Brief report

Genome-wide gene expression changes in an industrial clavulanic acid overproduction strain of *Streptomyces clavuligerus*

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Summary

To increase production of the important pharmaceutical compound clavulanic acid, a β-lactamase inhibitor, both random mutagenesis approaches and rational engineering of *Streptomyces clavuligerus* strains have been extensively applied. Here, for the first time, we compared genome-wide gene expression of an industrial *S. clavuligerus* strain, obtained through iterative mutagenesis, with that of the wild-type strain. Intriguingly, we found that the majority of the changes contributed not to a complex rewiring of primary metabolism but consisted of a simple upregulation of various antibiotic biosynthesis gene clusters. A few additional transcriptional changes in primary metabolism at key points seem to divert metabolic fluxes to the biosynthetic precursors for clavulanic acid. In general, the observed changes largely coincide with genes that have been targeted by rational engineering in recent years, yet the presence of a number of pre-viously unexplored genes clearly demonstrates that functional genomic analysis can provide new leads for strain improvement in biotechnology.

Introduction

Streptomyces clavuligerus is an important industrial microorganism, which produces the β-lactam antibiotic cephamycin C (Martín and Liras, 1989) and the β-lactamase inhibitor clavulanic acid (Saudagar *et al.*, 2008). Clavulanic acid is produced worldwide on a large scale, and co-formulated with amoxicillin in Augmentin (Brogden *et al.*, 1981). Two biotechnological strain optimization approaches have been utilized to increase the production of clavulanic acid by the bacterium: rational metabolic engineering and iterative optimization through random mutagenesis and screening.

In the rational approach, a specific gene is knocked out or overexpressed to divert metabolic fluxes towards the antibiotic biosynthetic pathways. Arguably, the best example comes from the work of Li and Townsend (2006), who re-engineered the *S. clavuligerus* glycolytic pathway by constructing a deletion mutant of the glyceraldehyde 3-phosphate dehydrogenase gene *gap1* to increase the pool of the clavulanic acid precursor glycerol 3-phosphate (G3P). This doubled clavulanic acid production compared to the wild-type. Overexpression of the regulatory proteins CcaR and ClaR also led to clavulanic acid overproduction (Hung *et al.*, 2007), and the two strategies have recently been combined successfully in a single strain (Jnawali *et al.*, 2010).

Yet, most or even all production strains that are used in industry have been obtained by classical strain improvement (Adrio and Demain, 2006), based on mutagenesis with mutagens such as nitrosoguanidine (NTG). Little is known about the exact genetic changes through which high production titers are achieved in these mutants.

Recently, we published the genome sequence of *S. clavuligerus* ATCC 27064 (Medema *et al.*, 2010). We now employed this information to perform a genome-wide transcriptome study on an industrial production strain, which has been generated from the ATCC 27064 type strain
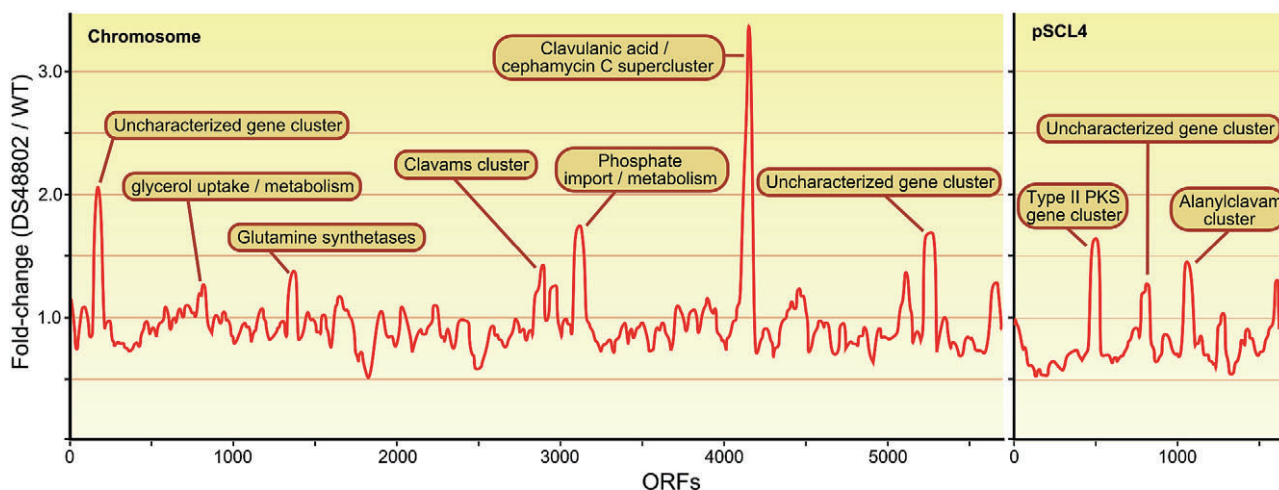
Fig. 1. Differential gene expression in *S. clavuligerus* DS48802 and ATCC 27064.
Sliding window plot (size = 50) of the difference in gene expression between *S. clavuligerus* DS48802 and wild-type ATCC 27064. Key upregulated operons or genes at the peaks are noted in the figure. See Table S2 for description of the ten gene clusters shown. For gene expression analysis, cultivations were performed in shake flasks directly inoculated with spore suspensions at 28°C and 280 r.p.m. The semi-synthetic growth medium used consisted of 30 g l^{-1} glycerol, 5 g l^{-1} wheat gluten, 3.5 g l^{-1} asparagine monohydrate, 1.5 g l^{-1} L-lysine, 0.7 g l^{-1} KH$_2$PO$_4$, 0.3 g l^{-1} MgSO$_4$·7H$_2$O, 0.2 g l^{-1} CaCl$_2$·2H$_2$O, 0.2 g l^{-1} FeSO$_4$·7H$_2$O, 10 g l^{-1} MOPS, 0.1 ml l^{-1} Basilodon and 1 ml l^{-1} trace elements solution at pH 7.0. The trace element solution consisted of 20.4 g l^{-1} H$_2$SO$_4$, 50 g l^{-1} citric acid H$_2$O, 16.75 g l^{-1} ZnSO$_4$·7H$_2$O, 1.6 g l^{-1} CuSO$_4$. 5 H$_2$O, 1.5 g l^{-1} MnCl$_2$·4H$_2$O, 2 g l^{-1} H$_3$BO$_3$ and 2 g l^{-1} Na$_2$MoO$_4$·2H$_2$O. After 70 h of cultivation, the cells were harvested by centrifugation, treated with RNAprotect (Qiagen) and directly frozen with liquid nitrogen and stored at −80°C. To isolate total RNA, the frozen mycelium was ground in a mortar, resuspended in TE buffer with 5 mg l^{-1} lysozyme and incubated for 5 min at room temperature. RNA isolation and purification were performed using phenol extraction (TRIzol reagent, Invitrogen) and RNeasy Kit (Qiagen). The RNA was quantified by measuring the absorbance at 260 nm. Biotinylated cDNA was prepared after fragmentation according to the standard Affymetrix protocol using GC rich (average 72%) primers from 10 µg total RNA. For hybridization, 5 µg and 7 µg biotinylated cDNA were used per Affymetrix gene Chip. Microarray data have been deposited at Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE24033.
Flux-balance analysis was performed using a recently published genome-scale metabolic model of *S. clavuligerus* (Medema *et al.*, 2010). In this study, we slightly changed our objective function and included both clavulanic acid and cephamycin C biosynthesis pathways. We dynamically changed the antibiotic concentration in the biomass composition based on experimental observations of Romero and colleagues (1984) and optimized the objective function for different concentrations of each antibiotic. Among the 785 genes that the model contains, 497 genes showed non-zero flux for at least one antibiotic concentration. We calculated Spearman correlation of fluxes of each reaction with increasing antibiotic concentrations. If an enzyme was involved in multiple reactions, we assigned the flux which had the highest r^2.

(Higgens and Kastner, 1971) by several iterations of mutagenesis and screening, and produces clavulanic acid at levels approximately 100× that of the wildtype. The majority of observed changes consist of increased transcript levels of the antibiotic biosynthesis gene clusters. These observations are in agreement with flux-balance analysis (FBA) predictions using a constraint-based genome-scale metabolic model constructed for *S. clavuligerus*. However, we also detected some potentially crucial transcript level changes in primary metabolism that could contribute to the increased production of clavulanic acid by redirection of fluxes, mimicking strategies utilized in rational approaches.

Results and discussion

Increased transcription of secondary metabolite biosynthesis gene clusters in strain DS48802

Comparing gene transcript levels of *S. clavuligerus* wild-type and DS48802 strains during stationary phase using microarrays, almost all genes ranking high in a differential transcriptome analysis belong to the complete clavulanic acid/cephamycin C supercluster, which is significantly overexpressed (between twofold and eightfold) in the DS48802 strain compared to the wild-type. Interestingly, the pathway-specific regulator genes *claR* and *ccaR* are also overexpressed in DS48802. They are located within the same supercluster and their products have been shown to regulate it positively (Alexander and Jensen, 1998; Paradkar *et al.*, 1998).

Additionally, the clavams gene cluster (*cvm1245* and *cas1*) and the 'paralogous' alanylclavam cluster (*orfABCD* and *ceaS1*/*pah1*/*bls1*/*oat1*) are significantly overexpressed (Fig. 1), as is the two-component system involving Cvm7p (SCLAV_p1079-p1080) that induces expression of the alanylclavam cluster (Tahlan *et al.*, 2007). This suggests the presence of a regulatory mechanism common to all these clusters. CcaR is an unlikely candidate for such a common regulatory factor, as it does not appear to control the paralogous cluster (Tahlan *et al.*, 2004); the pleiotropic regulator AdpA

(SCLAV_1957) is a more likely candidate, as it is known to induce clavulanic acid expression (Lopez-García *et al.*, 2010) and its gene is transcribed almost 2.5 times stronger in DS48802.

In contrast to, for example, the intrachromosomal amplification of the kanamycin biosynthesis gene cluster in *Streptomyces kanamyceticus* (Yanai *et al.*, 2006), hybridization of *S. clavuligerus* DS48802 genomic DNA to the microarrays revealed no amplifications of genes or gene clusters (data not shown). The overproduction that we observe therefore appears to be caused by transcriptional (and post-transcriptional) changes only.

Flux-balance analysis of increased clavulanic acid production correlates well with transcriptomic data

While many changes have occurred during the generation of the DS48802 strain through mutagenesis, it is important to note that changes in transcription levels of many genes may be due to random mutations that have no impact on antibiotic biosynthesis. In order to assess which changes could be causatively linked to antibiotic overproduction, we computationally predicted the metabolic fluxes during antibiotic overproduction, using a constraints-based genome-scale metabolic network model of *S. clavuligerus* (Medema *et al.*, 2010). We dynamically modelled the metabolic flux changes during increased production of clavulanic acid and cephamycin C with different rates of antibiotic production relative to the biomass, based on the experimental observations of Romero and colleagues (1984). Interestingly, the computational predictions made through dynamic FBA are largely in line with the observed expression changes. Eighty-seven genes were predicted to be upregulated (positively correlated with antibiotic production; $r > 0.6$) and 129 genes were predicted to be downregulated (negatively correlated; $r < -0.6$) at increasing antibiotic production levels. Forty per cent (15/37) of the genes that actually showed increased transcript levels (fold change > 2) were also predicted to do so according to FBA, and these include all genes encoding key biosynthetic enzymes known to be involved in clavulanic acid and cephamycin C biosynthesis. Even though 40% does not appear to be a very large percentage, these predictions are statistically very significant according to a Fisher's exact test ($P = 0.0005$; see Table S1). One should note that FBA predicts the flux for every reaction, not for every gene product, as multiple gene products can be involved in a single reaction. Therefore, in these cases the same flux was assigned to all gene products involved in that particular reaction, which is not necessarily the case in the actual gene expression, as only a single homologue or isoenzyme could be actively performing the reaction and thus would be differentially expressed. Out of the 47

different enzymatic reactions predicted to be upregulated (associated with 87 genes), 26 (55%) have at least one gene linked to them, which showed increased transcript levels. As both our FBA and gene transcript analysis pointed to an increased expression of the clavulanic acid and cephamycin core biosynthesis genes, we suggest that this is a crucial change required for antibiotic overproduction in this strain. Moreover, as the FBA indicated that the absolute fluxes required for high production of the secondary metabolites are minor compared to other fluxes involved in maintenance and cellular growth, a complete redirection of primary metabolism appears not to be necessary for overproduction.

Gene expression changes in primary metabolism

Nonetheless, because clavulanic acid is synthesized from the precursors G3P and L-arginine, which play important roles in primary metabolism, specific changes in the primary metabolism of DS48802 could have occurred during the various random mutagenesis rounds, so that the intracellular pools of these intermediates are increased.

Indeed, glycerol uptake and metabolism (SCLAV_0631-0632 and SCLAV_0877-0879) is clearly upregulated over twofold in DS48802, indicating an improved utilization of glycerol as a carbon source as well as increased production of the clavulanic acid precursor G3P (Fig. 2). Moreover, the aconitase and citrate synthase from the citric acid cycle appear to be downregulated. A likely explanation for this is that the carbon flux from G3P in this direction is reduced and is partly redirected to clavulanic acid biosynthesis. This situation is remarkably similar to the result of the rationally constructed *gap1* deletion that blocked G3P conversion into 1,3-bisphosphoglycerate, thus improving clavulanic acid biosynthesis by increasing the intracellular G3P pool (Li and Townsend, 2006). However, an advantage of the situation in DS48802, which seems to have an incomplete downregulation of the flux, could be that a considerable pool of acetyl-CoA is maintained, e.g. for the biosynthesis of ornithine from glutamate. DS48802 also seems to avoid the potential negative effects of a complete deletion of the aconitase and citrate synthase genes: a complete absence of these enzyme activities could lead to acidogenesis with negative consequences for secondary metabolite production as shown by Viollier and colleagues (2001a,b). Moreover, DS48802 still seems to be able to synthesize α-ketoglutarate (a co-substrate required for clavaminic acid biosynthesis; Salowe *et al.*, 1990), while achieving the benefits of higher acetyl-CoA and/or G3P pools that have made these genes attractive targets for rational engineering to improve antibiotic production (Viollier *et al.*, 2001a). A potentially important observation that we cannot
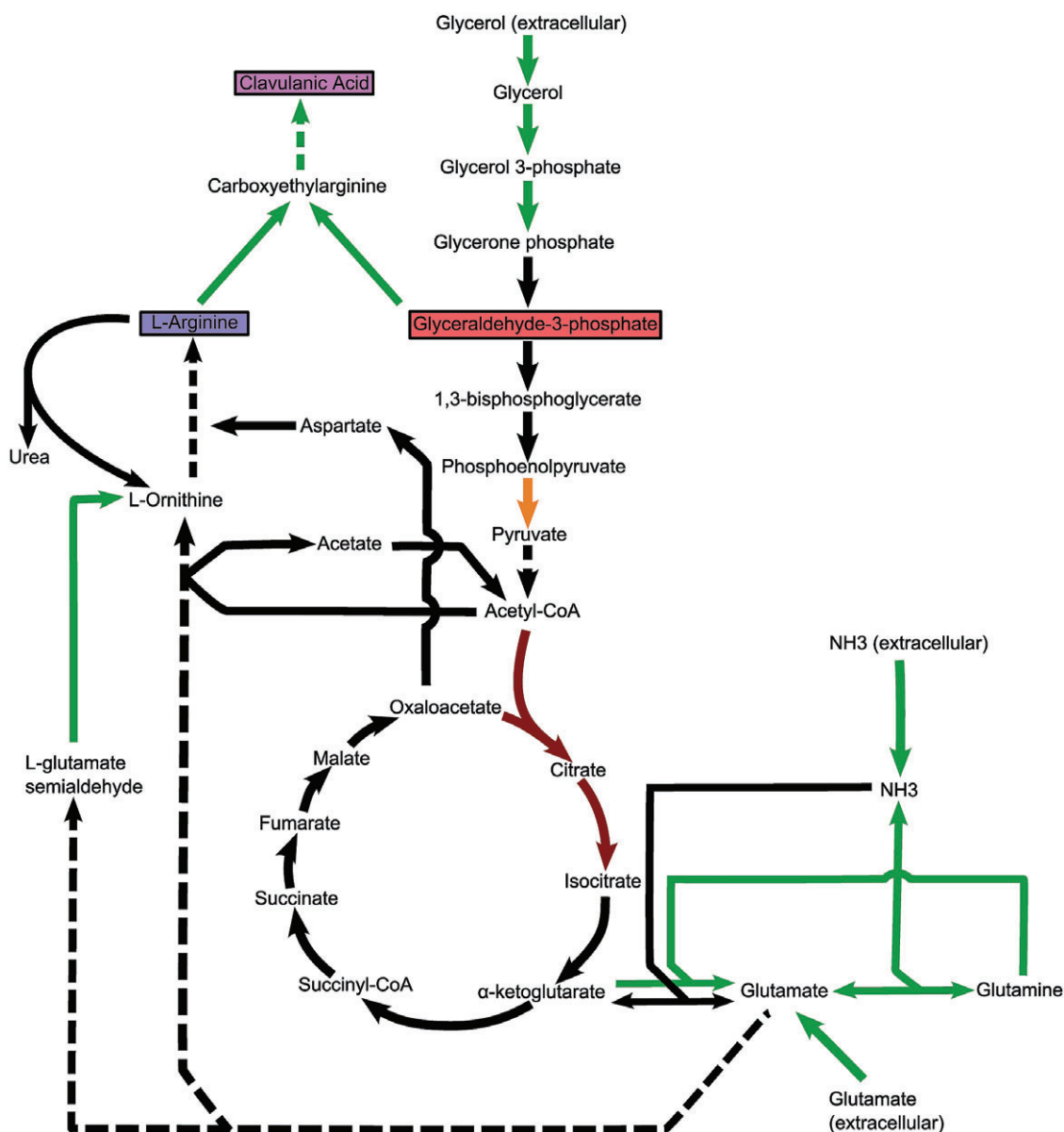
Fig. 2. Changes in *S. clavuligerus* primary and secondary metabolism affecting clavulanic acid production.
Changes in gene expression in *S. clavuligerus* DS48802 compared to the wild-type ATCC 27064 projected onto a metabolic map. Green arrows represent reactions catalyzed by genes expressed over twofold higher in DS48802 than in the wild-type. Red arrows represent reactions catalyzed by genes expressed over twofold lower in DS48802. The orange arrow represents the reaction catalyzed by pyruvate kinase, for which two isoenzymes exist which have changed in expression differently, one being downregulated (SCLAV_4329) and the other being upregulated (SCLAV_1203). Black arrows represent unchanged steps; solid arrows represent single biosynthetic steps; and dashed arrows represent multiple steps.

explain yet from the current data are the differential transcript level changes of the two pyruvate kinase isoenzyme genes, one being downregulated (SCLAV_4329) and the other being upregulated (SCLAV_1203).

We also observed a remarkable upregulation of glutamine synthetases I and II (SCLAV_1416 and SCLAV_1431), glutamate synthetases (SCLAV_1231) and glutamate importers (SCLAV_4660-4663). Glutamate can serve as a source for biosynthesis of the clavulanic acid

precursor arginine. This conversion takes place through the urea cycle involving ornithine as an intermediate (Rodríguez-García *et al.*, 2000), addition of which to the medium has been shown to strongly enhance clavulanic acid biosynthesis (Cheng *et al.*, 2003). Probably to overcome nitrogen and phosphate limitations, genes encoding the transporters for ammonia (SCLAV_4534) and phosphate (SCLAV_3166-3169) are also observed to be more highly transcribed in DS48802. This may be caused by the

increased expression of the pathway-specific activator genes *phoU* (SCLAV_3220, Ghorbel *et al.*, 2006) and *glnB* (SCLAV_4535, Drepper *et al.*, 2003), which both show over twofold increased transcription.

Conclusions

Our data show that a strain improvement program by random mutagenesis and screening has caused gene transcript changes in both primary and secondary metabolism. The overlap with results obtained by rational metabolic engineering through *claR/ccaR* overexpression and *gap1* deletion is intriguing. New leads from transcript changes observed in this study, such as the increased transcription of glutamine and glutamate synthetase genes, and of those encoding ammonium and phosphate transporters, can now be combined to rationally design novel high-producer strains. This approach might avoid the introduction of unwanted adverse effects from random mutagenesis, and provide strains suited for industrial application in a more efficient way. In this manner, functional genomics allows two key strategies applied in biotechnology – random mutagenesis and rational engineering – to become increasingly complementary.

Acknowledgements

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Statistical significance and comparison of transcriptional changes with FBA predictions using different cut-offs.
Table S2. Description and gene expression data of gene clusters upregulated in *S. clavuligerus* DS48802.

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