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Heterologous coexpression of *Vitreoscilla* hemoglobin and *Bacillus megaterium* glucanase in *Streptomyces lydicus* A02 enhanced its production of antifungal metabolites

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ABSTRACT

Streptomyces lydicus A02 is a novel producer of commercially important polyene macrocyclic antibiotic natamycin and a potential biocontrol agent to several plant fungal diseases, including wilt caused by *Fusarium oxysporum* f. spp. To improve the natamycin production and the antifungal activity of *S. lydicus* A02, we coexpressed gene *vgb* encoding *Vitreoscilla* hemoglobin (VHb) and *bglC* encoding *Bacillus megaterium* L103 glucanase, both under the control of the strong constitutive ermE* promoter, in *S. lydicus* A02. Our results showed that coexpressing VHb and glucanase improved cell growth, and the engineered strain produced 26.90% more biomass than the wild-type strain after 72 h fermentation in YSG medium. In addition, coexpressing genes encoding VHb and glucanase led to increased natamycin production, higher endogenous chitinase activity and exogenous glucanase activity, as well as enhanced antifungal activity in the engineered *S. lydicus* AVG02 and AGV02, regardless of the position of the two genes on the plasmids. Compared with model strains, few reports have successfully coexpressed VHb and other foreign proteins in industrial strains. Our results illustrated an effective approach for improving antifungal activity in an industrial strain by the rational engineering of combined favorable factors.

1. Introduction

Streptomyces species have been used extensively as biocontrol agents due to their ability to produce various antimicrobial compounds, such as antibiotics and hydrolytic enzymes like glucanases and chitinases [1–2]. Many studies have attempted to enhance the antifungal activity of *Streptomyces* against pathogens by increasing their antibiotic or hydrolytic enzyme production using traditional methods and genetic engineering, such as fermentation optimization, physical and chemical mutagenesis, and genetic manipulation of related regulatory genes [3–5].

Streptomyces lydicus strain A02 was isolated from the soil of a suburban forest field in Beijing (China), and presented stable and robust inhibitory activity against several plant pathogenic fungi, including *Fusarium oxysporum*, *Botrytis cinerea*, *Rhizoctonia cerealis* and *Monilinia laxa* [6]. The antifungal activity of *S. lydicus* A02 is mainly due to the production of natamycin, a tetraene macrolide antibiotic widely used as a food preservative and fungicide, with potential application for plant fungal disease control in agricultural production [7–10]. Glucanases and chitinases are pathogenesis-related (PR) proteins; they can degrade fungal cell walls and have been widely used as antifungal agents in plant protection [11,12]. The wild-type *S. lydicus* A02 strain exhibited no glucanase activity on CMC-Na plates by congo red staining and very weak chitinase activity when measuring the chitin hydrolysis zones [13]. Combining two biocontrol factors can enhance fungal resistance in plants or microorganisms [13–17]. For example, the expression of chitinase and β-1,3-glucanase genes in carrots enhanced resistance to *Alternaria dauci*, *A. radicina*, *Cercospora carotae* and *Erysiphe heraclei* [18]. Expression of chitinase from *Trichoderma harzianum* in natamycin-producing strain *S. lydicus* A01 increased the antagonistic effect of *S. lydicus* A01 on *B. cinerea* and *F. oxysporum* f. spp. [16,17].

Vitreoscilla hemoglobin (VHb) is a bacterial hemoglobin synthesized by *Vitreoscilla* under hypoxic conditions and has been broadly and successfully used in heterologous hosts to improve growth and productivity in a wide variety of organisms and bioproducts [19,20]. Although it is not yet known how VHb regulates metabolism, the putative function of VHb is to enhance respiration and energy metabolism by promoting oxygen delivery [21,22]. Heterologous expression of VHb has been found to increase the production of avermectin, chlortetracycline, ethanol

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and β-galactosidase [23–26]. Coexpression of VHb and foreign proteins can enhance the production of relative proteins in model strains, including *Escherichia coli* and *Pichia pastoris*. In *E. coli*, coexpression of VHb and D-amino acid oxidase (DAAO) showed a 1.5-fold improvement in DAAO activity compared with that of the DAAO-expressing control strain [27]. In *P. pastoris*, coexpression of lipase LIP2 gene *lip2* from *Yarrowia lipolytica* and VHb gene *vgb* under the control of AOX1 promoter resulted in a 1.84-fold increase in YlLIP2 production compared with that of VHb^- cells at lower dissolved oxygen levels [22], and coexpression of VHb and β-galactosidase and VHb and methionine adenosyltransferase under AOX1 promoter enhanced β-galactosidase activity and *S*-adenosylmethionine content [26,28]. However, there are few reports on the coexpression of *vgb* and other genes in industrial strains.

In our previous study, heterologous expression of glucanase in *S. lydicus* A02 had no effect on natamycin production but promoted antifungal activity against several plant pathogenic fungi [17]. In this study, we expressed the *vgb* gene in *S. lydicus* to meet the high oxygen demand of natamycin production, and then constructed recombinant *S. lydicus* AVG02 and AGV02 strains in which the coexpression of VHb and glucanase was each driven by the ermE* promoter. These constructs were used to characterize the effects of heterologous VHb and glucanase coexpression on natamycin production, chitinase activity, and glucanase activity in *S. lydicus*, as well as the antifungal activity of the engineered *S. lydicus* strain synergizing these three biocontrol factors.

2. Materials and methods

2.1. Strains and plasmids

The bacterial strain A02 (CGMCC 1654; China General Microbiology Culture Collection Center) was isolated from suburban vegetable and forest field soil, and was identified as *S. lydicus* based on the 16S rDNA sequence and phenotypic comparison [6]. *S. lydicus* strain AG02 (CGMCC 6814) is a recombinant *S. lydicus* A02 strain expressing a glucanase gene cloned from the biocontrol strain *Bacillus megaterium* L103 under the *S. erythraea* ermE* promoter [17]. The *S. lydicus* strains were grown at 29 °C on potato dextrose agar (PDA) slant for spore formation. E. coli strain DH5α was used as a host for genetic manipulation. Non-methylating E. coli strain ET12567 (pUZ8002) was used for DNA conjugal transfer from *E. coli* to *S.* lydicus. Conjugation was performed as described by Kitani et al. [29] and Paranthaman and Dharmalingam [30]. Plasmid pUC19 was used for routine cloning and subcloning experiments. The integration vector pIB139 from vector pSET152 containing Φ31 *int*, and *att*P [31] was used to introduce one or two genes into *S. lydicus*. Plasmid pRK404-VHb (provided by Dr Y. Wen, China Agricultural University, China) containing a vgb operon [GenBank: AF292694.1] was used for the construction of pVIB139. When necessary, media were supplemented with antibiotics (100 μg mL^{-1} of apramycin to LB medium, 60 μg mL^{-1} to selection medium, and 30 μg mL^{-1} to fermentation medium, as required). Synthesis of oligonucleotide primers and DNA sequencing of PCR products were performed by Invitrogen Biotechnology (China).

2.2. Construction of plasmid vectors with vgb gene under ermE* promoter

Primers VF 5′-GGAATTCCATATGATGTTAGACCAGCAAACCA-3′ and VR 5'- GCTCTAGATTATTCAACCGCTTGAGCGT-3' (underlined nucleotides were added to introduce *Nde*I and *Xba*I sites for cloning) were used to clone the *vgb* gene. The PCR fragment was digested with *Nde*I/*Xba*I and ligated into *Nde*I/*Xba*I-digested pIB139 to generate pVIB139, which harbored the ermEp* promoter and the intact *vgb* gene. Primers VF and VR1 5'-GGAATTCTTATTCAACCGCTTGAGCGT-3' (underlined nucleotides were added to introduce *Eco*RI site for cloning) were used to clone the *vgb* gene into *Nde*I/*Eco*RI-digested pIB139 to generate pIBV139, which has different restriction sites compared with pVIB139 for further vector construction. The cloned *vgb* gene was verified by DNA sequencing.

2.3. Construction of plasmid vectors with bglC gene under ermE* promoter

Primers GF (5′-GGAATTCCATATGATGAAACGGTCAATCTCG-3′) and GR (5′-GGAATTCCTAATCAGTTTTCCTTGA-3′) designed according to the endo-glucanase gene sequence of *B. megaterium* strain AP25 (GenBank accession number: HM130670.1), were used to clone glucanase gene *bglC* from *B. megaterium* L103 (underlined nucleotides were added to introduce *Nde*I and *Eco*RI sites for cloning). A 1482-bp segment was digested with *Nde*I/*Eco*RI and ligated into pIB139 digested with the same restriction enzyme to generate pIBG139, which harbored the ermEp* promoter and the intact *bglC* gene [17].

Primers GF and GR1 (5′-GCTCTAGACTAATCAGTTTTCCTTGA-3′) (underlined nucleotides were added to introduce *Xba*I sites for cloning) were used to clone glucanase gene *bglC* from vector pIBG139 [17]. A 1482-bp segment was digested with *Nde*I/*Xba*I and ligated into *Nde*I/*Xba*I-digested pIB139 generating pGIB139, which has different restriction sites compared with pIBG139 for further vector construction.

2.4. Combinatorial construction of vgb and bglC expressing plasmids

Primers ermF (5′-GCTCTAGACTAGTATGCATGCGAGTGTC-3′) and GR (underlined nucleotides were added to introduce *Xba*I and *Eco*RI sites for cloning) were used to clone the *bglC* gene [GenBank: KC245139.1] with the ermE* promoter from vector pIBG139. A 1679-bp segment containing *bglC* gene was digested with *Xba*I/*Eco*RI and ligated into *Xba*I/*Eco*RI-digested pVIB139, which harbored the ermEp* promoter and the intact *vgb* gene, generating pVGIB139. Plasmid pVGIB139 carried the *bglC* gene downstream of the *vgb* gene, each with an ermE* promoter.

Primers ermF and VR1 were used to clone the *vgb* gene with ermE* promoter from vector pIBV139. A 638-bp segment containing the *vgb* gene was digested with *Xba*I/*Eco*RI and ligated into *Xba*I/*Eco*RI-digested pGIB139, generating pGVIB139. Plasmid pGVIB139 carried the *vgb* gene downstream of the *bglC* gene, each with an ermE* promoter. The difference between pVGIB139 and pGVIB139 was the positions of *vgb* and *bglC* on the plasmids. Plasmids pVGIB139 and pGVIB139 were introduced into *S. lydicus* A02 via conjugation to generate AVG02 and AGV02, and plasmid pIB139 was also introduced into *S. lydicus* A02 as a control. The exconjugants were screened for apramycin resistance at 60 μg mL^{-1}. The genotype of the exconjugants was confirmed by PCR analysis with specific primers for the apramycin resistance gene aac(3)IV according to Li et al. [32]. Each amplified product was verified by DNA sequencing.

2.5. Fermentation and analysis of natamycin

Spores (5×10^7) of *S. lydicus* A02, AV02, AG02, AVG02 and AGV02 from PDA agar slant were inoculated into 50 ml of a seed culture medium [10], and incubated at 29 °C for 24 h on a rotary shaker (250 rpm). The YSG medium [33] was used for natamycin production analysis according to Wu et al. [10]. Sample extraction and HPLC (Japan Analytical Industry Co., Ltd., Japan) analysis of