was used, as described earlier [12,23], with some modifications. Cells were harvested from ATA slants and diluted in 0.9% NaCl solution to OD_{600} = 0.3 (base concentration), serial 10-fold dilutions were made in 0.9% NaCl, and 3 µL were inoculated onto Petri dishes with CDA medium without additives or with the addition of compounds (ODC inhibitors, or Spd, or together ODC inhibitors and Spd). The inoculated Petri dishes were incubated for 18 days at 26 °C; measurements were performed every 3 days after inoculation. The inhibitory effects of the compounds were evaluated by ratio of colony growth on CDA medium supplemented with APA, DFMO, Spd, APA + Spd or DFMO + Spd to the growth in the control (CDA medium without any additions), as described previously [21,24]. The formula: growth inhibition % = [(Dc − Dt)/Dc] × 100, where Dc indicates the colony diameter in control set, and Dt indicates the colony diameter in treatment set was used to determine the percent of fungal growth inhibition. The data recorded were measured in triplicate and repeated at least twice.

2.5. Preparation of Total RNA and cDNA Synthesis

Cells were harvested from ATA slant and diluted in 0.9% NaCl solution to OD_{600} = 0.01–0.05, inoculated onto Petri dishes with CDA medium, incubated for 15 days at 26 °C, then collected for RNA isolation. The total RNA preparation and cDNA synthesis were carried out as described previously [25,26].

2.6. qPCR Analysis

qPCR reactions were performed with previously designed pairs of primers for the expression analysis of genes for the metabolism of PAs (*odc*, *samdc*, *spds*, and *oaz*) and LOV biosynthesis (*lovA*, *lovB*, *lovC*, *lovD*, *lovE*, *lovF*, and *lovG*) (Table 1) [10,12]. Reactions and processing of the results were carried out in accordance with the protocol [26]. To normalize the data of mRNA expression levels, we used we used two reference genes, actin and the ubiquitin-conjugating enzyme E2 6 (Ube2 6; EC 2.3.2.23). Pairs of primers for these housekeeping genes were previously selected [10].

Table 1. Primers used for RT-PCR analysis.

Primer	Gene	Product, Function	Oligonucleotide (Sequence 5→3)	Source Sequence
Act_RT_F Act_RT_R	*actin*	A major component of the cytoskeleton	CCACGTTACCACTTTCAACTCC GAGGAGCGATGATCTTGACCT	XM_001209659.1, [10]
E2_6_RT_F E2_6_RT_R	*ube2 6*	Ubiquitin-conjugating enzyme E2 6	TGACCAGCGAAGAAATGACA TTATCTTTCATCCATTTCCA	XM_001211932.1, [10]
LovA_F LovA_R	*lovA*	Dihydromonacolin L monooxygenase	GCGATGTCAAGCCACTCCTCATTATG AGACCCAAGCTCCCAAGTACGTCAAG	AH007774.2, [12]
LovB_F1 LovB_R1	*lovB*	Lovastatin nonaketide synthase	GCCCCATTCTATAAAAACCTGAGGATTC AGTCCTCATTATTCGAGACTCGCAGC	AF151722, [12]
LovC_F1	*lovC*	Enoyl reductase	GCAGAGGAGGTCTTTGACTATCG	AH007774.2, [10]
LovC_R1			GACTCGACGTTGGTGATACAGTCG	
LovD_F1	*lovD*	2-Methylbutyryl/monacolin J transesterase	GGATCTGGACGGAGAGAACTG	
LovD_R1			CAGGGTTCCAGTTGGAAGAAC	
LovE_F LovE_R	*lovE*	GAL4-like transcription factor	TCGATGCGTCTACAGTGAGC TAGCTGTCCGGTGGATCAAG	
LovF_F LovF_R	*lovF*	Lovastatin diketide synthase	TTGCATCTTGCCATTCAGAG TCGAGTCAAATGAGTAGGA	
LovG_F	*lovG*	Thioesterase	GCTCCGTTCCCTTCCTCTGCA	AH007774.2, [12]
LovG_R			GGGGTGTTGAGTCTGCCAGTCG	
ODC_RT_F ODC_RT_R	*odc*	Ornithine decarboxylase	CCCCGGTGAGGAAGATGCGT TCGATCTCCGCCTTGGACGC	CH476600.1, [12]
SamDC_RT_F SamDC_RT_R	*samdc*	S-adenosylmethionine decarboxylase	TACACGACCTCGCCGTCATCCT CCTTCCAGATCTCCTCCGACACG	CH476605.1, [12]
SpdS_RT_F1 SpdS_RT_R1	*spds*	Spermidine synthase	GAAGGTCCTGGTCATTGGCGGT TCTTGAGGAACTCGAAGCCGTCG	CH476594.1, [12]
AZ_RT_F AZ_RT_R	*oaz*	Antizyme, Ornithine decarboxylase inhibitor	ATCTCAGTCTCCGAAGCGTCCTGG CGAGGATTTGTGACCGACATAAGTGG	XM_001214792.1, [12]

3. Results

3.1. Inhibitory Analysis of A. terreus Cell Growth by DFMO and APA

The analysis of ODC inhibition in *A. terreus* WT and HY strains was carried out on CDA synthetic medium, which did not contain PAs. The inhibition was assessed quantitatively by comparing the colony growth. ODC is the first and rate-limiting enzyme of PAs *de novo* synthesis in filamentous fungi; in this regard, inhibition of this enzyme on a synthetic medium without PAs can lead to a significant or complete loss of growth of fungal cells [16,21]. Inhibitory analysis was performed using DFMO, an irreversible suicide inhibitor of ODC, recently studied *versus* ODC from *A. terreus* [27], and APA, isosteric hydroxylamine-containing analogue of Put, showing more potent ODC inhibition than DFMO in some filamentous fungi [21,28]. Aliquots of fungal cells were inoculated onto CDA medium supplemented with compounds or without them (control) (Figure 1).

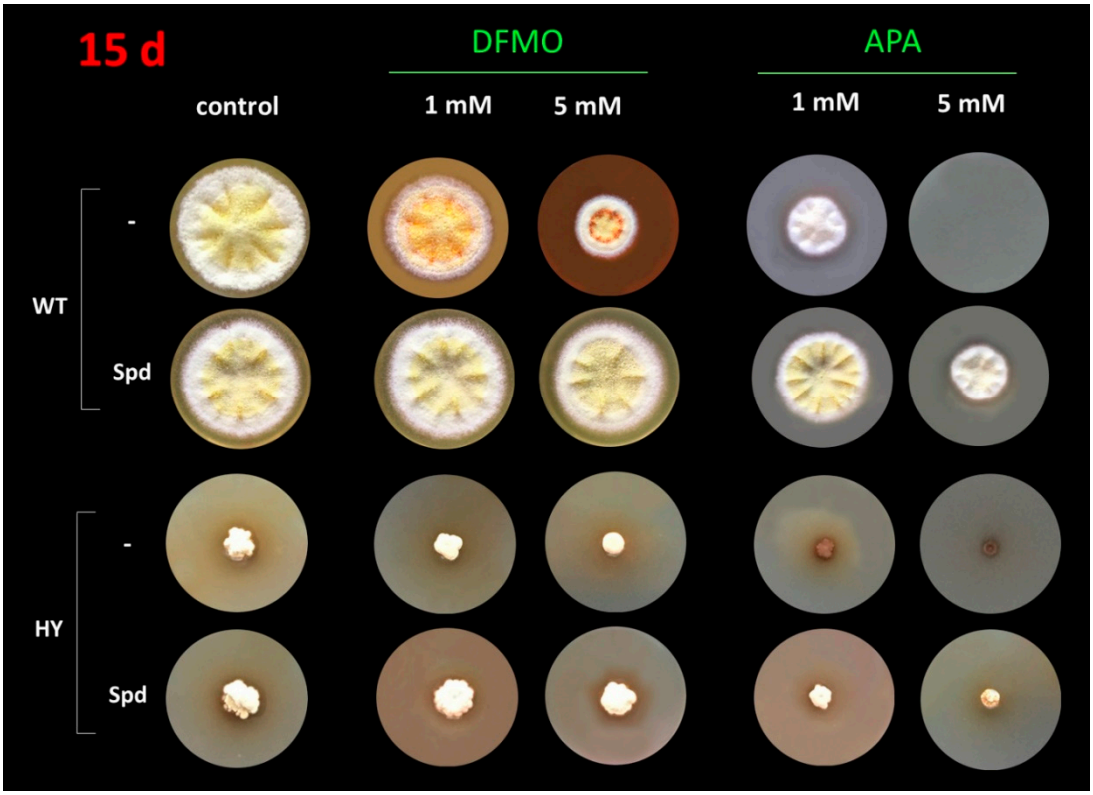

Figure 1. Growth inhibition of the WT (wild type) and HY (high-yielding lovastatin producer) *A. terreus* strains with 1 and 5 mM α-difluoromethylornithine (DFMO), 1 and 5 mM 1-aminooxy-3-aminopropane (APA) and the reversal of the inhibition with 0.5 mM spermidine (Spd) at 15 days after inoculation on agarized Czapek-Dox (CDA) medium.

The inhibitors were added in the concentration range 0.1–5 mM. To determine the specificity of inhibition on PAs biosynthetic pathway by removing the effect of growth inhibition, CDA/DFMO media or CDA/APA media were also supplmented with 0.5 mM Spd. To evaluate the effect of inhibition and removal of inhibition with Spd, fungal strains were also cultured on CDA media without additives and with the addition of only 0.5 mM Spd. The cultivation was carried out for 21 days at 26 °C. The inhibitory effect was determined quantitatively as the percentage of colony growth inhibition on CDA medium with the additions compared to the control medium (Figure 2 and Table S1).

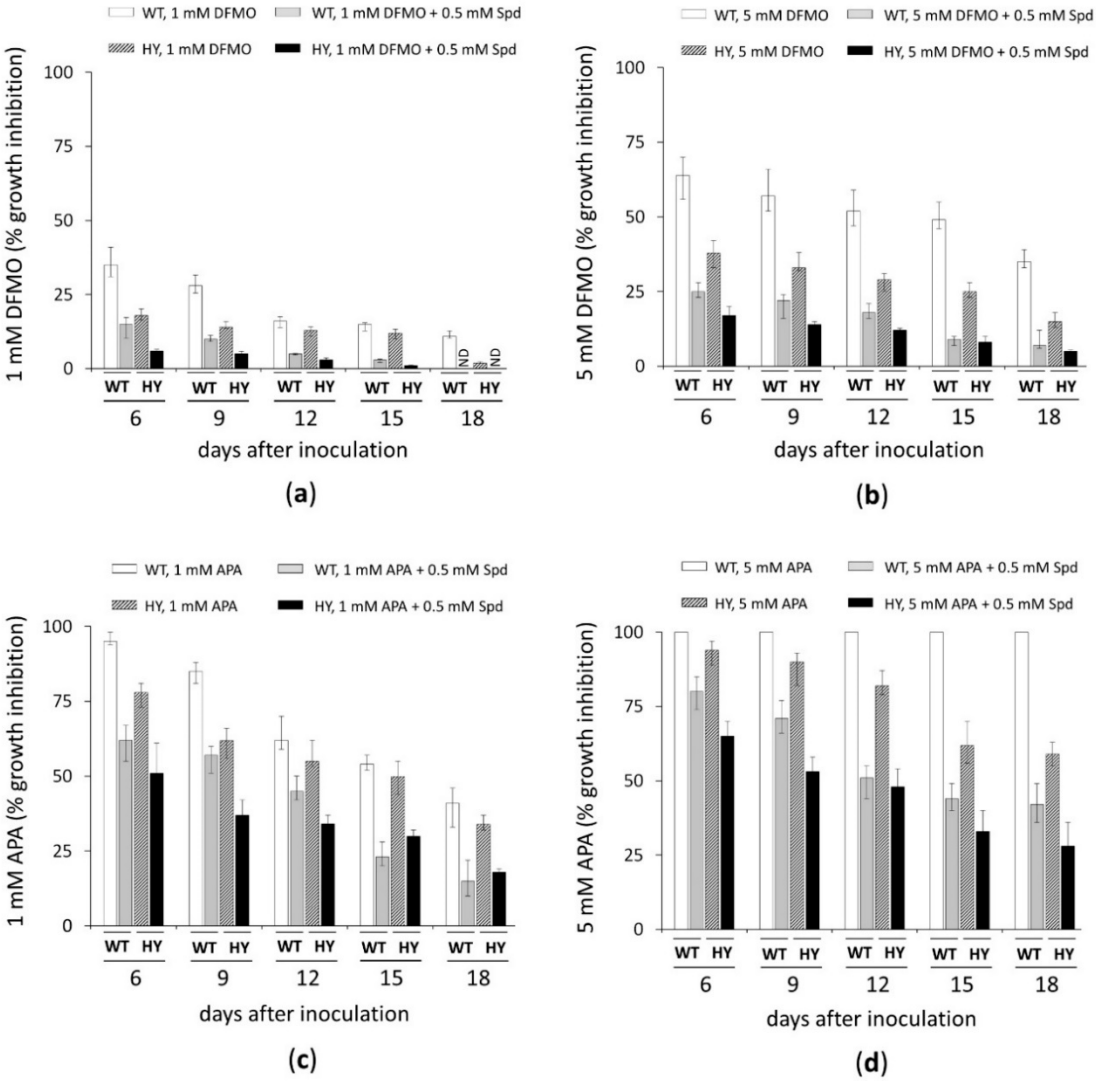

Figure 2. Growth inhibition of the *A. terreus* WT and HY strains with 1 and 5 mM DFMO, 1 and 5 mM APA and the reversal of the inhibition with 0.5 mM Spd on CDA medium. Growth inhibition (%) of *A. terreus* WT and HY strains with 1 mM DFMO (**a**), 5 mM DFMO (**b**), 1 mM APA (**c**), 5 mM APA (**d**), and reverse of the inhibition with 0.5 mM Spd (**a**–**d**). Data are means ± SD, $n = 3$. ND, not detected.

The maximum growth inhibition was observed at the early stages of cultivation for both strains and with the addition of both inhibitors (Figure 2). Further, by the 18th day, there was a gradual overcoming of the toxic effect, expressed in a decrease of growth inhibition. This happened in all cases, except for the effect of 5 mM APA on *A. terreus* WT, leading to the complete death of this strain, 100% inhibition (Figures 1 and 2d). At the same time, *A. terreus* HY exhibited increased resistance and showed growth on CDA medium with the addition of 5 mM APA (Figures 1 and 2d). Such increased resistance of the high-yielding LOV producer was shown against both studied ODC inhibitors (Figure 1). After 15 days of cultivation, *A. terreus* HY was more resistant to 5 mM DFMO, by 20–25%, and to 5 mM APA, by 40–45% (Figures 1 and 2b,d). Removal of the toxic effect of inhibitors when Spd was added together was approximately the same for both strains. The addition of Spd, together with DFMO or APA, led not only to the recovery (or partial recovery) of colony size, but also to the recovery of initial strain phenotype, colony color and morphology, for both strains (Figure 1). The lethal effect of 5 mM APA for the *A. terreus* WT strain was removed by supplementation of 0.5 mM Spd; the *A. terreus* WT strain growth on CDA/5 mM APA/0.5 mM Spd media after 15 days was approximately 50% of the control (Figures 1 and 2d).

3.2. Expression Levels of PAs Metabolism Genes and lovB in A. terreus WT and HY Strains

In order to explain the reasons of increased resistance of the high-yielding LOV producer to ODC inhibitors, we studied the expression levels of the most important PAs biosynthesis genes, such as *odc*, *samdc* (encodes S-adenosylmethionine decarboxylase, AdoMetDC; EC 4.1.1.50) and *spds* (encodes spermidine synthase, SpdS; EC 2.5.1.16). We also determined the expression level of the *oaz* gene (encodes the antizyme of ornithine decarboxylase, OAZ), a negative regulator of ODC lifespan in the fungi cell. To determine the biosynthetic status of the strains with regard to LOV production during cultivation on CDA medium, the expression levels of *lovA* (encodes cytochrome P450, dihydromonacolin L monooxygenase LovA; EC 1.14.14.124), *lovB* (encodes lovastatin nonaketide synthase LovB; EC 2.3.1.161), *lovC* (encodes trans-acting enoyl reductase LovC; EC 2.3.1.161), *lovD* (encodes 2-methylbutyryl/monacolin J transesterase LovD; EC 2.3.1.238), *lovE* (encodes GAL4-like transcription factor, LOV pathway-specific positive regulator LovE), *lovF* (encodes lovastatin diketide synthase LovF; EC 2.3.1.244), and *lovG* (encodes thioesterase LovG; EC 2.3.1.244) were also measured.. LovB is the so-called central enzyme of LOV biosynthesis, catalyzes the first 35 consecutive reactions for the polymerization and modification of 9 molecules of manoyl-CoA with the formation of dihydromonacalin L [29]. It turned out that during the cultivation on solid synthetic medium (CDA) in *A. terreus* HY strain *lovB* is upregulated, about 10-fold (Figure 3). Previously, we demonstrated that, under conditions of deep fermentation on the defined media, *lovB* is upregulated 30–35-fold as compared to *A. terreus* WT [11]. It is known that at different nutrient media the expression of *lov* genes in *A. terreus* can be regulated differently [30,31]. Thereby detection of *lovB* upregulation during the cultivation of *A. terreus* HY under not optimal (for LOV biosynthesis) conditions is rather important. We also found the upregulation of other studied *lov* genes in *A. terreus* HY strain on CDA medium. *lovA* was upregulated about 20-fold, *lovC* was upregulated about 1.5-fold, *lovD* was upregulated 10–15-fold, *lovE* was upregulated 7–9-fold, *lovF* was upregulated 5–6-fold, and *lovG* was upregulated about 6–8-fold (Figure 3). The upregulation of these *lov* genes on CDA medium was, as in the case of *lovB*, weaker compared to previously shown the increase in mRNA expression during the fermentation on the defined media, where *lovA* was upregulated more than 150-fold, *lovC* was upregulated 2–3-fold, *lovD* was upregulated 20–30-fold, *lovE* was upregulated 10–15-fold, *lovF* was upregulated 12–18-fold, and *lovG* was upregulated about 10–15-fold [11].

From the results obtained, it is seen that in the context of *lovB* upregulation, the *A. terreus* HY strain has also an increased expression of genes for key enzymes of PAs biosynthesis (Figure 3). In LOV high-yielding strain *odc* is upregulated, by 3–3.5-fold, *samdc* is upregulated, by 7–10-fold, *spds* is upregulated, by 3–4-fold (Figures 3 and 4). On the contrary, *oaz* is downregulated, by 4–6-fold (Figures 3 and 4). The combined effect of upregulation of *odc* and, at the same time, downregulation of *oaz*, can lead to an additional increase in the content of ODC in *A. terreus* HY cells (Figure 4). This can lead to increased resistance of the HY strain to ODC inhibitors. After 15 days, the addition of 5 mM APA inhibits the WT strain by 100%, the HY strain inhibits by 62%; the addition of 5 mM DFMO inhibits the WT strain by 49% and inhibits the HY strain by 25% (Figures 2 and 4).

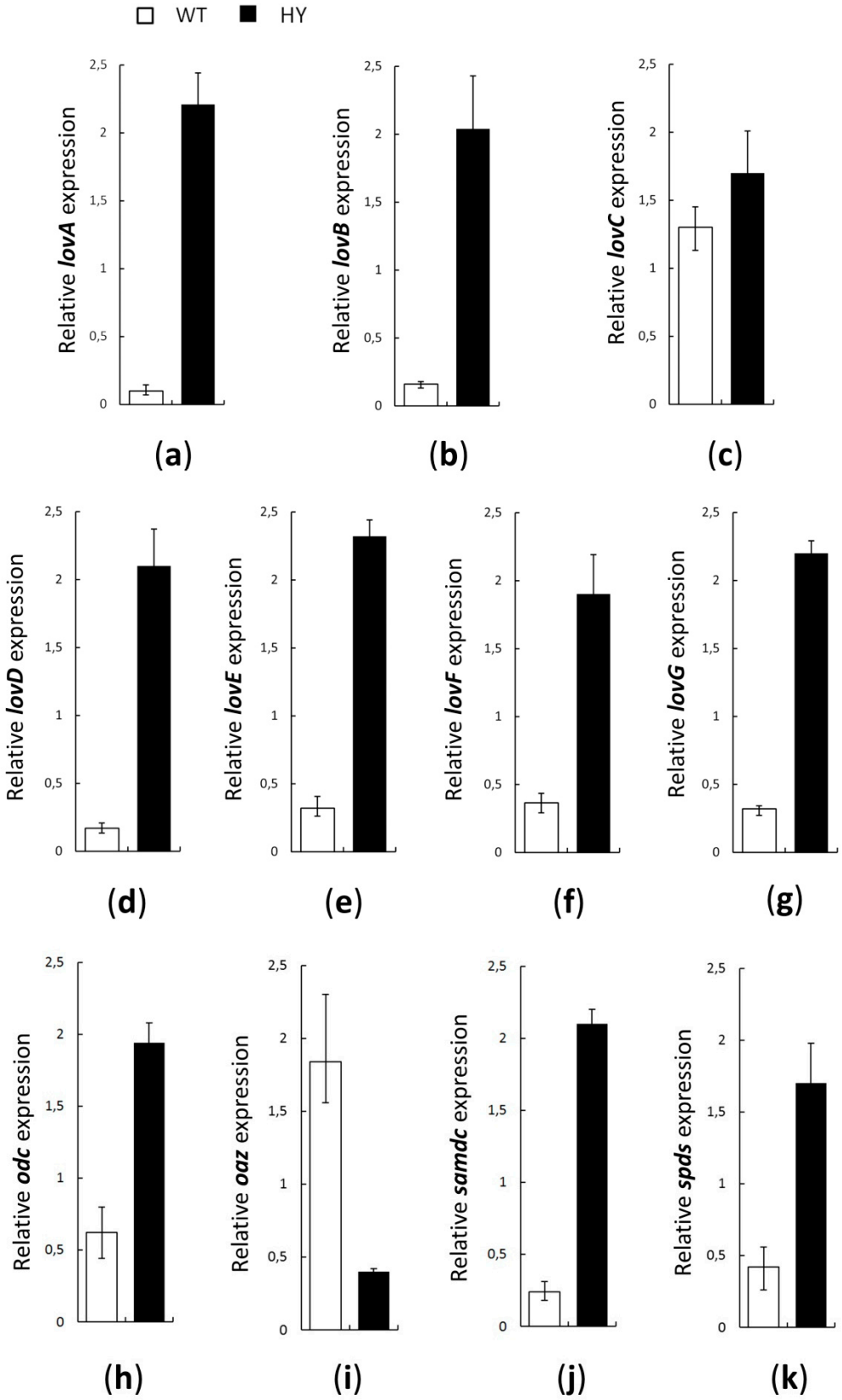

Figure 3. Relative gene expression in *A. terreus* WT and HY strains: *lovA* (**a**), *lovB* (**b**), *lovC* (**c**), *lovD* (**d**), *lovE* (**e**), *lovF* (**f**), *lovG* (**g**), *odc* (**h**), *oaz* (**i**), *samdc* (**j**), and *spds* (**k**), 15 days after inoculation on CDA medium. Data are means ± SD, $n = 3$.

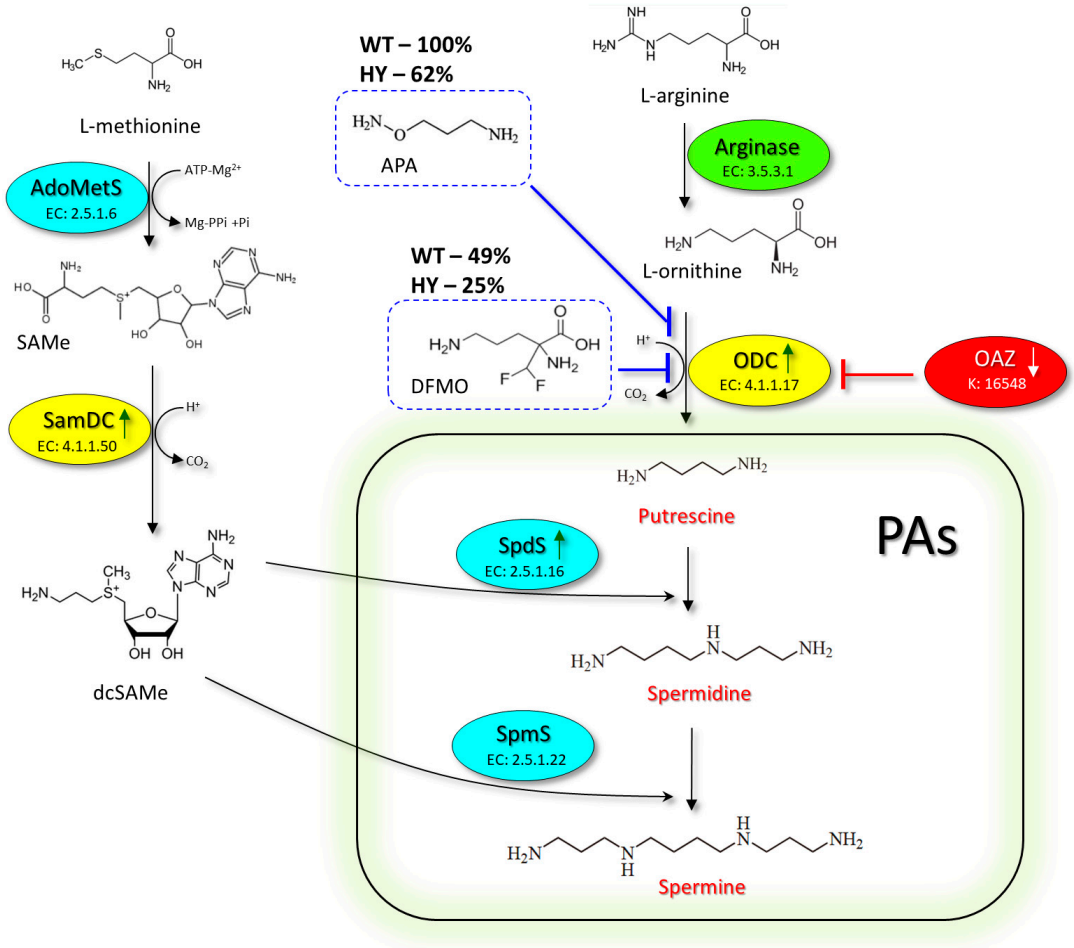

Figure 4. The putative pathway of polyamine (PAs) biosynthesis in *A. terreus* and growth inhibition (%) of WT (wild type) and HY (high-yielding lovastatin producer) strains by 5 mM inhibitors of ornithine decarboxylase (ODC). Fifteen days after inoculation on CDA medium. Green and up arrow next to the protein name means upregulation of corresponding gene in *A. terreus* HY strain; white and down arrow next to the protein name means downregulation of corresponding gene in *A. terreus* HY strain. SAMe—S-adenosylmethionine; dcSAMe—decarboxylated S-adenosylmethionine. OAZ—ornithine decarboxylase antizyme, AdoMetS—S-adenosylmethionine synthetase, SamDC—S-adenosylmethionine decarboxylase, SpdS—spermidine synthase, SpmS—spermine synthase, DFMO—α-difluoromethylornithine, APA—1-aminooxy-3-aminopropane.

4. Discussion

In our experiments, we found an increase in the resistance of *A. terreus* HY strain to inhibitors of ODC, a key enzyme of PAs biosynthesis (Figure 2). It is known that in the cells of various organisms a strict control of homeostasis for PAs content take place [32]. For this, there are both pathways of biosynthesis and catabolism of PAs, which have been studied, to a large extent, in mammals and a number of model organisms [33–35]. In filamentous fungi, some stages of PAs metabolism and its regulation are still not fully understood [36]. Previously, there was a misconception that filamentous fungi lack spermine [37]. Then, for numerous representatives of filamentous fungi, the presence of spermine was shown [38,39]. Our recent work on studying the composition of PAs in *A. chrysogenum* the presence of spermine as one of the main components of the PAs pool was also shown [21]. On the other hand, the pathways of putrescine biosynthesis have not yet been finally established in filamentous fungi [40]. In bacteria and higher plants, this diamine can be synthesized either directly from L-ornithine by the ODC enzyme, or through several successive steps, which are preceded by decarboxylation of L-arginine by the arginine decarboxylase enzyme (ADC; EC 4.1.1.19). From the other side, mammals

have only an ODC-dependent pathway for putrescine biosynthesis. In a number of fungi, DFMO, a suicidal inhibitor of ODC, completely suppresses cell growth [41,42]. At the same time, there are studies demonstrating increased sensitivity for some fungi to DFMA (α-difluoromethylarginine), a suicidal ADC inhibitor [43,44]. Since the ADC enzyme has not yet been found in fungi, it is assumed that the DFMA inhibition effect may result from the conversion of this compound to DFMO by the enzyme arginase (EC 3.5.3.1) [40,45]. In a recent work, the enzymatic properties of ODC from *A. terreus* were described [27]. It was shown that this enzyme, in addition to catalytic activity against L-ornithine, has residual catalytic activity against L-arginine [27]. In current work, the addition of 5 mM APA led to 100% inhibition of the growth of the WT strain (Figures 1 and 4). In addition, arginine-decarboxylating activity has been shown for fungi of the genus *Aspergillus*, leading to the formation of agmatine [46]. Therefore, 100% inhibition of *A. terreus* WT cell growth by 5 mM APA means that in *A. terreus* only ODC-dependent putrescine biosynthesis is functioning, or 5 mM APA nonspecifically inhibits fungal ADC, as demonstrated for high concentrations of APA versus ADC from *E. coli* [21]. From the other side, *A. terreus* HY strain exhibited increased resistance to ODC inhibitors (Figure 2). In particular, when 5 mM APA completely suppressed the growth of *A. terreus* WT strain, the growth inhibition of *A. terreus* HY was only 62%; when 5 mM DFMO inhibited *A. terreus* WT by 49%, inhibition of *A. terreus* HY was 25% (Figure 4).

The disruption of PAs regulation may trigger a number of pathological processes, leading, for example, to the depletion of the pool of S-adenosylmethionine (SAMe) used for the biosynthesis of PAs (Figure 4) [47,48]. In order to minimize deviations from the baseline PAs content, key biosynthetic enzymes such as ODC, SamDC and SpdS are tightly controlled at the levels of transcription, translation, and cell lifespan [32,49]. In this regard, the shift in PAs cell content is accompanied with several mutational events, corresponding to changes in at different levels of the regulation. Previously, we found an increased content of PAs in *A. chrysogenum* high-yielding producer of cephalosporin C (CPC) [21]. This strain also showed increased resistance to ODC inhibitors [21]. Possibly, the concentration of 5 mM ODC inhibitors was sufficient for complete depletion of PAs pool in *A. chrysogenum* WT strain and was insufficient to deplete the PAs pool in the *A. chrysogenum* HY strain. In the current study, we showed for *A. terreus* HY strain the increased resistance to DFMO and APA inhibitors of ODC, as well as *odc* upregulation and *oaz* downregulation.

The shift in PAs content in filamentous fungi strains obtained as a result of CSI programs, has recently been proposed [16,21]. As a result of multi-round random mutagenesis and screening, useful mutations are selected, leading to an increase in the yield of the target SM [16]. Along with this, a genetic load accumulates which represents side mutations and is expressed in a general decrease of strains fitness and a reduction in other vital processes [10,17,50]. In response to mutagenic effects, the cell activates its protective resources; PAs are also known to be able to protect cells from free radical damage through direct interaction with reactive oxygen species [51,52]. Recently, it was shown that PAs can promote homologous recombination during DNA repair complexed with RAD51, stimulating the formation of RAD51-ssDNA nucleofilaments [53]. Thus, an increase in the PAs content could be a side effect during multi-round mutagenesis, which made it possible to obtain a viable strain after the next exposure [16,21]. At the same time, the metabolism of PAs can intersect with SM biosynthesis at the level of consumption of common substrates, for example, SAMe [32]. The SAMe coenzyme is used both for transmethylation reactions during LOV biosynthesis and for aminopropylation during PAs biosynthesis (Figures 4 and 5). It is known that the biosynthesis of one LOV molecule requires two SAMe molecules [54]. For the synthesis of one molecule of Spd, one molecule of SAMe molecule is required; to synthesize one Spm molecule (from putrescine), two SAMe molecules are required [16]. The observed upregulation of genes for PAs biosynthesis (including *samdc* gene, coding SAMe-consuming enzyme), amid upregulation of *lov* genes (including *lovB* and *lovF* genes, coding SAMe-consuming enzymes), may lead to the depletion of the SAMe content during fermentation with 10–15 g/L yield of LOV (Figure 5).

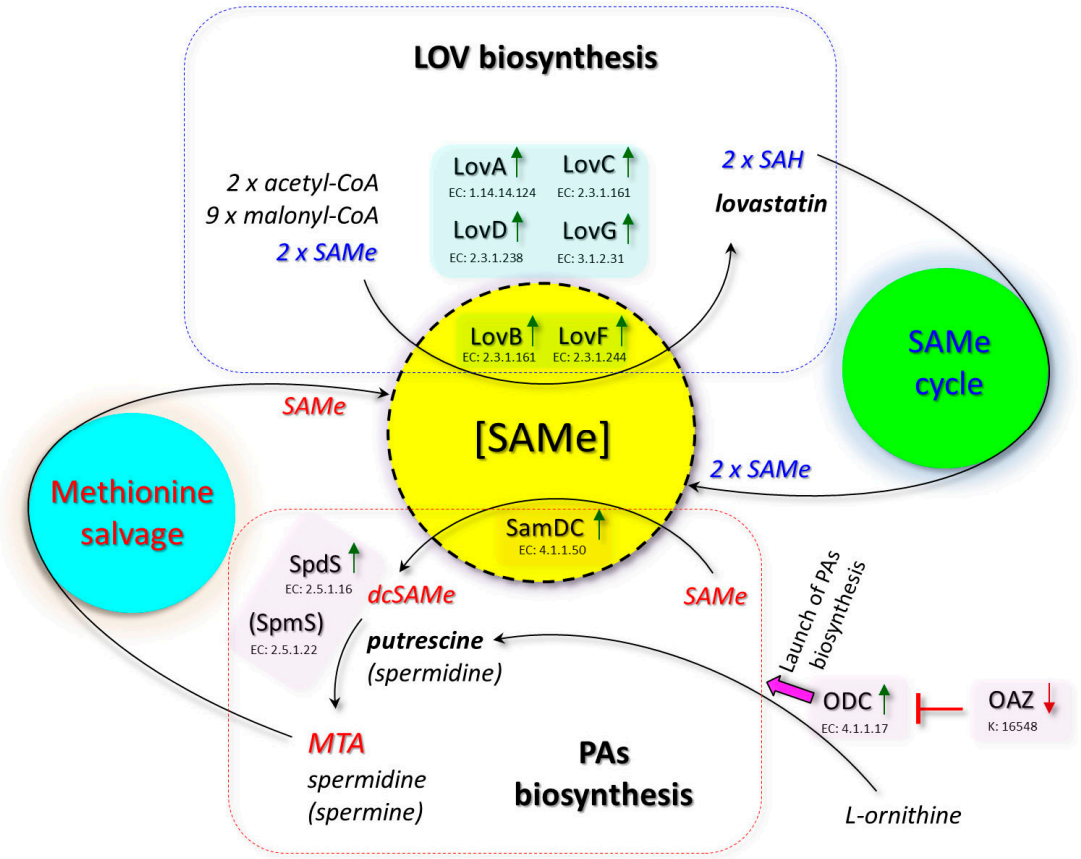

Figure 5. S-adenosylmethionine consumption for biosynthesis of lovastatin (LOV) and polyamines (PAs) in *Aspergillus terreus*. Green and up arrow next to the protein name means upregulation of corresponding gene in *A. terreus* high-yielding lovastatin producer (HY strain); red and down arrow next to the protein name means downregulation of corresponding gene in *A. terreus* HY strain. SAMe—S-adenosylmethionine; SAH—S-adenosylhomocysteine; dcSAMe—decarboxylated S-adenosylmethionine; MTA—5′-methylthioadenosine. LovA—dihydromonacolin L monooxygenase; LovB—lovastatin nonaketide synthase; LovC—enoyl reductase; LovD—2-methylbutyryl/monacolin J transesterase; LovF—lovastatin diketide synthase; LovF—thioesterase; ODC—ornithine decarboxylase; OAZ—ornithine decarboxylase antizyme, SamDC—S-adenosylmethionine decarboxylase, SpdS—spermidine synthase, SpmS—spermine synthase. Lovastatin dependent consumption and regeneration pathway of SAMe are shown in blue; polyamines dependent consumption and regeneration pathway of SAMe are shown in red.

It has also been shown that SAMe is required for the functioning of epigenetic factors, SAMe-dependent methyl-transferases of DNA and histones, the most important of which is LaeA, a positive regulator of LOV biosynthesis in *A. terreus* [55] or beta-lactams in *Penicillium chrysogenum* [19,56] and *A. chrysogenum* [20]. In this regard, maintaining an increased pool of PAs in improved strains partly takes up the resources required for the biosynthesis of the target SM. The introducing of exogenous PAs can lead by a feedback mechanism to a decrease in PAs endogenous biosynthesis [32] and the release of resources, for example, SAMe, for the production of the target SM. This may explain the additional increase in production in the improved strains during fermentation with PAs [16].

5. Conclusions

In our work, for the first time, an increased resistance of *Aspergillus terreus* lovastatin (LOV) high-yielding strain to the inhibitors of ornithine decarboxylase, a key enzyme of polyamine biosynthesis (PAs) in filamentous fungi, was found. This phenomenon may be of fundamental importance for

explaining the relationship between the biosynthesis of LOV and PAs in the fungal cell and of practical value for increasing the LOV yield during the optimizing of fermentation conditions with exogenously introduced PAs.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-3417/10/22/8290/s1, Table S1: Growth inhibition of the *A. terreus* WT and HY strains with 1 and 5 mM DFMO, 1 and 5 mM APA and the reversal of the inhibition with 0.5 mM Spd on CDA medium.

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