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Proteome-wide alterations in an industrial clavulanic acid producing strain of *Streptomyces clavuligerus*

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ABSTRACT

The usefulness of genetic/metabolic engineering for further improvement of industrial strains is subject of discussion because of the general lack of knowledge on genetic alterations introduced by iterative cycles of random mutagenesis in such strains. An industrial clavulanic acid (CA)-overproducer *Streptomyces clavuligerus* DEPA was assessed to understand proteome-wide changes that have occurred in a local industrial CA overproducer developed through succesive mutagenesis programs. The proteins that could be identified corresponded to 33 distinct ORFs for underrepresented ones and 60 ORFs for overrepresented ones. Three CA biosynthetic enzymes were overrepresented in *S. clavuligerus* DEPA; carboxyethylarginine synthase (Ceas2), clavaldehyde dehydrogenase (Car) and carboxyethyl-arginine beta-lactam-synthase (Bls2) whereas the enzymes of two other secondary metabolites were underrepresented along with two important global regulators [two-component system (TCS) response regulator (SCLAV_2102) and TetR-family transcriptional regulator (SCLAV_3146)] that might be related with CA production and/or differentiation. γ-butyrolactone biosynthetic protein AvaA2 was 2.6 fold underrepresented in *S. clavuligerus* DEPA. The levels of two glycolytic enzymes, 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase and phosophoglycerate kinase were found decreased while those of dihydrolipoyl dehydrogenase (E3) and isocitrate dehydrogenase, with two isoforms were found as significantly increased. A decrease of amino acid metabolism, methionine biosynthesis in particular, as well as S-adenosylmethionine synthetase appeared as one of the prominent mechanisms of success of *S. clavuligerus* DEPA strain as a prolific producer of CA. The levels of two enzymes of shikimate pathway that leads to the production of aromatic amino acids and aromatic secondary metabolites were also underrepresented. Some of the overrepresented stress proteins in *S. clavuligerus* DEPA included polynucleotide phosphorylase/polyadenylase (PNPase), ATP-dependent DNA helicase, two isoforms of an anti-sigma factor and thioredoxin reductase. Downregulation of important proteins of cell wall synthesis and division was recorded and a protein with β-lactamase domain (SCLAV_p1007) appeared in 12 isoforms, 5 of which were drastically overrepresented in DEPA strain. These results described herein provide useful information for rational engineering to improve CA production in *Streptomyces clavuligerus*.

1. Introduction

Clavulanic acid (CA) is a bicyclic compound with a β-lactam and an oxazolidine ring [1]. It has a weak antibacterial activity but is a very powerful class of β-lactamase inhibitor naturally produced by *Streptomyces clavuligerus*. CA is active against a wide spectrum of penicillin- and cephalosporin-resistant bacteria exerting its function by irreversibly binding to serine hydroxyl group in active sites of β-lactamases. Due to synergistic effect, it is co-formulated with conventional β-lactam antibiotics and prescribed clinically in combination with amoxycillin as Augmentin™ and with ticarcillin as Timentin™ [2]. *S. clavuligerus* fermentations are used for CA production in bioindustry as its large scale chemical synthesis is still not feasible [3].

Draft genome sequence of *S. clavuligerus* has revealed loci of

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three distinct gene clusters involved in biosynthesis of CA and 5S clavams (Fig. 1). Cephamycin and CA supercluster and clavam gene cluster are located on the chromosome, whereas paralog gene cluster lies on a large linear plasmid, pSCL4. The megaplasmid pSCL4 of *S. clavuligerus* is packed with at least 25 secondary metabolite biosynthetic gene clusters that are identical or resembling to those from other *Streptomyces* spp, supporting the hypothesis that many secondary metabolism biosynthetic gene clusters in bacteria are acquired by horizontal gene transfer. Together with the clusters on the chromosome, the total number of putative secondary metabolite gene clusters reaches to 48. These include 10 putative nonribosomal peptide synthetase (NRPS) gene clusters, eight putative PKS gene clusters, and six gene clusters putatively encoding NRPSs and PKSs or NRPS-PKS hybrids as well as 12 clusters putatively encoding one or more terpene synthases or cyclases [4].

Although whole characterization of ORFs in CA gene cluster has not yet been completed, essential biosynthetic genes were clearly identified [5–8]. Pathway leading to the biosynthesis of CA in *S. clavuligerus* is shown in Fig. 2. Among biosynthetic genes of CA cluster, *cas2* encodes for a rate-limiting enzyme, clavaminic acid synthase [9]. CA biosynthesis is controlled by pleiotropic regulators like BldG and BldA [10] and AdpA [11], and pathway-specific regulators (cluster situated regulators) CcaR, a transcriptional activator encoded by *ccaR* located in cephamycin C gene cluster and CA pathway-specific ClaR activator encoded by *claR* [12,13]. The roles of CcaR stimulating both cephamycin C and CA clusters and ClaR as the positive regulator of CA biosynthetic cluster have been well documented. In *ccaR*-deleted mutant, both "early" and "late" genes of CA biosynthesis pathway were downregulated while *claR* deletion led to low level of expression in "late" genes in CA cluster when compared to the parental strain [12–15]. Deletion of *bldA* led to underrepresentation of Cas2, OppA1 and GcaS proteins while deletion of *bldG* additionally resulted in the low formation of Bls2 [10]. Stringent response protein RelA positively affects CcaR expression indirectly while another response regulator, Orf-23 exerts its direct effect on CA biosynthesis by positively regulating ClaR expression [8]. γ-butyrolactones are also involved in regulation of CA biosynthesis in *S. clavuligerus* at different levels. Although no butyrolactone of A-factor type of *S. griseus* has been reported in *S. clavuligerus* [16,17], Brp is a butyrolactone receptor protein in this organism which is homologous to *S. griseus* ArpA and involved in negative regulation of antibiotic biosynthesis. It exerts its effect by binding to ARE boxes found in the *ccaR* and *adpA* promoters as well as its own promoter [11]. Binding of Brp to *ccaR* ARE box sequences has been demonstrated, and *brp*-deleted mutants overproduced CA significantly. AreB might affect *brp* expression which is downregulated during the first 36 h of cultivation and this decrease of Brp has been reported to result in a higher clavulanic acid production [17]. In an *areB*-deleted mutant, $ARE_{(ccaR)}$Brp complex can not be formed, leading to a general increase in CA levels [18] (Fig. 3).

Strain improvement strategies are commonly employed to achieve high-titers of industrial metabolites, including β-lactams [19,20]. For instance, an industrial *S. clavuligerus* strain with a 100-fold higher CA production capacity in comparison to its wild type counterpart was generated by random mutagenesis and screening [21]. Indeed, the integration of the tools of "classical" and "modern" approaches enables the researchers to stack multiple complex phenotypes [22]. For CA overproduction, the genetic and metabolic engineering approaches involving altering expression levels of biosynthetic or regulatory genes, increasing precursor flow into the pathway or eliminating competing reactions by oriented modifications have been applied mostly to the laboratory strains of *S. clavuligerus*, as summarized in Table S1 [11–13,23–29]. Because standard strains are able to produce only limited amounts of secondary metabolites, application of knowledge-based gene manipulations in industrial strains derived from random mutagenesis and selection might provide more productive strains [16,30]. Despite the fact that classical methodology is slow and laborious, its long history of success still fascinates researchers, especially with the availability of high throughput screening and analytical technologies today in the post-"omics" era [22]. Regarding "omics" of β-lactam overproducers, a comparison among the cytosolic proteomes of the wild-type *Penicillium chrysogenum* NRRL 1951, Wisconsin 54- 1255 (an improved, moderate penicillin producer), and AS-P-78 (a penicillin high producer) strains [31] has been reported. Also genome-wide gene expression in an industrial clavulanic acid overproducing *Streptomyces clavuligerus* [21] was already described. *S. clavuligerus* DEPA used for CA manufacturing process in Turkey produces at least 100-fold more CA relative to the wild type *S. clavuligerus*. In the present study, with the aim of providing insight into the modifications that this strain has undergone during the iterative cycles of mutagenesis program, *S. clavuligerus* NRRL3585 and DEPA strains were analyzed by comparative proteomics based on 2DE followed by protein identification via MALDI-TOF/MS.

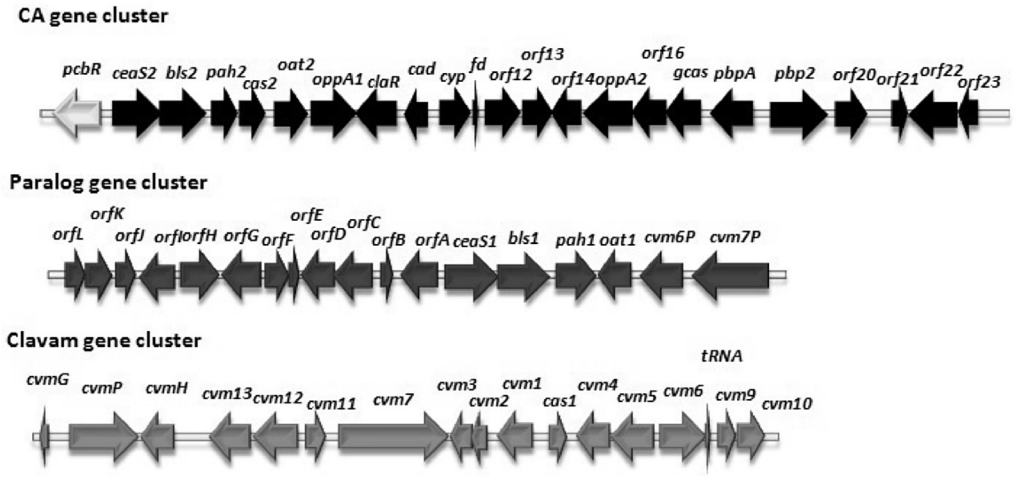

Fig. 1. Three distinct clusters involved in biosynthesis of CA and 5S clavams in *S. clavuligerus* (adapted from Hamed et al. [85]).