

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

Eser Ünsaldı^a, Aslıhan Kurt-Kızıldoğan^{a, b}, Birgit Voigt^c, Dörte Becher^c,
Gülay Özcengiz^{a, *}

^a Department of Biological Sciences, Middle East Technical University, 06800, Ankara, Turkey

^b Department of Agricultural Biotechnology, Ondokuz Mayıs University, 55139, Samsun, Turkey

^c Institute of Microbiology, Ernst-Moritz-Arndt-University of Greifswald, D-17487, Greifswald, Germany

ARTICLE INFO

Article history:

Received 3 August 2016

Received in revised form

17 October 2016

Accepted 23 October 2016

"With our special gratitude to Army for always being there with his endless pursuit of scientific research and support to his Army"

Keywords:

Clavulanic acid

Streptomyces clavuligerus

Industrial strain

Proteomics

2DE

MALDI-TOF/MS

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 successive 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 phosphoglycerate 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*.

© 2016 The Authors. Production and hosting by Elsevier B.V. on behalf of KeAi Communications Co. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

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 amoxicillin 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

* Corresponding author.

E-mail address: ozcengiz@metu.edu.tr (G. Özcengiz).

Peer review under responsibility of KeAi Communications Co., Ltd.

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 down-regulated 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.

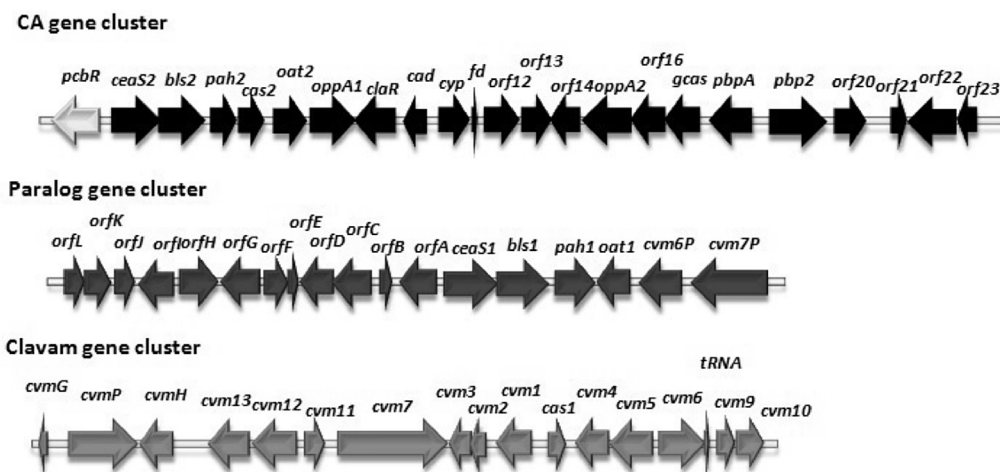


Fig. 1. Three distinct clusters involved in biosynthesis of CA and 5S clavams in *S. clavuligerus* (adapted from Hamed et al. [85]).

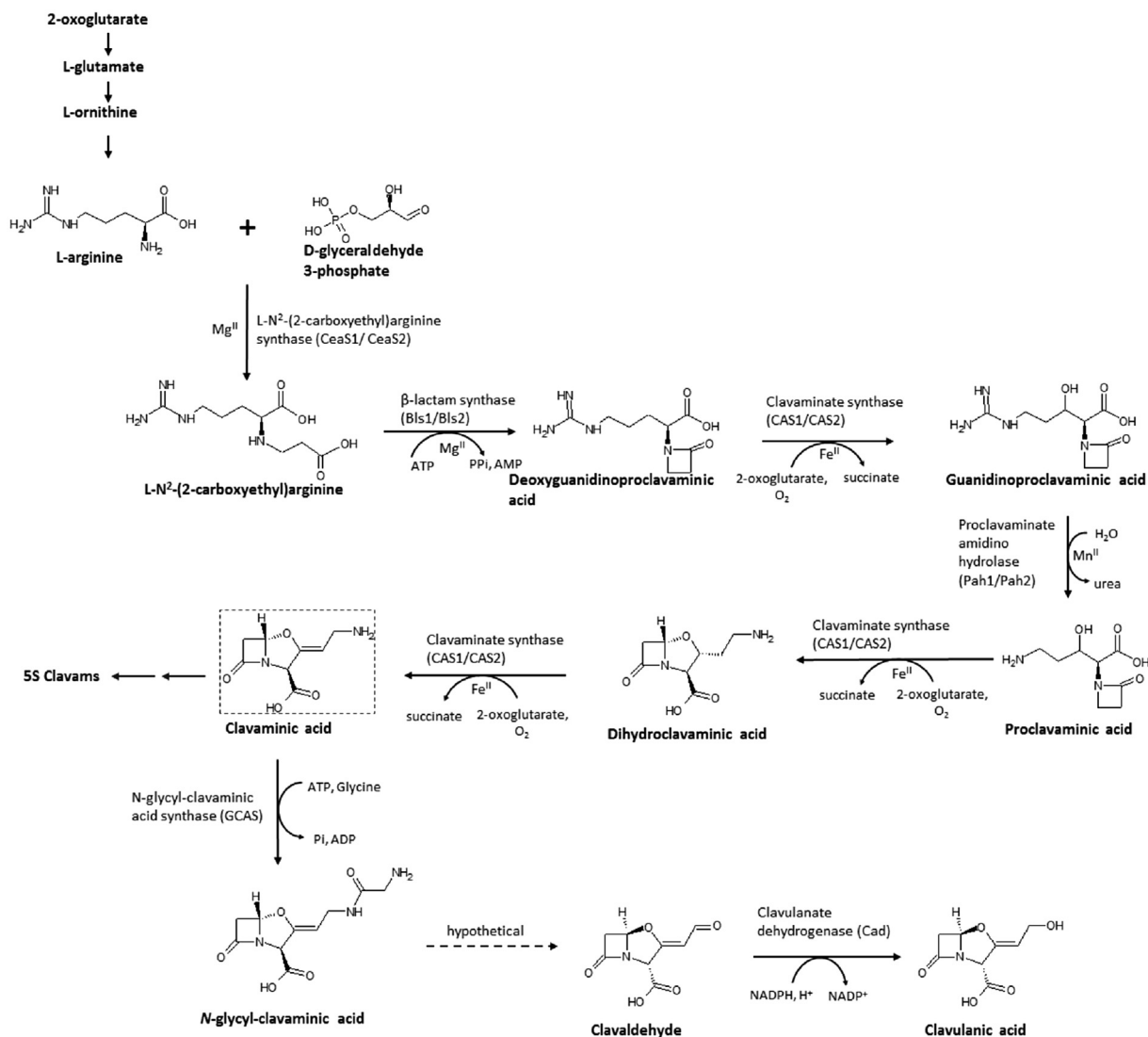


Fig. 2. Pathway leading to the biosynthesis of CA in *S. clavuligerus*. Clavaminic acid represents the branch point intermediate that separates CA biosynthesis from that of 5S clavams. Cofactors as well as co-substrates are also shown. Dashed arrow indicates a yet uncharacterized step in the pathway (adapted from Arulanantham et al. [86]; Hamed et al. [85] and Özcengiz and Demain [20]).

2. Materials and methods

2.1. Bacterial strains and culture conditions

S. clavuligerus strains NRRL3585 [32] and DEPA (DEPA Pharmaceuticals Co., İzmit, Turkey) were used as the wild type and industrial CA overproducer, respectively. The strains were grown at 28 °C at 220 rpm in baffled flasks in Tryptic Soya Broth (TSB) for stock preparation.

For the preparation of seed cultures, 200–600 μL of *S. clavuligerus* stock cultures were added into 50 mL of TSB medium and incubated for 24–48 h at 28 °C at 220 rpm. Optical density of seed cultures was measured according to the procedure by Malmberg et al. [33]. 0.5 mL of sample from seed culture was mixed with 3 mL of distilled water and 0.5 mL of 2.5 M HCl and then the mixture was homogenized via sonication (Ultrasonic Processor, Cole Parmer) for 3 × 30 s at 50% amplitude. When the OD₆₀₀ of homogenized mixture reached to 0.7–0.8, 25 mL of seed culture was centrifuged at 4000g for 10 min at 4 °C and the pellet was washed with fresh medium. The cells were re-suspended in 100 mL

of Starch-Asparagine (SA) medium, a defined medium favouring CA production [34] and the cultures were grown at 28 °C at 220 rpm for 48 h for proteome analyses.

2.2. Protein extraction

Protein extraction was slightly modified from Faurobert et al. [35]. Cell cultures harvested after 48 h were centrifuged at 6000g for 15 min and washed with fresh medium once. Immediately after that, the pellets were frozen in liquid nitrogen and stored at –80 °C until use. Frozen cultures were ground in liquid nitrogen in mortar and 1 g of ground cell culture was suspended in 3 mL of protein extraction buffer [500 mM Tris-HCl, 50 mM EDTA, 700 mM sucrose, 100 mM KCl, pH 8.0, freshly added 2% β-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride (PMSF)] in a 15 mL Falcon tube, vortexed gently and then incubated by shaking for 10 min on ice. An equal volume of Tris-buffered phenol (pH 8.0, Sigma) was added, the solution was mixed gently and incubated by shaking at RT for 10 min. After centrifugation at 3400g for 15–20 min, the phenolic phase was recovered and transferred to another tube. The phenolic

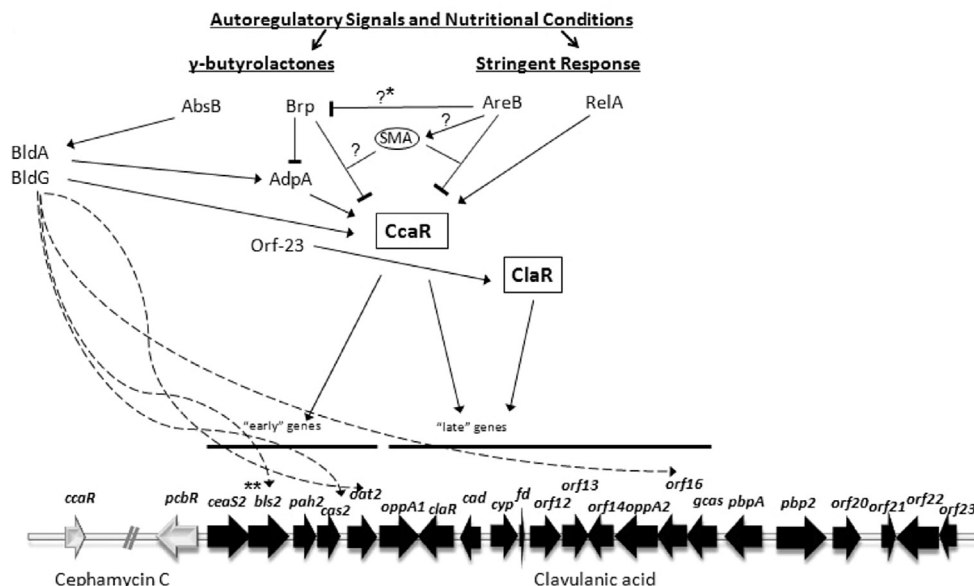


Fig. 3. Regulation of CA biosynthesis in *S. clavuligerus* NRRL3585 as based on previous reports cited in the text. Lines with pointed arrows show positive regulation while lines with perpendicular ends indicate suppression. Arrows with dashed lines point to the specific genes, the expression of which are dependent on BldA and BldG. *: first 36 h of cultivation, **: dependent only on BldG, SMA: Small Molecule Autoregulator yet unidentified.

phase was back extracted by adding 3 mL of protein extraction buffer and the mixture was shaken for 3 min at RT and vortexed. After centrifugation for 15–20 min at 3400g, the phenolic phase was recovered. 4 volume of precipitation solution (0.1 M ammonium acetate in cold acetone) was added to it, the solution was mixed and the proteins were precipitated overnight at -20°C . This was followed by centrifugation for 10 min at 3400g, washing three times with cold precipitation solution then with cold acetone. During each washing step, samples were incubated at -20°C for 30 min and then centrifuged for 10–15 min at 3400g. Pellet was dried either overnight at -20°C or under Speed-Vac.

2.3. Determination of protein concentration

Protein pellets were dissolved in rehydration buffer [36] and protein concentration was determined by using the modified Bradford assay as in Ramagli and Rodríguez [37] using bovine serum albumin as standard.

2.4. 2DE, image and data analyses

After samples were dissolved in rehydration buffer, linear IPG strips (18 cm, pH 4–7, BioRad) were rehydrated for 12–16 h by applying 400 μL of rehydration buffer containing 350 μg protein sample on BioRad PROTEAN IEF Cell. Isoelectric focusing (IEF) for the first dimension was performed with Immobilised pH Gradient (IPG) System “Ettan IPGphor3” (Amersham Biosciences, GE Healthcare). The voltage applied was as the following: 250 V (Gradient) for 1.5 h, 500 V (Gradient) for 1.5 h, 1000 V (Gradient) for 3 h, 5000 V (Gradient) for 4 h, 8000 V (Gradient) for 12 h, 500 V (Steep) for 2 h. After IEF, gels were either kept at -20°C or directly equilibrated at RT first with 4 mL of Equilibration Buffer Solution I (50 mM Tris-Cl pH 6.8, 6 M urea, 30% (v/v) glycerol, 1% SDS, 2% DTT) and secondly with 4 mL of Equilibration Buffer Solution II (50 mM Tris-Cl pH 6.8, 6 M urea, 30% (v/v) glycerol, 1% SDS, 2.5% iodoacetamide, 3.5 μM bromophenol blue) The isolated proteins were separated in 12% acrylamide/bis-acrylamide gels with a 4% stacking

gel using Bio-Rad Cell system (Bio-Rad, USA), applying approximately 27 mA per gel. To visualize the separated proteins, each gel was stained with colloidal Coomassie Blue G-250 [38]. Stained gels were digitized by using a scanner (Epson Perfection V750). The 2D image analysis software Delta2D version 3.4 (Decodon, Germany) was used for spot pattern analyses. Two biological replicates were used for each strain. Gels in each group (*S. clavuligerus* NRRL3585 and *S. clavuligerus* DEPA) were used to generate a fused (master) gel image which contained all the spots coming from each gel. Spot volume representing the spot abundance for each spot in the master images was used as a measure for the quantification of expression differences between the spots on the *S. clavuligerus* NRRL3585 and DEPA gels. Spot abundance on a gel was expressed as volume percentage (%V). The volume of all spots on an image amounts to 100% and hence the relative ratio of a spot to all spots on the gel can be described as its own %V. Image analysis software automatically normalizes %V of spots in each gel. By using the %V ratios for each spot on *S. clavuligerus* NRRL3585 and *S. clavuligerus* DEPA master gels, spot intensity showing 2.5-fold change in *S. clavuligerus* DEPA strain with respect to *S. clavuligerus* NRRL3585 was selected for identification by MALDI-TOF/MS analysis. After this, the statistical analyses for 2DE experiments were performed by using Delta2D 4.3 statistical tools to determine the statistical significance of the spots that showed 2.5-fold difference. For comparison of *S. clavuligerus* NRRL3585 and DEPA strains, paired *t*-test was used with Welch approximation and α value was equal to 0.05.

2.5. MALDI-TOF/MS analyses

Protein spots selected for identification were excised from 2DE gels with Proteome Works Spot Cutter System (Bio-Rad). In-gel trypsin digestion and extraction of the peptides were conducted by Ettan Spot Handling Workstation (GE Healthcare) as described in manufacturer’s protocol and by Kierul et al. [39]. To summarize the process, gels pieces were washed twice with 50 mM of NH_4HCO_3 containing 50% CH_3CN for 30 min and then washed once

with 75% CH₃CN for 10 min. Washed gel pieces were dried at 37 °C for 17 min. With the addition of 10 µl of trypsin solution (20 ng/µl trypsin) (Promega, Madison, WI, USA), gel pieces were incubated at 37 °C for 120 min. Extraction of the peptides was performed by covering them with 0.1% trifluoroacetic acid in 50% CH₃CN and then incubating at 40 °C for 30 min. Supernatants were transferred into another microtiter plate and extraction process was repeated. Supernatants were completely dried at 40 °C for 220 min. The peptides were dissolved in 2.0 µl matrix solution consisting of α -cyano-4-hydroxy cinnamic acid in 50% CH₃CN/0.5% trifluoroacetic acid. 0.7 µl of this mixture was spotted on the MALDI target. 4800 MALDI-TOF/TOF Proteomics Analyser (Applied Biosystems) was used for the peptide mass determination. Spectra were recorded in a mass range from 900 to 3700 Da with a focus mass of 2000 Da. 4000 Series Explorer software was used for spectrum calibration and analysis. TOF/TOF measurements were performed for the two highest peaks in a spectrum when possible.

2.6. Protein identification

For the database search, the Mascot search engine version 2.1 (Matrix Science) was used with *S. clavuligerus* sequence database retrieved from UniProt (<http://www.uniprot.org/>). The peak lists of each protein spot (peptide mass fingerprint and MS/MS data) obtained from MALDI TOF/MS measurement were analyzed with the aid of “Peptide Mass Fingerprint” and “MS/MS Ion Search” engines of MASCOT software (Matrix Science Inc., Boston, MA, USA) against the data retrieved from UniProt for *S. clavuligerus*. The results showing a probability score higher than 53 were assumed to be meaningful and used for protein identification.

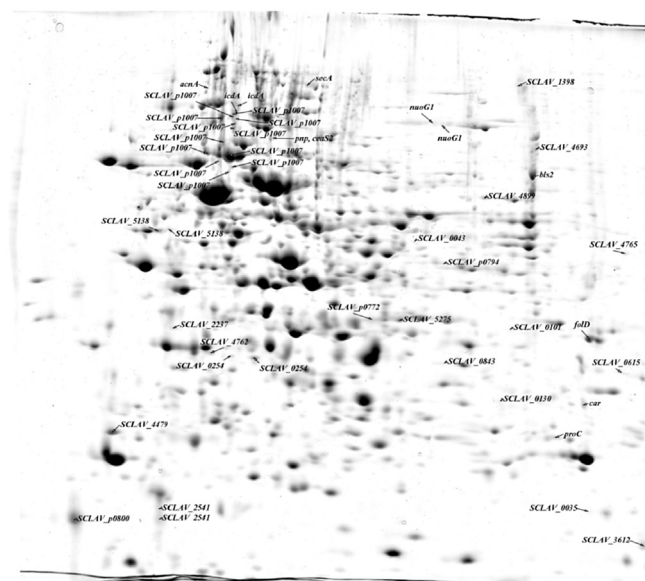
2.7. Databases for protein categorization, function and associations

Functional categories of the proteins were mostly determined by using COG database provided by NCBI (<ftp://ftp.ncbi.nih.gov/pub/COG/COG2014/static/lists/listStrbin.html>). Cellular localizations of the proteins were determined using PSORTb version 3.0.2 (<http://www.psort.org/psortb/>), Gpos-mPloc (<http://www.csbio.sjtu.edu.cn/bioinf/Gpos-multi/#>) and ngLoc (<http://genome.unmc.edu/ngLOC/index.html>) databases. Theoretical pI and Mw values of the proteins were calculated by using ExPASy pI/Mw tool [40]. Protein pathways and associations were predicted using STRING 10.0 (<http://string-db.org/>) which is a global source of genes from which protein associations can be inferred and predicted [41].

3. Results and discussion

The protein spots on 2DE gels of *S. clavuligerus* DEPA with at least 2.5 fold up- and down-regulation are shown in Figs. 4 and 5, respectively. Dual channel 2DE imaging (pl 4–7) of *S. clavuligerus* strains NRRL3585 and DEPA provides a picture of differential expression (Fig. S1). The proteins that could be identified corresponded to 33 distinct ORFs for overrepresented ones and 60 ORFs for underrepresented ones (supplementary Table S2 and Table S3, respectively). Six of the overrepresented and 10 of the underrepresented proteins appeared in two or more spots, differing in either their charge and/or mass, suggesting posttranslational modifications like phosphorylation, glycosylation and more delicate covalent modifications as well as intended proteolytic cleavage. On the other hand, the presence of multiple spots of the same protein on 2-D gels resulting from unintended protein degradation can not be ruled out, either.

The identified proteins were then functionally classified with respect to their biological functions. The relative functional distributions of up- and down-regulated proteins are presented in



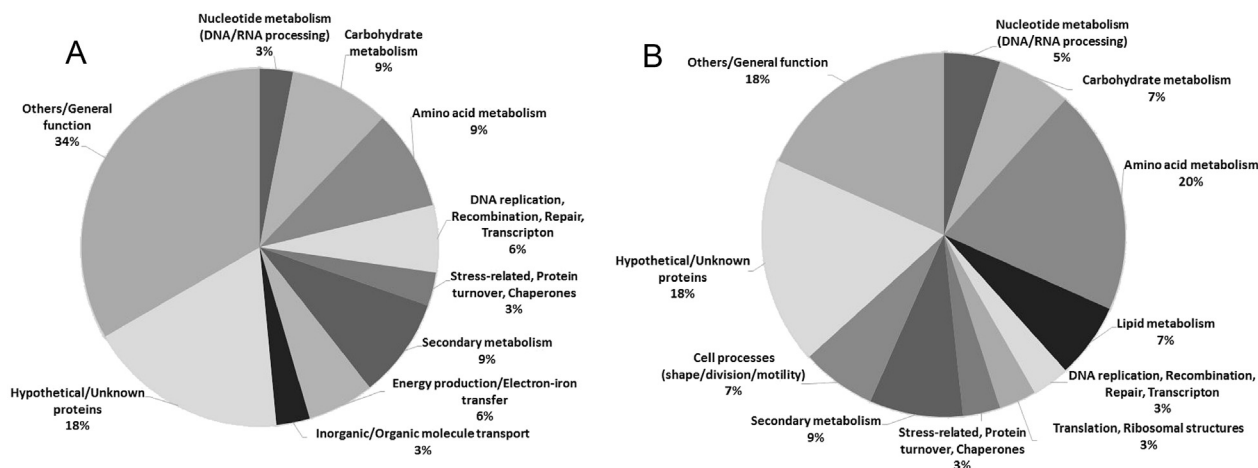


Fig. 6. Pie charts depicting the functional classification of overrepresented(A) and underrepresented(B) proteins in *S. clavuligerus* DEPA strain.

clavuldehyde dehydrogenase (Car) and carboxyethyl-arginine beta-lactam-synthase (Bls2) with 11, 8.2 and 20.6 fold increase, respectively. Both Cea2 and Bls2 are early biosynthetic enzymes that are required for both the CA and clavam biosynthesis. On the other hand, Car which catalyzes the last step of CA production is not involved in clavam biosynthesis. Interestingly, proclavaminate amidinohydrolase (Pah2) which is involved in early steps of CA biosynthesis as well as in clavam biosynthesis was 4.8 fold down-regulated. It is known that Pah is encoded by two paralogous genes: *pah1* and *pah2*. *pah2* and *pah1* mutants do not show considerable differences in the levels of CA compared to the parental strain [42]. It can then be speculated that the industrial strain might prefer Pah1 over Pah2 for CA production. Alternatively Pah may not be crucial, or yet unknown proteins might perform the same role in the pathway as Pah1/Pah2. As pathway specific activators, both CcaR and ClaR were expected to appear in the overrepresented protein list of *S. clavuligerus* DEPA; however, our proteome study did not identify these as differentially expressed proteins.

3.2. Other secondary metabolites

Underrepresented proteins of secondary metabolism other than CA biosynthesis included deacetoxycephalosporin C hydroxylase (7.6 fold) involved in cephamycin biosynthesis, MoeA5 protein (4.7 fold) involved in moenomycin biosynthesis, and a putative aminoglycoside 2-*N*-acetyltransferase Aac2 (22.7 fold). Aminoglycoside 2-*N*-acetyltransferases of AAC(2') family are generally engaged in the acetylation of aminoglycosides dibekacin, gentamicin, kanamycin, netilmicin, and tobramycin [43,44]. As the genes for self-resistance are generally known to be located in antibiotic biosynthetic gene clusters, the presence of a strongly downregulated aminoglycoside 2-*N*-acetyltransferase could be indicative of suppression of aminoglycoside biosynthesis in CA overproducer, yet no clusters encoding enzymes related to aminoglycoside biosynthesis were reported in the genome of *S. clavuligerus* [4]. It would be quite reasonable to postulate that the elimination of the production of other secondary metabolites is one of the mechanisms that *S. clavuligerus* DEPA exploits to increase CA biosynthesis, but the levels of these metabolites remain to be determined and compared with those in wild type strain.

3.3. Regulators of secondary metabolism

γ -butyrolactones are small extracellular autoregulators

interpreted as “microbial hormones” in streptomycetes having crucial roles in the secondary metabolism and morphological differentiation [45,46]. (SCLAV_0471) was 2.6 fold underrepresented in *S. clavuligerus* DEPA. *avaA2* codes for an Afs-like autoregulator synthase in *S. clavuligerus*. It has 31% amino acid identity to AfsA which is responsible for synthesis of classical autoregulatory γ -butyrolactone (A-factor) in *S. griseus* [47]. When A-factor is absent in cells, A-factor receptor protein, ArpA binds to *adpA* promoter and represses its transcription. However, when A-factor level exceeds a threshold value, it binds to ArpA and causes its dissociation from *adpA* promoter. As a result, AdpA can function as transcriptional activator of antibiotic biosynthesis [48]. No butyrolactones of A-factor type of *S. griseus* have been described in *S. clavuligerus* yet, and their role, or the role of AvaA2, remains to be studied.

From the 72 distinct putative TCS located in *S. clavuligerus* genome, only SCLAV_2102 was identified as 4.5 fold down-regulated in the DEPA strain, it might negatively regulate the formation of CA or CA precursors; however, the TCS *orf22/23* described as positive regulator of CA formation [49] was not affected in the industrial strain. TCSs, as AbrA1/A2 or AbrC1/C2/C3 have been described to control antibiotic biosynthesis in *S. coelicolor* [50] and TCS inactivation has been shown to stimulate the biosynthesis of gougerotin in *S. graminearum* [51], avermectin in *S. avermitilis* [52] and erythromycin in *Saccharopolyspora erythraea* [53], which supports a role for SCLAV_2102 in CA regulation.

3.4. Carbohydrate metabolism

In a previous study in which genome-wide gene expression changes in an industrial clavulanic acid overproduction strain of *S. clavuligerus* were investigated [21], the levels of glycolytic enzymes (2,3-bisphosphoglycerate-dependent phosphoglycerate mutase and phosphoglycerate kinase) in between glyceraldehyde-3-phosphate and phosphoenolpyruvate were found unchanged in their industrial strain. However, as found in our proteomics study, both enzymes were downregulated by 4.7 and 4.9 folds, respectively. Also found in our study was upregulation of aconitate hydratase by 4.6 fold in *S. clavuligerus* DEPA strain. This finding was not consistent with that of the microarray analysis [21]. In that study, the levels of two consecutive enzymes of TCA cycle, namely citrate synthase and aconitase, decreased over two fold in the industrial mutant by a possible reduction in carbon flux from G3P in this direction since the flux is partly redirected to clavulanic acid biosynthesis, as explained by the authors. On the other hand, there

was an incomplete downregulation of the flux with upregulated transcripts of pyruvate kinase, citrate synthase and *cis*-aconitate, and thus a considerable pool of acetyl-CoA is maintained, e.g. for the biosynthesis of ornithine from glutamate. We found dihydrolipoyl dehydrogenase (E3), one of the three components of the bacterial pyruvate dehydrogenase multienzyme complex, and isocitrate dehydrogenase, with two isoforms, as significantly over-represented proteins; however, the former was over two fold underrepresented and the level of the latter was unchanged in microarrays [21]. Notably, an additional protein spot possibly representing a different isoform of isocitrate dehydrogenase was 3 fold downregulated. Since these enzymes are related to the formation of metabolites which are used for a large variety of biosynthetic processes and include α -ketoglutarate family of amino acids with arginine as a precursor of CA, their elevated levels in a CA over-producer were as expected. Indeed, the differences between transcriptome and proteome data described herein are not all that surprising and systems-level analysis of metabolic pathways by the integration of genome, transcriptome, proteome, metabolome and fluxome data with *in silico* modelling and simulation will provide in-depth understanding of the whole cellular physiology in relation to CA production.

3.5. Amino acid metabolism

Another prominent mechanism of success of *S. clavuligerus* DEPA strain as a prolific producer of CA was demonstrated as downregulation of amino acid metabolism, methionine biosynthesis in particular. In this way, more aspartate would be directed towards L-arginine (CA precursor) biosynthesis instead of being also shared by methionine biosynthesis route. Cystathionine gamma-synthase (SCLAV_5668), as represented by 5 different spots on the *S. clavuligerus* DEPA gel forming a pearl-like pattern suggested an extensive posttranslational modification and these spots were at 3.5–83 fold decreased levels and most probably, along with other downregulated enzymes of methionine biosynthesis, served for lowering the metabolic flux to methionine. O-acetylhomoserine aminocarboxypropyltransferases which are historically known as O-acetylhomoserine thiolases (MetY) can accept methanethiol as the attacking nucleophile, resulting in the direct synthesis of methionine instead of a homocysteine intermediate [54]. Over 6 fold underrepresented levels of O-acetylhomoserine aminocarboxypropyltransferase (SCLAV_5559) identified in this study also provided evidence that streptomycetes utilize this single step conversion in addition to two step *trans*-sulfuration. The S-methylation of homocysteine is the final step in methionine biosynthesis. Either one of two non-homologous enzymes, namely cobalamin-dependent methionine synthase (MetH); or cobalamin-independent methionine synthase, also known as 5-methyltetrahydropteroyltriglutamate homocysteine methyltransferase (MetE) is theoretically employed for this reaction in *E. coli*. However since this organism has lost cobalamin biosynthetic pathway, it uses MetH only in the presence of exogenous cobalamin; moreover, it represses MetE in such conditions [54]. MetE catalyses direct transfer of a methyl group from the triglutamate derivative of 5-methyl-tetrahydrofolate to homocysteine [55]. In our study, MetE was represented by two isoforms, at up to 3 fold downregulated levels. Another cobalamin-independent enzyme also known as methionine synthase II (SCLAV_p1324) was also downregulated by 4 fold. While the primary function of S-adenosylmethionine synthetase (SAM; MetK) is to donate methyl groups to diverse metabolites coming from primary or secondary metabolism as well as proteins, nucleic acids and polysaccharides [56], it has also been shown to affect the secondary metabolism and

morphological differentiation in *Streptomyces* [57]. The introduction of multicopy *metK* genes as well as exogenous addition of SAM to *S. coelicolor* cells increased the production of actinorhodin [58]. Increased levels of SAM were also shown to increase the production of bicozamycin in *Streptomyces griseoflavus*, pristinamycin in *Streptomyces pristinaespiralis*, granaticin in *Streptomyces violaceoruber* [56]. In addition, SAM binds mRNA molecules to regulate the transcription in *B. subtilis* [57]. Kim et al. [59] showed that SAM itself can act on a transcriptional activator to increase the antibiotic levels in *Streptomyces lividans*. A decrease in the biosynthesis of secondary metabolites apart from CA in our industrial strain would be quite favorable for CA overproduction. Thus, it is very likely that a 2.6 fold reduction in the level of SAM synthetase together with a strongly downregulated levels of methionine biosynthesis contributed to the increased CA levels in *S. clavuligerus* DEPA.

Two other underrepresented enzymes of amino acid metabolism were 3-dehydroquinate synthase (AroB) and 3-phosphoshikimate 1-carboxyvinyltransferase (also known as 5-enolpyruvylshikimate-3-phosphate synthase; AroA) of shikimate pathway leading to aromatic amino acids and other aromatics. *Streptomyces* uses this pathway also to produce precursors for some secondary metabolites such as aromatic polyketide antibiotics. For instance, cyclohexanecarboxylic acid, a derivative of shikimic acid is used for the biosynthesis of ansatrienin (mycotrienin) in *Streptomyces collinus* [60], and another derivative of shikimic acid, dihydroxycyclohexanecarboxylic acid, is used for ascomycin (immunomycin; FK520) production in *Streptomyces hygroscopicus* var *ascomyceticus* [61].

3.6. Lipid metabolism

Two underrepresented proteins in lipid metabolism, 3-oxoacyl-[acyl-carrier-protein] reductase (SCLAV_1028) and enoyl-[acyl-carrier-protein] reductase (FabI), are the components of FAS II fatty acid biosynthetic system found in bacteria [62]. The questions of whether or not (i) Fas II and PKS pathways share protein components, and (ii) Fas II system plays an indirect role in providing building blocks for synthesis of PKS have been tried to be addressed almost exclusively in *Streptomyces* spp. due to the remarkable diversity of type II polyketides in these bacteria as well as the analogy between their PKSs and Fas II enzymes composed of discrete monofunctional proteins [63]. There still exist some contradictory findings. On the other hand, the possibility that downregulation of these two Fas II enzymes might limit PK synthesis either directly or indirectly in industrial CA producer cannot be ruled out.

3.7. Stress response

Tolerance to environmental stress is one of the desirable traits of industrial strains. The overrepresented relevant proteins included polynucleotide phosphorylase/polyadenylase (PNPase), ATP-dependent DNA helicase (SCLAV_4693), two isoforms of an anti-sigma factor (SCLAV_2541) and thioredoxin reductase (SCLAV_5275) with 11, 1344, 7.3, 3.3 and 3.3 fold upregulation, respectively. Being a component of RNA degradosome [64], PNPase seems to be a critical component of the cell under oxidative stress conditions. When *E. coli pnp* mutants were subjected to H₂O₂, the cells accumulated 8-oxoG in cellular RNA; however, when the cells were complemented with *pnp* gene, the amount of oxidized RNAs were minimized [65]. According to the UniProtKB database, ATP-dependent DNA helicase is a RecQ type ATP-dependent DNA helicase family responsible for genome maintenance [66]. Anti- σ factors bind to the related alternative σ factor, thereby preventing them from initiating the transcription of certain genes until the

required conditions are met [67]. Once reduced by thioredoxin reductase, thioredoxins have quite diverse functions within the cell, including cell division, detoxification/oxidative stress response, energy transduction, protein folding and degradation, transcription regulation, translation and some unknown functions [68]. COG database classifies thioredoxin reductase under the functional category of “Stress-Related, Protein Turnover, Chaperones”. However, thioredoxin reductase (SCLAV_5275) protein in this study has been identified as a thioredoxin oxidoreductase-like dithiol oxidase, namely HlmI, from the dithiopyrrolone antibiotic holomycin producer *S. clavuligerus*, which constructs intramolecular disulfide bridge from the acyclic ene-dithiol at a late stage in the holomycin biosynthetic pathway [69]. Indeed, it was shown much earlier that mutants of *S. clavuligerus* with ORF disruptions in different genes for CA biosynthesis overproduce holomycin [70]. In accordance with this finding, Li and Walsh [69] demonstrated that Δ ORF15 *S. clavuligerus* produced more holomycin than the wild type and the level of holomycin produced by Δ hlmI and Δ hlmI/ Δ ORF15 strains decreased by 10^2 – 10^3 folds in comparison to that of wild type and the Δ ORF15 mutant. Moreover, hlmI deletions in wild type and Δ ORF15 *S. clavuligerus* strains rendered both strains more susceptible toward holomycin. This suggested that holomycin may also exist in the inactive disulfide form and become reduced in the cellular environment yielding the active form of the antibiotic, HlmI possibly acting as a protective catalyst against holomycin in the producer. The transcriptomic analysis of a *ccaR*- and *claR*-deleted mutants further verified the cross-regulation of CA and holomycin biosynthetic pathways in that all the holomycin biosynthesis genes including *hlmI* were overexpressed in these mutants [14,15]. In view of all these findings, the increase in HlmI level in *S. clavuligerus* DEPA seems to be related with its functions other than holomycin biosynthesis.

A putative uncharacterized protein (SCLAV_0035) and Rhs element Vgr protein (SCLAV_0043) upregulated 3–4 fold in *S. clavuligerus* DEPA strain might provide clue for the existence of a contact-dependent type 6 secretion (T6SS)-like system in *S. clavuligerus*. Indeed, this organism is known to possess three main types of general protein secretion systems, namely the Sec pathway, the Exs secretion system (T7SS) and the Tat pathway [71]. STRING search revealed phage tail region proteins and Vgr proteins as the functional partners of SCLAV_0035. Rhs elements are accessory repetitive sequences which are the major source for chromosomal rearrangements in laboratory cultures. *vgr* (Val-Gly dipeptide repetition) is located upstream of the core regions and found only in *RhsE* and *RhsG* elements in *E. coli* [72]. Although T6SS has been known to be confined to Proteobacteria and basically important in pathogenesis, it also functions in resisting predation, sensing stress, regulating bacteria-bacteria interactions, in particular helping in competition for a specific niche in different bacteria [73,74]. Siderophore-interacting family proteins are contained within the biosynthetic gene clusters for siderophores and are responsible to reduce iron-siderophore complexes for releasing iron, hence cellular incorporation [75]. Almost 5 fold upregulation of SIB (SCLAV_0843) was indicative of an increased need for siderophore function in the industrial strain especially when non-classical biological functions of siderophores like non-iron metal transport, toxic metal sequestration, protection from oxidative stress and molecular signaling [76] are also considered. Moreover, siderophores are listed among the small molecules of primary metabolism or core physiology controlling antibiotic production although the relevant mechanisms remain to be elucidated [47]. Another upregulation was recorded in the Ycel family (SCLAV_4479) which are involved in osmotic and acid stresses [77,78].

3.8. Others

It is worth noting the significant downregulation of cell division protein FtsZ, teichoic acid (TA) biosynthesis protein, UDP-N-acetylmuramoyl-tripeptide–D-alanyl–D-alanine ligase and alanine racemase domain protein in *S. clavuligerus* DEPA, which might point to a general slowdown in cell wall synthesis and division compared to the *S. clavuligerus* NRRL3585. Interestingly, as many as 83 times decreased levels of TA biosynthetic protein and localization of the *tagC* gene encoding this protein on pSCL4 were observed while this megaplasmid did not seem to encode any functions essential to primary metabolism, as reported earlier [4].

One of the most striking difference between the industrial overproducer and *S. clavuligerus* DEPA was recorded for pSCL4 encoded protein with a β -lactamase domain (SCLAV_p1007). The protein appeared in 12 upregulated isoforms, five of which were drastically overrepresented in *S. clavuligerus* DEPA strain. A total of 22 proteins with a predicted β -lactamase domain was detected on the chromosome or megaplasmid of the organism [4]. Although *S. clavuligerus* β -lactamases have not yet been experimentally characterized, posttranslational modifications like lysine carboxylation in Class D [79,80] as well as proteolytic modification of zinc-based or metallo β -lactamases in Class B which are not inhibited by clavulanic acid [81] were reported. Phylogenetically diverse β -lactamases that exist in *Streptomyces* spp. and their relation with self-resistance were extensively reviewed very recently [82] with no specific information about SCLAV_p1007 identified in this study.

4. Conclusion and future prospects

Random mutagenesis is very slow and tedious for industrial fermentation researchers and might cause unintended changes to the entire system, but targeted approaches which do not have such limitations can be used to further improve the current high production strains as guided by current omics technologies and other tools of systems biotechnology. Comparative proteomics is a powerful tool in industrial biotechnology since the information obtained by comparing two or more genetically different strains or the same strain grown in different nutritional/environmental conditions can successfully lead to design new strategies for strain improvement even when a limited number of protein spots could be identified. On the other hand, each x-ome alone is not sufficient since the levels of RNAs, proteins, metabolites and fluxes vary independently, but various regulatory circuits coordinate them in a highly orchestrated fashion [83]. Especially for industrially important secondary metabolites, an integrated combined omics for inspection of correlations among different x-omes is essential to better link the components of the primary and secondary metabolism, define novel targets at gene and pathway levels and design strategies for metabolic engineering of organisms for increased secondary metabolite titers [84]. With some potentially crucial changes in the levels of certain proteins of primary and secondary metabolism, the present work shed light at some degree on proteome-wide changes that could contribute to increased production of CA in industrial producer developed by successive mutagenesis programs. Other mechanisms of CA overproduction could be specifically induced when the mutant is grown in a complex industrial culture medium rather than in defined SA. Our comparative proteomic analyses by LC-MS/MS of industrial cultures are underway with the hope of providing more alternative targets to design novel overproducers.

Acknowledgements

We acknowledge the Scientific and Technical Research Council

of Turkey [TÜBİTAK-KBAG, Grant No: 114Z648] and Middle East Technical University Research Fund [BAP-07-02-2014-007-050] for partially supporting this study. We would like to thank Dr. Servet Özcan and Dr. Volkan Yıldırım for their valuable suggestions during the study.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.synbio.2016.10.003>.

References

- [1] Reading C, Cole M. Clavulanic acid: a beta-lactamase-inhibiting beta-lactam from *Streptomyces clavuligerus*. *Antimicrob Agents Chemother* 1977;11: 852–7.
- [2] Saudagar PS, Survase SA, Singhal RS. Clavulanic acid: a review. *Biotechnol Adv* 2008;26:335–51.
- [3] Bentley PH, Berry PD, Brooks G, Gilpin ML, Hunt E, Zomaya II. Total synthesis of (±)-clavulanic acid. *J Chem Soc Chem Commun* 1977:748–9.
- [4] Medema MH, Trefzer A, Kovalchuk A, Van den Berg M, Muller U, Heijne W, et al. The sequence of a 1.8-Mb bacterial linear plasmid reveals a rich evolutionary reservoir of secondary metabolic pathways. *Genome Biol Evol* 2010;2: 212–24.
- [5] Jensen SE, Elder KJ, Aidoo KA, Paradkar AS. Enzymes catalyzing the early steps of clavulanic acid biosynthesis are encoded by two sets of paralogous genes in *Streptomyces clavuligerus*. *Antimicrob Agents Chemother* 2000;44:720–6.
- [6] Li R, Khaleeli N, Townsend TA. Expansion of the clavulanic acid gene cluster: identification and in vivo functional analysis of three new genes required for biosynthesis of clavulanic acid by *Streptomyces clavuligerus*. *J Bacteriol* 2000;182(14):4087–95.
- [7] Mellado E, Lorenzana LM, Rodríguez-Saliz M, Diez B, Liras P, Barredo JL. The clavulanic acid biosynthetic cluster of *Streptomyces clavuligerus*: genetic organization of the region upstream of the *car* gene. *Microbiology* 2002;148(Pt): 1427–38.
- [8] Song JY, Kim ES, Kim DW, Jensen SE, Lee KJ. A gene located downstream of the clavulanic acid gene cluster in *Streptomyces clavuligerus* ATCC 27064 encodes a putative response regulator that affects clavulanic acid production. *J Ind Microbiol Biotechnol* 2009;36:301–11.
- [9] Marsh EN, Chang MDT, Townsend CA. Two isozymes of clavamate synthase central to clavulanic acid formation: cloning and sequencing of both genes from *Streptomyces clavuligerus*. *Biochemistry* 1992;31(50):12648–57.
- [10] Ferguson NL, Peña-Castillo L, Moore MA, Bignell DRD, Tahlan K. Proteomics analysis of global regulatory cascades involved in clavulanic acid production and morphological development in *Streptomyces clavuligerus*. *J Ind Microbiol Biotechnol* 2016;43:537–55. <http://dx.doi.org/10.1007/s10295-016-1733-y>.
- [11] López-García MT, Santamarta I, Liras P. Morphological differentiation and clavulanic acid formation are affected in a *Streptomyces clavuligerus* *adpA* deleted mutant. *Microbiology* 2010;156:2354–65.
- [12] Pérez-Llarena FJ, Liras P, Rodríguez-García A, Martín JF. A regulatory gene (*ccaR*) required for cephamycin and clavulanic acid production in *Streptomyces clavuligerus*: amplification results in overproduction of both β -lactam compounds. *J Bacteriol* 1997;179:2053–9.
- [13] Pérez-Redondo R, Rodríguez-García A, Martín JF, Liras P. The *claR* gene of *Streptomyces clavuligerus*, encoding a LysR-type regulatory protein controlling clavulanic acid biosynthesis, is linked to the clavulanate-9-aldehyde reductase (*car*) gene. *Gene* 1998;211:311–21.
- [14] Álvarez-Álvarez R, Rodríguez-García A, Santamarta I, Pérez-Redondo R, Prieto-Domínguez A, Martínez-Burgo Y, et al. Transcriptomic analysis of *Streptomyces clavuligerus* Δ *ccaR::tsr*: effects of the cephamycin C-clavulanic acid cluster regulator CcaR on global regulation. *Microbiol Biotechnol* 2014;7(3):221–31. <http://dx.doi.org/10.1111/1751-7915.12109>.
- [15] Martínez-Burgo Y, Álvarez-Álvarez R, Rodríguez-García R, Liras P. The pathway specific regulator ClaR of *Streptomyces clavuligerus* has a global effect on the expression of genes for secondary metabolism and differentiation. *Appl Environ Microbiol* 2015;81(19):6637–48. <http://dx.doi.org/10.1128/AEM.00916-15>.
- [16] Paradkar A. Clavulanic acid production by *Streptomyces clavuligerus*: biogenesis, regulation and strain improvement. *J Antibiot* 2013;66(7):411–20.
- [17] Santamarta I, Pérez-Redondo R, Lorenzana LM, Martín JF, Liras P. Different proteins bind to the butyrolactone receptor protein ARE sequence located upstream of the regulatory *ccaR* gene of *Streptomyces clavuligerus*. *Mol Microbiol* 2005;56(3):824–35.
- [18] Santamarta I, López-García MT, Pérez-Redondo R, Koekman B, Martín JF, Liras P. Connecting primary and secondary metabolism: AreB, an IclR-like protein, binds the ARE *ccaR* sequence of *S. clavuligerus* and modulates leucine biosynthesis and cephamycin C and clavulanic acid production. *Mol Microbiol* 2007;66(2):511–4.
- [19] Adrio JL, Demain AL. Genetic improvement of processes yielding microbial products. *FEMS Microbiol Rev* 2006;30:187–214.
- [20] Özcengiz G, Demain AL. Recent advances in the biosynthesis of penicillins, cephalosporins and clavams and its regulation. *Biotechnol Adv* 2013;31: 287–311.
- [21] Medema MH, Alam MT, Heijne WHM, van den Berg MA, Muller U, Trefzer A, et al. Genome-wide gene expression changes in an industrial clavulanic acid overproduction strain of *Streptomyces clavuligerus*. *Microb Biotechnol* 2011;4(2):300–5.
- [22] Patnaik R. Engineering complex phenotypes in industrial strains. *Biotechnol Prog* 2008;24:38–47.
- [23] Paradkar AS, Mosher RH, Anders C, Griffin A, Griffin J, Hughes C, et al. Applications of gene replacement technology to *Streptomyces clavuligerus* strain development for clavulanic acid production. *Appl Environ Microbiol* 2001;67(5):2292–7.
- [24] Li R, Townsend CA. Rational strain improvement for enhanced clavulanic acid production by genetic engineering of the glycolytic pathway in *Streptomyces clavuligerus*. *Metab Eng* 2006;8(3):240–52.
- [25] Hung TV, Malla S, Park BC, Liou K, Lee HC, Sohng JK. Enhancement of clavulanic acid by replicative and integrative expression of *ccaR* and *cas2* in *Streptomyces clavuligerus* NRRL3585. *J Microbiol Biotechnol* 2007;17(9): 1538–45.
- [26] Jnawali NH, Lee HC, Sohng JK. Enhancement of clavulanic acid production by expressing regulatory genes in *gap* gene deletion mutant of *Streptomyces clavuligerus* NRRL3585. *J Microbiol* 2010a;20(1):146–52.
- [27] Jnawali NH, Yoo JC, Sohng JK. Improvement of clavulanic acid production in *Streptomyces clavuligerus* by genetic manipulation of structural biosynthesis genes. *Biotechnol Lett* 2010b;33:1221–6.
- [28] Jnawali HN, Liou K, Sohng JK. Role of σ -factor (*orf21*) in clavulanic acid production in *Streptomyces clavuligerus* NRRL3585. *Microbiol Res* 2011;166: 369–79.
- [29] Guo DK, Zhao YB, Yang KQ. Coordination of glycerol utilization and clavulanic acid biosynthesis to improve clavulanic acid production in *Streptomyces clavuligerus*. *Sci China Life Sci* 2013;56(7):591–600. <http://dx.doi.org/10.1007/s11427-013-4507-z>.
- [30] Nielsen J, Eliasson A, Borodina I. Metabolic engineering of *Streptomyces*. In: Smolke CD, editor. The metabolic pathway engineering handbook: fundamentals, vol. 24. CRC Press; 2009. p. 1–30.
- [31] Jami MS, Barreiro C, García-Estrada C, Martín JF. Proteome analysis of the penicillin producer *Penicillium chrysogenum*: characterization of protein changes during the industrial strain improvement. *Mol Cell Proteom* 2010;9(6):1182–98. <http://dx.doi.org/10.1074/mcp.M900327-MCP200>.
- [32] Higgins CE, Kastner RE. *Streptomyces clavuligerus* sp. nov., a beta-lactam antibiotic producer. *Int J Syst Bacteriol* 1971;21(4):326–31. <http://dx.doi.org/10.1099/00207713-21-4-326>.
- [33] Malmberg LH, Hu WS, Sherman DH. Precursor flux control through targeted chromosomal insertion of the lysine epsilon-aminotransferase (*lat*) gene in cephamycin C biosynthesis. *J Bacteriol* 1993;175(21):6916–24.
- [34] Aharonowitz Y, Demain AL. Nitrogen nutrition and regulation of cephalosporin production in *Streptomyces clavuligerus*. *Can J Microbiol* 1979;25(1): 61–7.
- [35] Faurobert M, Pelpoir E, Chaïb J. Phenol extraction of proteins for proteomic studies of recalcitrant plant tissues. *Methods Mol Biol* 2007;355:9–14.
- [36] Yin P, Wang YH, Zhang SL, Chu J, Zhuang YP, Wang ML, et al. Isolation of soluble proteins from an industrial strain *Streptomyces avermitilis* in complex culture medium for two-dimensional gel electrophoresis. *J Microbiol Meth* 2008;73(2):105–10.
- [37] Ramagli LS, Rodriguez LV. Quantitation of microgram amounts of protein in two-dimensional polyacrylamide gel electrophoresis sample buffer. *Electrophoresis* 1985;6(11):559–63.
- [38] Neuhoff V, Arold N, Taube D, Ehrhardt W. Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis* 1988;9(6):255–62.
- [39] Kierul K, Voigt B, Albrecht D, Chen XH, Carvalhais LC, Borriss R. Influence of root exudates on the extracellular proteome of the plant growth-promoting bacterium *Bacillus amyloliquefaciens* FZB42. *Microbiology* 2015;161(Pt1): 131–47.
- [40] Gasteiger E, Hoogland C, Gattiker A, Duvaud S, Wilkins MR, Appel RD, et al. Protein identification and analysis tools on the ExPASy Server. In: Walker JM, editor. The proteomics protocols handbook. Totowa, NJ: Humana Press Inc.; 2005. p. 571–607.
- [41] von Mering C, Huynen M, Jaeggi D, Schmidt S, Bork P, Snel B. String: a database of predicted functional associations between proteins. *Nucleic Acids Res* 2003;31(1):258–61.
- [42] Jensen SE, Wong A, Griffin A, Barton B. *Streptomyces clavuligerus* has a second copy of the proclavamate amidinohydrolase gene. *Antimicrob Agents Chemother* 2004;48(2):514–20.
- [43] Hegde SS, Javid-Majd F, Blanchard JS. Overexpression and mechanistic analysis of chromosomally encoded aminoglycoside 2'-N-Acetyltransferase (AAC (2')-Ic) from *Mycobacterium tuberculosis*. *J Biol Chem* 2001;276(49): 45876–81.
- [44] Vetting MW, Hegde SS, Javid-Majd F, Blanchard JS, Roderick SL. Aminoglycoside 2'-N-acetyltransferase from *Mycobacterium tuberculosis* in complex with coenzyme A and aminoglycoside substrates. *Nat Struct Mol Biol* 2002;9(9):653–8.
- [45] Liras P, Gomez-Escribano JP, Santamarta I. Regulatory mechanisms controlling antibiotic production in *Streptomyces clavuligerus*. *J Ind Microbiol Biotechnol*

- 2008;35:667–76.
- [46] Takano E. γ -Butyrolactones: *Streptomyces* signalling molecules regulating antibiotic production and differentiation. *Curr Opin Microbiol* 2006;9(3):287–94.
- [47] Niu G, Chater KF, Tian Y, Zhang J, Tan H. Specialised metabolites regulating antibiotic biosynthesis in *Streptomyces* spp. *FEMS Microbiol Rev* 2016;40(4):554–73.
- [48] Yamamoto S, He Y, Arakawa K, Kinashi H. γ -Butyrolactone-dependent expression of the *Streptomyces* antibiotic regulatory protein gene *srrY* plays a central role in the regulatory cascade leading to lankacidin and lankamycin Production in *Streptomyces rochei*. *J Bacteriol* 2008;190(4):1308–16.
- [49] Jnawali HN, Oh TJ, Liou K, Park BC, Sohng JK. A two-component regulatory system involved in clavulanic acid production. *J Antibiot* 2008;61(11):651–9. <http://dx.doi.org/10.1038/ja.2008.92>.
- [50] Yepes A, Rico S, Rodríguez-García A, Santamaría RI, Díaz M. Novel two-component systems implied in antibiotic production in *Streptomyces coelicolor*. *PLoS One* 2011;6(5):e19980. <http://dx.doi.org/10.1371/journal.pone.0019980>.
- [51] Wei J, Tian Y, Niu G, Tan H. GouR, a TetR family transcriptional regulator, coordinates the biosynthesis and export of gougerotin in *Streptomyces gramineus*. *Appl Environ Microbiol* 2014;80(2):714–22.
- [52] He F, Liu W, Sun D, Luo S, Chen Z, Wen Y, et al. Engineering of the TetR family transcriptional regulator SAV151 and its target genes increases avermectin production in *Streptomyces avermitilis*. *Appl Microbiol Biotechnol* 2014;98(1):399–409.
- [53] Wu H, Wang Y, Yuan L, Mao Y, Wang W, Zhu L, et al. Inactivation of SACE_3446, a TetR family transcriptional regulator, stimulates erythromycin production in *Saccharopolyspora erythraea*. *Syst Synth Biol* 2016;1(1):39–46.
- [54] Ferla MP, Patrick WM. Bacterial methionine biosynthesis. *Microbiology* 2014;160(8):1571–84.
- [55] Whitfield CD, Steers EJ, Weissbach H. Purification and properties of 5-methyltetrahydropteroyltrimethylglutamate-homocysteine transmethylase. *J Biol Chem* 1970;245(2):390–401.
- [56] Yoon GS, Ko KH, Kang HW, Suh JW, Kim YS, Ryu YW. Characterization of S-adenosylmethionine synthetase from *Streptomyces avermitilis* NRRL8165 and its effect on antibiotic production. *Enzyme Microb Technol* 2006;39(3):466–73.
- [57] Shin SK, Xu D, Kwon HJ, Suh JW. S-adenosylmethionine activates *adpA* transcription and promotes streptomycin biosynthesis in *Streptomyces griseus*. *FEMS Microbiol Lett* 2006;259(1):53–9.
- [58] Okamoto S, Lezhava A, Hosaka T, Okamoto-Hosoya Y, Ochi K. Enhanced expression of S-adenosylmethionine synthetase causes overproduction of actinorhodin in *Streptomyces coelicolor* A3(2). *J Bacteriol* 2003;185(2):601–9.
- [59] Kim DJ, Huh JH, Yang YY, Kang CM, Lee IH, Hyun CG, et al. Accumulation of S-adenosyl-L-methionine enhances production of actinorhodin but inhibits sporulation in *Streptomyces lividans* TK23. *J Bacteriol* 2003;185(2):592–600.
- [60] Wu TS, Duncan J, Tsao SW, Chang CJ, Keller PJ, Floss HG. Biosynthesis of the ansamycin antibiotic ansatrienin (mycotrienin) by *Streptomyces collinus*. *J Nat Prod* 1987;50(1):108–18.
- [61] Wilson DJ, Patton S, Florova G, Hale V, Reynolds KA. The shikimic acid pathway and polyketide biosynthesis. *J Ind Microbiol Biotechnol* 1998;20(5):299–303.
- [62] Zhang YM, Lu YJ, Rock CO. The reductase steps of the type II fatty acid synthase as antimicrobial targets. *Lipids* 2004;39(11):1055–60.
- [63] Cronan JE, Thomas J. Bacterial fatty acid synthesis and its relationships with polyketide synthetic pathways. *Methods Enzymol* 2009;459:395–433.
- [64] Bralley P, Jones GH. Organization and expression of the polynucleotide phosphorylase gene (*pnp*) of *Streptomyces*: processing of *pnp* transcripts in *Streptomyces antibioticus*. *J Bacteriol* 2004;186(10):3160–72.
- [65] Wu J, Jiang Z, Liu M, Gong X, Wu S, Burns CM, et al. Polynucleotide phosphorylase protects *Escherichia coli* against oxidative stress. *Biochemistry* 2009;48(9):2012–20.
- [66] Cobb JA, Bjergbaek L, Gasser SM. RecQ helicases: at the heart of genetic stability. *FEBS Lett* 2002;529(1):43–8.
- [67] Helmann JD. Anti-sigma factors. *Curr Opin Microbiol* 1999;2(2):135–41.
- [68] Kumar JK, Tabor S, Richardson CC. Proteomic analysis of thioredoxin-targeted proteins in *Escherichia coli*. *P Natl Acad Sci U. S. A* 2004;101(11):3759–64.
- [69] Li B, Walsh CT. *Streptomyces clavuligerus* HmlI is an intramolecular disulfide-forming dithiol oxidase in holomycin biosynthesis. *Biochemistry* 2011;50(21):4615–22.
- [70] de la Fuente A, Lorenzana LM, Martín JF, Liras P. Mutants of *Streptomyces clavuligerus* with disruptions in different genes for clavulanic acid biosynthesis produce large amounts of holomycin: possible cross-regulation of two unrelated secondary metabolic pathways. *J Bacteriol* 2002;184(23):6559–65.
- [71] Chater KF, Biró S, Lee KJ, Palmer T, Schrempf H. The complex extracellular biology of *Streptomyces*. *FEMS Microbiol Rev* 2010;34(2):171–98.
- [72] Hill CW. Large genomic sequence repetitions in bacteria: lessons from rRNA operons and Rhs elements. *Res Microbiol* 1999;150(9–10):665–74.
- [73] Schwarz S, Hood RD, Mougous JD. What is type VI secretion doing in all those bugs? *Trends Microbiol* 2010;18(12):531–7.
- [74] Costa TR, Felisberto-Rodrigues C, Meir A, Prevost MS, Redzej A, Trokter M, et al. Secretion systems in Gram-negative bacteria: structural and mechanistic insights. *Nat Rev Microbiol* 2015;13(6):343–59.
- [75] Li K, Chen WH, Bruner SD. Structure and mechanism of the siderophore-interacting protein from the fuscachelin gene cluster of *Thermobifida fusca*. *Biochemistry* 2015;54(25):3989–4000.
- [76] Johnstone TC, Nolan EM. Beyond iron: non-classical biological functions of bacterial siderophores. *Dalton Trans* 2015;44(14):6320–39.
- [77] Handa N, Terada T, Doi-Katayama Y, Hirota H, Tame JRH, Park SY, Kuramitsu S, et al. Crystal structure of a novel polyisoprenoid-binding protein from *Thermus thermophilus* HB8. *Protein Sci* 2005;14(4):1004–10.
- [78] Zammit CM, Mangold S, Jonna Vr, Mutch LA, Watling HR, Dopson M, et al. Bioleaching in brackish waters—effect of chloride ions on the acidophile population and proteomes of model species. *Appl Microbiol Biotechnol* 2012;93(1):319–29.
- [79] Li J, Cross JB, Vreven T, Meroueh SO, Mobashery S, Schlegel HB. Lysine carboxylation in proteins: OXA-10 β -lactamase. *Proteins* 2005;61(2):246–57.
- [80] Che T, Bethel CR, Pusztai-Carey M, Bonomo RA, Carey PR. The different inhibition mechanisms of OXA-1 and OXA-24 β -lactamases are determined by the stability of active site carboxylated lysine. *J Biol Chem* 2014;289(9):6152–64.
- [81] Koh TH, Yamaguchi K, Ishii Y. Characterisation of the metallo- β -lactamase VIM-6 and its genetic support. *Int J Antimicrob Agents* 2008;32(5):446–9.
- [82] Ogawara H. Self-resistance in *Streptomyces*, with special reference to β -lactam antibiotics. *Molecules* 2016;21(5). pii: E605.
- [83] Lee SY, Lee D-Y, Kim TY. Systems biotechnology for strain improvement. *Trends Biotechnol* 2005;23(7):349–58. <http://dx.doi.org/10.1016/j.tibtech.2005.05.003>.
- [84] Hwang KS, Kim HU, Charusanti P, Palsson BØ, Lee SY. Systems biology and biotechnology of *Streptomyces* species for the production of secondary metabolites. *Biotechnol Adv* 2014;32:255–68.
- [85] Hamed RB, Gomez-Castellanos J, Henry L, Ducho C, McDonough MA, Schofield CJ. The enzymes of β -lactam biosynthesis. *Nat Prod Rep* 2013;30:21–107. <http://dx.doi.org/10.1039/c2np20065a>.
- [86] Arulanantham H, Kershaw NJ, Hewitson KS, Hughes CE, Thirkettle JE, Schofield CJ. ORF17 from the clavulanic acid biosynthesis gene cluster catalyzes the ATP-dependent formation of N-glycyl-clavaminic acid. *J Biol Chem* 2006;281(1):279–87.