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Transcriptomics and quantitative physiology of ß-lactam-producing *Penicillium chrysogenum*

Diana Harris

Transcriptomics and quantitative physiology of ß-lactam-producing *Penicillium chrysogenum*

Proefschrift

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Diana Marianne HARRIS

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General Introduction

Introduction

Industrial biotechnology

In 1919 the Hungarian Kark Ereky first coined the term "biotechnology" as all the lines of work by which products are produced from raw materials with the aid of living organisms [16,35]. However, biotechnology has been around for millennia. As early as the 7th millenium BC, a mixed fermented beverage of rice, honey and fruit was produced in China [60]. Similarly, archaeologists have found indications for the production of wine and beer in Iran and Egypt at 6000 and 3000 BC respectively. In later days, bakers' yeast was used for the leavening of bread as well. Nowadays, biotechnology is not only used for the production of food and beverages but also for the production of commodity chemicals, medicines and for agricultural processes. With the discovery of DNA by Watson and Crick [102] and the techniques that have become available since then, it has become possible to modify genes, cells and living tissue in a predictable and controlled manner to generate changes in the genetic make-up of an organism; this has opened up the way towards modern biotechnology.

Modern biotechnology can be used in a wide range of fields, reflected by the distinction using colours that has been made in order to describe the different areas of biotechnology. The four main areas of biotechnology are red for health care, green for agricultural applications, blue for marine biotechnology and white for industrial biotechnology [29].

Industrial (the term 'white biotechnology' is unlikely to gain world-wide acceptance due to racial connotations, especially in the USA) biotechnology, also called the third wave of biotechnology after green and red biotechnology, is the use of microorganisms or derivatives of microorganisms (such as enzymes) for the production of (commodity) chemicals. Industrial biotechnology can be used in a wide range of sectors, ranging from the use of enzymes for pulp and paper bleaching, to the production of bio based plastics, food flavouring compounds and the production of fuels. Two recent successful examples of industrial biotechnology are the engineering of *Escherichia coli* to produce 1,3-propanediol and the engineering of bakers' yeast, *Saccharomyces cerevisiae*, to produce the antimalaria drug artemisinin.

1,3-propanediol is traditionally produced from petrochemicals. As a polymer it has numerous applications, including fibres and fabric, films and resins. In nature, various microorganisms produce 1,3-propanediol from glycerol. However, at the time this research was initiated, glycerol prices were unfavourable for the economic

production of 1,3-propanediol from it and synthesis from sugars was preferable. DuPont and Genencor therefore decided to modify the non-producing *E. coli* in such a way that it would produce 1,3-propanediol from glucose. In order to do so at least 14 genetic modifications were required, including introduction of genes from 1,3 propanediol producing organisms and removal of *E. coli* endogenous genes. This process has resulted in production levels of at least 135 g/L and the start of commercial production in November 2006 [65].

The antimalaria drug artemisinin is highly effective against the multidrug resistant malaria parasite, *Plasmodium falciparum*, but must be derived from a plant and is therefore in short supply and very expensive. Microbiological production of the direct precursor, artemisinic acid could be a cost-effective, environmentally friendly and reliable source of artemisinin, provided that sufficiently high production levels can be obtained. In order to achieve this, *S. cerevisiae* was engineered by extending the mevalonate pathway of yeast with the biosynthetic pathway for artemisinic acid from the plant *Artemisia annua L* (sweet wormwood). In addition, the flux through the endogenous yeast mevalonate pathway was increased by overexpression of the genes and their transcription factor. Finally the flux through the branch of this pathway leading to ergosterol was decreased [74]. Microbiological production of artemisinin is now possible and has brought the cheap production of this compound one step closer.

One of the oldest and very successful examples of industrial biotechnology is the production of the antibiotic penicillin by the filamentous fungus *Penicillium chrysogenum*. When this antibiotic was discovered in 1929 by Alexander Fleming [40], production levels were very low and a mixture of different penicillins was produced. Now, almost 80 years since its discovery, both classical strain improvement and the use of modern DNA techniques have increased production tremendously and it is now also possible to produce antibiotics that are not naturally produced by *P. chrysogenum*.

History of antibiotic production

Coming back from a holiday, Alexander Fleming, a microbiologist at St. Mary's Hospital in London, noticed that one of his Petri dishes with *Staphylococcus aureus* bacteria was contaminated with a mould, but more interestingly that no bacterial growth surrounded this mould. This finding resulted in the first paper describing the antibacterial mode of action of penicillin [40]. In this paper, Fleming showed that the filtered broth in which *Penicillium* had been grown had inhibitory, bactericidal and bacteriolytic properties. For reasons of simplicity he named this broth filtrate

penicillin as the mould had been identified as a member of genus *Penicillium*. *Penicillium* is derived from the Latin name for brush, *penicillus*, which describes the appearance of the spore heads of the fungus (Figure 1).

Figure 1: Conidial growth of *Penicillium chrysogenum*.
Image kindly made available by CBS Fungal Diversity Centre

After his initial discovery, Fleming tried to repeat the experiment, which proved to be difficult. As it turned out, Fleming's discovery had been serendipitous. That summer had been particularly cold, which resulted in the slow growth of the bacteria. This allowed the fungus, which has a lower optimal temperature for growth, to grow and produce penicillin without being previously overgrown by the bacteria [48]. Later experiments also showed that not all filamentous fungi, and not even all species of *Penicillium*, produce antibiotics. Fleming classified his fungus as *Penicillium rubrum,* but it was later identified as *Penicillium notatum,* and eventually renamed *P. chrysogenum*. Fleming's series of experiments showed that penicillin was mainly active against gram-positive bacteria and was non-toxic to rabbits. Although Fleming had realized the potential of penicillin as an antiseptic on surface wounds, he did not follow up this work for much longer. In 1932 a paper was published by Clutterbuck *et al* [26] in which they tried to purify penicillin. Although they managed to extract penicillin with ether, they were unable to recover it from the solvent without losing most of its activity. As a result, they also discontinued the work. A third paper on penicillin was published Roger Reid from the Pennsylvania

State College in 1935. As he was unable to isolate any other fungi with antibacterial properties, he continued to work with Fleming's strain and further characterised penicillin, but also encountered the instability of the antibiotic [73]. It took another 5 years before the work on penicillin finally took off. In 1940, pathologist Howard Florey and biochemist Ernst Chain and co-workers from Oxford University published their first article on "Penicillin as a chemotherapeutic agent". They had finally managed to extract active penicillin and to test its inhibitory power. In this paper they described toxicity tests in various rodents. They concluded that penicillin was active *in vivo* against at least three of the organisms that were also inhibited *in vitro* [23]. This work was followed by a more extensive paper in 1941 containing detailed descriptions of the conditions required for production of substantial amounts of penicillin, and in which they also described the first successful clinical tests [1].

With the outbreak of World War II, the need for antibiotics had increased dramatically and the interest in penicillin from government and pharmaceutical companies in the UK and USA increased. Soon various large programmes for the large-scale production of penicillin were initiated, with governments and industry collaborating. Within these consortia much effort was spent in finding strains with higher productivity and to improve fermentation and extraction techniques. A breakthrough was achieved by the isolation of a strain of *P. chrysogenum*, NRRL 1951, from a cantaloupe by Mary Hunt (affectionately nicknamed "Mouldy Mary") from the Northern Regional Research Laboratories (NRRL). NRRL 1951 not only produced high titres, but also was able to do so in submerged cultures [72].

Dutch penicillin research started secretly in 1944 under German occupation. The common story tells that the news of the new "wonder drug" reached researchers via clandestine listening to a BBC radio transmission and a message in the propaganda newspaper *De Vliegende Hollander*. Nevertheless, some uncertainties around this story remain. Following the first BBC broadcast on penicillin in September 1942, transcripts from the BBC Written Archive show that at least 12 other programmes were broadcast on this subject between 1942 and 1944 [10,17]. It cannot be said which of these programmes initiated the interest of NG&SF. Secondly; a search of the *Vliegende Hollander* archive did not retrieve any publications on penicillin. On the other hand, another publication from that time, *De Wervelwind*, does (personal communication, Dr. L.A. Robertson, [17]).

Anyhow, interest was raised and as the Nederlandsche Gist- en Spiritusfabriek in Delft (NG&SF, later Gist-Brocades and now DSM) had ample experience with fermentations, it was decided to request about twenty *Penicillium* strains from the Centraal Bureau for Schimmelcultures and start experiments. To prevent the occupiers from finding out, the code name Bacinol was used. Comparisons with vials that were included in the allied food and medicine drops in 1945 showed that

indeed the quality of the Bacinol produced equalled that of American penicillin. Due to this successful research during the occupation, the NG&SF was well-placed to start commercial production immediately at the end of the war and bring penicillin to the Dutch market in 1946 [17,96].

ß-Lactam antibiotics

The term "antibiotic" was coined by Selman Waksman, the head of a team that discovered a range of antibiotics, including streptomycin, in 1941 [34]. Antibiotics are substances produced by, or semisynthetic substances partially derived from, a microorganism and able in dilute solution to inhibit or kill another microorganism [61]. In general, the term antibiotic is used for any compound capable of inhibiting or killing a microorganism, despite its origin, microbial or chemical. Often the nucleus of a compound is produced by fermentation and subsequently chemically modified in order to increase its efficacy. Since the initial success of penicillin and streptomycin in treating infections, much effort has been spent in finding other antibiotics. This search was mainly driven by the fact that penicillin was not active against all bacterial pathogens and resistance to the existing antibiotics was developing. Currently more than 20000 antibiotics have been identified [31] of which there are more than 160 different antibiotics commercially available and the world market exceeds US$30 billion [30,42].

Class of antibiotic	Mode of action
Aminoglycosides	Inhibit protein synthesis
ß-lactams:	
• Carbapenems	
• Ceph-3-ems	Inhibit cell wall synthesis
• Clavams	
• Monolactams	
• Penams	
Chloramphenicols	Inhibit protein synthesis
Fluoroquinolones	Interfere with DNA synthesis
Lincosamides	Inhibit protein synthesis
Macrolides	Inhibit protein synthesis
Nitrofurans	Inactivate essential cell components
Tetracyclines	Inhibit protein synthesis

Table 1: Different classes of antibiotics and their mode of action

Each antibiotic has its own niche for use, depending on the type of infection and the desired mode of administration. A large number of antibiotics inhibit cell wall synthesis or protein synthesis, while others interfere with DNA or RNA synthesis (Table 1).

ß-lactam antibiotics form the most important group of antibiotic compounds and take up about 65% of the world antibiotic market [33]. ß-lactams can be classified in 5 groups: penams, ceph-3-ems, clavams, monolactams and carbapenems. The first two groups include the penicillins, cephalosporins and cephamycins and are the best-characterised and also the most important ß-lactams in terms of sales and production [7]. The latter groups have only been identified relatively recently. The clavam group contains clavulanic acid, a ß-lactamase inhibitor, which is used in combination with penicillins. Carbapenems and monolactams have a broad antimicrobial spectrum and most of them are resistant to several ß-lactamases [27]. Cephalosporins were discovered in 1950 in seawater near the outlet of a sewer in Cagliari, Sardinia by Giuseppe Brotzu and are predominantly produced by *Acremonium chrysogenum* (previously *Cephalosporium acremonium*) [15]. Cephamycins are 7-methoxycephalosporins, due to the presence of this methoxy group they are active against penicillin-resistant bacteria [57]. ß-lactams can be naturally produced by a wide variety of microorganisms ranging from filamentous fungi to both gram-positive and gram-negative bacteria [57]. Whereas penicillins are solely produced by filamentous fungi, cephalosporins can be produced by organisms belonging to all groups. For the remaining groups of ß-lactams only bacterial producers have been reported so far (Figure 2) [13,14].

Soon after the discovery of the natural penicillins, resistant strains of bacteria emerged. This eventually led to the development of new classes of ß-lactam antibiotics. Nowadays the majority of all ß-lactams in use are so-called semisynthetic antibiotics derived from intermediates produced by fermentation. A common intermediate for the production of semisynthetic antibiotics is 6-aminopenicillanic acid (6-APA), which is obtained by the enzymatic cleavage of penicillin-G. From 6-APA, a whole range of semisynthetic penicillins (SSPs) can be obtained relatively easily. 6-APA also forms the precursor for semisynthetic cephalosporins (SSCs), which requires the expansion of the five membered ring of penicillins to a six membered ring. Common precursors for SSCs are adipyl-7-aminodesacetoxycephalosporanic acid (adipyl-7-ADCA) and adipyl-7-aminocephalosporanic acid (adipyl-7-ACA) [94].

The common factor in ß-lactam antibiotics is the characteristic nucleus, the ß-lactam ring. Penicillins, cephalosporins and cephamycins share the first steps of their biosynthesis (Figure 4). Biosynthesis starts with the condensation of the three amino acids cysteine, valine and α-aminoadipic acid to form the tripeptide ACV.

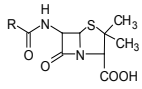
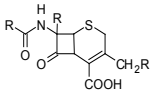
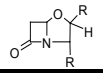
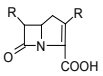
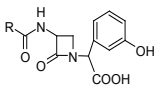
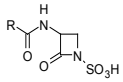
Classes of naturally occurring ß-lactams	Antibiotics	Producing microorganisms (examples)		
		Fungi	Bacteria	
			Gram +	Gram -
Penam 	Penicillins	*Penicillium chrysogenum* *P. notatum* *Aspergillus nidulans*		
Ceph-3-em 	Cephalosporins Cephamycins Cephabacins Clavulanic acid Chitinovrins	*Acremonium chrysogenum* *Paecilomyces persinicus*	*Streptomyces clavuligerus* *Nocardia lactamdurans*	*Flavobacterium* sp. *Lysobacter lactamgenus*
Clavam 	Clavulanic acid		*Streptomyces clavuligerus*	
Carbapenem 	Thienamycins Olivanic acid Epithienamycins		*Streptomyces clavuligerus* *S. olivaceus*	*Erwinia carotovora* *Seratia* sp.
Monolactam 	Nocardicines		*Nocardia uniformis* *Subsp tsuyamanensis*	
	Monobactams			*Agrobacterium radiobacter* *Pseudomonas acidophila*

Figure 2: Naturally occurring classes of ß-lactams [13,14]

This reaction is catalysed by the non-ribosomal peptide synthase ACVS encoded by *pcbAB* [8,9]. In the next step the classic ß-lactam ring structure is formed by isopenicillin-N synthase (*pcbC*) [20]. Isopenicillin-N forms the branch point for the penicillins and cephalosporins. Penicillins can be easily produced from isopenicillin-N by the exchange of the α-aminoadipic acid moiety for a CoA activated side chain, such as phenylacetic acid or phenoxyacetic acid, by acyl-CoA: isopenicillin-N acyltransferase (*penDE*), which results in the production of penicillin-G or penicillin-V [8,9].

For cephalosporin and cephamycin biosynthesis, isopenicillin-N is epimerised by isopenicillin-N epimerase, encoded by *cefD*, to form penicillin-N. In the following step the 5-membered thiazolidine ring is expanded to a 6-membered dihydrothiazine ring by the deacetoxycephalosporin-C synthetase (DAOCS, commonly named expandase, encoded by *cefE*). The deacetoxycephalosporin-C formed by this is hydroxylated by deacetylcephalosporin-C synthase (DACS, hydroxylase, *cefF*) to form deacetylcephalosporin-C. In *S. clavuligerus* two enzymes catalyse this reaction, while one bifunctional enzyme can catalyse both reactions in *A. chrysogenum*. At deacetylcephalosporin-C the biosynthesis of cephalosporins and cephamycins split. For cephalosporin-C biosynthesis deacetylcephalosporin-C is acetylated via acetyl-

CoA: deacetylcephalosporin-C acetyltransferase (*cefG*) forming the end product cephalosporin-C.

For the formation of cephamycins the C-3' hydroxyl group is carbamoylated by deacetylcephalosporin-C O-carbamoyltransferase (commonly named carbamoyltransferase) encoded by the *cmcH* gene. Finally a C-7 methoxy group is added by *O*-carbamoyldeacteylcephalosporin-C-7-methoxyl transferase (*cmcH* and *cmcH*) to form cephamycin-C (Figure 4).

As is common for most secondary metabolite genes, the ß-lactam biosynthesis genes are clustered [53]. In *P. chrysogenum*, the penicillin biosynthesis genes *pcbAB*, *pcbC* and *penDE* form a single cluster located on chromosome I. The cephalosporin biosynthesis genes in *A. chrysogenum* are organized in two clusters, the so-called early and late cephalosporin cluster. The *pcbAB, pcbC, cefD1* and *cefD2* genes are located on chromosome VII, whereas the *cefEF* and *cefG* are located separately on chromosome I. In contrast, in *S. clavuligerus* all biosynthesis genes, the *pcbC, pen, cefF* and *cmcH* genes are located in one cluster (Figure 3, reviewed by [14,45,57,83]).

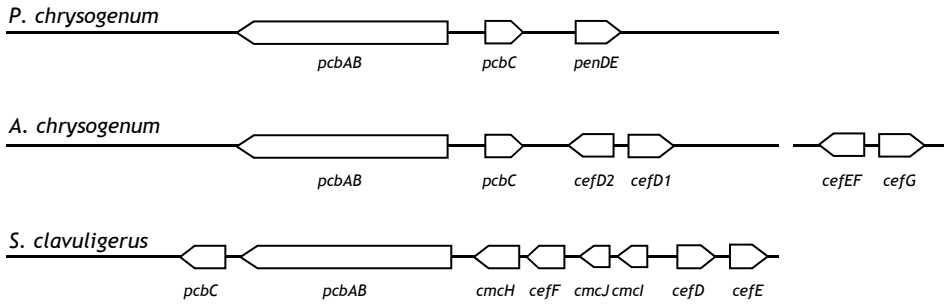


Figure 3: Clusters of genes for ß-lactam biosynthesis in three microorganisms

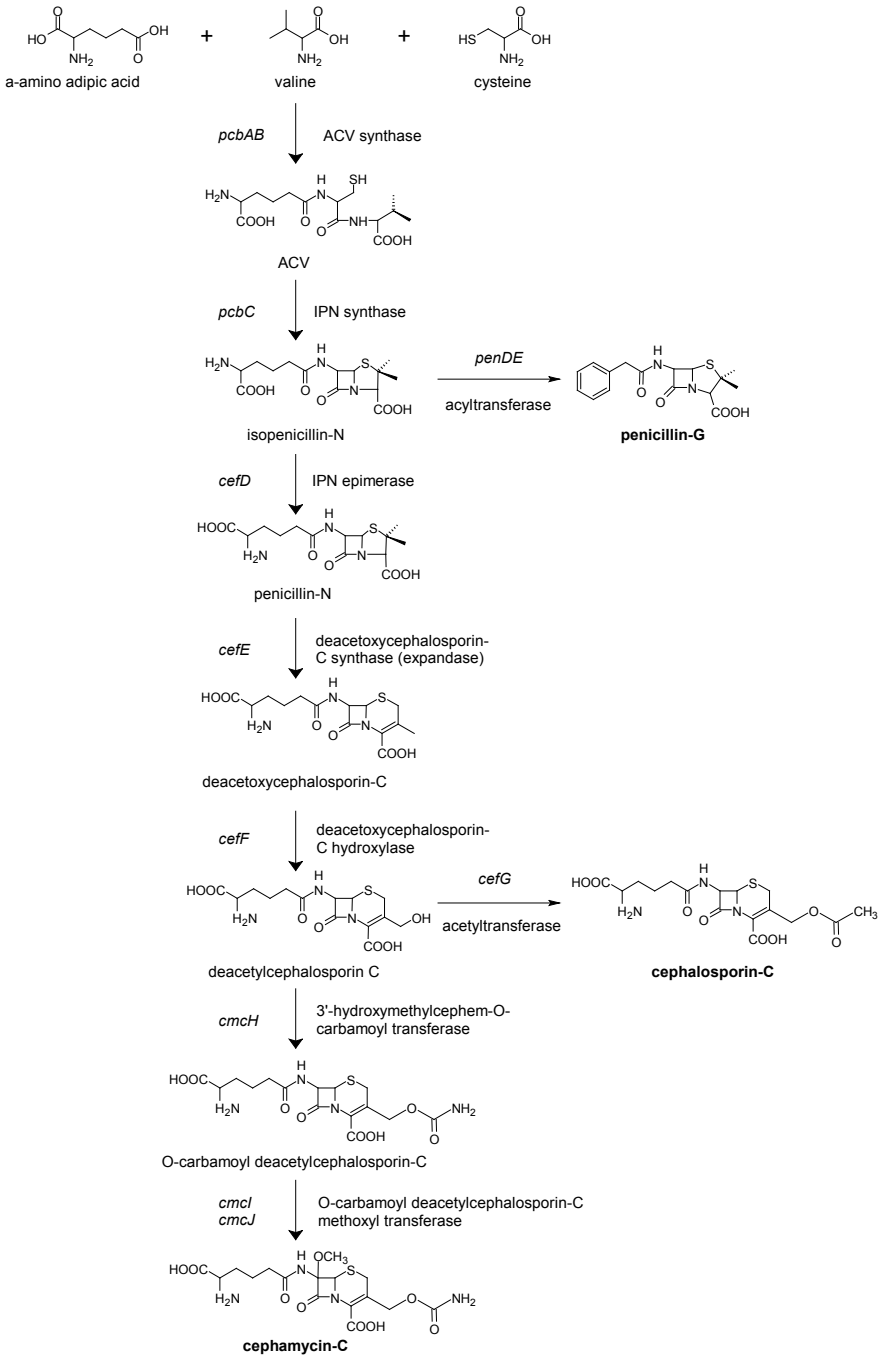


Figure 4: Biosynthesis of the ß-lactams: penicillin, cephalosporin and cephamycin [13,91]

P. chrysogenum and its genome

Penicillium species are filamentous fungi, are widespread in nature and are among the most common sources of fungal food spoilage. For example, *Penicillium italicum* and *P. digitatum* are common spoilers of citrus fruits, whereas *P. expansum* is known to be responsible for apple rotting. On the other hand, *P. roquefortii* and *P. camembertii* are the fungi responsible for the flavours of the well-known French cheeses, Roquefort and Camembert respectively. *P. chrysogenum* is probably the most common *Penicillium* species and is known to act as a food spoilage agent as well as being a common presence indoors, on damp building materials, walls and wallpaper and in furniture dust [84].

P. chrysogenum is a member of the spore-forming *Ascomycetes*. *Ascomycetes* produce ascospores in a saclike structure called the ascus (hence the name sac fungi). Except for sexual reproduction via the ascospores, fungi from this class can also reproduce asexually by means of conidia spores formed on the extension of hyphae (conidiophores). Spores are generally resistant to harsh conditions and may travel long distances. In the lab, they also provide an easy way to maintain strains.

The life cycle of *P. chrysogenum* starts and ends with the spores. Once a spore encounters suitable physical conditions (e.g. aqueous with sufficient nutrients), germination is initiated. Germination can be divided into three phases, swelling, germ tube emergence and elongation. Germination starts with the spherical growth of the spore in which new cell material is formed. After a certain time polarity is established and a germ tube emerges from the spore. The germ tube develops into a hypha. Full-grown *P. chrysogenum* consists of a complex network of entwined hyphal elements. Each hypha contains a series of cells in a chain and only separated by septa. Cell material can flow freely between the cells via these septae. In general a hypha can be divided into three areas, each with its own function. At the tip of the hypha is the apical compartment involved in tip extension. These cells are not separated by septae. Just behind the septae are the cells in the subapical compartment. These cells have a composition very similar to the cells in the apical compartment and are probably involved in supplying the apical compartment with cell material. Further away from the hyphal tip is the hyphal element with cells containing large vacuoles. These cells are not directly involved in the tip extension process but are believed to provide sufficient intracellular pressure for transport of protoplasm towards the tip.

With the publication of the genome of the Wisconsin54-1255 strain, *P. chrysogenum* has finally entered the genomic era, opening up new possibilities for research [95]. The genome of *P. chrysogenum* contains 12,960 open reading frames distributed over

32.19 Mb, which is comparable to related fungi [41,69,104]. All ORFs have been given a systematic name as follows: Pc for the organism, a two-digit supercontig number, followed by g for gene and a five-digit number matching the order of the ORFS on the contig (i.e. Pc21g21390, ACVS). Of the 12,941 predicted proteins, approximately 40% could be related to the functional protein classes related to metabolism, energy, cellular transport and protein fate [81]. Another 40 % of the ORFs fall into the functional category unclassified, opening up myriad possibilities for functional characterisation of genes. In addition to the known ß-lactam biosynthesis genes of *P. chrysogenum*, the genome contains additional ORFs with homology to other ß-lactam genes. For instance, while penicillin-N has not been detected in cultures of *P. chrysogenum*, the genome contains a putative isopenicillin N epimerase, the first gene in cephalosporin biosynthesis from isopenicillin-N. Similarly, using a multi-step semiautomatic approach for annotation of description and functional categories, many transporters and transcription factors have been identified. Combined effort from the *Penicillium* research community can only result in an improved annotation of the genome. Transcriptome studies followed by functional characterisation will be an important tool for this.

Improving the process

Classical strain improvement

After identification of the natural isolate NRRL 1951 that was able to produce satisfactory yields of penicillin in submerged cultures, research to produce strains with improved characteristics was initiated, and continues. Soon after the discovery of NRRL 1951, a large programme, headed by the University of Wisconsin, resulted in the isolation of a number of strains with improved production [2]. Another important source of improved strains are those derived from the Panlabs strain improvement program [56]. The classical strain improvement programmes included a brute force approach using random mutagenesis of the best existing production strains by means of radiation or toxic chemicals, followed by the screening of thousands of descendants and eventual selection of the strain with improved characteristics. Although tedious and labour intensive, this method is still used to a large extent in industry today, because it has been proven to be very successful. Although it is impossible to obtain a full picture owing to industrial confidentiality, it is assumed that most industrial strains currently in use are descendants from these early strains from the Wisconsin family [66]. The Wisconsin54-1255 strain is now an international laboratory standard [6,37,71]. Although the primary aim of strain improvement programmes will of course always be to obtain increased titres of the product of interest, this is not the only strain characteristic selected for. Other characteristics selected for include: a) utilization of cheaper substrates, b) elimination of side

products, c) improved sporulation, d) improved morphology for better fermentation handling, e) elimination of unwanted catabolism of precursors and f) shortening of the fermentation time (increasing production rates) [27,31,32,68,70].

One of the first observations when analysing the improved strains was the increased copy number (up to 50 fold) of the biosynthesis genes compared to the NRRL-1951 and Wisconsin54-1255 strains [8,89]. Later, it was also shown that the genes were clustered together and amplified in tandem repeats [38,66]. Analysis of the gene cluster showed that the amplified region is 56.8 kb large and contained 16 putative open reading frames of which 8 were expressed under penicillin producing conditions. One of the ORFs expressed was a putative saccharopine dehydrogenase, which might be related to the biosynthesis of the α-aminoadipic acid precursor. The cluster also contained a putative transcriptional regulator [39].

Additionally, mRNA levels of the biosynthesis genes have increased more than could be expected from gene-dosage alone, suggesting that expression has become deregulated [70,89].

A major breakthrough in the improvement of penicillin production was the discovery of the effect of the growth medium on productivity [31]. Original cultures of penicillin produced a range of different natural antibiotics. In 1946 it was discovered that corn steep liquor had a positive effect on the total penicillin production [63,64] and that it predominantly resulted in the production of penicillin-G. Later work showed that this was the result of the catabolic product phenylacetate from phenylalanine in the corn steep liquor [11,12,43,85]. However, even at an early stage it was observed that only a small fraction of the added phenylacetic acid was recovered in penicillin-G and that the majority was oxidized [50,86]. As phenylacetate is an important cost factor in commercial penicillin-G production, reduced precursor oxidation was among the first priority targets for classical strain improvement [56]. Phenylacetate catabolism occurs via the homogentisate pathway in which phenylacetate is first oxidised to *ortho*-hydroxyphenylacetate (mandelate) via phenylacetate hydroxylase and via 4 more steps to fumarate and acetoacetate which can enter central metabolism (Figure 5).

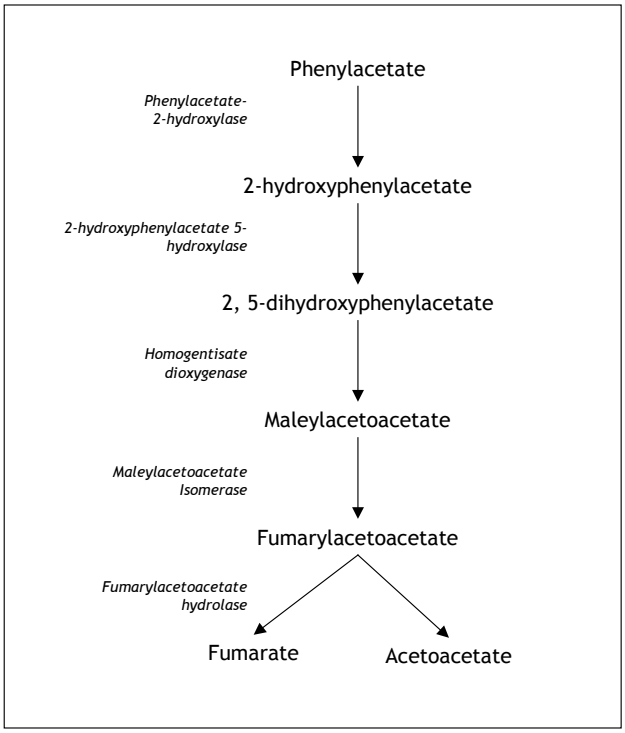


Figure 5: Phenylacetic acid metabolism via the homogentisate pathway [79]

In contrast to the producing strains of *P. chrysogenum* the low penicillin-producing *Aspergillus nidulans* can grow on phenylacetate as the sole carbon source. Phenylacetate hydroxylase mutants of *A. nidulans* were not able to grow on phenylacetate as a sole carbon source anymore. The mutation did result in a 3-5 fold increase in penicillin production, indicating that catabolism competes with penicillin biosynthesis for phenylacetate [62]. Subsequent analysis on the phenylacetate hydroxylase gene of *P. chrysogenum* showed that a point mutation occurred early in the strain improvement programmes at the University of Wisconsin, resulting in improved penicillin-G production, better efficiency of phenylacetate utilisation and less growth [79]. Similar results were later found when comparing the original Fleming strain to early industrially-used strains, indicating that another point mutation in the phenylacetate hydroxylase gene was responsible for the loss of the ability to grow on phenylacetate as a carbon source, as was the case for the Fleming strain [80]. Despite these improvements, in current production processes small amounts of *ortho*-hydroxyphenylacetate are still produced, suggesting that the phenylacetate hydroxylase has retained low activity and offering a possibility for further improvement via metabolic engineering [79].

Metabolic engineering of ß-lactam production

The emergence of recombinant DNA technology during the 1970s allowed more precise genetic modification of organisms. This led to the emergence of a new field in biotechnology, called metabolic engineering. Metabolic engineering was defined as "the improvement of cellular activities by manipulations of enzymatic, transport and regulatory functions of the cell with the use of recombinant DNA techniques" [3].

Metabolic engineering can be applied with different aims [67]:

1. Extension of substrate range for growth and product formation
2. Introduction of new product formation pathways
3. Reduction of by-product formation
4. Improvement of productivity or yield
5. Engineering of cellular physiology for process improvement

Each metabolic engineering effort starts with a thorough analysis of the microorganism of choice and the (desired) pathway. Based on the possibilities and the bottlenecks in the current process, targets for genetic engineering are pinpointed. Subsequently the engineered strains are synthesised and analysed for their performance compared to the original strain. From the results, new targets are identified and the cycle continues (Figure 6). Each phase is of equal importance. In extensively studied organisms such as *S. cerevisiae and E. coli*, design can often be based on exhaustive analysis of published sources. However, for less studied and genetically less accessible organisms such as *P. chrysogenum*, the analysis and synthesis phases remain important research activities.

Metabolic modelling

In silico analysis of the integral metabolic pathways using metabolic modelling and metabolic flux analysis has become an invaluable tool for metabolic engineering. Metabolic modelling is based on reconstruction of all metabolic reactions occurring in a microorganism. Due to the complexity of the cell, such a model is inevitably based on a combination of knowledge and assumptions. With the limited knowledge of the metabolism of *P. chrysogenum,* it is therefore possible to construct multiple metabolic models depending on the assumptions made. Several studies have addressed the metabolic bottlenecks in high-producing penicillin strains using such an approach [49,52,97,98]. However, as different strains of *P. chrysogenum* and different metabolic networks with different assumptions were used, the results of these studies are inconclusive.

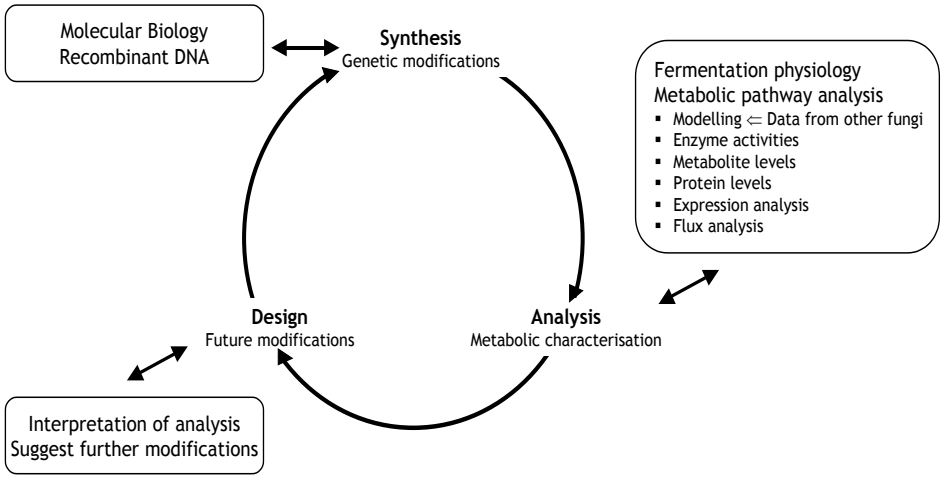


Figure 6: The metabolic engineering cycle [67]

Nevertheless some important indications can be obtained from them. For instance all studies indicate that NADPH is an important factor for penicillin biosynthesis due to the large requirement for this cofactor in cysteine biosynthesis. Because of the overproduction of penicillin, stoichiometrically high amounts of cysteine, are required. Depending on the biosynthesis route for cysteine, 40-60 % more NADPH is required by high-producing cultures than by non-producing cultures. Furthermore, producing strains of *P. chrysogenum* have an unusually large flux through the pentose phosphate pathway, the assumed largest provider of NADPH [24,49,52,98]. Recently, a positive relationship between the flux through the pentose phosphate pathway and penicillin productivity has been observed [54]. Similarly, metabolic flux analysis studies have shown that penicillin production is accompanied by a large, unexplained drain of energy in the form of ATP [97]. Obviously this high energy requirement will influence the maximum theoretical yield of penicillin-G on any carbon source. Clarifying this extra ATP requirement and subsequent genetic modifications might result in a higher penicillin yielding strain.

In the following sections various metabolic engineering strategies applied to ß-lactam production will be highlighted. Hitherto, most of these strategies were based on analysis of the strain lineages obtained via classical strain improvement programmes.

Increased gene-dosage of biosynthesis genes

An obvious first choice for the improvement of ß-lactam production using molecular biology was to increase expression of the biosynthesis genes. The first successful example of this approach was in a high-producing cephalosporin-C strain of *Acremonium chrysogenum*. Researchers at Lilly Laboratories overexpressed the *cefEF* gene encoding for the bifunctional protein with expandase and hydroxylase activity into a high-producing strain of *A. chrysogenum*, resulting in a 10-15 % increase in cephalosporin-C production and reduced production of the intermediate penicillin-N [88]. Later it was shown that the plasmid used for the transformation additionally contained the *cefG* gene and that expression of this gene alone resulted in a two- to threefold increase in the cephalosporin titre. In addition, the *cefG* acetyltransferase activity was shown to be rate-limiting for cephalosporin-C production. It remains to be seen whether expression of the *cefEF* gene alone will also result in increased production [46,47,59].

Similarly, at Gist-Brocades, production of penicillin-V was increased by combined overexpression of the *pcbC* and *penDE* genes (IPNS and AAT) in the low-producing strain Wisconsin54-1255 [100] after overexpression of the *pcbC* gene in an industrial strain of *P. chrysogenum* had failed [87]. Theilgaard *et* al have later reported on a systematic study in Wisconsin54-1255 in which different combinations of overexpression of the biosynthesis genes were tested. Transformants in which the whole gene cluster had been amplified showed the largest increase in penicillin production [90]. Until now, no reports have been published showing a successful example of this approach in high-producing strains of *P. chrysogenum*. As was shown previously, an increase in copy number was linked to an increase in penicillin biosynthesis. At a certain copy number, however, there seems to be a limit to the increase in yield, which might explain why this approach has not been successful in industrial strains with previously increased copy numbers [66,91]. In addition, it is likely that in industrial strains other factors, such as amino acid supply, become rate limiting.

Increased supply of precursors

A second approach towards increased ß-lactam production would be to increase the supply of amino acid precursors. As described earlier, biosynthesis of penicillins, cephalosporins and cephamycins starts with the condensation of the three amino acids cysteine, valine and α-aminoadipic acid. The latter compound is an intermediate in the lysine biosynthesis pathway. In order to increase the α-aminoadipic acid pools two approaches have been followed. On the one hand targeted disruption of the first gene in the lysine biosynthetic pathway following the formation of α-aminoadipic acid, *lys2* encoding α-aminoadipic acid reductase, resulted in doubled penicillin production in a Wisconsin54-1255 derivative strain [22]. The second approach consisting of overexpression of the first gene of lysine

biosynthesis, homocitrate synthase (*lys1*), did not result in increased penicillin levels [5]. In the case of α-aminoadipic acid, channelling of this compound towards penicillin biosynthesis via downstream gene inactivation is thus successful (*lys2*). On the other hand, upstream amplification of a specific gene (*lys1*) did not result in increased penicillin production, possibly because other bottlenecks still occur after removal of the first [21].

Heterologous expression of genes to extend product range

Except for improving existing pathways in ß-lactam-producing organisms, metabolic engineering can also be employed to transfer biosynthesis routes from one organism to another. One of the first examples of this in the case of ß-lactam production was the production of penicillin-G by *A. chrysogenum*. *P. chrysogenum* and *A. chrysogenum* share the first two steps towards the biosynthesis of ß-lactams, but the routes diverge at the point of isopenicillin-N. In *P. chrysogenum,* the α-aminoadipyl side chain is exchanged for phenylacetic acid (in the case of penicillin-G) by acyltransferase, whereas in *A. chrysogenum,* isopenicillin-N is isomerised to penicillin-N, which can then be subjected to expansion of the ring by the expandase activity of *cefE*. *A. chrysogenum* does not contain an acyltransferase capable of performing the side chain exchange and can therefore not produce penicillin-G/V. By heterologous expression of the *penDE* gene of *P. chrysogenum, A. chrysogenum* was converted so that it produced a mixture of penicillin-G and cephalosporin-C when fed with phenylacetic acid [44].

Although this was an interesting proof-of-principle, it had little industrial significance, as *P. chrysogenum* is a superior ß-lactam producer over *A. chrysogenum*. Of more applied interest would be the transferral of cephalosporin biosynthesis to *P. chrysogenum*.

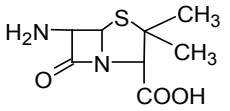
Until recently, production of the semisynthetic cephalosporin precursors 7-ACA and 7-ADCA involved expensive and environmentally unfriendly chemical steps with the penicillin nucleus 6-APA as a starting point [94]. A breakthrough in the fermentative production of these compounds was achieved in the early 1990's with the expression of the bacterial *cefD* and *cefE* genes into *P. chrysogenum*. By expression of the isopenicillin-N isomerase from *Streptomyces lipmanii* (*cefD*) and of the expandase from *S. clavuligerus* (*cefE*), *P. chrysogenum* was converted to produce deacetoxycephalosporin-C and penicillin-V simultaneously [18,19]. This initial study showed that it was feasible to transfer the cephalosporin biosynthetic pathway from bacteria to filamentous fungi, but that there were still a number of improvements to be made. In order to produce 7-ADCA from deacetoxycephalosporin C, the natural α-aminoadipyl side chain needs to be removed, a reaction for which no enzyme has yet been found [103]. As an alternative approach, it was shown in

1960 that feeding *P. chrysogenum* with adipic acid resulted in the production of adipyl-6-APA [4], and it had been suggested that cleavage of this side chain would be relatively easy [28]. Crawford *et al*. therefore fed adipic acid to recombinant *P. chrysogenum* strains carrying the *cefE* gene of *S. clavuligerus*, which resulted in the production of adipyl-7-ADCA. When the strains were transformed with the *cefEF* gene of *A. chrysogenum* [82] they would produce adipoyl-7-ACA [28]. The adipoyl side chain could be easily removed by an acylase derived from *Pseudomonas,* resulting in the SSC precursors ADCA and ACA respectively. In this way the high biosynthetic potential of *P. chrysogenum* could now be exploited for the production of cephalosporins. Although initial production levels were already promising, classical strain improvement approaches increased production even further. Extensive physiological characterisation of such a strain of *P. chrysogenum* carrying the expandase gene of *S. clavuligerus* showed that at the optimal growth rate, biomass yields, maintenance and specific productivity were quite similar to the penicillin-G producing host strain, suggesting that the expansion reaction does not pose a large extra energetic burden [75-78]. Due to its large economic and environmental benefits, the process was selected for full scale production in 2000 at the Anti-Infectives site of DSM in Delft [94].

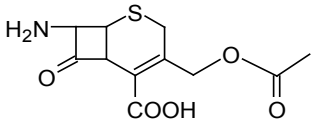
Recently, the complete cephalosporin biosynthesis route of *A. chrysogenum* has been transferred to *P. chrysogenum* [92]. As a host for this process they used a mutant strain which accumulates high levels of isopenicillin-N due to an inactive acyltransferase as the result of a point mutation in the *penDE* gene [36]. Although all four genes (*cefD1, cefD2, cefEF and cefG*) were expressed in *P. chrysogenum,* the recombinant strains only excreted deacetylcephalosporin-C and no cephalosporin-C. Further analysis showed that both of these compounds accumulate intracellularly, resulting in the hypothesis that the recombinant *P. chrysogenum* lacks a cephalosporin exporter [92]. The recently identified multidrug efflux pump *cefT,* located in the cephalosporin gene cluster, might be the candidate for this. Indeed, amplification of this gene in *A. chrysogenum* has been shown to double cephalosporin production [93].

Simultaneously with these experiments in *P. chrysogenum*, production of 7-ADCA and 7-ACA by *A. chrysogenum* has also been explored. Japanese researchers have managed to convert a cephalosporin-C producing strain of *A. chrysogenum* to a 7-ACA producing strain via the insertion of D-amino acid oxidase and cephalosporin acylase from *Fusarium solani* and *Pseudomonas diminuta* respectively [51]. The main disadvantage of this process was the low yield of 7-ACA due to the low *in vivo* activity of the acylase and the production of two by-products, 7-ADCA and 7-aminodeacetylcephalosporanic acid (7-ADAC). Following the approach for

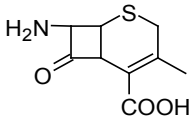
P. chrysogenum, Velasco and co-workers expressed the *cefE* gene of *S. clavuligerus* into *A. chrysogenum*. At the same time they inactivated the native *cefEF* gene in order to prevent the formation of other cephalosporin intermediates. This process resulted in high levels of deacetoxycephalosporin-C, which can subsequently be converted to 7-ADCA via two bioconversion steps [101].



6-APA



7-ACA



7-ADCA

Figure 7: Three penicillin and cephalosporin precursor molecules

Techniques

Two techniques have been essential for the work described in this thesis. All physiological data presented have been obtained using chemostat cultivations. Also, after the genome sequence of *P. chrysogenum* became available half way through this project [95], it was possible to use microarrays to study the transcriptome of chemostat-cultivated strains of *P. chrysogenum*.

Chemostat cultivations

Although chemostat cultivations are not in all respects representative of industrial scale fed-batch fermentations of *P. chrysogenum*, they do offer important academic advantages. Compared to industrial processes, penicillin production is low in continuous cultures because of the instability of the compound. Secondly, industrial strains tend to be genetically unstable, resulting in the loss of antibiotic

production during prolonged cultivation. This limits the possible duration of the continuous culture experiments [25,55,98]. Nevertheless, the ability to specifically study one parameter at a time, together with the reproducibility of chemostats, offers valuable advantages in quantitative physiology studies. Chemostats are characterised by a continuous inflow of fresh medium into an ideally mixed fermenter and a continuous equal outflow of spent broth including mycelium. At the same time, the fermenter is sparged with air and the pH is maintained at a constant level by automatic addition of acid or alkaline. These factors ensure a controlled and constant environment for the growing microorganisms over time. In addition, as all parameters are controlled by the operator, experiments are highly reproducible. In most cases, the medium is composed of pure salts, sugars and trace elements with a constant quality, allowing a defined and reproducible inflow of medium components. The medium should contain all essential factors for growth, with one component growth limiting and all others in excess. In the case of the experiments presented here, carbon (glucose) was the limiting nutrient. The rate at which the limiting nutrient is provided will determine the specific growth rate, μ [h^{-1}]. Once a steady state is reached, the specific growth rate equals the dilution rate of the culture (D is the fraction of the volume that is replaced per unit of time and is dictated by the volume of the fermenter and the inflowing rate).

Due to the filamentous nature of *P. chrysogenum* and its tendency to attach itself to anything below the liquid surface, obtaining a true chemostat culture is not straightforward. The system that has been used throughout this thesis has been successfully used for the cultivation of *P. chrysogenum* for almost ten years [99]. In order to prevent unwanted wall growth, all tubes below the liquid surface were continuously flushed with air. Effluent was removed discontinuously using a special overflow device and overpressure in the fermenter. The time interval between effluent removals was fixed in such a way that approximately 1% of the culture volume was removed each time. Although effluent removal is thus discontinuous, the fluctuations in the volume are so small that continuous effluent removal is approached.

Microarrays

With the availability of the genome sequence of *P. chrysogenum*, it became possible to use the DNA microarray platform available in the Industrial Microbiology group of Delft University of Technology with this industrially relevant microorganism. With microarrays, it is possible to measure, at a whole genome scale, the mRNA levels in a cell. Although mRNA levels indicate which genes are transcribed under certain conditions they will only provide part of the picture of what is going on in a cell. For a more complete interpretation, protein and metabolite levels are also required. Nevertheless, as mRNA forms the first step in the cascade from genome

to metabolite, transcript levels do provide information about possible changes in metabolism occurring from one situation to another.

Although there are many different microarray systems, all studies described in this thesis used the Affymetrix GeneChip® platform. Affymetrix GeneChips® consist of over 500.000 25-mer oligonucleotide probes chemically synthesised on a coated quartz surface of 1.6 cm^2. Each probe uniquely matches one gene and each gene is represented by 11 of these perfectly matching oligonucleotides. To enhance the performance of the system, each perfect match is accompanied by an imperfect match in which the middle nucleotide mismatches the original nucleotide. This mismatch is used to detect any false or contaminating signals (for more detailed information see [58]). Affymetrix microarrays are made in large numbers in a controlled environment and are highly reproducible. Because of the strict requirements for sample preparation and hybridisation, it is therefore possible to treat each sample independently and extend datasets without having to run a control sample each time.

Aim, scope and outline of the thesis

With an over 1000-fold improvement in specific productivity since its discovery, penicillin is one of the most successful examples of industrial biotechnology. Although classical strain improvement programmes have been a major contributor to this success, the wish for a more rational approach towards improvements has driven the work described in this thesis. As most ß-lactam biosynthesis routes share the first steps in their pathway, the production of penicillin-G in chemostat cultivations of *P. chrysogenum* has been chosen as a model system. Compared to common laboratory organisms such as bakers' yeast, the information on *P. chrysogenum* is relatively limited, which also reduces the range of possibilities for a rational approach. The majority of the work described in this thesis can therefore be assigned to the analysis phase of the metabolic engineering cycle.

Chapters 2 and 3 are a direct follow-up of metabolic modelling work published by the Bioprocess Technology group of the Department of Biotechnology of the Delft University of Technology [97,98]. Penicillin production requires the condensation of three amino acids. These amino acids, cysteine, valine and α-amino adipic acid, need to be provided by the central metabolism of the cell. In addition to the carbon precursors, biosynthesis of cysteine and valine also require sufficient reducing power in the form of NADPH. Depending on the biosynthesis pathway for cysteine, the total stoichiometric demand of NADPH is 7-10 mole NADPH per mole penicillin. In the strain used in this work, penicillin production increased the total NADPH demand by 40-60%. On the basis of these modelling studies, it was

concluded that the bottleneck for penicillin production was not in the supply of carbon precursors, but might be related to the availability of NADPH. NADPH is a conserved moiety and can act as a cofactor in many enzymatic reactions. It is generally accepted that NADPH can be oxidised to NADP in various reactions during the formation of amino acids, lipids and nucleic acids. On the other hand, reduction of NADP to NADPH generally occurs in the central carbon metabolism. With the aim of identifying possible targets for metabolic engineering in order to increase the supply of NADPH for penicillin production, the main possible enzymatic reactions oxidising and reducing NADPH have been characterised (chapter 2).

A second conclusion of the modelling studies was the unexpectedly high requirement for energy in the form of ATP for penicillin production (corresponding to 73 mole ATP per mole penicillin). Under most cultivation conditions, the required carbon and energy are usually provided by one source (in this case glucose). As the carbon source is an important cost factor for industrial production, chapter 3 describes investigations into whether co-feeding glucose-limited chemostat cultures of *P. chrysogenum* with an auxiliary energy substrate can increase penicillin yields. Auxiliary substrates are compounds that can solely be used for providing free energy and cannot act as a substrate for assimilation. Many studies have shown that this concept works for the production of biomass. However, whether feeding an auxiliary substrate would also work in the production of a secondary metabolite whose biosynthesis requires a large input of free energy has not previously been investigated. Formate was used as the additional energy source. Formate (HCOOH) can be oxidised by formate dehydrogenase to CO_2. This reaction requires the reduction of NAD to NADH, which could be used in the electron transport chain to generate ATP. Two scenarios can be devised for this extra energy. It can be used to increase the biomass yield on glucose with maintenance of the specific penicillin production rate, or the biomass yield could remain constant and the specific production rates go up. In both cases, the total penicillin yield on glucose would increase.

The work presented in the second half of this thesis was made possible by the availability of the genome sequence of *P. chrysogenum*. In order to improve their production strains, and also with the aim of finding new compounds, DSM decided to sequence the whole genome of the international lab standard Wisconsin54-1255. The genome and the Affymetrix GeneChips® were made available to us in Spring 2005, and allowed us to undertake a whole series of transcriptome studies of chemostat based cultivations of *P. chrysogenum*. The main aim of these studies was to identify key factors involved in penicillin production. As a central set up advantage was taken of the fact that in the absence of a side-chain precursor (i.e.

phenylacetic acid) no penicillin is produced. In this way, one can easily distinguish between non-producing and producing conditions and compare the changes. A disadvantage of this set up is that phenylacetic acid itself can induce effects other than the production of penicillin and it is impossible to distinguish between these effects. To circumvent this, a second strain was grown under the same conditions, in the presence and absence of the side-chain precursor. This second strain was derived directly from the high-producing strain, and should only lack the penicillin biosynthesis genes. Chapter 4 describes the results of the independent triplicate chemostat cultures and transcriptome data from these two strains under two conditions and shows how the overlaps in the transcriptome data were used to determine the effects of phenylacetic acid consumption and penicillin production.

The final chapter in this thesis concerns the fermentative production of the cephamycin precursor adipoyl-7-aminocarbamoylcephalosporanic acid. Like the fermentative production of adipoyl-7-aminodesacetoxycephalosporanic acid, the penicillin biosynthesis pathway has been extended with heterologous cephamycin biosynthesis genes. By introducing the *cefEF* gene from *A. chrysogenum* (encoding for expandase/hydroxylase activity) and the *cmcH* gene from *S. clavuligerus,* and by adding adipic acid to the feed, a high-producing *P. chrysogenum* strain has been engineered to produce a cephamycin. Chapter 5 describes the construction and performance of this strain in shake flasks. In addition, the strain and the observed adipic acid consumption have been characterised by means of chemostat cultivation and transcriptome analysis.

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Chapter 2

Enzymic analysis of NADPH metabolism in ß-lactam-producing *Penicillium chrysogenum*: presence of a mitochondrial NADPH dehydrogenase

Diana M. Harris, Jasper A. Diderich, Zita A. van der Krogt, Marijke A.H. Luttik, Léonie M. Raamsdonk, Roel A.L. Bovenberg, Walter M. van Gulik, Johannes P. van Dijken, Jack T. Pronk

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Abstract

Based on assumed reaction network structures, NADPH availability has been proposed to be a key constraint in ß-lactam production by *Penicillium chrysogenum*. In this study, NADPH metabolism was investigated in glucose-limited chemostat cultures of an industrial *P. chrysogenum* strain. Enzyme assays confirmed the $NADP^+$-specificity of the dehydrogenases of the pentose-phosphate pathway and the presence of $NADP^+$-dependent isocitrate dehydrogenase. Pyruvate decarboxylase/ $NADP^+$-linked acetaldehyde dehydrogenase and $NADP^+$-linked glyceraldehyde-3-phosphate dehydrogenase were not detected. Although the NADPH requirement of penicillin-G-producing chemostat cultures was calculated to be 1.4-1.6-fold higher than that of non-producing cultures, *in vitro* measured activities of the major NADPH-providing enzymes were the same. Isolated mitochondria showed high rates of antimycin A-sensitive respiration of NADPH, thus indicating the presence of a mitochondrial NADPH dehydrogenase that oxidises cytosolic NADPH. The presence of this enzyme in *P. chrysogenum* might have important implications for stoichiometric modelling of central carbon metabolism and ß-lactam production and may provide an interesting target for metabolic engineering.

Keywords

Penicillium chrysogenum; penicillin-G; NADPH; redox metabolism; chemostat cultures; metabolic engineering; respiratory chain; mitochondria

Abbreviations

αAAA	α-aminoadipic acid
LLD-ACV	L-α-aminoadipyl-L-cysteinyl-D-valine
PAA	phenylacetic acid
rcf	relative centrifugal force
TCA cycle	tricarboxylic acid cycle

Introduction

The filamentous fungus *Penicillium chrysogenum* is applied on a large scale for the production of ß-lactam antibiotics such as penicillin-G and penicillin-V. Over the past fifty years, classical strain improvement programs have increased penicillin productivity by more than 1000-fold [53]. Further improvement of ß-lactam production by *P. chrysogenum* via knowledge-based metabolic engineering will not only have to address the product formation pathway, but also its integration in the central metabolic network [53].

The Metabolic Engineering cycle consists of three essential parts: analysis, design and synthesis [34]. In extensively studied microorganisms such as *Escherichia coli* and *Saccharomyces cerevisiae*, design can often be based directly on an extensive body of scientific literature. However, for metabolic engineering of less studied organisms such as *P. chrysogenum*, analysing the structure of metabolic networks remains a primary research activity. Various stoichiometric models have been developed and conventional and ^{13}C-based metabolic flux analysis have been performed for quantitative analysis of the metabolic network of penicillin-producing *P. chrysogenum* [9,17,21,55,61]. The key precursors for penicillin production are the amino acids L-cysteine, valine and α-aminoadipic acid [2,51]. The knowledge on the biosynthetic routes of these amino acids in *P. chrysogenum* is sufficient for a reliable construction of this part of the metabolic models [13,22,27,36]. However, construction of stoichiometric models also requires detailed information on the fate of conserved moieties such as ATP, NADH and NADPH. The physiology of *P. chrysogenum* has been studied in much less detail than that of other laboratory organisms and a complete genome sequence is not yet available. Therefore, models and thus conclusions derived from these are based on experimental data for the yeast *S. cerevisiae* complemented with limited knowledge of the biochemistry of catabolic, anabolic and product pathways of *P. chrysogenum* [35]. In addition to assumptions on cofactor specificities, some models [17,21,55] include assumptions on 'elusive' parameters such as P/O ratio, growth and non-growth associated maintenance requirements [59,62].

Due to the intrinsic complexity of cofactor regeneration and the assumptions that have been made in the case of *P. chrysogenum*, cofactor mass balances are far from complete and may lead to erroneous flux estimates when included in metabolic models [60]. An approach to circumvent these problems is to use the ^{13}C-labelling technique, which is based on metabolic reactions that split and form carbon bonds in well-known ways. As discussed by van Winden *et al*. [59] this technique is not without its own pitfalls. Incomplete knowledge of the biochemical networks, catalytic properties of enzymes and their subcellular compartmentalisation may complicate interpretation of data from ^{13}C-labelling studies [59]. Despite these

challenges, several model-based analyses of the *P. chrysogenum* metabolic network led to the consistent conclusion that NADPH availability may be a major constraint in ß-lactam production. This conclusion is primarily based on a strong positive correlation between model-based estimates of intracellular NADPH demand under different conditions and the observed biomass-specific rates of ß-lactam production [17,52,55]. If NADPH availability does indeed limit ß-lactam productivity in industrial strains, this could lead to new and promising strategies for metabolic engineering. However, before embarking on such studies, it is relevant to verify some of the underlying assumptions.

The main uncertainties concerning NADPH metabolism of *P. chrysogenum* are the identity of the main NADPH-generating reactions and the question if and to what extent NADPH can be used in dissimilation in addition to its well-known roles in assimilation. Various metabolic modelling, ^{13}C-isotopomeric analysis and flux analysis studies under a variety of growth conditions have pointed to the pentose-phosphate pathway as a major source of NADPH [9,17,21,55,61]. However, biochemical information on possible other sources and sinks of NADPH in *P. chrysogenum* is lacking. In *S. cerevisiae* cells lacking glucose-6-phosphate dehydrogenase activity, $NADP^+$-linked acetaldehyde dehydrogenase activity is indispensable for provision of NADPH [15]. With respect to a possible role of NADPH in dissimilation, it is known that several yeasts, including *Kluyveromyces lactis* and *Candida utilis* can couple the oxidation of cytosolic NADPH to the respiratory chain via a mitochondrial NADPH dehydrogenase [40,57]. Mitochondrial NADPH dehydrogenase activity is also present in plants [31] and a gene encoding an mitochondrial NADPH dehydrogenase has recently been identified in the filamentous fungus *Neurospora crassa* [6]. Clearly, such a dissimilatory role of NADPH – which has hitherto not been included in stoichiometric models of *P. chrysogenum* – could have important implications for cofactor balancing. Moreover, such a system might compete for NADPH with ß-lactam synthesis and thus might provide an interesting target for metabolic engineering.

The goals of the present study were to verify key assumptions on sources and sinks of NADPH in previous modelling studies on *P. chrysogenum* and to investigate whether, in this fungus, NADPH is solely an assimilatory reduction equivalent or whether it can also serve a dissimilatory function via a respiratory-chain-linked NADPH dehydrogenase (Figure 1).

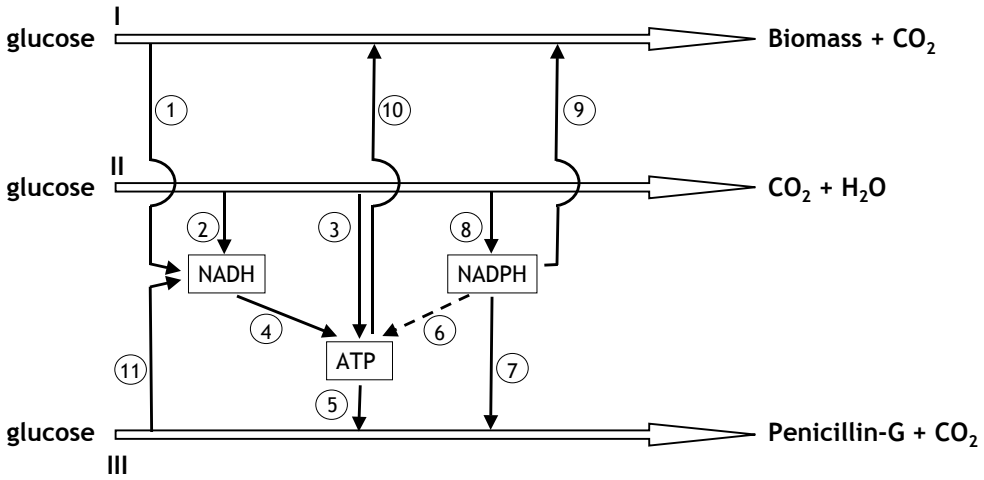

Figure 1: Fluxes of ATP and reducing equivalents in growing cells of *P. chrysogenum* that produce penicillin-G. I: assimilation of glucose to biomass; II: dissimilation of glucose via respiration; III: penicillin-G biosynthesis. 1. net production of NADH during assimilatory reactions, 2. production of NADH in dissimilatory reactions, 3. generation of ATP via substrate-level phosphorylation, 4. generation of ATP via mitochondrial oxidation of NADH, 5. ATP consumption for penicillin production, 6. NADPH oxidation via the mitochondrial respiratory chain, 7. NADPH consumption in penicillin synthesis, 8.production of NADPH via the pentose-phosphate pathway and $NADP^+$-linked isocitrate dehydrogenase, 9. consumption of NADPH during formation of amino acid, lipids and nucleic acids, 10. ATP consumption for synthesis of cell-monomers and their polymerisation, 11. NADH production in penicillin synthesis.

Materials and Methods

Strain

A high-yielding penicillin-G strain of *Penicillium chrysogenum* (code name DS17690) was obtained from DSM-Anti-Infectives (Delft, The Netherlands).

Media

The mineral medium used was set at pH 5.5 using KOH pellets and contained per litre of demineralised water 3.5 g $(NH_4)_2SO_4$, 0.8 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, and 10 mL of a trace element solution. This trace element solution contained 15 g·L^{-1} $Na_2EDTA{\cdot}2H_2O$, 0.5 g·L^{-1} $Cu_2SO_4{\cdot}5H_2O$, 2 g·L^{-1} $ZnSO_4{\cdot}7H_2O$, 2 g·L^{-1} $MnSO_4{\cdot}H_2O$, 4 g·L^{-1} $FeSO4{\cdot}7H_2O$, and 0.5 g·L^{-1} $CaCl_2{\cdot}2H_2O$ and was set at pH 6.0 by means of NaOH pellets. Production of penicillin-G was induced by adding 0.58 g·L^{-1} phenylacetic acid (PAA) to the medium.

Medium preparation

The appropriate amount of PAA was dissolved in demineralised water. After the pH was set to 6.5 with KOH pellets, the PAA solution was autoclaved for 40 min at 121 °C. All other components were dissolved in demineralised water and added to the PAA solution in the medium vessel via filter sterilisation with a sterile 0.2 µm capsule filter (Supor®DCF™; Pall Corporation, East Hills, USA). Glucose was heat-sterilised separately (110 °C) and added to the medium in a concentration of 7.5 $g \cdot L^{-1}$. After filter sterilisation and during the chemostat experiments the medium vessel was mixed continuously by a magnetic stirrer.

Chemostat cultivation

Aerobic glucose-limited chemostat cultivation was performed at 25 °C in 3 - L turbine stirred bioreactors (Applikon, Schiedam, The Netherlands) with a working volume of 1.8 L. The pH was maintained at 6.5 via automated addition of 2 M NaOH (ADI 1030 biocontroller, Applikon, Schiedam, The Netherlands). The fermenter was sparged with air at a flow rate of 0.9 $L \cdot min^{-1}$ using a Brooks mass-flow controller (Brooks Instruments, Hatfield, USA) and stirred at 750 rpm. The dissolved-oxygen concentration was continuously monitored with an oxygen electrode (Applisens, Schiedam, The Netherlands) and was always above 50% of air saturation. Continuous cultivation was initiated after 50-60 hours of batch cultivation. The addition of medium was controlled by a peristaltic pump and the dilution rate was set at 0.03 h^{-1}. Effluent was removed discontinuously by means of a special overflow device, which has been described previously [56]. The time interval between effluent removals was fixed in such a way that each time approximately 1 % of the culture volume was removed. To prevent excessive foaming, silicone antifoam (10 % vol/vol, BDH) was discontinuously added at timed intervals (6 seconds on, every 7 min, 1.0 $ml \cdot h^{-1}$).

Determination of culture dry weight

Culture samples (10 mL) were filtered over preweighed glass fibre filters (Type A/E, Pall Life Sciences, East Hills, USA). The filters were washed with demineralised water and dried for 20 minutes at 600 W in a microwave oven and subsequently weighed.

Substrate and metabolite analysis

Glucose concentrations in the medium were determined by HPLC analysis with an Aminex HPX-87H column (Biorad, Hercules, USA) at 60 °C with 5 mM H_2SO_4 as the mobile phase. Phenylacetic acid and penicillin-G concentrations were determined

by isocratic HPLC analysis with a Platinum EPS C18 column (Alltech, Deerfield, USA) at 30 °C. The mobile phase consisted of 5 M acetonitrile with 5 mM KH_2PO_4 and 6 mM H_3PO_4.

Gas analysis

The exhaust gas of chemostat cultures was cooled in a condenser and dried with a Perma Pure dryer (type MD-110-48P-4, Perma pure, Toms River USA). Oxygen and carbon dioxide concentrations were determined with a NGA 2000 analyser (Rosemount Analytical, Orville, USA). Off-gas rates were determined from an average of 10 measurements using a SAGA digital flow meter (Ion Science, Cambridge, UK). Specific rates of carbon dioxide and oxygen consumption were calculated as described previously [58].

Subcellular fractionation

Mitochondria were isolated from glucose-limited chemostat cultures by a procedure based on that described previously for the yeasts *Saccharomyces cerevisiae* and *Kluyveromyces lactis* [5,26,40]. Approximately 1.5 g dry weight was harvested by filtration over a glass fibre filter. Each filter was washed with 100 mL of a potassium phosphate buffer (50 mM, pH 7.5). The pellets were pooled and resuspended in buffer A (50 mM potassium phosphate, pH 7.5, 2.5 mM $MgCl_2$) containing 2 M sorbitol in a final volume of 35 mL. To this suspension, 35000 units of Lyticase (from *Arthrobacter luteus*, >3000U·(mg protein)$^{-1}$, Sigma-Aldrich), dissolved in 0.5 mL of the same buffer, were added. The suspension was subsequently incubated under gentle shaking for 35 min at 25 °C. This incubation period resulted in sufficiently susceptible spheroplasts for controlled cell lysis. All subsequent steps were performed on ice or in a cooled (4 °C) centrifuge. Spheroplasts were harvested by centrifugation (10 min, 3000 rcf). To remove the Lyticase, the pellet was washed in the same buffer (5 min, 3000 rcf) and resuspended to a final volume of 20 mL of buffer A. Subsequently 60 mL of buffer B (50 mM potassium phosphate, pH 7.5, 2.5 mM $MgCl_2$, 1 g·L^{-1} BSA) containing 0.2 M sorbitol was added drop-wise while the suspension was slowly stirred with a magnetic stirrer bar. The spheroplasts were broken by 10 strokes in a Potter-Elvehjem homogeniser (100 rpm, clearance 28 µm). After centrifugation (5 min, 3000 rcf) the homogenate was separated from the whole cells and debris and centrifuged again (10 min, 12000 rcf). The resulting pellet containing mitochondria and other organelles was resuspended in 5 mL of buffer B containing 0.65 M sorbitol and stored on ice. Protein concentrations of the fractions were determined according to the Lowry method [25] and corrected for sorbitol interference and bovine serum albumin present in the pellet buffer. Bovine serum albumin (fatty acid free; Sigma) was used as a standard. The success of subcellular fractionation was verified by measuring glucose-6-phosphate dehydrogenase and

citrate synthase in all fractions in the presence and absence of 0.5 % (vol/vol) triton X-100.

Oxygen uptake studies with mitochondrial preparations

Oxygen consumption by isolated mitochondria was measured at 25 °C with a Clark-type oxygen electrode. The assay mixture contained 50 mM potassium phosphate buffer (pH 7.5), 5 mM $MgCl_2$ and 0.65 mM sorbitol (4 mL final volume). Reactions were started with succinate (5 mM), glycerol-3-phosphate (5 mM) or L-malate plus pyruvate (5 mM each). As commercial preparations of NADH and NADPH are contaminated with ethanol [41], NADH was generated *in situ* by addition of 5 mM glucose, 0.2 mM NAD^+ and 5 U glucose dehydrogenase (EC 1.1.1.47, from *Bacillus megaterium*). NADPH was generated by addition of glucose-6-phosphate (2.5 mM), $NADP^+$ (0.2 mM) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49, from yeast). Oxygen uptake rates were calculated via a dissolved oxygen concentration of 0.26 mM in air-saturated buffer at 25 °C. Respiratory control values were determined by adding 0.25 mM ADP. The sensitivity of oxygen consumption to inhibitors of the respiratory chain was measured by adding 1.2 µM antimycin A (from a 0.5 mM stock solution in methanol) or 1 mM potassium cyanide. Addition of methanol alone did not significantly affect respiration.

Preparation of cell extracts

Cells were harvested from glucose-limited chemostat cultures (approximately 0.06 g dry weight) by centrifugation (10 min, 4600 rcf). The pellet was washed with (10 min, 4600 rcf) and resuspended in a 10 mM potassium phosphate buffer with 2 mM EDTA (pH 7.5) and stored at –20 °C until further use. Upon defrosting the pellet was washed in a 100 mM potassium phosphate buffer with 2 mM $MgCl_2$ (4 °C) and subsequently resuspended in 5 mL of the same buffer containing 1 mM 1,4-dithiothreitol (DTT). Cells were disrupted using a pre-cooled (4 °C) cell disrupter from Constant Systems Ltd (Daventry, UK) at 1.5 kbar with three repetitive passages. The cell extract was separated from the cell debris by centrifugation (20 min, 48000 rcf). The supernatant was stored on ice.

Enzyme assays

Enzyme activities were determined with an Hitachi model 100-60 spectrophotometer (Tokyo, Japan) at 25 °C in an assay mixture of 1 mL by measuring the (dis)appearance of NAD(P)H at 340 nm (ε = 6.3 $mM^{-1} \cdot cm^{-1}$). Each dehydrogenase was tested for specificity for both cofactors in identical concentrations; the preferred cofactor (NAD^+ or $NADP^+$) is included in the protocol. To test linearity of the assay, all reactions were carried out with two concentrations of cell extract. In those cases

in which a particular activity could not be detected either a commercial enzyme or another cofactor was added to check that the reaction conditions were suitable for determination of the enzyme activity. Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) was assayed in a reaction mixture containing 50 mM Tris-HCl pH 8.0, 5 mM $MgCl_2$, 0.8 mM $NADP^+$ and cell extract. The reaction was started with 10 mM glucose-6-phosphate. Isocitrate dehydrogenase activity (EC 1.1.1.41; 1.1.1.42) was assayed in a reaction mixture containing 100 mM potassium phosphate buffer pH 7.0, 2.5 mM $MgCl_2$, 0.5 mM AMP, 0.4 mM $NADP^+$ and cell extract. The reaction was started by 5 mM D,L-isocitrate. Glutamate dehydrogenase (EC 1.4.1.2, 1.4.1.3) was measured in an assay containing 50 mM Tris-HCl pH 8.5, 1 mM EDTA, 250 mM ammonium acetate, 0.15 mM NADPH and cell extract. The assay was started with 10 mM α-ketoglutarate. Malate dehydrogenase (EC 1.1.1.37) was assayed in a reaction containing 100 mM potassium phosphate buffer, pH 8.0, 0.15 mM NADH and cell extract. The reaction was started with 1 mM oxaloacetate. Malic enzyme (EC 1.1.1.40) was assayed in a reaction mixture containing 100 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 0.4 mM $NADP^+$ and cell extract. The reaction was started with 100 mM L-malate, pH 7.5. 6Phosphogluconate dehydrogenase (EC1.1.1.44) was assayed in a mixture containing 50 mM glycyl-glycine buffer, pH 8.0, 0.8 mM $NADP^+$ and cell extract. The reaction was started with 4 mM 6-phosphogluconate. Acetaldehyde dehydrogenase (EC 1.2.1.10) was measured in a reaction mix containing 100 mM potassium phosphate buffer pH 8.0, 15 mM pyrazole, 0.4 mM 1,4 dithiothreitol (DTT), 10 mM KCl, 0.4 mM NAD^+ and cell extract. The reaction was started with 0.1 mM freshly prepared acetaldehyde. Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12, 1.2.1.13) was assayed in a reaction mix containing 100 mM triethanolamine, pH 7.6, 1 mM ATP, 1 mM EDTA, 1.5 mM $MgSO_4$, 0.15 mM NADH, 22.5 U 3phosphoglycerate kinase (in 3 M $(NH_4)_2SO_4$, from yeast, EC 2.7.2.3) and cell extract. The reaction was started with 5 mM 3-phosphoglyceric acid. Pyruvate decarboxylase (EC 4.1.1.1) was measured in a reaction mixture containing 40 mM imidazole-HCl, pH 6.5, 5 mM $MgCl_2$, 0.2 mM thiamine pyrophosphate chloride, 0.15 mM NADH, 88 U alcohol dehydrogenase (lyophilisate from yeast, EC 1.1.1.1) and cell extract. The reaction was started with 50 mM pyruvate. Citrate synthase (EC 2.3.3.1) was measured at 412 nm (ε = 13.6 $mM^{-1} \cdot cm^{-1}$) in a mixture containing 100 mM Tris-HCl pH 8.0, 1 mM acetyl-coenzyme A, 80 mM potassium chloride, 0.1 mM 5,5'-dithio-bis(2-nitrobenzoic acid) in Tris-HCl (DTNB) and cell extract. The reaction was started with 0.2 mM oxaloacetic acid. Glucose oxidase (EC 1.1.3.4) was measured in the supernatant at 420 nm (ε = 43.2 $mM^{-1} \cdot cm^{-1}$) in a mixture containing 50 mM potassium phosphate buffer, pH 5.5, 10 $U \cdot mL^{-1}$ peroxidase (lyophilisate from horse radish, EC 1.11.1.7), 10 $U \cdot mL^{-1}$ 2,2'-azino-bis(3-ethyl-benzthiazoline-6-sulfonic acid) (ABTS) and supernatant. The reaction was started with 100 mM glucose. Protein

concentrations were determined according to the Lowry method [25]. Bovine serum albumin (fatty acid free; Sigma) was used as a standard.

Electron microscopy

Mitochondria were fixed in 6 % (vol/vol) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 60 min at 0 °C, followed by post-fixation in a mixture of 0.5 % (wt/vol) OsO_4 and 2.5 % (wt/vol) $K_2Cr_2O_7$ in the cacodylate buffer for 90 min at 0 °C. Samples were embedded in Epon 812 after dehydration in a graded ethanol series. Ultrathin sections were examined in a Philips CM10 Transmission Electron Microscope.

Results

Calculation of NADPH turnover in relation to penicillin-G productions

Penicillium chrysogenum DS17690 was grown in aerobic, glucose-limited chemostat cultures at a dilution rate of 0.03 h^{-1}. Production of penicillin-G was induced by the addition of phenylacetic acid (PAA) to the feed medium (Table 1). Omission of PAA resulted in the absence of penicillin-G production, however, several by-products were formed such as isopenicillin-N, 6-amino-penicillanic acid (6-APA), 8-hydroxypenillic acid (8-HPA) and 6-oxopiperidine-2-carboxylic acid (OPC) [54]. Consistent with earlier reports [54], production of penicillin-G was accompanied by a significant reduction of the biomass yield relative to non-producing chemostat cultures (Table 1).

	Non-producing	Producing	p-value
Biomass yield (g biomass·(g glucose)$^{-1}$)	0.38 ± 0.00	0.35 ± 0.01	0.006
q_{CO2} (mmol·(Cmol biomass)$^{-1}$·h^{-1})	32.8 ± 1.8	40.5 ± 6.4	0.120
q_{O2} (mmol·(Cmol biomass)$^{-1}$·h^{-1})	35.0 ± 4.9	42.4 ± 8.7	0.270
q_{pen} (mmol·(Cmol biomass)$^{-1}$·h^{-1})	0.00 ± 0.00	0.56 ± 0.03	0.000

Table 1: Growth parameters of *P. chrysogenum* under producing and non-producing conditions in aerobic, glucose-limited chemostat cultures grown at a dilution rate of 0.03 h^{-1}. Results are the averages ± S.D (σ_{n-1}) of three independent chemostat cultures. Calculations are based on a molecular weight of biomass of 28.00 g·$Cmol^{-1}$ [55]. P-values indicate the significance level as determined by a two-tailed, equal variance t-test, p-values ≤ 0.05 were considered significant.

Previous experimental and theoretical analysis of ß-lactam production by *P. chrysogenum* identified NADPH availability as a possible constraint in product formation [17,21,55]. Therefore, prior to an enzymic analysis of NADPH metabolism, we estimated the *in vivo* turnover rates of NADPH in the chemostat cultures. An important factor in the overall NADPH requirement for penicillin-G production from glucose, sulfate and ammonia is the pathway for L-cysteine biosynthesis. Two routes have been reported in fungi, the direct sulfhydrylation and the transsulfuration route [3], which require 5 or 8 moles NADPH per mole of L-cysteine, respectively. Since the contribution of each of these two pathways to *in vivo* L-cysteine production is unclear, the NADPH requirement for penicillin-G production cannot be precisely calculated.

Based on published biomass composition data for *P. chrysogenum* [55], the stoichiometric NADPH demand for biomass formation was calculated to be 0.287 mol·(Cmol biomass)$^{-1}$ and 0.286 mol·(Cmol biomass)$^{-1}$ for scenarios in which L-cysteine was synthesised via the transsulfurylation route and via the direct sulfhydrylation route, respectively. The corresponding NADPH requirements for penicillin-G biosynthesis are 10 mol·(mol penicillin)$^{-1}$ and 7 mol·(mol penicillin)$^{-1}$, respectively [10].

Based on the experimentally observed biomass and penicillin-G yields (Table 1) it was calculated that the NADPH turnover of penicillin-G-producing cultures was 40-60 % higher than that of non-producing cultures (Table 2). These calculations were based on the assumption that, in *P. chrysogenum*, NADPH is exclusively used as an electron donor in assimilatory processes and cannot be used in dissimilation.

	Non-producing	Producing	Producing / Non-producing
Direct sulfhydrylation	0.016	0.022	1.4
Transsulfuration	0.016	0.025	1.6

Table 2: Theoretical required NADPH fluxes (µmol·(mg protein)$^{-1}$·min^{-1}) in non-producing and producing cultures as affected by the biosynthetic route of L-cysteine. Biomass compositions as reported previously were used for the calculations, i.e. molecular weight of biomass 28.00 g·Cmol^{-1}, protein content 33 % (w/w) and cysteine content in protein 0.46 mol·mol^{-1} [55].

Absence of glucose oxidase activity in glucose-limited chemostat cultures

It is generally assumed that glucose metabolism in *P. chrysogenum* proceeds via glycolysis as the main catabolic pathway and that the pentose-phosphate pathway acts as a route for the provision of NADPH and pentose phosphates. However, at least in theory, glucose can also be oxidised extracellular via glucose oxidase

(EC 1.1.3.4). δ-Gluconolactone and gluconic acid thus formed may be taken up, phosphorylated and then further metabolised. Induction of glucose oxidase requires high concentrations of glucose [23,33,45] and therefore the synthesis of this enzyme is unlikely to occur in glucose-limited chemostat cultures. Indeed, glucose-oxidase activity was neither detected in cell extracts nor in culture supernatants, thus ruling out a bypass of the NADPH-providing reactions of the pentose-phosphate pathway via this direct oxidative pathway.

Activities of key $NADP^+$ – dependent enzymes in cell extracts

The increased cellular demand for NADPH in penicillin-G-producing chemostat cultures requires an increased flux through pathways that supply this reduction equivalent. Activities of both glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were detected in cell extracts of the *P. chrysogenum* chemostat cultures. In addition, a $NADP^+$-dependent isocitrate dehydrogenase was detected (Table 3). Activities of pyruvate decarboxylase and $NADP^+$-dependent acetaldehyde dehydrogenase were negligible. $NADP^+$-linked glyceraldehyde-3-phosphate dehydrogenase activity was also absent. Glutamate dehydrogenase, which acts as a major sink of NADPH, also exhibited a weak activity for NADH (5 % of that of NADPH, corresponding to less than 0.01 μmol·(mg protein)$^{-1}$·min^{-1}). In cell extracts, no NAD-dependent activities were detected for glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and isocitrate dehydrogenase.

To investigate whether the increased rates of NADPH turnover in the penicillin-G-producing cultures (Table 2) were accompanied by increased levels of $NADP^+$-dependent dehydrogenases, we analysed the activities of these enzymes in producing as well as non-producing cultures. No significant differences in the specific activities of these enzymes were found (Table 3).

Isolation of respiration-competent mitochondrial preparations

Procedures for isolation of respiration-competent mitochondria from yeasts and filamentous fungi invariably involve enzymic degradation of the cell wall, followed by controlled lysis of the resulting spheroplasts. Critical parameters for the isolation of functional mitochondria are nature and osmotic value of the buffer system, the lysing enzyme, pre-treatment of the cells with reducing agents and the disruption conditions [4,5,24,37,40,46]. In contrast to the yeasts *Saccharomyces cerevisiae* and *Kluyveromyces lactis* [26,40], the cell wall of chemostat-grown *P. chrysogenum* was not sufficiently susceptible to the lysing enzyme Zymolyase (from *Arthrobacter luteus*, ICN Biochemicals). Also Glucanex (from *Trichoderma harzianum,* Sigma-Aldrich) did not yield efficient spheroplast formation under these conditions (results not shown). The lysing enzyme Lyticase from *Arthrobacter luteus* has

been used to isolate functional mitochondria in yeast [7,49]. It also resulted in rapid spheroplast formation with *P. chrysogenum* (Figure 2). An optimum between spheroplast formation and premature lysis was obtained by evaluating different Lyticase concentrations. As phosphate is essential for respiratory control [19], the complete isolation procedure was performed in a potassium phosphate buffer.

Enzyme	Non-producing	Producing
Glucose-6-phoshate dehydrogenase	0.28 ± 0.02	0.29 ± 0.05
6-Phosphogluconate dehydrogenase	0.24 ± 0.03	0.23 ± 0.02
Isocitrate dehydrogenase	0.06 ± 0.01	0.06 ± 0.02
Glutamate dehydrogenase	0.23 ± 0.02	0.21 ± 0.07
Acetaldehyde dehydrogenase	<0.005 ± 0.00	<0.005 ± 0.00

Table 3: Specific activities (μmol·(mg protein)$^{-1}$·min^{-1}) of enzymes possibly involved in the oxidation and reduction of NADP(H). Cells were grown in aerobic, glucose-limited chemostat cultures at a dilution rate of 0.03 h^{-1} under producing and non-producing conditions. All enzymes were assayed in the presence of NADP(H). Experimental results are the averages ± S.D (σ_{n-1}) of two independent chemostat cultures.

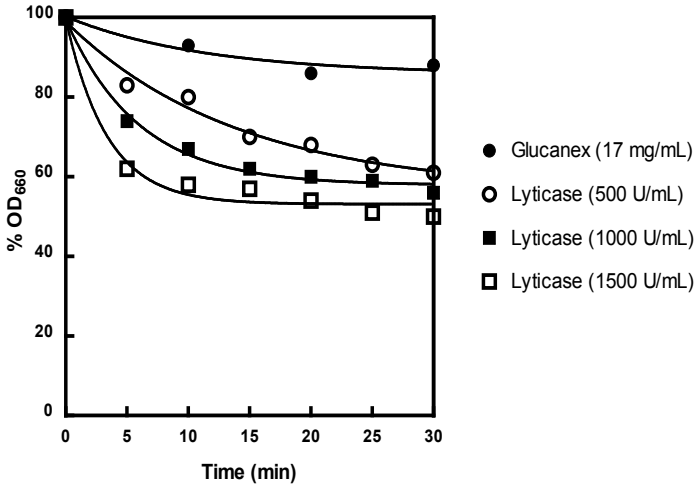

Figure 2: Spheroplast formation by Glucanex (Sigma) and Lyticase (Sigma) at different concentrations. Spheroplast formation was measured as the decrease in optical density due to lysis in water.

Enzyme	Fraction	Non-producing			Producing		
		Specific activity	Total activity	Relative activity	Specific activity	Total activity	Relative activity
Glucose-6-phosphate dehydrogenase	Homogenate	0.10	17964	100 %	0.15	21523	100 %
	Cytosolic fraction	0.10	17069	95 %	0.14	17916	83 %
	Particulate fraction	0.00	317	2 %	0.05	286	1 %
Citrate synthase	Homogenate	0.01	3990	100 %	0.01	4299	100 %
	Cytosolic fraction	0.00	0	0 %	0.00	0	0 %
	Particulate fraction	0.48	7838	196 %	0.35	4618	107 %

Table 4: Glucose-6-phosphate dehydrogenase and citrate synthase activities in the three fractions of a mitochondria isolation. Activities were determined after disruption of the organelles by 0.5% triton X-100. Specific activities in $\mu mol \cdot mg^{-1} \cdot min^{-1}$. Note that the activity of citrate synthase is enriched in the mitochondrial fractions.

Localisation studies of glucose-6-phosphate dehydrogenase as a cytosolic marker and citrate synthase as a mitochondrial marker [38], showed that the cytosolic and particulate fractions were sufficiently separated. Less than 2% of the glucose-6-phosphate-dehydrogenase activity in spheroplast homogenates was present in the particulate fraction, showing that contamination of the particulate fraction by cytosol was negligible. Similarly, no citrate synthase activity was found in the cytosolic fraction, thus indicating the absence of contamination of the cytosol by mitochondria (Table 4). Citrate synthase could only be detected in the particulate fraction upon disruption of the particular fraction with 0.5% triton X-100, which indicates that the mitochondria present in the particulate fraction were intact. This is further supported by electron micrographs that showed intact membranes (Figure 3). Due to the complexity and relatively low yields of the procedure, the particulate fractions were not further purified but used directly for quantification of respiratory activities.

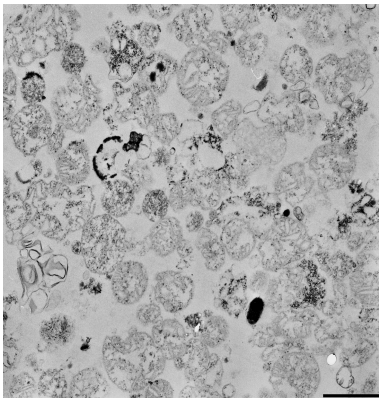

Figure 3: Electron micrograph of mitochondria from cells grown in aerobic, glucose-limited chemostat cultures. The bar represents 1 µm.

Respiratory activities of crude mitochondrial preparations

The mitochondrial preparations exhibited succinate-dependent uptake of oxygen, with a high degree of respiratory control (i.e. increase of respiration rates after addition of ADP, [8]. Succinate-dependent respiration was completely blocked by 1 mM KCN, an inhibitor of cytochrome c oxidase (complex IV) (Figure 4).

Substrate-dependent oxygen uptake was also observed with exogenous NADH and NADPH, but with a low degree of respiratory control (Table 5). To confirm that oxidation of NADPH occurred via mitochondrial respiration chain rather than via, for example, soluble oxidoreductases [1,43,44] the effect of antimycin A, a

specific inhibitor of the bc1-complex (complex III), on the oxidation of NADPH was investigated. NADPH-dependent oxygen uptake was completely inhibited by 1.2 µM antimycin A, thus confirming that the NADPH-dependent oxygen uptake was the result of mitochondrial respiration.

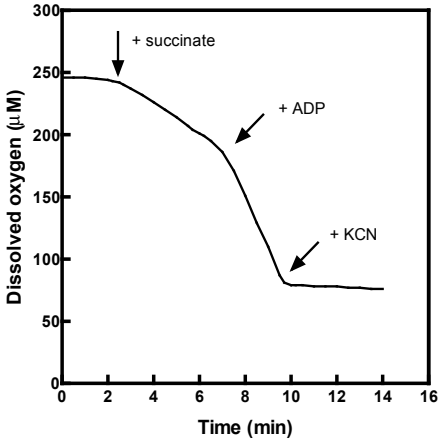

Figure 4: Oxygen uptake, determined by a Clark-type oxygen electrode, by isolated mitochondria of *P. chrysogenum* upon the addition of succinate as substrate. Respiratory control is evident from the increase in oxygen uptake rate upon addition of 0.25 mM ADP. Respiration is completely blocked by 1 mM potassium cyanide (KCN).

The mitochondrial preparations also exhibited low respiration rates towards glycerol-3-phosphate and a mixture of pyruvate and malate. The latter combination of substrates generates NADH in the mitochondrial matrix [39]. Substrate-dependent oxygen uptake rates by mitochondria from cultures producing penicillin-G were higher than those of mitochondria from non-producing cells (Table 5). While this seems consistent with the higher *in vivo* rates of oxygen uptake by the penicillin-G producing chemostat cultures (Table 1), the possibility cannot be excluded that this difference originates from the isolation procedure. The relative activities for the different substrates were similar in crude mitochondrial preparations isolated from penicillin-G producing and non-producing cells. NADH and NADPH resulted in the highest oxygen consumption rates, followed by succinate, and only low respiration rates were observed with glycerol-3-phosphate and a mixture of malate and pyruvate (Table 5).

Substrate	Non-producing			Producing		
	Respiration rate	Respiratory control	n	Respiration rate	Respiratory control	n
NADH	0.06 ± 0.02	1.0 ± 0.1	4	0.11 ± 0.02	1.2 ± 0.1	2
NADPH	0.05 ± 0.00	1.1 ± 0.1	2	0.12 ± 0.02	1.0 ± 0.0	3
Glycerol-3-phosphate	0.01 ± 0.00	0.9 ± 0.0	2	0.03 ± 0.01	1.0 ± 0.1	2
Succinate	0.03 ± 0.01	2.2 ± 0.3	2	0.06 ± 0.04	2.6 ± 0.6	3
Malate + pyruvate	< 0.01 ± 0.00	1.1 ± 0.1	2	0.03 ± 0.01	1.9 ± 0.5	2

Table 5: Specific rates of substrate-dependent oxygen consumption as μmol·(mg protein)$^{-1}$·min^{-1} by isolated mitochondria of mycelium from producing and non-producing cultures of *P. chrysogenum*. Mitochondria were isolated from aerobic, glucose-limited chemostat cultures under producing and non-producing conditions (dilution rate: 0.03 h^{-1}). The respiration rates (μmol·(mg protein)$^{-1}$·min^{-1}) were measured in the presence of 0.25 mM ADP. Respiratory control values represent the ratio of respiration rates in the presence and absence of ADP. n represents the number of independent chemostat cultures used for the respective studies. Experimental results are the averages ± S.D (σ_{n-1}) of the number of measurements.

Discussion

A first key objective of this study was to verify several assumptions on NADPH-providing reactions in earlier studies on stoichiometric modelling of the metabolic network of *Penicillium chrysogenum* [17,21,55]. In general, these assumptions were shown to be correct: the dehydrogenases of the pentose-phosphate pathway are NADP$^+$-dependent and NADP$^+$-dependent isocitrate dehydrogenase may act as an additional source of NADPH.

Two potential alternative sources of NADPH, an NADP$^+$-dependent glyceraldehyde-3-phosphate dehydrogenase recently found in the yeast *Kluyveromyces lactis* [63] and an NADP$^+$-dependent pyruvate-dehydrogenase bypass as occurring in *Saccharomyces cerevisiae* [42], were not detected under the experimental conditions. In cell extracts, we found only trace activities of NADP$^+$-dependent malic enzyme (EC 1.1.1.40), even though this enzyme has been reported to be present in filamentous fungi [64,65]. Various enzymes in polyol metabolism may act with NADP$^+$ as a cofactor, including glycerol dehydrogenase (EC 1.1.1.72) and mannitol dehydrogenase (EC 3.1.3.22) [20]. A mannitol cycle has been postulated to play an important role in various fungi for regeneration of NADPH, at the expense of NADH and ATP [18]. However, increasing the NADPH demand in the cell did not lead to increased activity of this cycle, whereas other NADPH providing reactions did increase in activity [47]. Moreover, since mannitol is excreted by *P. chrysogenum* [16] it seems unlikely that a mannitol cycle is operative in *P. chrysogenum*.

NADPH turnover rates were estimated to differ by maximally 40 – 60 % in penicillin-G-producing and non-producing chemostat cultures (Table 2). This change

was not reflected by increased *in vitro* activities of the major $NADP^+$-dependent dehydrogenases in cell extracts (Table 3). Apparently, any increased *in vivo* activity of these enzymes was not caused by an up-regulation of the corresponding genes. Instead, an increased rate of NADPH turnover is probably achieved by changing levels of substrates, products and/or effectors of these dehydrogenases. This is relevant for metabolic engineering of ß-lactam production, as it implies that increased fluxes through the product formation pathway will affect metabolite levels in central metabolism.

For a further analysis of NADPH metabolism, a protocol was developed for isolation of respiratory competent mitochondria from *P. chrysogenum* chemostat cultures. Respiration by crude mitochondrial fractions was completely inhibited by antimycin A and potassium cyanide, a specific inhibitor of complex III and complex IV, respectively. Sensitivity to antimycin A and potassium cyanide is commonly taken as evidence for the absence of an alternative oxidase [7,50], although caution should be taken with calculations of specific activities of this enzyme via such inhibition studies [11,30]. The complete inhibition of respiration by antimycin A and potassium cyanide indicates that, under the growth conditions applied, an alternative oxidase is not expressed in *P. chrysogenum*.

In vivo analysis of the distribution of glucose-6-phosphate over glycolysis and pentose-phosphate pathway has yielded unexpectedly high *in vivo* fluxes through the pentose-pathway (up to 75 %, [9]). In fact it was concluded that the flux through the pentose-phosphate pathway was higher than the calculated demand for NADPH in biosynthesis [9]. This observation already suggested the involvement of an unknown, probably non-biosynthetic mechanism for oxidation of NADPH [53]. In the present study, a mitochondrial respiratory-chain linked NADPH dehydrogenase is identified as a very strong candidate for this role. In contrast to mammalian mitochondria, which are unable to directly oxidize cytosolic NADH and NADPH [6,32,40], mitochondria from plants and from several fungi contain 'external' NADH and/or NADPH dehydrogenases that link the oxidation of cytosolic NAD(P)H to the respiratory chain. The present study clearly demonstrates that, similar to *K. lactis* [14,40] and *Neurospora crassa* [28,29], *P. chrysogenum* mitochondria contain an external NADPH dehydrogenase, the activity of which is completely inhibited by the complex III inhibitor antimycin A.

The presence of a mitochondrial NADPH dehydrogenase might have important implications for metabolic engineering of *P. chrysogenum*. Firstly, this enzyme could allow NADPH to fulfil a dual function: (i) electron donor for assimilatory reactions (i.e., biomass and ß-lactam biosynthesis) and (ii) electron donor for dissimilation. Hitherto, modelling of central metabolism in *P. chrysogenum* has not included this second role. This puts an upper limit to the activity of the oxidative pentose-phosphate pathway, similar to the situation in the yeast *S. cerevisiae*, which lacks

a mitochondrial NADPH dehydrogenase [12,48,57]. Thus, the presence of this activity could provide a potential complication in metabolic modelling of ß-lactam-producing *P. chrysogenum*. As an example: involvement of NADPH dehydrogenase in *in vivo* NADPH oxidation could imply that the relative difference of NADPH turnover in penicillin-G producing and non-producing cultures is lower than the 40 – 60 % calculated in the results section.

A second implication for metabolic engineering is that mitochondrial NADPH dehydrogenase may compete for cytosolic NADPH with ß-lactam biosynthesis. Given the high NADPH requirement of penicillin-G biosynthesis, this would suggest the mitochondrial NADPH dehydrogenase of *P. chrysogenum* to be a potentially relevant target for further studies. This work would require identification of the structural gene encoding for NADPH dehydrogenase.

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Chapter 3

Formate as an auxiliary substrate for glucose-limited cultivation of *Penicillium chrysogenum*: impact on penicillin-G production and biomass yield

Diana M. Harris, Zita A. van der Krogt, Walter M. Van Gulik,
Johannes P. van Dijken, Jack T. Pronk

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Abstract

Production of ß-lactams by the filamentous fungus *Penicillium chrysogenum* requires a substantial input of ATP. During glucose-limited growth this ATP is derived from glucose dissimilation, which reduces the product yield on glucose. The present study has investigated whether penicillin-G yields on glucose can be enhanced by co-feeding of an auxiliary substrate that acts as an energy source, but not as a carbon substrate. As a model system, a high-producing industrial strain of *P. chrysogenum* was grown in chemostat cultures on mixed substrates containing different molar ratios of formate and glucose. Up to a formate-to-glucose ratio of 4.5 mol·mol^{-1}, an increasing rate of formate oxidation via a cytosolic NAD^+-dependent formate dehydrogenase increasingly replaced the dissimilatory flow of glucose. This resulted in increased biomass yields on glucose. Since, at these formate-to-glucose ratios, the specific penicillin-G production rates remained constant, the volumetric productivity increased. Metabolic modelling studies indicated that formate transport in *P. chrysogenum* does not require input of free energy. At formate-to-glucose ratios above 4.5 mol·mol^{-1}, the residual formate concentrations in the cultures increased, probably due to kinetic constraints in the formate-oxidizing system. The accumulation of formate coincided with a loss of the coupling between formate oxidation and the production of biomass and penicillin-G. These results demonstrate that, in principle, mixed-substrate feeding can be used to increase the yield on carbon source of 'assimilatory' products such as ß-lactams.

Introduction

The filamentous fungus *Penicillium chrysogenum* is applied on a large scale (> 60,000 t·y^{-1}, [8,32]) for the industrial production of ß-lactam antibiotics such as penicillin-G and penicillin-V and for the production of the cephalosporin precursor adipoyl-7-ADCA. ß-lactam antibiotics are formed in a multi-step process in which the first two steps are common for penicillins and cephalosporins. The three amino acids cysteine, valine and α-aminoadipic acid, derived from central metabolism, are condensed to form the tripeptide ACV (α-aminoadipyl-cysteinyl-valine). The next step is a ring closure that leads to the characteristic penam structure of isopenicillin-N (IPN), the branch point intermediate at which penicillin biosynthesis diverges from cephalosporin biosynthesis. Penicillin-G is formed from IPN by exchanging its α-aminoadipic acid side chain for phenylacetic acid, using phenylacetyl-CoA as side-chain donor.

Overproduction of secondary metabolites can have a large impact on central metabolism if it requires significant amounts of carbon precursors, reducing equivalents (NADH and NADPH) and free energy equivalents (ATP). Previous studies on penicillin-G production in a high-producing industrial strain of *P. chrysogenum* have shown that constraints in central metabolism may reside in the supply and regeneration of the cofactor NADPH rather than in the supply of the carbon precursors, α-aminoadipic acid, cysteine and valine [35]. Moreover, a careful model-based analysis of chemostat data revealed that penicillin-G production in this strain appeared to be associated with an unexpectedly high additional energy dissipation (corresponding to 73 moles of ATP per mole penicillin-G) [34].

In glucose-limited, penicillin-G producing cultures of *P. chrysogenum,* three main carbon flows can be distinguished (Figure 1): (i) dissimilation of glucose to provide free energy equivalents and reducing power, (ii) assimilation of glucose to cell material and (iii) production of penicillin-G via its carbon precursors (Figure 1). These three flows are linked via closed balances of the conserved moieties NAD^+/NADH, $NADP^+$/NADPH and ATP/ADP/AMP. As indicated in Figure 1, this situation implies that biomass formation and penicillin-G production compete for glucose, NADPH and ATP.

Chemo-organoheterotrophic microorganisms such as *P. chrysogenum* use organic substrates such as glucose both as a carbon source and as a source of free energy. Since part of the glucose has to be used for the generation of ATP equivalents in dissimilation, it can be called an energy-deficient substrate [4]. Due to this intrinsic free energy deficiency, experimentally obtained biomass yields on glucose are generally lower than the maximum biomass yield that could be theoretically reached when assimilatory reactions are fully optimised [5,9].

Many previous studies have demonstrated that co-feeding with an auxiliary energy substrate can compensate for the energy deficiency of carbon substrates. Auxiliary substrates are compounds that can be dissimilated to provide free energy requirements, but cannot be used as a carbon source for growth. An increase of the carbon-source-to-biomass conversion efficiency has been demonstrated for many combinations of carbon sources and auxiliary substrates, including acetate/thiosulfate [13], acetate/formate [10] and glucose/formate [9,12,15]. Formate is a suitable model auxiliary energy substrate for yeasts and fungi, as many of these organisms contain an NAD^+-linked formate dehydrogenase (EC1.2.1.1, FDH) that catalyses the oxidation of formate to CO_2, but are unable to assimilate formate [3,4,10,16,26,31,35]. The NADH formed in the FDH reaction can be coupled to the mitochondrial respiratory chain and thus lead to ATP production.

The stoichiometry of this process (P/O ratio) depends on the subcellular localization of FDH. In most eukaryotes, FDH appears to be a cytosolic enzyme [9,26], with the notable exception of plants where it is located in the mitochondria

and chloroplasts [17,23,24]. At saturating rates of formate consumption, glucose-limited growth should switch from an energy-limited to an energy-excess or carbon-limited situation.

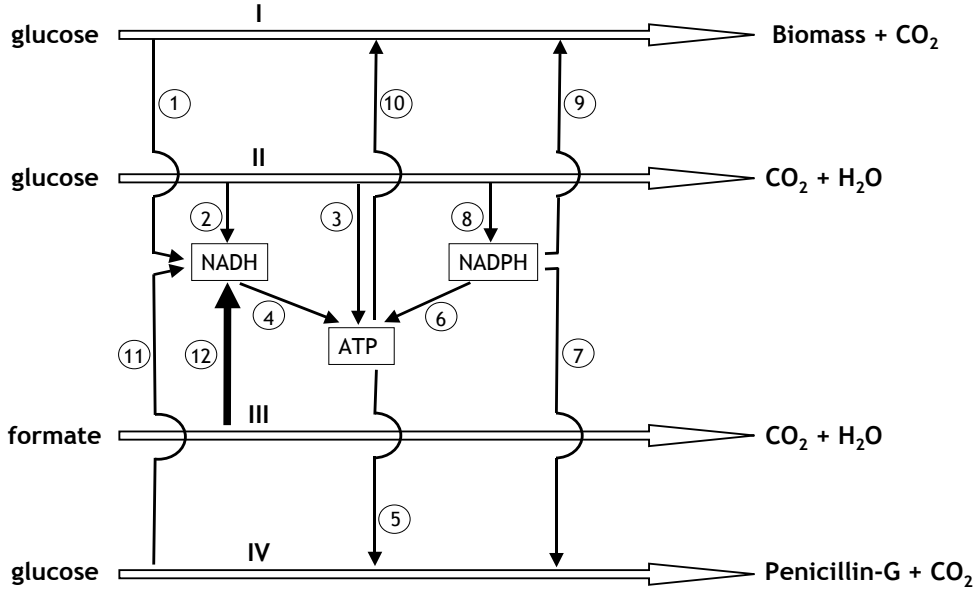

Figure 1: Fluxes of ATP and reducing equivalents in penicillin-G producing, growing cells of *P. chrysogenum*. I: assimilation of glucose to biomass; II: respiratory dissimilation of glucose; III: oxidation of formate; IV: penicillin-G biosynthesis. 1. net production of NADH during assimilatory reactions, 2. production of NADH in dissimilatory reactions, 3. generation of ATP via substrate-level phosphorylation, 4. generation of ATP via mitochondrial oxidation of NADH, 5. ATP consumption for penicillin production, 6. NADPH oxidation via the mitochondrial respiratory chain, 7. NADPH consumption in penicillin synthesis, 8. production of NADPH via the pentose-phosphate pathway and NADP+-linked isocitrate dehydrogenase, 9. consumption of NADPH during formation of amino acids, lipids and nucleic acids, 10. ATP consumption for synthesis of biomass monomers and their polymerisation, 11. NADH production in penicillin synthesis, 12. formation of NADH via formate dehydrogenase

Although the auxiliary substrate approach has been extensively studied in relation to biomass production from 'energy-deficient' carbon substrates, it has not, to our knowledge, been systematically investigated in microbial systems that produce a secondary metabolite whose biosynthesis requires a large net input of free energy. The aim of the present study is to investigate how the use of formate as an auxiliary substrate in aerobic, glucose-limited chemostat cultures of a penicillin-G high-yielding strain of *P. chrysogenum* affects β-lactam biosynthesis and the yield of biomass on the carbon substrate glucose.

Materials and Methods

Strain

A penicillin-G high-producing industrial strain of *Penicillium chrysogenum* (code name DS17690) was obtained from DSM-Anti-Infectives (Delft, The Netherlands). This strain was a re-isolation of the strain previously used in our lab (DS12975) [14,34,35].

Media and medium preparation

The mineral medium was prepared as described previously [14] and contained per litre of demineralised water: 7.5 g glucose, 3.5 g $(NH_4)_2SO_4$, 0.8 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 10 ml of a trace element solution and a varying concentration of formate. The trace element solution contained 15 g·L^{-1} $Na_2EDTA{\cdot}2H_2O$, 0.5 g·L^{-1} $Cu_2SO_4{\cdot}5H_2O$, 2 g·L^{-1} $ZnSO_4{\cdot}7H_2O$, 2 g·L^{-1} $MnSO_4{\cdot}H_2O$, 4 g·L^{-1} FeSO4·$7H_2O$, and 0.5 g·L^{-1} $CaCl_2{\cdot}2H_2O$. Production of penicillin-G was induced by adding 0.58 g·L^{-1} phenylacetic acid (PAA) to the medium.

Chemostat cultivation

Aerobic glucose-limited chemostat cultivation was performed at 25 °C in 3 - L turbine stirred bioreactors (Applikon, Schiedam, The Netherlands) with a working volume of 1.8 L. The pH was maintained at 6.5 via automated addition of 2 M NaOH (ADI 1030 biocontroller, Applikon, Schiedam, The Netherlands). The fermenter was sparged with air at a flow rate of 0.9 L·min^{-1} using a Brooks mass-flow controller (Brooks Instruments, Hatfield, USA) and stirred at 750 rpm. The dissolved oxygen concentration was continuously monitored with an oxygen electrode (Applisens, Schiedam, The Netherlands). Dissolved oxygen tension was not allowed to decrease below 40% of air saturation as this results in reduced penicillin-G production in the steady state cultures. Continuous cultivation was initiated after 50-60 hours of batch cultivation. The feed medium was supplied continuously by a peristaltic pump (Masterflex, Cole Parmer, USA) and the dilution rate was set at 0.03 h^{-1} for all chemostat experiments. Effluent was removed discontinuously by means of a special overflow device, which has been described previously [36]. The time interval between effluent removals was fixed in such a way that each time approximately 1% of the culture volume was removed. To prevent excessive foaming, silicone antifoam (10% vol/vol, BDH) was discontinuously added at timed intervals.

Determination of culture dry weight

Culture samples (10 ml) were filtered over preweighed glass fibre filters (Type A/E, Pall Life Sciences, East Hills, USA). The filters were washed with demineralised

water and dried for 20 minutes at 600 W in a microwave oven and were subsequently weighed.

Substrate and metabolite analysis

Glucose concentrations in the medium were determined by HPLC analysis using an Aminex HPX-87H column (Biorad, Hercules, USA) at 60 °C with 5 mM H_2SO_4 as the mobile phase. Phenylacetic acid and penicillin-G concentrations were determined by isocratic HPLC analysis using a Platinum EPS C18 column (Alltech, Deerfield, USA) at 30 °C. The mobile phase consisted of 5 M acetonitrile with 5 mM KH_2PO_4 and 6 mM H_3PO_4.

Gas analysis

The exhaust gas of chemostat cultures was first passed through a condenser kept at 4°C. The fraction of the gas that was send to the off-gas analyser was subsequently dried with a Perma Pure dryer (type MD-110-48P-4, Perma Pure, Toms River USA). Oxygen and carbon dioxide concentrations were determined with a NGA 2000 analyser (Rosemount Analytical, Orville, USA). Off-gas flow rates were determined from an average of 10 measurements using a SAGA digital flow meter (Ion Science, Cambridge, UK). Specific rates of carbon dioxide and oxygen consumption were calculated as described previously [37].

Subcellular fractionation and oxygen uptake studies with mitochondrial preparations

Mitochondria were isolated via controlled lysis of cells from glucose-limited chemostat cultures as described previously [14]. Spheroplasts were formed using Lyticase (from *Arthrobacter luteus*, >3000U·(mg protein)$^{-1}$, Sigma-Aldrich). The spheroplasts were subsequently lysed via lowering of the osmotic pressure and subsequent shear stress in a Potter-Elvehjem homogeniser. After centrifugation, the homogenate was separated from the whole cells and debris and centrifuged again. The resulting pellet, containing mitochondria and other organelles was resuspended in 0.65 M sorbitol and stored on ice. Protein concentrations of the fractions were determined [21] and corrected for sorbitol interference and bovine serum albumin present in the pellet buffer. The success of subcellular fractionation was verified by measuring glucose 6-phosphate dehydrogenase and citrate synthase in all fractions in the presence and absence of 0.5% (vol/vol) Triton X-100. Oxygen consumption by isolated mitochondria was measured as described before [14]. The assay mixture contained 50 mM potassium phosphate buffer (pH 7.5), 5 mM $MgCl_2$ and 0.65 mM sorbitol (4 ml final volume). Reactions were started with 5 mM potassium formate.

Preparation of cell extracts and enzyme activity assays

Enzyme activities were determined in cell extracts, which were prepared as described before [14]. Glucose 6-phosphate dehydrogenase (EC 1.1.1.49) was assayed in a reaction mixture containing 50 mM Tris-HCl pH 8.0, 5 mM $MgCl_2$, 0.8 mM $NADP^+$ and cell extract. The reaction was started with 10 mM glucose 6-phosphate. Citrate synthase (EC 2.3.3.1) was measured at 412 nm (ε = 13.6 $mM^{-1} \cdot cm^{-1}$) in a mixture containing 100 mM Tris-HCl pH 8.0, 1 mM acetyl-coenzyme A, 80 mM potassium chloride, 0.1 mM 5,5'-dithio-bis(2-nitrobenzoic acid) in Tris-HCl (DTNB) and cell extract. The reaction was started with 0.2 mM oxaloacetic acid. Formate dehydrogenase (EC 1.2.1.2, FDH) was assayed in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 2 mM NAD^+ and cell extract or the different cell fractions. The reaction was started with 50 mM potassium formate (pH 7.0). Cofactor specificity was tested by performing the same assay with 2 mM $NADP^+$. Protein concentrations were determined [21] with bovine serum albumin (fatty acid free; Sigma) as a standard.

Metabolic modelling

Metabolic modelling studies were carried out using a published stoichiometric model for growth and product formation of *P. chrysogenum* [35]. The model was extended with a cytosolic NAD^+-specific formate dehydrogenase reaction and two alternative formate uptake systems, either passive diffusion of the undissociated acid or active transport requiring 1 mole of ATP per mole of formate imported. The ATP-stoichiometry parameters which were used in the model were the same as those estimated previously for this *P. chrysogenum* strain [34]. The thus obtained determined stoichiometric model required 8 measured net conversion rates to calculate all reaction rates and remaining net conversion rates, namely biomass growth rate, penicillin-G production, formate consumption, formation of by-products associated with penicillin production (6-APA, 8-HPA and OPC) and formation of by-products associated with biomass growth (peptides and polysaccharides). Because by-product formation was not quantified, previously measured values for the same strain, cultivated at the same dilution rate in glucose-limited chemostat cultures carried out in an identical chemostat set-up were used [35].

Results

Formate oxidation by *Penicillium chrysogenum*

Glucose-limited chemostat cultures of a penicillin-G high-yielding strain of *Penicillium chrysogenum* readily oxidized formate to CO_2 when this C_1 substrate was included in the medium reservoir. At a fixed glucose concentration of 7.5 $g \cdot L^{-1}$, the

formate concentration in the medium reservoir could be increased up to 430 mM before washout of the cultures occurred (Figure 2A). At formate concentrations in the feed of up to 200 mM, the residual formate concentrations in the cultures were below 15% of those in the feed, reflecting a modest affinity of *P. chrysogenum* for formate. At higher formate concentrations in the feed, a stronger increase of the residual formate concentrations occurred (Figure 2B).

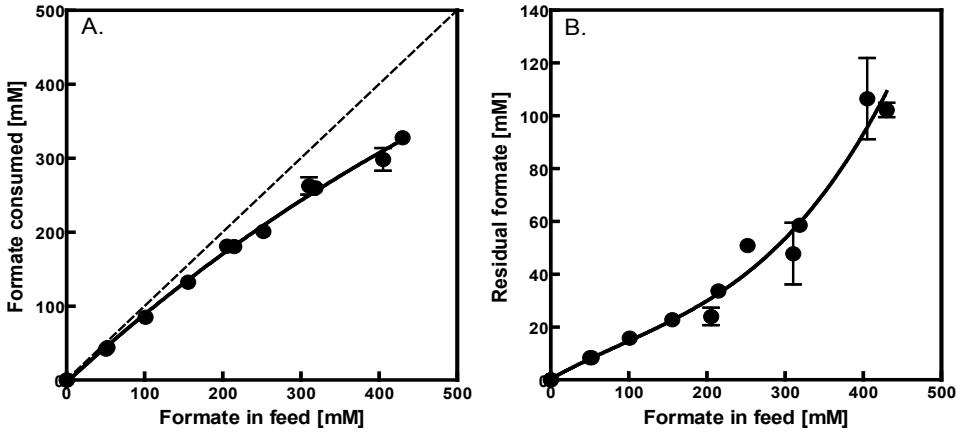

Figure 2: Formate consumption (panel A) and residual formate concentrations (panel B) for different formate concentrations in the feed medium. Results are the averages ± S.D (σ_{n-1}) of measurements on at least two consecutive days during steady state. Cells were grown in aerobic, glucose-limited chemostat cultures at a dilution rate of 0.03 h^{-1} and at varying formate concentrations. Each data point represents an independent chemostat culture. The dashed line indicates theoretical complete consumption.

Formate dehydrogenase (FDH) activity was measured in cell extracts of *P. chrysogenum* chemostat cultures grown in the presence and absence of formate. In cell extracts prepared from cultures grown without formate, no NAD^+-dependent FDH activity could be detected. A clear induction of NAD^+-dependent FDH activity occurred when formate was included in the feed. This activity increased linearly with the rate of formate consumption by the chemostat cultures. No $NADP^+$-dependent FDH activity was detected (data not shown).

To study the subcellular localisation of formate dehydrogenase, subcellular fractionation studies were performed. Localisation studies of glucose 6-phosphate dehydrogenase as a cytosolic marker and citrate synthase as a mitochondrial marker [24] showed that the cytosolic and particulate fractions were sufficiently separated. Less than 2% of the glucose 6-phosphate-dehydrogenase activity in spheroplast homogenates was present in the particulate fraction, showing that contamination of the particulate fraction by cytosol was negligible. Similarly, no citrate synthase activity was found in the cytosolic fraction, thus indicating the absence of contamination of the cytosol by mitochondria. Consistent with a cytosolic

localisation, FDH activity was only detected in the soluble fraction. To further test the hypothesis that FDH in *P. chrysogenum* is extramitochondrial, we performed oxygen-uptake studies with crude mitochondrial preparations. A mitochondrial FDH would produce NADH in the mitochondrial matrix. Malate/pyruvate specific oxygen uptake studies showed that the mitochondria were capable of generating and oxidising intra-mitochondrial NADH. However, the same mitochondrial preparations, isolated from formate-oxidising chemostat cultures, did not exhibit formate-dependent oxygen uptake rates.

Growth and product formation as a function of formate supply

To assess the effect of the co-consumption of formate on the energetics of growth and product formation, the yield of biomass on glucose, the rate of penicillin-G production and the rates of oxygen consumption and carbon dioxide production were quantified in chemostat cultures grown at different formate-to-glucose ratios at a dilution rate of 0.03 h^{-1}. This dilution rate was chosen as the specific penicillin production rate of the *P. chrysogenum* strain used appeared optimal at this dilution rate [35]. In all chemostat cultivations the carbon balances closed for more than 90%. From previous experiments under comparable conditions it was calculated that the missing carbon is most likely due to the excretion of polymeric by-products (proteins and polysaccharides) and by-products of penicillin biosynthesis [22,35]. Consistent with the stoichiometry of formate oxidation by FDH, the specific CO_2 production rate increased with increasing formate consumption (Figure 3A). Simultaneously, the specific O_2 consumption rate also increased, albeit not as steeply as the CO_2 production rate, thus leading to an increase of the respiratory quotient (RQ, Figure 3B).

Up to a molar formate-to-glucose ratio of 4.5 (based on consumed substrate), the biomass yield on glucose increased while the biomass-specific penicillin-G production rate showed no significant change (Figure 4). This resulted in an increase of the volumetric productivity of these cultures by up to 20% at a molar formate-to-glucose ratio of 4.5 (corresponding to a formate concentration in the feed of 200 mM).

At even higher consumed formate-to-glucose ratios, the specific penicillin production rate gradually decreased to about 50% of the reference rate at the highest formate to glucose ratio. The reduced penicillin-G productivity could not be attributed to a limiting supply of the side-chain precursor PAA, because the decrease of the specific Pen-G production at formate concentrations in the feed above 200 mM coincided with an increase of the residual PAA concentration. At the optimal formate-to-glucose ratio, the fraction of the glucose carbon that ended up in either biomass or penicillin-G had increased from 49% (in the reference cultures without formate) to 62%.

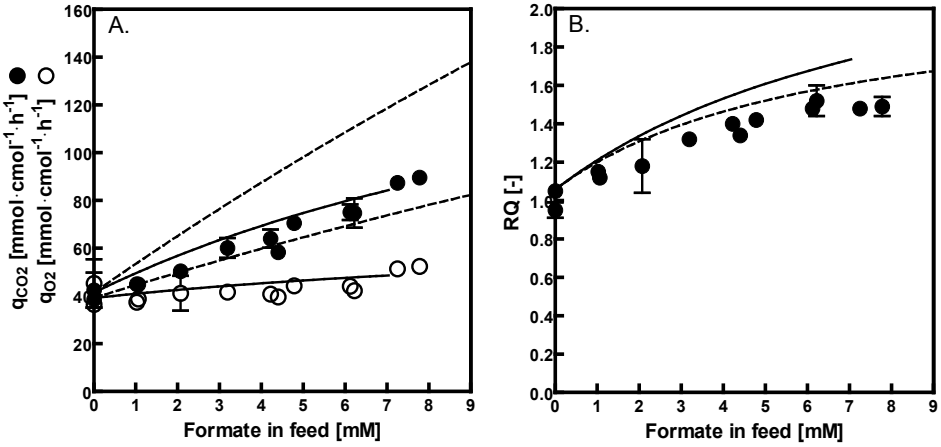

Figure 3: Specific respiratory rates of *P. chrysogenum* cells grown in glucose-limited aerobic chemostat cultures with increasing formate consumption. Results are the averages ± S.D (σ_{n-1}) of measurements on at least two consecutive days during steady state. Cells were grown at a dilution rate of 0.03 h^{-1} at varying formate concentrations in the feed. $R_{f/g}$ denotes the consumed molar formate to glucose ratio. Calculations were based on a molecular weight of biomass of 28.00 g·Cmol^{-1} [35]. Theoretical values were calculated assuming that no free energy input is required for formate transport (solid line) and by assuming that 1 ATP per formate is transported (dashed line). The ends of the lines coincide with the maximal predicted formate to glucose ratio in the case that uncoupling does not occur (see section on metabolic modelling).

Metabolic modelling of co-metabolism of formate and glucose

For a quantitative evaluation of the effect of co-consuming formate as an auxiliary energy source, metabolic modelling was performed using a previously developed stoichiometric metabolic network model for this strain [35]. Calculations were based on previously published P/O ratios for cytosolic and mitochondrial NADH [34]. For these calculations it was assumed that the biomass-specific penicillin-G production rate was not affected by formate co-consumption. The experimental data show that this assumption is valid for consumed molar formate-to-glucose ratios below 5, but not for higher ratios (Figure 4).

Since the energetics of formate transport by *P. chrysogenum* may have a substantial impact on the efficiency of formate as an auxiliary substrate, two distinct model scenarios were evaluated. In the first scenario, formate uptake was assumed not to require a net input of free energy, but instead to take place via diffusion of the free acid or electroneutral symport of the anion with a proton. In a second scenario, uptake of formate was assumed to require 1 ATP equivalent. This scenario would describe symport of the free acid with a proton, followed by expulsion of the proton via the plasma-membrane ATPase (which requires hydrolysis of 1 ATP, [18]) or, alternatively, primary transport of formate coupled to ATP hydrolysis. With a P/O ratio of 1.1 for cytosolic NADH [34], the second scenario would only allow

for a marginal ATP production from the oxidation of formate. A comparison of the biomass yields as well as the oxygen consumption and carbon dioxide production rates predicted by the two model scenarios and the actually observed values (Figure 3A and Figure 4) clearly shows that the experimental data are not consistent with a formate uptake mechanism that requires a net input of ATP. Instead, they give an excellent fit when an energy-independent mechanism is assumed to be responsible for formate transport in *P. chrysogenum*. Further analysis was therefore based on a model that incorporated energy-independent formate uptake.

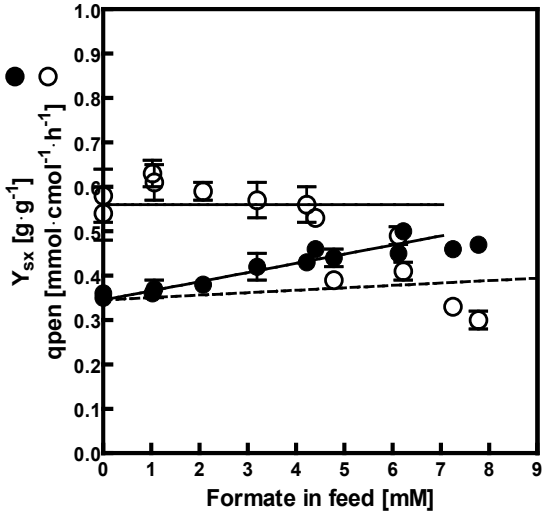

Figure 4: Effects of increased formate co-consumption on biomass yield on glucose (Y_{sx}) and specific penicillin production rate (q_{pen}). Results are the averages ± S.D (σ_{n-1}) of measurements on at least two consecutive days during steady state. Cells were grown in aerobic, glucose-limited chemostat cultures at a dilution rate of 0.03 h^{-1} at varying formate concentrations in the feed. $R_{f/g}$ denotes the consumed molar formate to glucose ratio. Calculations were based on a molecular weight of biomass of 28.00 g·Cmol^{-1} [35]. Each data point was derived from an independent chemostat culture. Theoretical values were calculated at a dilution rate of 0.03 h^{-1} by assuming that no free energy input is required for formate transport (solid line) and by assuming that 1 ATP per formate is transported (dashed line). The ends of the lines coincide with the maximal predicted formate to glucose ratio in the case that uncoupling does not occur (see section on metabolic modelling).

In theory, an optimal scenario for utilization of formate as an auxiliary energy source can be described as a situation in which (i) all glucose used for dissimilation is replaced by formate, (ii) some glucose is used for NADPH generation and (iii) the remainder of the glucose is used for production of biomass and penicillin-G. On stoichiometric grounds, the model predicted that this situation should be reached at a formate-to-glucose consumption ratio of 7.4 mol·mol^{-1}. At this ratio all NADH required in oxidative phosphorylation is generated via formate oxidation and glucose only fulfils an assimilatory role, assuming that no changes

occurred in ATP metabolism. In practice, this situation was not reached as, at formate-to-glucose ratios above 4.5, formate accumulated in the cultures and probably interfered with cellular metabolism.

Discussion

Formate as an auxiliary substrate for improving penicillin production.

Formate oxidation by glucose-limited, penicillin-G producing chemostat cultures of *Penicillium chrysogenum* led to an increased biomass yield while, over a limited range of formate-to-glucose ratios, the biomass-specific rate of penicillin-G remained constant. At the optimum formate-to-glucose ratio, this resulted in a 20% increase of the penicillin-G volumetric productivity. These results provide proof of principle that the productivity of 'assimilatory', ATP-requiring products such as ß-lactam antibiotics can be improved by the use of an auxiliary energy substrate. However, several factors preclude immediate industrial application.

For any industrial process based on the co-feeding of an auxiliary substrate to be economically viable, it has to be cheaper than the carbon source on an electron-pair (NADH) basis. While formate is a useful model substrate for laboratory experiments, it does not meet this cost requirement. For industrial applications oxidation of methanol (via a linear oxidation pathway involving NAD^+-dependent methanol, formaldehyde and formate dehydrogenases) would be much more interesting as it can yield up to 3 mole NADH per mole methanol [15,28,38]. Such an approach would require metabolic engineering, as NAD^+-linked methanol dehydrogenases have been characterised in gram-positive bacteria [2,16], but not in fungi. Recently this was successfully demonstrated for *Corynebacterium glutamicum* [30]. The typical 'eukaryotic' pathway of methanol oxidation via an H_2O_2-generating methanol oxidase (reviewed by [25]) leads to a loss of one NADH per methanol. Ideally, a methanol-oxidizing pathway should be expressed in the mitochondrial matrix, as this would lead to a maximal P/O ratio due to involvement of the proton-translocating Complex I NADH dehydrogenase [19,27]. However, even when a methanol-oxidizing pathway can be engineered into *P. chrysogenum*, the possible kinetic limitations of this fungus in formate oxidation, which were revealed in the present study need to be addressed.

In the present study, the theoretical maximum increase of biomass and penicillin-G yields on glucose, calculated for saturating formate feeds, could not be reached in practice. Instead, co-feeding of increased formate-to-glucose ratios above a certain value resulted in a decrease of the specific penicillin-G production rates. Several factors may have contributed to this phenomenon. Firstly, the high rates of NAD^+-dependent formate oxidation may have led to an increased intracellular NADH concentration, which, in turn, may have affected

NAD^+-dependent reactions delivering precursors for the synthesis of penicillin-G. Indeed, it has been reported that glycolysis in microorganisms may be inhibited by addition of formate via an effect of the FDH reaction on the NADH/NAD^+ ratio [6,7,17,29]. Secondly, it has been found that the affinity of *Saccharomyces cerevisiae* FDH for formate is negatively affected by high NADH/NAD^+ ratios showing that FDH from *S. cerevisiae* obeys sequential bi-bi two-substrate kinetics [11,26,33]. If the same holds for *P. chrysogenum* this kinetic mechanism may have contributed to the rapid increase of the residual formate concentrations at high formate-to-glucose concentrations, which may have caused further toxic effects. Such toxicity may have involved specific inhibitory effects of formate on key enzymes in biomass or penicillin-G production or, alternatively, more general mechanisms such as weak-acid uncoupling.

A previously developed metabolic model of *P. chrysogenum* [35] was used to analyse the energetics of formate utilisation by *P. chrysogenum*. The power of stoichiometric modelling of metabolic networks was demonstrated by the clear discrimination between energy-dependent and energy-independent formate-transport mechanisms. The recent completion of the genome sequence of *P. chrysogenum* (R. Bovenberg, DSM, personal communication) will be instrumental in extending the model used in the present study to a genome-scale metabolic model [1,20], thereby further refining its predictive value. However, extension of the model with additional reactions will not enable the prediction of kinetic phenomena such as substrate accumulation, 'backpressure' of high NADH/NAD^+ ratios or weak acid uncoupling. Since the construction of genome-scale kinetic models of metabolism represents a formidable challenge, a combination of modelling and experimentation will continue to be essential for rational optimisation of metabolic networks for increased product formation in industrially relevant microorganisms.

Acknowledgements

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Chapter 4

Exploring and dissecting genome-wide transcriptional responses of *Penicillium chrysogenum* to phenylacetic acid consumption and penicillin-G production

Diana M. Harris, Zita A. van der Krogt, Paul Klaassen, Leonie M. Raamsdonk, Susanne Hage, Marco A. van den Berg, Roel A.L. Bovenberg, Jack T. Pronk, Jean-Marc Daran

Manuscript in preparation

Abstract

In studies on β-lactam production by *Penicillium chrysogenum*, addition and omission of a side-chain precursor is commonly used to generate producing and non-producing scenarios. To dissect effects of penicillin-G production and of its side-chain precursor phenylacetic acid (PAA), a derivative of a penicillin-G high-producing strain without a functional penicillin-biosynthesis gene cluster (*pcbAB-pcbC-penDE*) was constructed. The copy number of this cluster was first reduced to one via spontaneous recombination. The remaining copy was removed by targeted deletion, thereby completely abolishing β-lactam biosynthesis. In glucose-limited chemostat cultures of the high-producing and cluster-free strains, PAA addition caused a small reduction of the biomass yield, consistent with PAA acting as a weak-organic-acid uncoupler. A low rate of penicillin-G-independent PAA consumption indicated activity of a PAA-degrading pathway. Microarray-based analysis on chemostat cultures of the high-producing and cluster-free strains, grown in the presence and absence of PAA, showed that: (i) Absence of a penicillin gene cluster resulted in transcriptional upregulation of another secondary metabolite gene cluster, possibly involved in production of an aristolochene-related compound, (ii) The homogentisate pathway for PAA catabolism is strongly transcriptionally upregulated in PAA-supplemented cultures (iii) Several genes involved in nitrogen and sulfur metabolism were transcriptionally upregulated under penicillin-G producing conditions only, suggesting a drain of amino-acid precursor pools. Furthermore, the number of candidate genes for penicillin transporters was strongly reduced, thus enabling a focusing of functional analysis studies. This study demonstrates the usefulness of combinatorial transcriptome analysis in chemostat cultures to dissect effects of biological and process parameters on transcriptional regulation.

Introduction

Since the discovery of the production of antibiotics by the filamentous fungus *Penicillium chrysogenum* by Fleming in 1929 [29], much effort has been invested in selection and synthesis of strains with improved productivity [49,57]. This research has made decisive contributions to the successful large-scale production of ß-lactam antibiotics after World War II. After isolation of the high-producing wild-type *P. chrysogenum* strain NRRL 1951 from a cantaloupe [5], random mutagenesis with irradiation or chemicals, followed by selection for superior production strains, enabled an over 1000–fold increase of penicillin productivity [72].

The penicillin biosynthesis pathway has been well characterised both genetically and biochemically. Biosynthesis starts with the condensation of the three amino acids cysteine, valine and α-aminoadipic acid to form the tripeptide ACV. This reaction is catalysed by the non-ribosomal peptide synthase ACVS encoded by *pcbAB* [7,8,86]. In the next step, the classic ß-lactam ring structure is formed by isopenicillin-N synthase (*pcbC*, IPNS) [12]. Isopenicillin-N forms the branch point for the penicillins and cephalosporins. Penicillins can be easily produced from isopenicillin-N by the exchange of the α-aminoadipic acid moiety for a CoA activated side-chain, such as phenylacetic acid or phenoxyacetic acid, by acyl-CoA: isopenicillin-N acyltransferase (*penDE*), which results in the production of penicillin-G or penicillin-V [7,8]. These three biosynthesis genes were shown to be physically linked in a penicillin biosynthesis gene cluster (*pcbAB-pcbC-penDE*) [7,17,18,35,65]. Present as a single copy in early strains, this gene cluster was shown to be present as amplified tandem repeats in later, high-producing strains [26,54]. Although increases in copy number have indeed been shown to result in improved productivity, saturation occurs at very high copy numbers [54,72], presumably due to limitations elsewhere in metabolism. In *P. chrysogenum* the biosynthesis pathway is compartmentalised. The enzymes involved in the first steps of biosynthesis, ACVS and IPNS, are localised in the cytosol, whereas the final steps, acyltransferase and the activation of the side-chain precursor by phenylacetyl-CoA ligase, take place in peroxisomes [75].

With some exceptions (e.g. clear increases in the copy numbers of penicillin-G biosynthesis genes [26]), the molecular basis for high-level β-lactam production remains to be elucidated. Several cellular processes have been implicated in improved productivity, including a better utilisation of precursors, higher expression of biosynthesis genes, higher gene-dosage and mutations in the regulatory genes controlling gene expression or other steps of the biosynthesis [35]. For rational and successful metabolic engineering, identification of these mutations will be of extreme benefit. With the availability of the *P. chrysogenum* genome sequence [77], it is now possible to study penicillin-G production at a genome-wide scale.

Penicillin-G production requires an exchange of the α-aminoadipic acid side-chain of isopenicillin-N for the side-chain precursor phenylacetic acid (PAA) [34]. PAA is a weak acid (pK_a = 4.3) and as such is likely to be toxic to cells depending on its concentration and the culture pH. Such toxicity may involve specific inhibitory effects of PAA on key enzymes in biomass or penicillin-G production or, alternatively, more general mechanisms such as weak-acid uncoupling [38,39]. Penicillin-G producing *P. chrysogenum* can metabolise PAA via at least two routes: incorporation in the penicillin-G molecule or catabolism via the homogentisate pathway to acetoacetate and fumarate [4,22,23,25,53]. Although this catabolic route has been described in *P. chrysogenum* [62,63] little is known about the pleiotropic effects of PAA on *P. chrysogenum*. Only a few studies dedicated to the uptake of PAA have been reported and these show contrasting results. Hillenga *et al*. [39] and Eriksen *et al*. [21] both suggest that in production strains, and at moderate concentrations of PAA, this side-chain precursor enters the cell by simple diffusion across the plasma membrane. In contrast, at low external PAA concentrations, a high-affinity transporter has been proposed to contribute to PAA uptake by the Wisconsin54-1255 strain [21,24].

The specific rate of β-lactam production by *P. chrysogenum* is strongly dependent on the availability of a suitable side-chain precursor. In many studies on the physiological impact of β-lactam production, a comparison is made between cultures grown in the absence and presence of a side-chain precursor (e.g. phenylacetic acid, phenoxyacetic acid or adipic acid) [38,44,81]. While this approach has contributed to our insight in the energetics and kinetics of penicillin-G production, it does not allow a clear distinction between effects caused by, on the one hand, β-lactam production and, on the other hand, PAA consumption and/or toxicity. A suitable experimental approach would be to compare two strains with similar backgrounds under well-defined culture conditions by using chemostat cultures, in which one strain would only be lacking the penicillin biosynthesis cluster.

Mutants of *P. chrysogenum* impaired in penicillin biosynthesis have been described [11,28,30]. Most of these mutants were derived by random mutagenesis from the low-producing strain Wisconsin54-1255, which contains one copy of the penicillin biosynthesis cluster. Although these mutants were very useful for studying gene expression and gene/enzyme relationships, for identification of the factors important for penicillin-G production and PAA consumption a strain obtained from a high-producing strain background by targeted deletion is more beneficial. To this end we constructed a strain in which the tandem-repeated penicillin biosynthesis cluster was specifically deleted, whereas the strain background was retained. In such a way it is possible to identify, by a combinatorial approach, those genes affected by phenylacetic acid and those specifically important for penicillin-G biosynthesis.

Materials and Methods

Strains

Penicillium chrysogenum strain DS17690 is a high producing strain and derived from the strain improvement program of DSM [36,44]. DS50661 lacks the penicillin biosynthesis cluster and was constructed from DS17690 as described in this paper.

Preparation of protoplasts

Preparation of *P. chrysogenum* protoplasts was performed as described by Cantoral *et al.* [10], using Glucanex (Novo Nordisk, Bagsvaerd, Denmark) instead of Novozyme as the cell wall degrading enzyme. Protoplasts were separated from the mycelium, washed and plated on mineral medium agar [16], without phenylacetic acid, but supplemented with 15 $g \cdot L^{-1}$ agar to solidify and 1 M sucrose for osmotic stabilization. Regenerating colonies were transferred to plates without sucrose to induce sporulation. Spores were collected, washed with 0.85 % NaCl, diluted and plated out on YEPD agar plates (10 $g \cdot L^{-1}$ Yeast Extract, 10 $g \cdot L^{-1}$ Peptone, 20 $g \cdot L^{-1}$ glucose and 15 $g \cdot L^{-1}$ agar). Isolated colonies were transferred to mineral medium agar plates serving as stock culture plates.

Genomic DNA isolation

To isolate genomic DNA, *P. chrysogenum* strains were grown in mineral-medium shake-flask cultures for 48 h at 25°C and 280 rpm. Cells were harvested, washed with 0.85 % NaCl and the pellet was frozen in liquid N_2. Frozen cells were grinded using a pestle and mortar, transferred to a plastic tube and an equal volume of phenol: $CHCl_3$:isoamylalcohol (25:24:1) was added. This mixture was vortexed vigorously, centrifuged and the aqueous phase was transferred to a fresh tube. This procedure was repeated twice, each time using a fresh volume of phenol:$CHCl_3$:isoamylalcohol (25:24:1). Finally, DNA was isolated from the aqueous phase by ethanol precipitation according to standard procedures.

Estimation of penicillin biosynthetic gene cluster copy numbers

Genomic DNA (3 µg) was digested with *Eco*RI, separated on a 0.6% agarose gel and transferred to a nylon membrane by vacuum Southern Blotting [68]. Fragments of the *pcbC* and *niaA* genes were used as probes. The former probe gives an indication of the copy number of penicillin biosynthetic genes and the latter probe is a single copy gene (encoding nitrite reductase) in *P. chrysogenum* DS17690. The probe sequences were amplified using gene specific primers 1-4 (Table 1A) and labelled with the ECL non-radioactive hybridisation kit (Amersham, Little Chalfont, UK) according to the supplier's instructions. The ratio between the intensity of

both signals (*pcbC*:*niaA*) was used to estimate the relative gene copy number of the penicillin gene cluster. The parent strain DS17690 and the single-copy lab strain Wisconsin54-1255 were used as controls.

Deletion of a single-copy penicillin biosynthetic gene cluster

To delete a single copy of the penicillin gene cluster that remained after spontaneous homologous recombination events (see Results section) a double homologous recombination strategy was applied. As double homologous crossover is a rare event in *P. chrysogenum,* three constructs were generated with 3, 5 and 7 kb homologous flanks on each side of the *amdS* gene respectively (Figure 1B). The oligonucleotides used are listed in Table 1A. Following PCR amplification, the fragments were cloned in pCRXL (Invitrogen, Carlsbad, USA) via TOPO T/A cloning (Invitrogen). Subsequently, all three 5' flanking sequences (3, 5 and 7 kb) were digested with *Acc*65I and *Not*I followed by ligation in pBluescript II SK+ (Invitrogen) pre-digested with *Acc*651 and *Not*I (Table 1B). The resulting plasmids carrying the 5'-flanking fragments were then digested with *Not*I to facilitate cloning of the 3' flanking sequences, which were pre-digested with *Not*I and *Eco*521 (Table 1B). The obtained 3, 5 and 7 kb flanking-plasmids all had a unique *Not*I site between the 5' and 3' flanking sequences, which was used to insert the *amdS* gene as selection marker. This was obtained by digesting pHELY-A1 [78] with *Not*I and isolating the 3.1 kb *PgpdA-AnamdS* expression cassette. The *amdS*-containing deletion fragments were isolated after digestion with *Kpn*I and transformed to the single copy penicillin gene cluster strains. Transformants were selected for their ability to grow on acetamide-containing plates and afterwards screened for antibiotic production by replica plating colonies on mineral medium and overlaying them after 4 days of growth with the ß-lactam sensitive indicator organism, *Escherichia coli* strain ESS2231 [46]. Isolates that did not show an inhibition zone were analysed via colony PCR with three primer sets: *amdS*, for the selection marker (primers 13 and 14); *penDE*, as indicator for the presence or absence of the penicillin biosynthetic gene cluster (primers 15 and 16) and *niaD* as an internal control (primers 17 and 18; Table 1).

Media and media composition

The mineral medium was prepared as described [36] and contained per litre of demineralised water 7.5 g glucose, 3.5 g $(NH_4)_2SO_4$, 0.8 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 10 mL of a trace element solution. The trace element solution contained 15 g·L^{-1} $Na_2EDTA \cdot 2H_2O$, 0.5 g·L^{-1} $Cu_2SO_4 \cdot 5H_2O$, 2 g·L^{-1} $ZnSO_4 \cdot 7H_2O$, 2 g·L^{-1} $MnSO_4 \cdot H_2O$, 4 g·L^{-1} $FeSO_4 \cdot 7H_2O$, and 0.5 g·L^{-1} $CaCl_2 \cdot 2H_2O$. Production of penicillin-G was induced by adding 0.58 g·L^{-1} phenylacetic acid (PAA) to the medium. To ensure similar residual

Table 1: Strain construction. Panel A: Primers used in this study (restriction sites are underlined). LF and RF, Left and Right flanking region respectively, location of the flanking region relative to the *amdS*-cassette (see Figure 1A). Size of the flanking region in kb. ID primer, uniquely numbered identifier of the primer used.

ID primer	Gene	Direction	Sequence
1	pcbC	FWD	GAT TGG CGC TCC TCG TTC ACC
2	pcbC	REV	CCA TTA TTT TTC TAG TCG ACA TGG CAT CGA TTC CCA AGG CCA ATG TCC CC
3	niaA	FWD	CAC AGA GAA TGT GCC GTT TCT TTG G
4	niaA	REV	TCA CAT ATC CCC TAC TCC CGA GCC
5	LF 7 kb	FWD	GTT ACA CGC TTT GAT TCT GTG GGT ACC GAT GTT ATA TTC AGC TAC
6	LF	REV	CCC AAT AGC GGC CGC AGT TGA TAA TAT CAA TAT CTA AAA CTC CC
7	LF 5 kb	FWD	GGC ATA TAC GAG CAT GGT ACC AGG GAC AGA TGC CCA TCC TTG
8	LF 3 kb	FWD	GTA TAA AAG GGG AGG GTA CCG GGA AAG ATT TGT GGG CCT G
9	RF	FWD	GTA TGT AGC TGC GGC CGC CTC CGT CTT CAC TTC TTC GCC CGC ACT
10	RF 3 kb	REV	CCG CCT TCC TCA CTA ACC GGC CGG CAG GTA CCG ATG GAC TCA GCA TTA TC
11	RF 5 kb	REV	CTC TAG AAT GCT ACG GCC GTT CGA GGT ACC TTA TAG GAA AAA GGT AG
12	RF 7 kb	REV	CCT TTT CGC TGA GCG GCC GCA ATC ACA GGT ACC GTT TTT GTC GTC
13	amdS	FWD	ATG CCT CAA TCC TGG GAA GAA CTG
14	amdS	REV	CTT GAC GTA GAA GAC GGC ACC GGC
15	penDE	FWD	CCC GCA GCA CAT ATG CTT CAC ATC CTC TGT CAA GGC
16	penDE	REV	ATG ACA AAC ATC TCA TCA GGG
17	niaD	FWD	CAC AGA GAA TGT GCC GTT TCT TTG G
18	niaD	REV	TCA CAT ATC CCC TAC TCC CGA GCC
19	pcbAB	FWD	GAA GAC GTC ATA CTT ATT CTC TG
20	pcbAB	REV	CGG CAT CGG ATA AAG AGA TCT GG

Panel B: Primer sets used for construction of double homologous crossover cassettes

Flank	Size (kb)	Forward primer		Reverse primer	
		ID primer	Restriction enzyme site introduced	ID primer	Restriction enzyme site introduced
Left	7	5	Acc65I	6	NotI
Left	5	7	Acc65I	6	NotI
Left	3	8	Acc65I	6	NotI
Right	3	9	NotI	10	Eco52I, Acc65I
Right	5	9	NotI	11	Eco52I, Acc65I
Right	7	9	NotI	12	Eco52I, Acc65I

concentrations of PAA, 0.30 g·L^{-1} was added to the medium of the cluster-free strain DS50661.

Chemostat cultivation

Aerobic glucose-limited chemostat cultivation was performed at 25ºC in 3-litre turbine-stirred bioreactors (Applikon, Schiedam, The Netherlands) with a working volume of 1.8 L. The pH was maintained at 6.5 via automated addition of 2 M NaOH (ADI 1030 biocontroller, Applikon, Schiedam, The Netherlands). The fermented was sparged with air at a flow rate of 0.9 L·min^{-1} using a Brooks mass-flow controller (Brooks Instruments, Hatfield, USA) and stirred at 750 rpm. The dissolved-oxygen concentration was continuously monitored with an oxygen electrode (Applisens, Schiedam, The Netherlands). Continuous cultivation was initiated after 50-60 h of batch cultivation. The feed medium was supplied continuously by a peristaltic pump (Masterflex, Cole Parmer, USA) and the dilution rate was set at 0.03 h^{-1} for all chemostat experiments. Effluent was removed discontinuously by means of a special overflow device, which has been described previously [82]. The time interval between effluent removals was fixed in such a way that each time approximately 1 % of the culture volume was removed. To prevent excessive foaming, silicone antifoam (10 % vol/vol, BDH Chemicals Ltd, Poole, UK) was discontinuously added at timed intervals. The offgas was cooled by a condensor at 4ºC after drying with a Perma Pure dryer (type MD-110-48P-4, Perma Pure, Toms River, USA) oxygen and carbon dioxide concentrations were determined with a NGA 2000 analyser (Rosemount Analytical, Orville, USA). Off-gas flow rates were determined from an average of 10 measurements using a SAGA digital flow meter (Ion Science, Cambridge, UK). Specific rates of carbon dioxide and oxygen consumption were calculated as described previously [84].

Determination of culture dry weight

Culture samples (10 mL) were filtered over preweighed glass fiber filters (Type A/E, Pall Life Sciences, East Hills, USA). The filters were washed with demineralised water and dried for 20 min at 600 W in a microwave oven and were subsequently weighed.

Substrate and metabolite analysis

Glucose concentrations in the medium were determined by HPLC using an Aminex HPX-87H column (Biorad, Hercules, USA) at 60 °C with 5 mM H_2SO_4 as the mobile phase. Phenylacetic acid and penicillin-G concentrations were determined by isocratic HPLC using a Platinum EPS C18 column (Alltech, Deerfield, USA) at 30ºC. The mobile phase consisted of 5 M acetonitrile with 5 mM KH_2PO_4 and 6 mM H_3PO_4.

Sampling and RNA extraction procedures

60 mL of culture broth was sampled and rapidly filtered over a glass fiber filter (Type A/E, Pall Life Sciences, East Hills, USA). The filter with mycelium was wrapped in aluminium foil, quenched in liquid nitrogen and stored at -80ºC until further use. For total RNA extraction, half of the pellet was grounded by mortar and pestle under constant cooling with liquid nitrogen. The powder was dissolved in 5 mL of Trizol reagent (Invitrogen) and 1 mL chloroform (Sigma) and mixed well. The two phases were separated by centrifugation (4600 g, 15 min). Total RNA was isolated using a phenol-chloroform extraction method, which consisted of two extraction steps in acid-phenol/chloroform/isoamyl alcohol (5:1, pH 4.8, Ambion, Foster City, USA), followed by a chloroform extraction step. Each time the phases were separated by centrifugation (4600 g, 15 min). Total RNA was precipitated for 30 min at -20ºC in 96% ethanol and 0.3 M sodium acetate. The RNA was collected by centrifugation at 23000 g for 15 min and dissolved in RNAse free H_2O.

Microarray analysis: probe preparation and target hybridisation

Double stranded cDNA synthesis was carried out using 10 µg of total RNA and the components of the One Cycle cDNA Synthesis Kit (Affymetrix, Santa Clara, USA). The double-stranded cDNA was purified with the GeneChip Sample Cleanup Module (Affymetrix/Qiagen) followed by *in vitro* transcription and labelling using the GeneChip IVT labeling Kit (Affymetrix). Finally, labelled cRNA was purified (GeneChip Sample Cleanup Module, Affymetrix/Qiagen) prior to fragmentation. 15 µg of fragmented, biotinylated cRNA was hybridised to Affymetrix custom-made *Penicillium chrysogenum* GeneChip® microarrays (array code DSM_PENa520255F) at 45ºC for 16 h as described in the Affymetrix users' manual. Washing and staining of arrays were performed using the GeneChip® Fluidics Station 400 and scanning with the Affymetrix GeneArray Scanner 3000.

Data analysis

Acquisition and quantification of array images were performed using Affymetrix GeneChip Operating Software (GCOS version 1.2). Before comparison, all arrays were globally scaled to a target value of 100 using the average signal from all gene features. To the 15,531 transcript features on the arrays, a filter was applied to extract 13,746 open reading frames of which there were 13,485 different genes. This discrepancy was due to several genes being represented more than once. To represent the variation in triplicate measurements, the coefficient of variation (S.D. divided by the mean) was calculated. When the genes were ranked according to increasing average intensity, the average coefficient of variation showed a sharp increase for the genes with the lowest expression. Therefore, all genes in which the average expression in all conditions was below 12 were removed from the dataset.

Subsequently all remaining values below 12 were set to a value of 12. To assess differential expression, the Significance Analysis of Microarrays (SAM version 1.21) add-in to Microsoft™ Excel was used for comparisons of replicate array experiments [59,73]. The fold-change threshold and the false discovery rate values were set at 2 and 1% respectively. The genes with significantly changed expression in one of the comparisons were arranged in groups by overlapping them in Microsoft™ Excel.

Enrichment of MIPS categories (version 1.3) was assessed by Fisher's Exact test employing hypergeometric distribution with a p-value cut-off of 10^{-4} (with a Bonferroni correction). The probability was calculated as follows: the p-value of observing z genes, belonging to the same functional category is:

$$P = \sum_{x=z}^{\max(N,M)} \frac{\binom{N}{x} \cdot \binom{G-N}{M-x}}{\binom{G}{M}}$$

, where N is the total number of genes in a category, M is the total number of differentially expressed genes in the cluster and G is the total number of *P. chrysogenum* genes.

Promoter analysis was performed using the web-based software Multiple Em for Motif Elucidation (MEME [6]) incorporated in the software package Genedata Phylosopher (Genedata, Basel, Switzerland). The promoters (from -800, 0) of each set of co-regulated genes were analysed for overrepresented decanucleotides. Promoters with an E-value < 10^{-5} and without long stretches of A and T (>40% GC content) were included in the analyses. Consensus sequences were depicted using the web based application WebLogo, version 2.8.2 [1,14].

Results and Discussion

Generation of a penicillin gene cluster-free derivative of a penicillin-G high-producing *Penicillium chrysogenum* strain

To enable studies on the genome-wide impact of penicillin-G biosynthesis in *Penicillium chrysogenum*, penicillin gene clusters (*pcbAB-pcbC-penDE*) were removed from the penicillin-G high-producing strain *P. chrysogenum* DS17690. Since the ß-lactam gene amplifications are in direct repeats of a larger amplified region on the same chromosome [26] spontaneous recombinations between different repeats can result in loss of gene cluster copies [54]. Isolates that underwent spontaneous recombinations were obtained via protoplast formation and subsequent sporulation of regenerating colonies. 27 Random isolates were selected for Southern analysis to estimate the relative gene copy numbers of the penicillin biosynthesis gene *pcbC* and the single-copy *niaA* gene. The same strains were tested for penicillin-V production in shake-flask cultures grown on mineral medium with the side-chain

precursor phenoxyacetic acid. Half of the random isolates tested had both reduced *pcbC:niaA* ratios and reduced penicillin-V titres. One selected strain was subjected to a second round of protoplast formation and screening. Again, 27 random isolates were selected and analysed (Figure 1, panel B). Several putative single-copy penicillin gene cluster candidates (indicated by arrows in Figure 1, panel B) were identified based on a similar *pcbC:niaA* ratio to that of the single-copy Wisconsin54-1255 strain [7,26,54,79]. Three of these strains were tested for penicillin-V production, with the parental *P. chrysogenum* DS17690 parent, the intermediate parent DS47274, the laboratory strain Wisconsin54-1255 and a non-producing isolate of the latter strain, npe10 [11], as controls. All three isolates showed a drastically reduced penicillin-V titre, comparable to that of the single-copy strain Wisconsin54-1255. It was therefore assumed that these three isolates are derivatives of *P. chrysogenum* DS17690 that carry a single copy of the penicillin biosynthesis gene cluster (Figure 1, panel C). To delete the last remaining copy of the penicillin gene cluster, a double homologous recombination strategy was applied. Sequences adjacent to the ß-lactam biosynthetic gene cluster (3' from the *pcbAB* gene and 3' from the *penDE* gene) were used as homologous flanking sequences to target the *amdS* selection marker to this locus and to delete the entire 17 kb region containing the cluster of ß-lactam biosynthetic genes (*pcbAB-pcbC-penDE*) in the same integration event. Homologous integration by double crossover will generate transformants able to use acetamide as the sole nitrogen source (due to the presence of the *amdS* gene) but unable to produce any penicillins. Moreover, genomic DNA of strains that have undergone a successful double crossover should not hybridise to ß-lactam gene specific probes. Of 27,076 transformants tested, 22 (0.08%) gave no inhibition zone when overlaid with a penicillin sensitive *E. coli* strain and were selected for further analyses. All 22 putative mutants were negative in the diagnostic PCR for the ß-lactam biosynthetic gene *penDE*. Two of these strains gave no signal for *amdS*, suggesting that they had spontaneously lost the marker gene. Southern analysis of the obtained *P. chrysogenum* strains was subsequently carried out. As a probe, a DNA fragment of 425 bp from the *pcbAB* 3'-flanking sequence was PCR amplified (Table 1, primers 19 and 20). All strains with intact penicillin biosynthesis gene sequences (*i.e.* the industrial parent strain as well as the single-copy copy strain) showed a 9.2 kb BamHI hybridising fragment and a 5.3 kb band in case of digestion with HindIII. Putative ß-lactam cluster-free DS17690 derivatives showed a 6.4 kb BamHI hybridising fragment and a 10.2 kb band if the DNA was digested with HindIII. To confirm their inability to biosynthesise ß-lactams all 22 mutants were inoculated in liquid mineral medium with phenylacetic acid as precursor. Indeed, none of the mutants was capable of producing penicillin-G (Figure 1, panel D). It was concluded that all 22 strains are derivatives of the *P. chrysogenum* DS17690 strain in which all

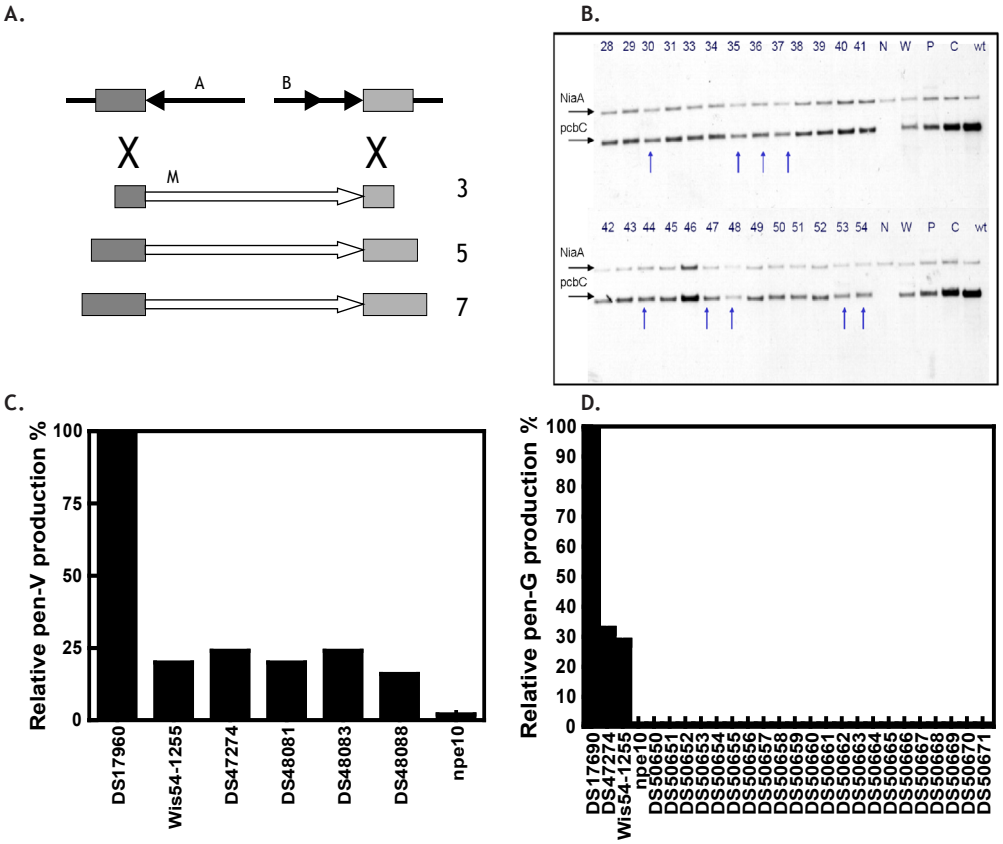


Figure 1: Obtaining a penicillin-biosynthesis-gene-cluster-free strain. Panel A: Double homologous recombination strategy to delete the final biosynthesis gene cluster. A denotes the *pcbAB* gene, B the *pcbC* and *penDE* genes and M the marker gene *amdS*. Panel B: Southern blot analysis to determine relative gene-copy number. Arrows indicate putative 'single copy' penicillin biosynthetic gene cluster candidates. Each number represents a single mutant. N, the non-producing isolate npe10; W, the lab strain Wisconsin54-1255, P, the parent strain DS47274, C, strain DS47276 with ± 8 copies and wt, the high-producing strain DS17690. Panel C: Relative penicillin-V production by putative single copy isolates (DS47274; DS48081; DS48083; DS48088) in shake flasks. Panel D: Relative penicillin-G production by putative zero-amplicon mutants (DS50650-DS50671) in shake flasks.

copies of the penicillin gene cluster have been deleted. Isolate DS50661 was used as cluster-free strain in this study.

Physiology of a penicillin-G high-producing strain and a cluster-free derivative in chemostat cultures

The high-producing DS17690 strain and the cluster-free strain (DS50661) were grown in aerobic, glucose-limited chemostat cultures at a dilution rate of 0.03 h^{-1}, in the presence and absence of the penicillin-G side-chain precursor PAA. For each combination of strain and PAA presence or absence, at least three independent chemostat cultures were analysed. In the absence of PAA, no penicillin-G was produced by the DS17690 strain, however intermediates such as isopenicillin-N were still produced [36]. Under the same conditions, the cluster-free strain did not produce any ß-lactam intermediates. The small difference between the biomass yields cultures of the high-producing and cluster-free strains grown in the presence of PAA confirms an earlier report [80] that penicillin-G biosynthesis imposes an energetic burden on *P. chrysogenum*. However, the PAA-induced reduction of the biomass yield of the cluster-free strain (Table 2) suggests that part of the biomass yield decrease that is observed upon induction of penicillin-G production by PAA addition [80], may in fact be caused by the side-chain-precursor PAA itself, e.g. via uncoupling of the plasma membrane [38,39].

Although PAA could not be used for penicillin-G production in the cluster-free strain, it was still consumed at circa 25% of the rate observed in the DS17690 strain. The PAA consumption rate in the cluster-free strain corresponded quantitatively to the PAA consumption that was not incorporated into penicillin-G in the high-producing strain (Table 2). As reported previously for industrial strains of *P. chrysogenum*, this could be the result of oxidation to 2-hydroxyphenylacetic acid by phenylacetate hydroxylase and subsequent catabolism via the homogentisate pathway [21,62].

Experimental design of transcriptome analysis and global transcriptional responses to penicillin-G production and PAA consumption

Genome-wide transcriptome analysis was carried out on mycelium of both strains grown in glucose-limited chemostat cultures in the presence and absence of PAA at a dilution rate of 0.03 h^{-1}. The average coefficient of variation of the transcriptome data derived from independent triplicate cultures did not exceed 0.21 (Table 2), which is similar to the reproducibility obtained with chemostat cultures of the non-filamentous yeast *Saccharomyces cerevisiae* [59]. The level of the *actA* [19] and *gdh2* transcripts, which are commonly used loading standards for Northern analysis, varied by less than 14% over the four situations tested. Chemostat experiments were designed to dissect transcriptional responses to PAA and penicillin-G production. Addition of PAA to growing DS17690 strain may induce two types of responses.

	Y_{sx}[a] ($g \cdot g^{-1}$)	q_{pen}[b] ($\mu mol \cdot g^{-1} \cdot h^{-1}$)	q_{PAA} ($\mu mol \cdot g^{-1} \cdot h^{-1}$)	q_{CO2} ($mmol \cdot g^{-1} \cdot h^{-1}$)	q_{O2} ($mmol \cdot g^{-1} \cdot h^{-1}$)	Avg CV[c]	PcACTA[d]	PcGDH2[e]	n
DS17690 – PAA	0.37 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	1.15 ± 0.08	1.19 ± 0.11	0.21	4190 ± 170	1240 ± 120	3
DS17690+ PAA	0.35 ± 0.01	19.81 ± 1.47	24.04 ± 2.38	1.42 ± 0.11	1.42 ± 0.17	0.18	3560 ± 360	1140 ± 270	4
DS50661 – PAA	0.39 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	1.16 ± 0.05	1.16 ± 0.11	0.17	3450 ± 390	1020 ± 170	3
DS50661 + PAA	0.36 ± 0.01	0.00 ± 0.00	5.60 ± 0.38	1.44 ± 0.10	1.43 ± 0.08	0.14	3030 ± 680	1060 ± 180	3

Table 2: Physiological and microarray quality parameters for aerobic, glucose-limited chemostat cultures grown at a dilution rate of 0.03 h^{-1}. *P. chrysogenum* strains DS17690 (penicillin-G high producing) and DS50661 (lacking a functional penicillin gene cluster) were grown in the presence and absence of phenylacetic acid (PAA) in independent glucose-chemostat cultures at D=0.03 h^{-1}. Results are the averages ± S.D. (σ^{n-1}). a biomass yield on glucose (g of biomass per g of glucose consumed). b biomass specific penicillin-G production rate. In addition to penicillin-G, intermediates and by-products are formed, which accounts for ~6% of the consumed PAA [81]. c the average coefficient of variation (standard deviation divided by the mean) for all genes except the genes with the mean below 12. d encoding actin; average signal and standard deviation. e encoding glutamate dehydrogenase (NAD+ dependent); average signal and standard deviation

Firstly, the presence of PAA itself may affect cellular processes outside β-lactam biosynthesis and thus affect the transcriptome of the fungus. Secondly, availability of a side-chain precursor enables the penicillin-G formation, thus resulting in the induction and/or repression of the transcription of genes that are (in)directly related to β-lactam production. To dissect these two types of responses, we used the cluster-free strain DS50661 as a filter for the PAA response, as this strain neither produces penicillin-G nor intermediates. Comparison of the transcript data of the two strains grown under the same conditions (DS17690+PAA versus DS50661+PAA; DS17690-PAA versus DS50661-PAA) will provide information on the effect of the removal of the penicillin cluster (and of possible unintended genetic changes resulting from the gene-cluster removal procedure) (Figure 2).

In total, four pair-wise comparisons between the two strains and the two conditions were performed (Figure 2), yielding a total of 1755 transcripts (representing 13% of the *P. chrysogenum* genome) that were differentially expressed in at least one of the comparisons based on the statistical criteria applied in this study (|fold difference| ≥ 2; false discovery rate 1%, see Methods section). The majority of the genome (8001 transcripts) did not show significant changes between the four conditions and transcript levels of 30% of the genome (3990 ORFs) was below the detection limit in all four situations (Figure 2).

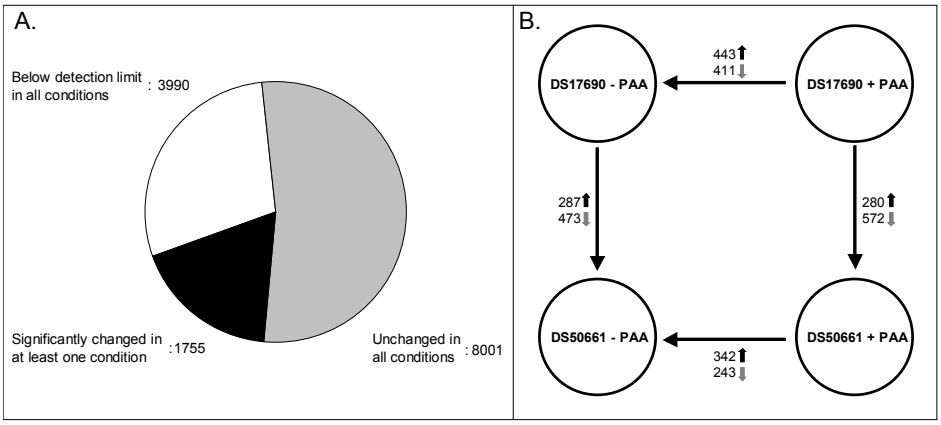
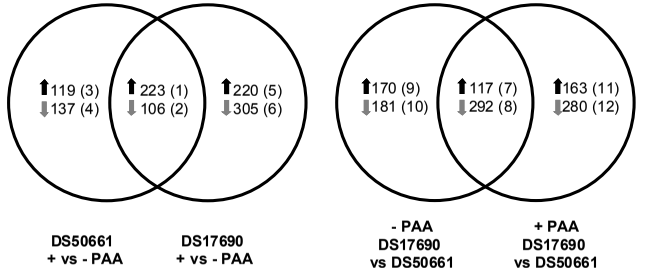


Figure 2: Global transcriptional response of DS17690 and DS50661 strains to the presence and absence of PAA. Total RNA was obtained from *P. chrysogenum* strains DS17690 and DS50661, grown in the presence and absence of phenylacetic acid (PAA) in independent glucose-limited chemostat cultures at D=0.03 h^{-1} and hybridised to Affymetrix GeneChip® microarrays. Panel A: Pie chart of overall transcript differences of the DS50661 and DS17690 strains grown in the absence and presence of PAA. Panel B: Results of the pairwise comparisons of the two strains and the two conditions. Red arrows indicate genes with a higher transcript level in the respective pairwise comparison, green arrows indicate genes with a lower transcript level.

Figure 3, legend next page:

A.



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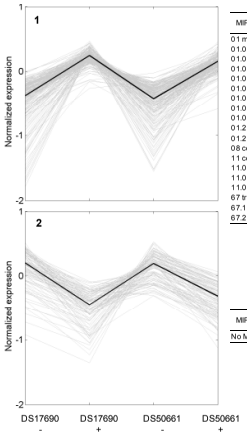
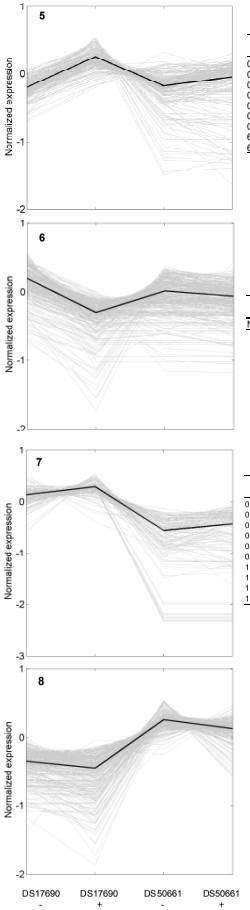


MIPS_categories:	P-value:	Frequency of occurence:
01 metabolism	$1.14 \cdot 10^{-07}$	111 of 2472
01.01 amino acid metabolism	$2.16 \cdot 10^{-05}$	23 of 467
01.01.10.05 degradation of amino acids of the cysteine-aromatic group	$6.72 \cdot 10^{-06}$	8 of 62
01.01.10.05.06 degradation of tyrosine	$7.99 \cdot 10^{-05}$	4 of 15
01.05 o-compound and carbohydrate metabolism	$1.40 \cdot 10^{-12}$	51 of 1089
01.05.01 c-compound and carbohydrate utilization	$3.04 \cdot 10^{-11}$	40 of 760
01.05.01.01 c-compound, carbohydrate catabolism	$2.32 \cdot 10^{-05}$	25 of 377
01.05.01.01.09 aerobic aromatec atabolism	$3.10 \cdot 10^{-07}$	8 of 42
01.06 lipid-, fatty-acid and isoprenoid metabolism	$2.78 \cdot 10^{-09}$	26 of 410
01.20.35 biosynthesis of secondary products derived from l-phenylalanine and l-tyrosine	$1.33 \cdot 10^{-04}$	6 of 49
01.20.35.01 biosynthesis of phenylpropanoids	$1.33 \cdot 10^{-04}$	6 of 49
08 cellular transport and transport mechanisms	$1.51 \cdot 10^{-05}$	35 of 910
11 cell rescue, defense and virulence	$7.33 \cdot 10^{-10}$	39 of 809
11.07 detoxification	$1.87 \cdot 10^{-11}$	28 of 379
11.07.01 detoxification involving cytochrome p450	$4.76 \cdot 10^{-08}$	6 of 28
11.07.05 detoxification by export	$7.89 \cdot 10^{-05}$	11 of 165
67 transport facilitation	$2.90 \cdot 10^{-10}$	40 of 819
67.19 allantoin and allantoate transporters	$7.60 \cdot 10^{-06}$	8 of 63
67.26 drug transporters	$1.68 \cdot 10^{-05}$	14 of 183

MIPS_categories:	P-value:	Frequency of occurence:
No MIPS categories significantly overrepresented		



MIPS_categories:	P-value:	Frequency of occurence:
01 metabolism	$9.86 \cdot 10^{-4}$	86 of 2472
01.02 nitrogen and sulfur metabolism	$2.89 \cdot 10^{-10}$	18 of 172
01.02.01 nitrogen and sulfur utilization	$1.54 \cdot 10^{-09}$	15 of 126
01.05 ocompound and carbohydrate metabolism	$1.54 \cdot 10^{-05}$	36 of 1069
01.20 secondary metabolism	$3.90 \cdot 10^{-06}$	18 of 379
08 cellular transport and transport mechanisms	$3.03 \cdot 10^{-06}$	34 of 910
08.19 cellular import	$1.82 \cdot 10^{-10}$	26 of 366
67 transport facilitation	$2.80 \cdot 10^{-09}$	36 of 819
67.19 allantoin and allantoate transporters	$6.53 \cdot 10^{-07}$	9 of 63

MIPS_categories:	P-value:	Frequency of occurence:
No MIPS categories significantly overrepresented		

MIPS_categories:	P-value:	Frequency of occurence:
01 metabolism	$4.38 \cdot 10^{-08}$	46 of 2472
01.02 nitrogen and sulfur metabolism	$1.09 \cdot 10^{-04}$	8 of 172
01.02.01 nitrogen and sulfur utilization	$9.89 \cdot 10^{-05}$	7 of 126
01.20 secondary metabolism	$7.48 \cdot 10^{-07}$	15 of 379
01.20.37 biosynthesis of peptide derived compounds	$5.97 \cdot 10^{-11}$	1 0 of 59
01.20.37.05 biosynthesis of beta-lactams	$3.87 \cdot 10^{-13}$	1 0 of 37
11 cell rescue, defense and virulence	$4.97 \cdot 10^{-06}$	19 of 809
11.05 disease, virulence and defense	$3.52 \cdot 10^{-05}$	11 of 289
11.05.05 virulence, disease factors	$2.22 \cdot 10^{-07}$	11 of 171
11.05.05.01 toxins	$7.43 \cdot 10^{-09}$	1 0 of 95

MIPS_categories:	P-value:	Frequency of occurence:
40.02 plasma membrane	$3.78 \cdot 10^{-06}$	4 of 10
99 unclassified proteins	$8.02 \cdot 10^{-06}$	208 of 7742

Figure 3, previous page: Cross-sections, profiles and overrepresented functional categories of the pair-wise comparisons. Transcript data from independent chemostat cultures of P. chrysogenum strains DS17690 and DS50661 grown at D=0.03 h^{-1} in the presence and absence of phenylacetic acid (PAA) were compared in four pairwise comparisons (DS17690 + PAA versus DS17690 – PAA; DS50661 + PAA versus DS50661 – PAA; DS17690 + PAA versus DS50661 + PAA and DS17690 – PAA versus DS50661 – PAA. Genes whose transcript level was significantly different in at least one of the four pairwise comparisons were overlapped as shown in panel A, resulting in 12 different groups of genes: 1-6: comparing the response to PAA in DS17690 and DS50661 1. genes with a higher transcript level in the presence of PAA both in DS17690 and DS50661; 2. genes with a lower transcript level in the presence of PAA both in DS17690 and DS50661; 3. genes with higher transcript level in the presence of PAA in DS50661 and not in DS17690; 4. genes with lower transcript level in the presence of PAA in DS50661 and not in DS17690; 5. genes with higher transcript level in the presence of PAA in DS17690 and not in DS50661; 6. genes with lower transcript level in the presence of PAA in DS17690 and not in DS50661; 7-12 compare the effect of the cluster removal; 7. genes with a higher transcript level in DS17690 compared to DS50661 irrespective of the presence of PAA; 8. genes with a lower transcript level in DS17690 compared to DS50661 irrespective of the presence of PAA; 9. genes with a higher transcript level in DS17690 only in the absence of PAA; 10. genes with a lower transcript level in DS17690 only in the absence of PAA; 11. genes with a higher transcript level in DS17690 only in the presence of PAA; 12. genes with a lower transcript level in DS17690 only in the presence of PAA. Panel B shows the gene-transcript profiles of the groups of specific interest with the results of the hypergeometric distribution analysis for enrichment of functional categories. The thick line represents the average of the mean normalized transcript data of the genes comprising the cluster. The y-axis represents 10log transcript values. Functional categories are mentioned together with their P-value and the number of genes in the respective functional category in the group of genes with a higher transcript level compared to the prevalence of this functional category in the whole genome. Due to a large redundancy in the functional categories some categories might appear without having a significant biological relevance.

The set of differentially expressed genes was distributed over 12 groups following a two-way comparison (Figure 3). Groups 1 and 2 (Figure 3) contained genes whose transcript levels were consistently higher or lower in the presence of PAA, irrespective of the strain background. Similarly, groups 7 and 8 harboured genes whose transcript levels were consistently higher or lower in the DS17690 strain, irrespective of the presence of PAA (Figure 3). Groups 5 and 6 represent genes that show a higher and a lower transcript level in the presence of PAA, but only in the DS17690 strain. These latter profiles are consistent with genes whose transcription is responsive to the production of penicillin-G.

Enrichment of functional categories in these clusters was assessed according to the MIPS functional categories annotation [64] (Figure 3). To identify possible regulatory networks, a systematic search for possible protein-binding motifs in promoter sequences was performed on the different clusters (Figure 8).

Transcriptional responses to removal of the penicillin biosynthesis gene cluster

A total number of 409 genes (117 in group 7 and 292 in group 8; Figure 3) showed significantly different transcript levels in cultures of the high-producing and cluster-free strains, irrespective of the presence of the side-chain precursor PAA. As expected, transcripts of the three biosynthesis genes, *pcbAB, pcbC* and *penDE* could not be detected in the cluster-free strain. As a result of the strain construction the other genes in the amplified region (Pc21g21280-Pc21g21420) are present as a single copy in the DS50661 strain. The observation that several of these genes showed reduced expression levels in the DS50661 strain would therefore be consistent with a gene-dosage effect (Figure 4). In addition, as confirmed by studies on a different industrial strain of *P. chrysogenum* [27] and on the laboratory strain Wisconsin54-1255 [79], not all genes in the amplified region are transcriptionally induced under penicillin-G producing conditions. In addition to an enrichment of functional categories related to ß-lactam biosynthesis (01.20; 01.20.37.05; 11; 11.05, 11.05.05; and 11.05.05.01), group 7 (increased transcript levels in the high-producing strain DS17690) was also enriched for functional categories 01.02 and 01.02.01, which are related to nitrogen and sulfur metabolism (Figure 3). Upregulation of the synthesis of (sulfur-containing) amino acids may be indicative for an increased synthesis of the amino-acid β-lactam precursors. The observation that higher transcript levels of these genes were also observed in the DS17690 strain when it was grown in the absence of the side-chain precursor PAA may indicate that even the low net production of ß-lactam intermediates under these conditions has an impact on transcriptional regulation of precursor biosynthesis.

Among the 292 genes that were transcribed at higher levels in the penicillin-biosynthesis gene cluster-free strain than in the DS17690 strain (group 8) 17 genes

were physically linked in the same chromosomal region (Figure 5). Although the increased transcript level of these genes was also observed in the presence of PAA, it was most pronounced in its absence. Annotation of many of these genes and their clustering suggests a role in secondary metabolite production (Figure 5B) [43]. Among those 17 genes, the paralog of the aristolochene synthase gene, *Ari1*, from *Penicillium roqueforti* [60], which shares 97% identity, was identified (Pc12g06310). Synthesis of aristolochene represents the first step in the synthesis of sesquiterpenoids [52]. In *Penicillium roqueforti*, aristolochene is a precursor of PR toxin [67], for which the further biosynthesis pathway remains unidentified.

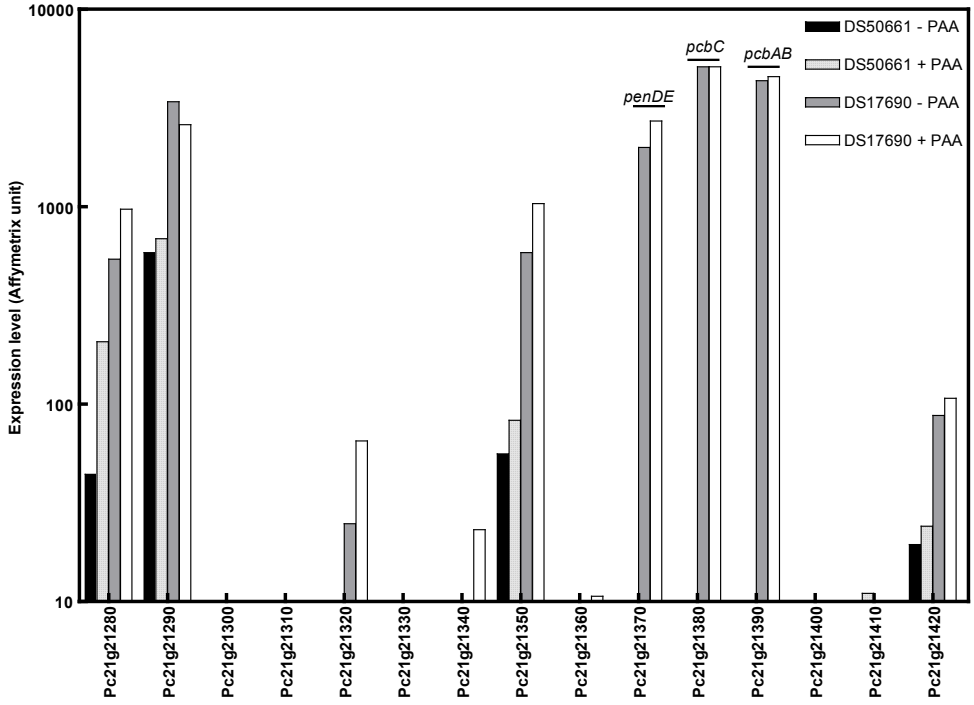


Figure 4: Transcript profiles of the amplified region in penicillin-G-producing strains of *Penicillium chrysogenum*. Transcript level of the penicillin biosynthesis genes embedded in a region that is present in tandem repeats in penicillin-G-high-producing strains, the amplified region (Pc21g21280-Pc21g21420, [27,77,79]). Total RNA was obtained from *P. chrysogenum* strains DS17690 and DS50661, grown in the presence and absence of phenylacetic acid (PAA) in independent glucose-chemostat cultures at D=0.03 h^{-1} and hybridised to Affymetrix GeneChip® microarrays.

Transcriptional responses to the side-chain precursor phenylacetic acid (PAA)

329 genes (groups 1 and 2, Figure 3) showed a consistently different transcript level in the presence and absence of PAA. Remarkably, genes belonging to the penicillin biosynthesis gene cluster were not differentially expressed in the presence

and absence of PAA, with the *pcb* genes still being highly expressed in the absence of PAA. Only the gene encoding the PAA-CoA ligase (*phl*, Pc22g14900) [47] showed a significantly higher transcript level in the presence of PAA (increase of 70 and 100% relative to cultures lacking PAA for the DS17690 and DS50661 strains, respectively). Recent functional characterisation of *phl* revealed that upon its deletion, the PAA-CoA ligase activity decreased by only 40% [47], indicating the existence of one or more additional PAA-CoA ligases. Five genes with similarity to aryl- or fatty-acid-CoA ligases were expressed to a higher level in cultures of both strains grown in the presence of PAA. The two closest *phl* homologues (Pc22g24780 and Pc22g20270) showed over 65% identity with *Aspergillus* proteins of unknown function, as well as a strong similarity with *Arabidopsis thaliana* 4-coumarate-CoA ligase (30%) (Figure 6). Further inspection of the predicted amino acid sequences of both these genes identified the presence of PTS1 peroxisomal targeting sequences. Based on these observations, Pc22g24780 and Pc22g20270 represent interesting candidates for further functional analysis aimed at identifying alternative PAA CoA-ligases in *phl*Δ strains.

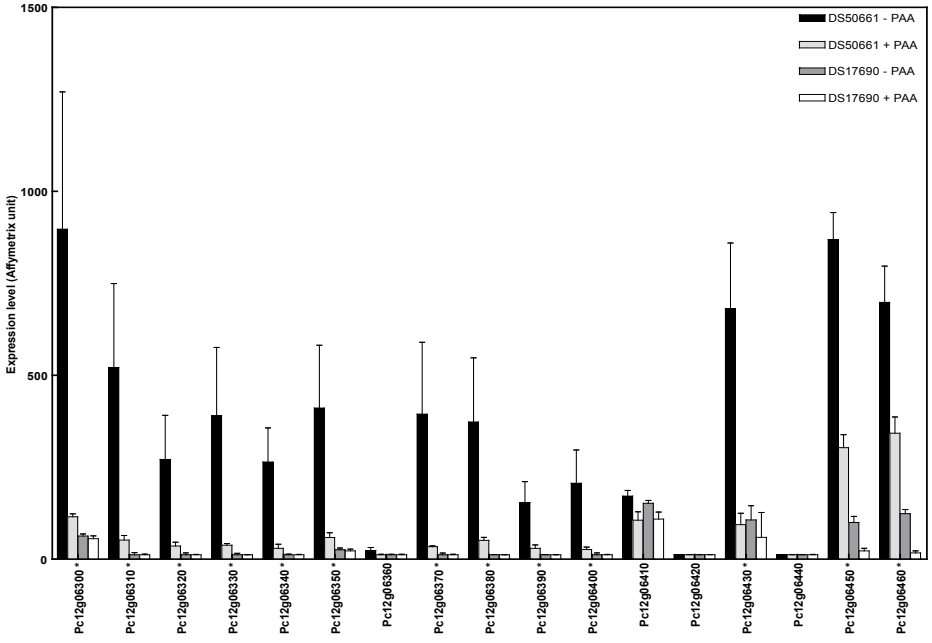


Figure 5: Clustered genes with a higher transcript level in the DS50661. Transcript levels of a cluster of genes of which many exclusively had higher transcript levels in the cluster-free strain DS50661 than in the penicillin-high-producing strain DS17690 irrespective of the side chain precursor. Annotation of many of these genes and their clustering suggests a role in secondary metabolite production. Total RNA was obtained from *P. chrysogenum* strains DS17690 and DS50661, grown in the presence and absence of phenylacetic acid (PAA) in independent glucose-chemostat cultures at D=0.03 h^{-1} and hybridised to Affymetrix GeneChip® microarrays. *, genes with a significantly higher transcript level (fold change >2, FDR 1%) in DS50661 compared to DS17690

Both strains showed penicillin-G independent metabolism of PAA. In the DS17690 strain, this was evident from the observation that PAA consumption exceeded penicillin-G production (Table 2). Indeed, the PAA hydroxylase gene (*pahA*) [62] that encodes the first step of the PAA catabolism through the homogentisate pathway was highly induced in cultures grown in the presence of PAA (from +10- to over 100-fold). However, it has been reported, that already early in the strain improvement lineage a mutation (L181F) in this gene results in a dramatic reduction of enzyme activity [62]. Interestingly, a second gene (Pc16g01770), whose predicted protein sequence shared 42% identity with the *pahA* product also showed strongly elevated transcript levels (≥ + 20-fold) in the presence of PAA. This second gene is 82% identical to *Aspergillus nidulans* PhacB [25], which encodes a 3-hydroxyphenylacetate 6-hydroxylase and 3,4-dihydroxyphenylacetate 6-hydroxylase cytochrome P450 monooxygenase capable of converting PAA into 2-hydroxyphenylacetate. This second gene may well be responsible for residual PAA catabolism in industrial strains that carry a loss-of-function mutation in *pahA*. Based on genome annotation and the transcript profiles of cultures grown in the presence and absence of PAA, the entire homogentisate pathway, which ultimately leads to the formation of fumarate and acetoacetate, could be mapped. Genes encoding a homogentisate dioxygenase (Pc12g09040); a maleylacetoacetate isomerase (Pc12g09020) and a fumarylacetoacetase (Pc12g09030) were tentatively identified, completing the identification of the metabolic pathway. In contrast to the other PAA-utilizing pathway (penicillin-G synthesis) the five genes of the homogentisate pathway all showed a strong transcriptional upregulation in presence of PAA (ranging from +6.6-fold to +90-fold). Apparently, despite the reduction of the pathway's activity, transcriptional regulation of the homogentisate pathway is still functional in high-producing strains of *P. chrysogenum*. As indicated by their gene identity codes, the ORFs Pc12g09020, Pc12g09030 and Pc12g09040 form a chromosomal cluster. A similar clustering of the homogentisate pathways genes has been observed in *Aspergillus nidulans* [32]. An additional gene of this cluster, Pc12g09010, that shares 51% of identity with *A. nidulans* AN1893.3 and displays similarity with a putative transcription factor from *Neosartorya fischeri,* was also upregulated in presence of PAA in both DS17690 and DS50661 (+2.6-fold and +3.8 respectively). This observation makes it tempting to speculate that Pc12g09010 participates in transcriptional regulation of PAA catabolism (Figure 6). The characterisation of the genes encoding the pathway (a homogentisate dioxygenase (Pc12g09040); a maleylacetoacetate isomerase (Pc12g09020) and a fumarylacetoacetase (Pc12g09030)), the physical clustering of these genes and the presence of a co-clustered putative transcription factor represent interesting targets for metabolic engineering to eliminate residual rates of PAA consumption and to alleviate a potential protein burden [66] imposed by high-level induction of this pathway.

Transport mechanisms for ß-lactam antibiotics and side-chain precursors, both across the fungal plasma membrane and between intracellular compartments, remain incompletely understood. The functional category analysis of genes that showed an increased transcript level in cultures grown in the presence of PAA showed a clear enrichment of the transport-related functions (Figure 3). 42 Genes in the functional categories 'cellular transport and transport mechanisms' and 'transport facilitation' showed a significantly increased transcript level in cultures grown with PAA in both strains (Figure 3, group 1). The uptake of undissociated phenylacetic acid in *P. chrysogenum* has been reported to occur via passive diffusion [39]. However, by analogy to the well-studied non-filamentous yeast *Saccharomyces cerevisiae,* in which PAA is exported by the ATP-binding cassette transporter Pdr12 [37], its anion form is likely to be actively exported into the medium as a detoxification mechanism. From the available functional annotation of the *Penicillium* genome, 2 out the 42 genes (Pc22g14600 and Pc22g20390) display motif signatures of ABC transporters, as well as sequence similarity to the *Aspergillus nidulans atrB* [2] and *atrD* [3] proteins, respectively. Only Pc22g14600 belongs to the ABC-G transporters cluster [77] that also includes Pdr12, which makes Pc22g14600 a very attractive candidate for further characterization.

Activation of PAA and the final biosynthetic step in the penicillin-G biosynthesis pathway, the exchange of the aminoadipic acid side-chain for PAA, both occur in the peroxisome. This metabolic compartmentation of penicillin-G production makes transport of isopenicillin-N, PAA and penicillin-G across the peroxisomal membrane an integral and important part of penicillin-G biosynthesis. Only one of the transporter genes that showed an increased transcript level in cultures grown with PAA (Pc21g09430) showed a clear link with peroxisomes. This gene shows strong similarity to the *Saccharomyces cerevisiae ANT1* gene that encodes a peroxisome-localised protein involved in adenine nucleotide transport, medium-chain fatty acid metabolism, and peroxisome proliferation [83].

The genes that showed a consistently lower transcript level in cultures grown in the presence of PAA (Figure 3, Group 2 (106 genes)), failed to show a clear enrichment of any functional category and, moreover, showed a high incidence of genes with unknown function and/or similarity with a gene of unknown function in another organism.

Dissection of transcriptional responses to PAA and to penicillin-G production

For future studies into the mechanism, compartmentation and regulation of penicillin-G biosynthesis, it would be helpful to dissect transcriptional responses of side-chain precursor availability and penicillin-G biosynthesis itself. The cluster-free strain described above synthesises neither penicillin-G nor any of its intermediates, even when grown in presence of PAA. Consequently, genes that show a transcriptional

response to PAA that is specific for the high-producing strain DS17690 are likely to be related to penicillin-G production rather than to the presence of PAA per se (Groups 5 and 6, Figure 3).

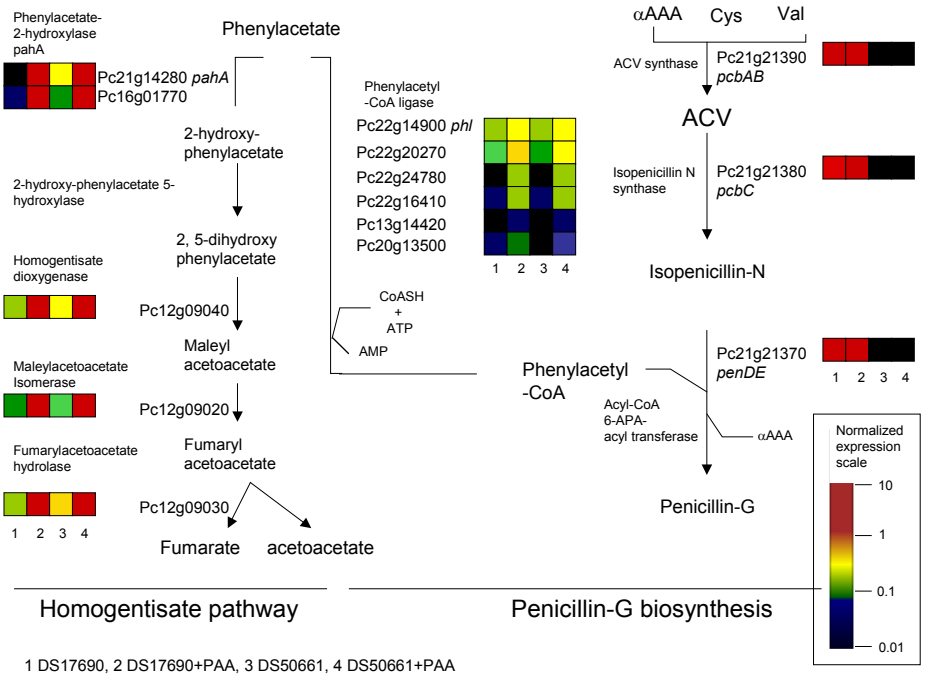


Figure 6: Detoxification of phenylacetic acid via incorporation into penicillin-G or the homogentisate pathway. Those genes (putatively) related to penicillin, biosynthesis and phenylacetate catabolism are depicted. Total RNA was obtained from *P. chrysogenum* strains DS17690 and DS50661, grown in the presence and absence of phenylacetic acid (PAA) in independent glucose-chemostat cultures at D=0.03 h^{-1} and hybridised to Affymetrix GeneChip® microarrays. The colour bar indicates the range of the mean normalized transcript level value per gene. Transcript level values range from 12-5000 Affymetrix Units. 1, DS17690 – PAA; 2, DS17690 + PAA, 3, DS50661 – PAA; 4, DS50661 + PAA

This approach yielded 220 genes (Group 5, Figure 3) whose transcript level was specifically higher in penicillin-G producing cultures of the DS17690 strains and 305 genes (Group 6, Figure 3) that showed a lower transcript level in these conditions. Group 5 was enriched for genes whose annotation involved functions in nitrogen and sulfur metabolism, secondary metabolism and transport (Figure 3). Among the 18 genes assigned to the nitrogen and sulfur metabolism FunCAT annotation, six were sharing significant homology with dioxygenases (sulfur dioxygenase, taurine dioxygenase, 2-oxoglutarate-dependent dioxygenase, arylsulfatase, Fe(II)-dependent sulfonate alpha-ketoglutarate dioxygenase, Figure 7). Although these enzymes participate in the mobilisation of sulfur from alternative sources (e.g., in the absence of sulfate [20]), the analogy with the low-sulfate response

as recorded in *S. cerevisiae* [9,69] cannot be extended further. For example, consistent with the excess sulfate included in the growth media, we did not observe a transcriptional upregulation of key enzymes in sulfate assimilation (e.g. genes involved in methionine biosynthesis). However, the transcript level of the sulfate transporter gene *sutB* [76] was specifically increased by 60 % in the penicillin-G producing cultures of the DS17690 strain. Moreover, the *sutB* transcript level was +4.4-fold higher in the DS17690 strain than in the DS50661 strain when grown in the presence of PAA. A similar transcript profile was observed for Pc18g03480, which has a strong similarity with high-affinity methionine permease genes. The second *P. chrysogenum* sulfate transporter gene *sutA* [76] was transcribed at very low levels under all conditions tested. Specific transcriptional upregulation of several genes involved in sulfur and nitrogen assimilation in penicillin-G producing scenarios suggests that a drain of amino acid precursors (cysteine and possibly valine) may affect intracellular pools of these amino acids. Indeed, metabolic flux analysis of *P. chrysogenum* grown under producing and non-producing conditions showed that the flux from 3-phosphoglycerate to serine and cysteine biosynthesis was 6-fold higher under penicillin-G-producing conditions [81]. One gene involved in serine and cysteine synthesis (Pc21g03190, encoding a putative hydroxypyruvate dehydrogenase) showed an increased transcript level in penicillin-G producing cultures of the DS17690 strain. This identifies the analysis, and possibly engineering, of cysteine and valine biosynthesis as relevant activities in applied research on β-lactam production.

The transporter for penicillin-G in *P. chrysogenum* is still unknown. Although it cannot be excluded that the penicillin transporter is among the 700 constitutively transcribed transporters, the 36 transporters in group 5 form an interesting group for initial analysis. Out of the 36 transporter-encoding genes found in group 5, 18 were assigned to transport of a wide range of nitrogen sources, including urea (2 genes), allantoate (7 genes), various amino acids including lysine and methionine (8 genes) and oligopeptides (1 gene). Whereas lysine and methionine transport might be related to the sulfur status of the cells (see above) the role of the other genes is more elusive. However, two observations on transport-related genes provide interesting leads for follow-up studies. Firstly, Pc22g11250, whose transcript profile correlated perfectly with the production of penicillin-G, shows strong similarity with the *A. niger* gene An15g07460, which encodes an oligopeptide transporter. Interestingly transport of ß-lactams through human intestinal epithelium involves an oligopeptide (di- and tripeptide) transporter [50]. We are currently investigating the possibility that this transporter is involved in penicillin-G export.

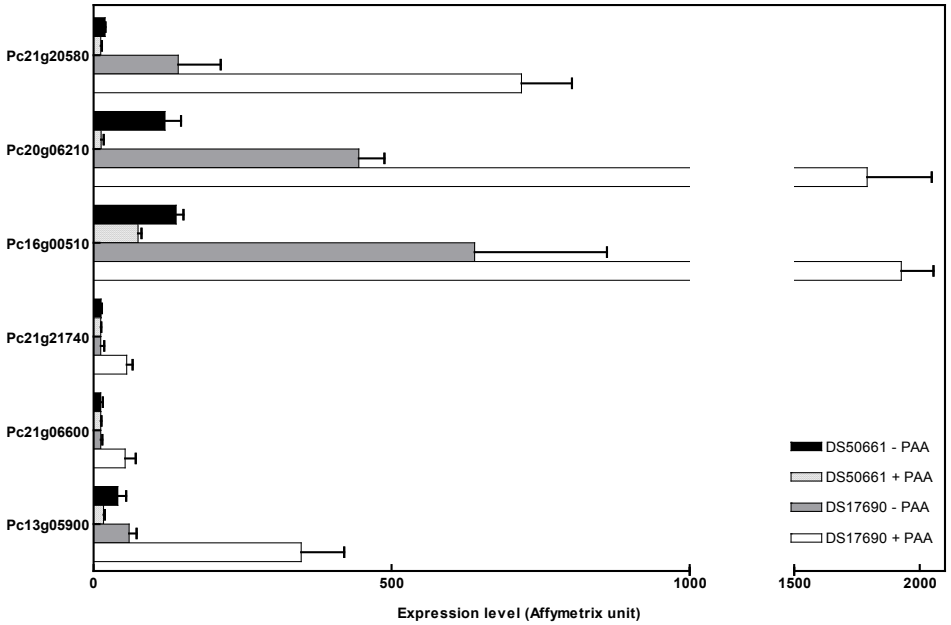


Figure 7: Transcript levels of putative sulfonatase genes specifically responding to penicillin-G production. Group 5 containing genes exclusively responding to penicillin-G biosynthesis contains six putative sulfonatases. Total RNA was obtained from *P. chrysogenum* strains DS17690 and DS50661, grown in the presence and absence of phenylacetic acid (PAA) in independent glucose-chemostat cultures at D=0.03 h^{-1} and hybridised to Affymetrix GeneChip® microarrays.

The second example *a priori* has no relationship with the penicillin synthesis; a group of 8 genes, whose products all show similarities with the yeast allantoate transporter Dal5, exhibited a significant upregulation under penicillin-G producing conditions. These genes belong to a larger genome-wide family of 30 members. Although these 30 transporters share the same description "strong similarity to Dal5", they display very different expression profiles. Without functional analysis studies on these genes, any biological interpretation of this observation would remain speculative.

Along with the penicillin-G synthesis, 18 genes that could be involved in secondary metabolism were also expressed to a higher level under penicillin-G producing conditions. This group harboured two genes Pc21g23730 and Pc21g20650 that exhibit strong similarities with a feruoyl-CoA synthetase from *A niger* and a 4-coumarate-CoA ligase from *Arabidopsis thaliana*, respectively. While the transcript levels of these genes, remained lower than those of the two PAA-inducible putative aryl-CoA ligases mentioned above, this does not rule out a possible contribution of their gene products to *in vivo* PAA activation, which is further supported by the putative peroxisomal targeting signal that both harbour.

Analysis of upstream regulatory sequences

The 800 nucleotides upstream of the ATG of groups of genes that showed a similar transcriptional regulation were analysed for *cis*-regulatory elements. MEME analysis identified two motifs in group 1 and 1 motif in group 2 that met the statistical criteria applied (Figure 8; see Materials and Methods for details). Although these motifs had a good coverage of the genes in the group, the most strongly regulated genes, those involved in PAA catabolism, did not contain this motif in their 800 nucleotide upstream region. In addition, some of the motifs identified are shared by the different co-regulated groups and none match any of the known binding sites of the limited set of described transcription factors (Figure 8). Possibly the long history of strain improvement of these strains has resulted in a loss of conserved motifs. Nevertheless, a similar analysis performed on a set of 53 co-regulated genes responding to PAA in the lab strain Wisconsin54-1255 and the DS17690 strain (cluster 1, described in [77]) identified the same motifs as found in the present study (motif 1 from group 1 and motif 2 from group 7). Increasing the length of the motif to be identified resulted in the same core conserved nucleotides (data not shown). In addition, as the groups of co-regulated genes are quite large, the discriminating power of MEME may not be sufficient to identify the actual conserved and regulating motifs. Testing a small subset of the genes, the 7 genes known to be directly involved in PAA catabolism, indeed resulted in different motifs that had a very high coverage over the genes tested. However, none of these motifs passed the stringent statistical criteria applied. These preliminary results suggest that motif identification in filamentous fungi may require different approaches than hitherto applied in more intensively studied microorganisms.

Conclusions

80 years after Fleming's discovery of the antibacterial activity of penicillin, research on *Penicillium chrysogenum* has now become accessible to genomics approaches [77]. In the present study, we have integrated microarray-based transcriptome analysis with chemostat cultivation. This approach, which has already shown to be fruitful in other organisms such as *Saccharomyces cerevisiae* [9,15,69,71], *Trichoderma reesei* [61] and *Escherichia coli* [40,48], enables an investigation of the effect of individual culture parameters on genome-wide transcriptional regulation. Reproducibility of transcript data is often cited as an additional advantage of chemostat-based microarray analysis. Although steady-state chemostat cultivation of filamentous fungi is experimentally more challenging than chemostat cultivation of non-filamentous microorganisms, the excellent reproducibility of the transcript data obtained with *P. chrysogenum* indicates that this does not preclude accurate and reproducible chemostat-based transcriptome analysis. In aerobic, glucose-

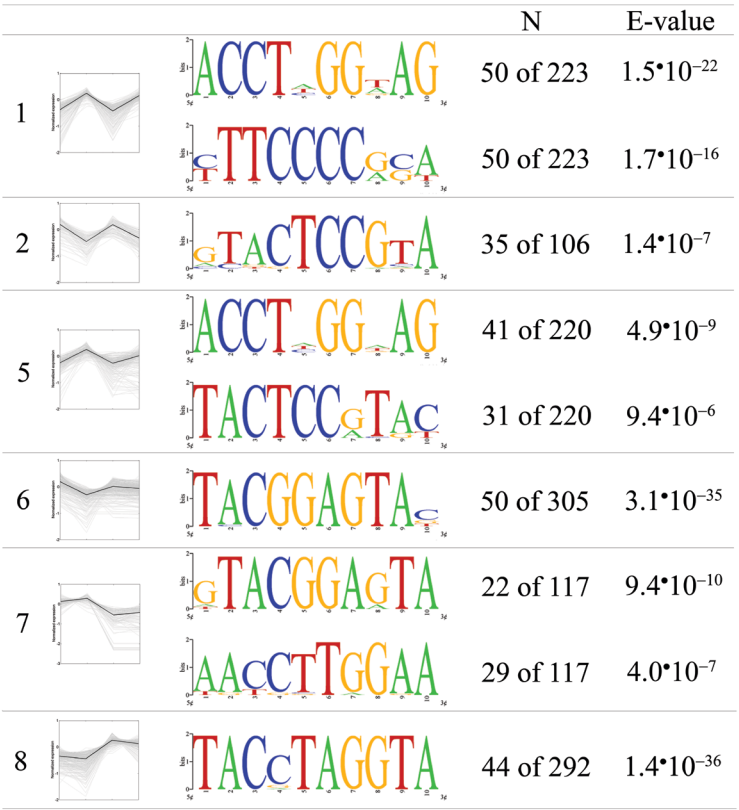


Figure 8: Predominant motifs identified in 800 nucleotide upstream region. Promoter analysis of the 800 nucleotide upstream region of sets of co-regulated genes, identified by overlapping the results of the four pairwise comparisons (groups 1, 2, 5, 6, 7, 8), using MEME. Motifs with an E-value $<10^{-5}$ and without long stretches of A and T (>40% GC content) were included in the analyses. Consensus sequences were depicted using the web based application WebLogo, version 2.8.2 [1,14]. N denotes the number of genes with the motif compared to the number of genes in the co-regulated group.

limited chemostat cultures of *S. cerevisiae*, ca. 86 % of its 6400 genes [59] showed a detectable transcript level. Of the much larger genome of *P. chrysogenum*, cultivation under similar conditions yielded a detectable transcript for only 67 % of the genes. Furthermore, of the 1755 genes that showed a differential transcript level under at least one of the conditions, 53% has an unknown function. This percentage is similar to the percentage of unclassified proteins throughout the whole genome sequence [77]. These observations illustrate the formidable challenges that remain to be addressed in the functional analysis of the genomes of filamentous fungi. The identification of gene function in *P. chrysogenum* is likely to benefit tremendously from the rapid sequencing, annotation and analysis of the genomes of other filamentous fungi, such as those of *N. crassa* [31], *A. fumigatus* [55], *A. nidulans* [32] , *A. oryzae* [51], *A. niger* [41,56], *T. reesei* [42]. For example, the recent characterisation of a new sulfate transporter gene, *astA*, in *A. nidulans*,

homologous to the *S. cerevisiae* Dal5 transporter [58] enabled us to tentatively interpret the involvement of a similar gene in *P. chrysogenum* as being part of a broader sulfur-related response.

Carefully designed transcriptomics experiments can help to prioritise targets for functional analysis based on at least three criteria: (i) the absence of a detectable transcript level rules out that the gene product contributes to either fitness or industrial performance under the experimental conditions, (ii) transcriptional regulation can provide insight into the possible role of gene products in an experimental context, although relations between transcript profiles and contribution to fitness are not necessarily straightforward [33,70,85], and (iii) the availability of possible sequence-derived information on the putative catalytic, structural or regulatory role(s) of gene products that suggest a role in fitness and/or industrial performance. Based on these criteria, several priority targets for future functional analysis studies have been identified in the present study (see Results and Discussion section).

The present study demonstrates how a simple combinatorial design of chemostat experiments, involving two *P. chrysogenum* strains, can be applied to dissect effects of side-chain-precursor availability and β-lactam production. While similar approaches have previously been applied to dissect effects of oxygen availability and nutrient limitation in *S. cerevisiae* [9,45,69], this is to our knowledge the first application of such an approach to a product-forming system. Our experimental design required a strain of *P. chrysogenum* that lacked a functional penicillin gene cluster. A cluster-free strain previously described [28], was derived from the low-producing Wisconsin54-1255 strain. As we sought to maximize the difference between producing and non-producing scenarios, a new cluster-free strain (DS50661) was derived from a penicillin-G-high-producing strain background (DS17690). The approach presented in this paper should be applicable to the production of other secondary metabolites in systems where production can be controlled by the addition of a precursor molecule. In *P. chrysogenum*, this might for example include the production, by engineered strains, of the cephalosporin precursors 7-amino-deacetoxycephalosporanic acid (7-ADCA) [13] and deacetylcephalosporin C [74].

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Chapter 5

Engineering of *Penicillium chrysogenum* for fermentative production of a novel carbamoylated cephem antibiotic precursor

Diana M. Harris, Ilja Westerlaken, Dick Schipper, Zita A. van der Krogt, Andreas K. Gombert, John Sutherland, Leonie M. Raamsdonk, Marco A. van den Berg, Roel A.L. Bovenberg, Jack T. Pronk, Jean-Marc G. Daran

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Abstract

Penicillium chrysogenum was successfully engineered to produce a novel carbamoylated cephalosporin that can be used as a synthon for semi-synthetic cephalosporins. To this end, structural genes for *Acremonium chrysogenum* expandase/hydroxylase and *Streptomyces clavuligerus* carbamoyltransferase were expressed in a penicillin-G high-producing strain of *P. chrysogenum*. Growth of the engineered strain in the presence of the side-chain precursor adipic acid resulted in production of adipoyl-7-amino-3-carbamoyloxymethyl-3-cephem-4-carboxylic acid (adACCA) and of several adipoylated pathway intermediates. A combinatorial chemostat-based transcriptome study, in which the adACCA- producing strain and a strain lacking key genes in ß-lactam synthesis were grown in the presence and absence of adipic acid, enabled the dissection of transcriptional responses to adipic acid per se and to adACCA production. In chemostat cultures of both strains, adipic acid served as an additional carbon source. Transcriptome analysis supported an earlier proposal, based on ^{13}C-labelling studies, that adipic acid catabolism in *P. chrysogenum* occurs via ß-oxidation and enabled the identification of putative genes for enzymes involved in mitochondrial and peroxisomal ß-oxidation pathways. Several of the genes that showed a specifically altered transcript level in adACCA-producing cultures were previously implicated in oxidative stress responses. As strain improvement programmes lead to increased specific productivity and yields, a deeper understanding of these stress responses is likely to be important to also achieve high adACCA titres with engineered strains of *P. chrysogenum*.

Abbreviations

ACV	δ-(L-α-aminoadipyl)-L-cysteine-D-valine
adACCA	adipoyl-7-amino-3-carbamoyloxymethyl-3-cephem-4-carboxylic acid
adADCA	adipoyl-7-aminodesacetoxy cephalosporanic acid
adAHCA	adipoyl-7-aminohydroxycephalosporanic acid
ad6APA	adipoyl-6-aminopenicillanic acid
6APA	6-aminopenicillanic acid
IPN	isopenicillin-N

Introduction

More than 20000 antibiotics have currently been identified [17]. The world market of the over 160 antibiotics that are commercially available exceeds US$ $3x10^{10}$ [16,26]. ß-lactams form the most important group of antibiotic compounds and take up about 65% of the world antibiotic market [20]. ß-Lactams can be naturally produced by a wide variety of microorganisms ranging from filamentous fungi to Gram-positive and Gram-negative bacteria [43] and can be classified in five groups: penams, ceph-3-ems, clavams, monolactams and carbapenams [7]. The first two groups include the penicillins, cephalosporins and cephamycins and are the best characterised and also the most important ß-lactams in terms of sale and production [2]. Due to their broader spectrum of activity, high potency, low toxicity and resistance to ß-lactamase cephalosporins are superior to penicillins [2]. However, as their production processes are costly and complex, their distribution in the global market is limited in terms of volume [13]. Naturally occurring cephalosporins are produced by *Acremonium chrysogenum*, but their efficacy is relatively low. Therefore, all clinically important cephalosporins are produced semi-synthetically from the building blocks 7-aminodeacetoxycephalosporanic acid (7-ADCA) and 7-aminocephalosporanic acid (7-ACA).

A breakthrough in the production of cephalosporins was achieved when the well-established ß-lactam-producing filamentous fungus *Penicillium chrysogenum* was engineered for the production of 7-ADCA [13,56,70]. *P. chrysogenum* cannot naturally produce cephalosporins and cephamycins, but is a superior penicillin producer. Since its discovery [24], strain improvement programmes have resulted in an increase of penicillin productivity by over 1000–fold [66]. By introduction of the *cefE* gene from *Streptomyces clavuligerus* [13], *P. chrysogenum* was equipped with the ability to expand the characteristic penicillin 5-membered thiazolidine ring to a 6-membered dihydrothiazine ring. This *cefE*-encoded deacetoxycephalosporin-C synthetase activity (commonly named expandase) enabled *P. chrysogenum* to produce cephalosporins. Co-feeding of adipic acid as a side-chain precursor resulted in the production of adipoyl-7-ADCA. The adipoyl side chain can be easily removed via enzymatic catalysis. This process has led to a tremendous improvement of 7-ADCA production in terms of end product purity, energy efficiency, reduced use of organic solvents and, obviously, cost reduction [70]. In view of these economic and environmental advantages, it will become increasingly important to integrate chemical steps into biological processes.

Approximately two-thirds of the semisynthetic cephalosporins in use are derived from 7-ACA. In 2002, the worldwide production of 7-ACA-derived antibiotics was ~3000 ton/year [2]. 7-ACA can be produced by fermentation with *P. chrysogenum* via a similar approach as used for the fermentative production of 7-ADCA. Equipping

P. chrysogenum with the *A. chrysogenum* dual function expandase/hydroxylase gene (*cefEF*) and the acetyltransferase (*cefG*) gene resulted in the production of adipoyl-7-ACA [13]. However, as cephalosporins are notoriously instable and several chemical conversions are needed to form active pharmaceutical intermediates (API's) [20], an intermediate that is carbamoylated on the 3-position of the dihydrothiazine ring would offer important advantages.

Carbamoylation of cephalosporins naturally occurs in the biosynthesis process of cephamycins. Cephamycins are produced by various species of *Streptomyces* and *Nocardia* [43]. The biosynthesis pathways for penicillins, cephalosporins and cephamycins have been well characterised and share the same initial steps (Figure 1). The route from deacetylcephalosporin-C to cephamycin-C proceeds via two steps in *S. clavuligerus and N. lactamdurans*. The genes involved have been characterised and cloned [11,12] and the route consists of a carbamoylation via deacetylcephalosporin-C O-carbamoyltransferase (*cmcH*) and a methoxylation via a two-protein O-carbamoyldeacetylcephalosporin-C 7-methoxyl transferase (*cmcI* and *cmcJ*). Provided that carbamoyltransferase can accept adipoyl-7-amino-deacetylcephalosporanic acid as a substrate, extension of the adipoyl-7-ADCA pathway in *P. chrysogenum* [13] with a carbamoyltransferase reaction could in theory yield adipoyl-7-aminocarbamoyl cephalosporanic acid (adACCA), which would represent a (new) precursor for semi-synthetic cephalosporins such as Ceftriaxone, Cefazolin and Ceftazidine [72]. This process could either use the bifunctional expandase/hydroxylase (*cefEF*) from *A. chrysogenum* or the separate expandase (*cefE*) and hydroxylase (*cefF*) from *S. clavuligerus*.

The goal of the present study was to explore the metabolic engineering of *P. chrysogenum* for the production of the important semisynthetic cephalosporin precursor adACCA. In this way, new fermentation processes for the production of this compound can be designed, resulting in a reduction of the use of environmentally unfriendly chemicals as well as a cost reduction. Genetic engineering for this purpose involved the introduction into *P. chrysogenum* of the *cefEF* gene from *A. chrysogenum* and the *cmcH* gene of *S. clavuligerus*. To investigate the physiological impact of the introduction of this novel pathway, physiological and genome-wide transcriptional studies were performed in batch and chemostat cultures of the engineered *P. chrysogenum* strains grown in the absence and presence of the side-chain precursor adipic acid. To distinguish between effects of the side-chain precursor per se and effects of adACCA production, parallel experiments were run with a congenic strain that lacks three key genes for β-lactam biosynthesis.

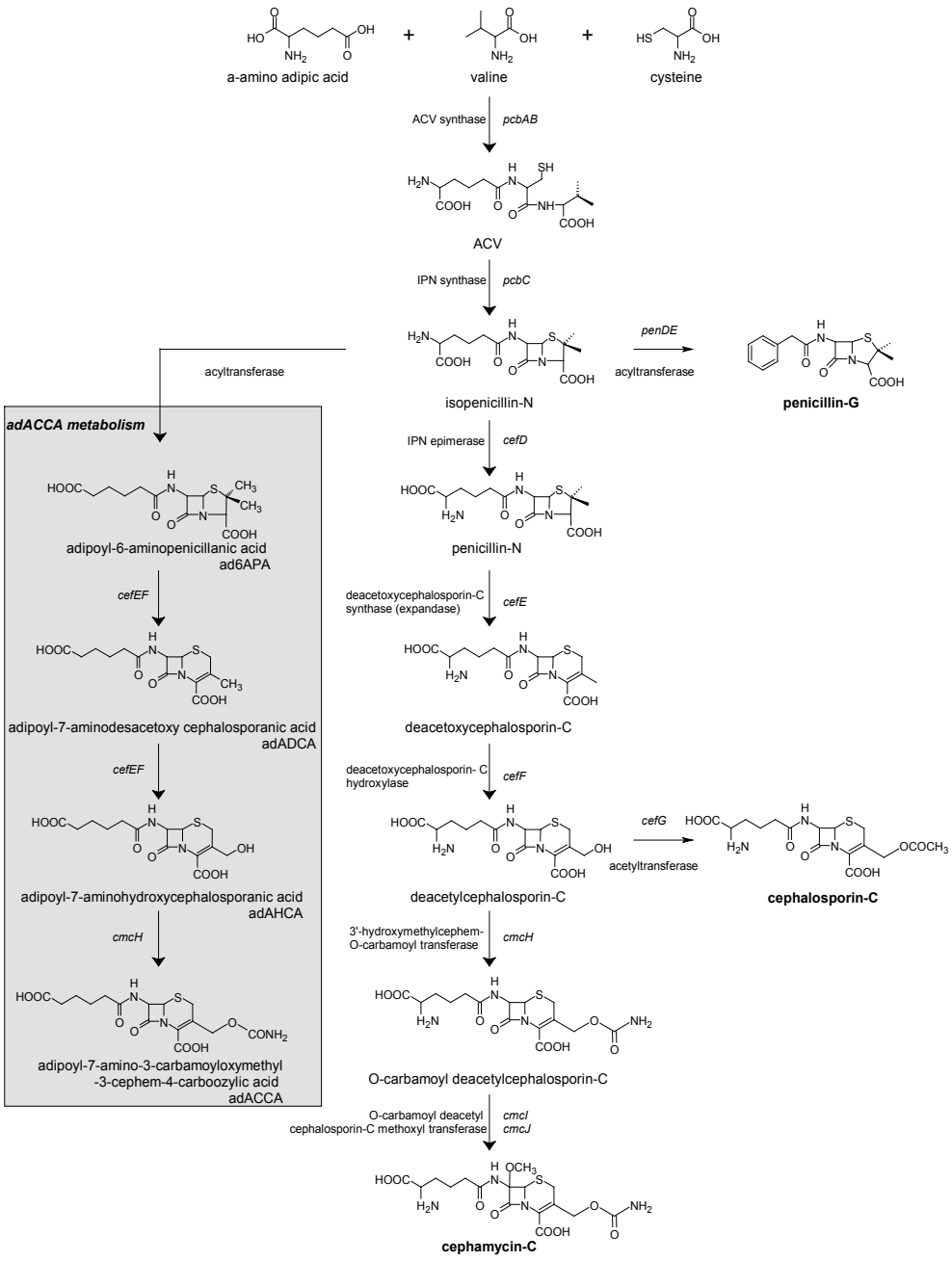


Figure 1: The common pathway for the production of penicillins, cephalosporins and cephamycins in bacteria and fungi. The genes are indicated in italics, the engineered route towards adACCA is shown in the grey box, ACV, δ-(L-α-aminoadipyl)-L-cysteine-D-valine

Materials and Methods

Microbial strains and media

P. chrysogenum DS17690 is a penicillin-G high-producing strain from the DSM lineage [29,37]. *P. chrysogenum* DS50661 (*amdS*) strain is a derivative from DS17690 that misses the penicillin biosynthesis genes (*pcbAB, pcbC* and *penDE*) [30]. Construction of *P. chrysogenum* DS49834 ($pcbC_{pr}$ - *cefEF* - $penDE_{ter}$ $pcbC_{pr}$ - *cmcH* - $penDE_{ter}$), an engineered adACCA-producing strain, is described in this paper.

P. chrysogenum transformants were selected on acetamide-containing media [61] and grown in shake flasks on a defined ß-lactam production medium [15] that was modified by replacing phenyl acetic acid by 0.5 $g{\cdot}L^{-1}$ sodium adipate as side-chain precursor. Product formation was analysed after 168 h of incubation at 25°C and 250 rpm.

Escherichia coli strains DH10b and TOP10 were used for general cloning procedures and obtained from Invitrogen (Carlsbad, USA). Standard growth was performed in 2xYT medium with the appropriate antibiotics [59].

Chemostats cultures were grown on a mineral medium, which was prepared as described [29] and contained per litre of demineralised water 7.5 g glucose, 3.5 g $(NH_4)_2SO_4$, 0.8 g KH_2PO_4, 0.5 g $MgSO_4{\cdot}7H_2O$, 10 mL of a trace element solution. The trace element solution contained 15 $g{\cdot}L^{-1}$ $Na_2EDTA{\cdot}2H_2O$, 0.5 $g{\cdot}L^{-1}$ $Cu_2SO_4{\cdot}5H_2O$, 2 $g{\cdot}L^{-1}$ $ZnSO_4{\cdot}7H_2O$, 2 $g{\cdot}L^{-1}$ $MnSO_4{\cdot}H_2O$, 4 $g{\cdot}L^{-1}$ $FeSO_4{\cdot}7H_2O$, and 0.5 $g{\cdot}L^{-1}$ $CaCl_2{\cdot}2H_2O$. Production of adACCA was induced by adding 5.0 $g{\cdot}L^{-1}$ adipic acid (ADA) to the medium. To account for the fact that adipic acid was not required for ß-lactam biosynthesis 4.3 $g{\cdot}L^{-1}$ was added to the medium of strain DS50661.

Chemostat cultivation

Aerobic glucose-limited chemostat cultivation was performed at 25ºC in 3 - L turbine stirred bioreactors (Applikon, Schiedam, The Netherlands) with a working volume of 1.8 L. The pH was maintained at 6.5 via automated addition of 2 M NaOH (ADI 1030 biocontroller, Applikon, Schiedam, The Netherlands). The fermented was sparged with air at a flow rate of 0.9 $L{\cdot}min^{-1}$ using a Brooks mass-flow controller (Brooks Instruments, Hatfield, USA) and stirred at 750 rpm. The dissolved-oxygen concentration was continuously monitored with an oxygen electrode (Applisens, Schiedam, The Netherlands). Continuous cultivation was initiated after 50-60 hours of batch cultivation. The feed medium was supplied continuously by a peristaltic pump (Masterflex, Cole Parmer, USA) and the dilution rate was set at 0.03 h^{-1} for all chemostat experiments. Effluent was removed discontinuously by means of a special overflow device, which has been described previously [74]. The time interval between effluent removals was fixed in such a way that each time approximately

1 % of the culture volume was removed. To prevent excessive foaming, silicone antifoam (10 % vol/vol, BDH) was discontinuously added at timed intervals. The offgas was cooled by a condensor at 4ºC after which it was dried with a Perma Pure dryer (type MD-110-48P-4, Perma Pure, Toms River USA). Oxygen and carbon dioxide concentrations in the offgas were determined with a NGA 2000 analyzer (Rosemount Analytical, Orville, USA). Off-gas flow rates were determined from an average of 10 measurements using a SAGA digital flow meter (Ion Science, Cambridge, UK). Specific rates of carbon dioxide and oxygen consumption were calculated as described previously [76].

Molecular biology techniques

DNA Cloning

Common techniques in gene cloning [59] were used. PCR fragments were routinely cloned in pCR2.1 TOPO (Invitrogen, Carlsbad, USA) for sequence verification of several clones before continuing. The DNA isolation from *P. chrysogenum* Wisconsin54-1255, *A. chrysogenum* ATCC14553 and *S. clavuligerus* ATCC 27064 were performed as described previously [30].

Cloning of cefEF, encoding expandase-hydroxylase

The expandase/hydroxylase gene *cefEF* was PCR amplified from *Acremonium chrysogenum* genomic DNA using the EXHY-FWD and EXHY-REV primers (Table 1), introducing *Nde*I and *Nsi*I sites upstream and downstream of the gene respectively for cloning. The fragment was cloned into the plasmid pISEWAn digested with the same restriction enzymes, resulting in the plasmid pICEFWA. The construction of the pISEWAn is described in detail in Supplemental Material I. The expression of *cefEF* was controlled by the *pcbC* (encoding IPN synthase from *P. chrysogenum*) promoter and the *penDE* (encoding acyltransferase of *P. chrysogenum*) terminator.

Primer	Sequence (5' - 3')
EXHY-FWD	AAA ACC ACA GCA TCC ATA TGA CTT CCA AGG TCC CCG TCT TTC GTC TC
EXHY-REV	ATG CAT TGG CTC GTC ATG AAG AGC CTA CTA AGT GGC TAT AGG AG
CMCH-FWD	ACA GAC CAT ATG CTC GTC GTT GCA TTC AAG
CMCH-REV	GAC GGC ATG CAT TCA GGA ACC GGC TAT TCG C

Table 1: Primers used in this study

Cloning of cmcH, encoding 3'-hydroxymethylcephem O-carbamoyltransferase

The *cmcH* gene was PCR amplified from *S. clavuligerus* using the CMCH-FWD and CMCH-REV primers (based on Genbank accession number AF073897, Table 1), introducing *Nde*I and *Nsi*I sites upstream and downstream of the gene respectively for cloning. Digestion with *Nde*I and *Nsi*I enabled to clone *cmcH* into the plasmid pISEWAn digested with the same restriction enzymes resulting in the plasmid pIScCTWA. As for the *cefEF* gene, the expression of *cmcH* was controlled by the *pcbC* (encoding IPN synthase from *P. chrysogenum*) promoter and the *penDE* (encoding acyltransferase of *P. chrysogenum*) terminator.

Transformation of Penicillium chrysogenum

All expression cassettes were obtained free from the plasmid backbone by double digestion of the plasmids pICEFWA and pIScCTWA with *Fsp*I and *Not*I. The *amdS* fragment was isolated after digestion with *Hin*dIII from plasmid pHELY-A1 [72]. Transformation of *P. chrysogenum* DS17690 was carried out as described previously [64]. The different expression cassettes were simultaneously transformed resulting in the adACCA producing strain DS49834.

Analytical methods

Determination of culture dry weight

Culture samples (10 mL) were filtered over preweighed glass fibre filters (Type A/E, Pall Life Sciences, East Hills, USA). The filters were washed with demineralised water and dried for 20 minutes at 600 W in a microwave oven and were immediately weighed.

Substrate and metabolite analysis

Glucose and adipic acid concentrations in the medium and in the filtrates of culture samples were determined by HPLC analysis using an Aminex HPX-87H column (Bio-Rad, Hercules, USA) at 60 °C with 5 mM H_2SO_4 as the mobile phase.

β-lactam analysis

Filtrates of cultures were analysed by HPLC, MS and NMR for the production of adipoyl-7-amino-3-carbamoyloxymethyl-3-cephem-4-carboxylic acid (=adACCA). A chemical standard for adACCA was used to quantify the HPLC signals (Supplemental Material I). Quantitative ^{1}H NMR experiments were performed at 600 MHz on a Bruker Avance 600 spectrometer. To a known quantity of filtrate, a known quantity of internal standard (maleic acid), dissolved in phosphate buffer was added prior to lyophilisation. The residue was dissolved in D_2O and measured at 300 K. The delay

between scans (30 s) was more than 5 times T_1 of all compounds, so the ratio between the integrals of the compounds of interest and the integral of the internal standard is an exact measure for the quantity of the penicillins and cephalosporins.

ß-lactam bioassay

Colony purified *P. chrysogenum* strains were transferred to fresh penicillin production agar plates (as in [15], solidified with 15 $g \cdot L^{-1}$ bacto agar) and incubated for 72-96 h at 25ºC. The ß-lactam sensitive *E. coli* ESS2231 strain [55] was cultivated in 2xYT medium [59] to mid-log phase and diluted in pre-warmed 2xYT 0.8% agar and carefully distributed over the fungal colonies. Penase (DIFCO, Franklin Lakes, USA) was added to digest all penicillins. After overnight incubation at 37ºC ß-lactam production is visible as a clear zone (halo) around the producing colonies.

Calculation of the carbamoyl phosphate demand

The total carbamoyl phosphate demand was calculated under non-producing and producing conditions using experimental data from chemostat cultivations and literature data for RNA, DNA and protein contents [31,47,58,63] in Microsoft™ Excel.

Microarray methods

Sampling of cells from chemostats, probe preparation and hybridisation to Affymetrix GeneChip® microarrays were performed as described previously [71]. Independent triplicate cultures were sampled for each growth condition. Samples were filtered within seconds and quenched in liquid nitrogen. Total RNA isolation using Trizol reagent and acid phenol-chloroform for extraction was followed by cDNA synthesis and cRNA synthesis. Hybridised arrays were scanned and analysed using the Affymetrix GeneChip® Operating Software (GCOS, Affymetrix, Santa Clara, USA). All arrays were globally scaled to a target value of 100 using the average signal from all gene features using GCOS. Differential expression was assessed using the Significance Analysis of Micro arrays (SAM version 1.21) add-in to Microsoft™ Excel [51,68]. The fold-difference threshold and the false discovery rate values were set at 2 and 1% respectively. The genes with significantly changed expression in one of the comparisons were arranged in groups via overlapping them in Microsoft™ Excel. Enrichment of MIPS categories (version 1.3) was assessed by Fisher's Exact test employing hypergeometric distribution with a *p*-value cut-off of $2 \cdot 10^{-4}$ (with a Bonferroni correction) [38].

Results and Discussion

Construction of a *Penicillium chrysogenum* strain producing adACCA

In order to produce carbamoylated cephalosporins with *P. chrysogenum*, the penicillin-G high-producing strain DS17690 was transformed with various expression constructs. The *cmcH* and *cefEF* constructs were introduced via co-transformation with the *amd*S selection marker. The integration of the *amd*S marker enables the *P. chrysogenum* transformants to grow on selection medium containing acetamide as the sole nitrogen source [61]. Acetamide-positive clones were transferred to a second acetamide-containing plate and subsequently transferred to induce sporulation. Subsequently, the clones were colony purified and expandase activity was assayed in 202 *amdS*-positive strains using a cephalosporin-specific bioassay. A total number of 41 DS17690 derivatives tested positive.

All 41 expandase-positive strains were tested in shake flasks for adACCA production, which is indicative for the presence of an active *cmcH* gene product. NMR analysis of culture filtrates showed that 10 expandase positive transformants were capable of producing adACCA (see Figure 2A). This result was confirmed by HPLC analysis (data not shown) using chemical synthesized adACCA as a standard (Supplemental Material II). Further confirmation for the identity of the final product was obtained from its chemical stability. Cephalosporins are notoriously instable at pH 13 and/or at 80ºC, while penicillin-G is instable at pH 4. adACCA-containing filtrates were assayed for stability under different conditions. The pH was adjusted with 4N HCl or 4N KOH to either pH 4 or pH 13. The samples were then incubated for 8 h at 4, 25 and 80ºC and subsequently analysed by NMR. Consistent with the characteristics of cephalosporins, adACCA was stable at 4 and 25 ºC degrees and pH 4 and 6 (Figure 2B).

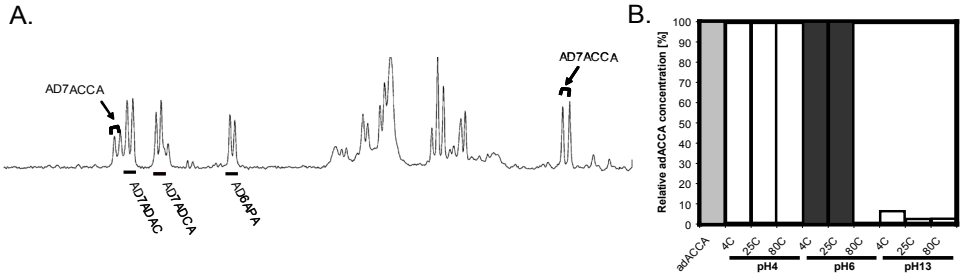


Figure 2: adACCA production in shake flasks. Panel A, NMR spectrum of ad7ACCA producing DS17690 *P. chrysogenum* transformants. Panel B, Relative stability of adACCA at various conditions. The pH of adACCA-containing filtrates was adjusted with 4N HCl or 4N KOH to either pH 4 or pH 13. The samples were then incubated for 8 h at 4, 25 and 80ºC and subsequently analysed by NMR.

For analysis of by-product formation filtrates of well-grown cultures were separated from biomass via filtration and analysed by NMR. The biomass was washed directly twice with ice cold physiological salt (0.85% NaCl), frozen in liquid nitrogen, freeze dried, resuspended in water, and also analysed by NMR. Besides adACCA the strains also produced the intermediates isopenicillin-N (IPN), 6-aminopenicillinic acid (6APA), adipoyl-6-aminopenicillinic acid (ad6APA), adipoyl-3-deacetoxy-7-aminocephalosporanic acid (ad7ADCA) and adipoyl-7-aminohydroxycephalosporanic acid (adAHCA). While the early cephalosporin intermediate ad6APA accumulated extracellularly, ad7ADCA and adAHCA further down in the pathway were mainly retained intracellularly (data not shown). The final product adACCA (adipoyl-7-amino-3-carbamoyloxymethyl-3-cephem-4-carboxylic acid) was the only ß-lactam that was completely recovered in the extracellular fraction (data not shown).

Physiology of adACCA producing strain DS49834 in chemostat cultivations

The adACCA-producing transformant *P. chrysogenum* DS49834 was used for physiological characterisation in glucose-limited chemostat cultures. In shake-flask cultures this strain produced 0.70 ± 0.03 mM (0.28 ± 0.01 $g \cdot L^{-1}$) adACCA. Also in glucose-limited chemostat cultures, fed with the side-chain precursor adipic acid, adACCA was produced (Table 2). Only a small fraction (circa 4%) of the consumed adipic acid was recovered as adACCA. Similar to what was found in shake flasks, substantial amounts of intermediates in the pathway, such as ad-6-APA and adAHCA were formed, accounting for circa 8% of the consumed adipic acid (data not shown). The remaining 88% of the consumed adipic acid could not be recovered in product or by-products. In studies on penicillin-G production, it has been reported [29,73] that induction of ß-lactam biosynthesis by addition of a side-chain precursor resulted in a reduction of the biomass yield on glucose as compared to non-producing conditions. This decrease has been attributed to a large free-energy requirement for ß-lactam biosynthesis [29,73]. Conversely, adipate addition to glucose-limited cultures of *P. chrysogenum* DS50661 resulted in an increase of the apparent biomass yield on glucose (Table 2). This suggested that, in addition to its role as a side-chain precursor, adipic acid served as an additional carbon and energy source for growth of *P. chrysogenum*. In a strain that lacked the capacity to produce ß-lactams (DS50661), due to deletion of the penicillin biosynthesis genes [30], adipic acid metabolism was even more pronounced and led to a 17% increase of the biomass yield on glucose (Table 2). Metabolism of adipic acid has also been reported to occur at low specific growth rates in engineered strains of *P. chrysogenum* that produce the semi-synthetic cephalosporin adADCA [57]. Based on ^{13}C labelling studies it has been proposed that adipic acid degradation in *P. chrysogenum* occurs via ß-oxidation [65].

	Y_{sx}[a] ($g \cdot g^{-1}$)	q_{adACCA}[b] ($\mu mol \cdot g^{-1} \cdot h^{-1}$)	q_{ADA} ($\mu mol \cdot g^{-1} \cdot h^{-1}$)	q_{CO2} ($mmol \cdot g^{-1} \cdot h^{-1}$)	q_{O2} ($mmol \cdot g^{-1} \cdot h^{-1}$)	Avg CV[c]	PcACTA[d]	PcGDH2[e]	n
DS49834 – ADA	0.35 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	1.28 ± 0.12	1.34 ± 0.08	0.14	3720 ± 400	1110 ± 130	3
DS49834 + ADA	0.36 ± 0.01	1.40 ± 0.21	38.05 ± 6.23	1.41 ± 0.06	1.47 ± 0.07	0.14	3160 ± 80	1260 ± 210	4
DS50661 – ADA	0.40 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	1.12 ± 0.06	1.11 ± 0.09	0.16	3450 ± 390	1020 ± 170	3
DS50661 + ADA	0.47 ± 0.02	0.00 ± 0.00	107.73 ± 7.65	1.33 ± 0.07	1.40 ± 0.07	0.11	3660 ± 350	990 ± 240	3

Table 2: Physiological and micro array quality parameters for aerobic, glucose-limited chemostat cultures grown at a dilution rate of 0.03 h^{-1}. *P. chrysogenum* strains DS49834 and DS50661 were grown in the presence and absence of adipic acid (ADA) in independent glucose-chemostat cultures at D=0.03 h^{-1} (n in table).Results are the averages ± S.D. (σ_{n-1}). [a] biomass yield on glucose (g of biomass per g of glucose consumed). [b] biomass specific adACCA production rate. In addition to adACCA, intermediates and by-products are formed, which account for ~8% of the consumed adipic acid. [c] average coefficient of variation (standard deviation divided by the mean) for all genes except the genes with the mean expression level below 12. [d] encoding actin; average signal and standard deviation. [e] encoding glutamate dehydrogenase (NAD^+ dependent); average signal and standard deviation

Experimental design and global transcriptional response towards adACCA production and adipic acid degradation

Genome-wide transcriptome analysis was carried out on the adACCA-producing strain *P. chrysogenum* DS49834 and on strain DS50661, which cannot produce β-lactams due to the absence of the *pcbAB, pcbC* and *penDE* genes [30]. Both strains were grown in glucose-limited chemostat cultures at a dilution rate of 0.03 h^{-1}, in the presence and absence of the adACCA side-chain precursor adipic acid. The average coefficient of variation of the transcriptome data derived from independent triplicate cultures did not exceed 0.16 (Table 2). Moreover, the level of the *actA* [19] and *gdh2* transcripts, common loading standards for Northern analysis, varied by less than 11% over the four situations tested.

Based on the physiological analysis described above, addition of adipic acid to an adACCA-producing strain of *P. chrysogenum* can lead to two modes of adipic acid metabolism: production of adipoylated ß-lactams and use of adipic acid as a carbon and energy source for growth. To distinguish between responses that are related to these two modes of adipic acid metabolism, we used strain DS50661. This strain cannot produce ß-lactams and hence, any transcriptional effects of the addition of adipic acid are independent from its role as a β-lactam side-chain precursor. By subtracting these responses to adipic acid addition from those observed in the adACCA-producing strain, it should be possible to identify responses that are directly linked to cephalosporin production (Figure 3B and Figure 4). Two pairwise transcriptome comparisons between the two strains and two conditions were performed (Figure 3), yielding a total of 288 transcripts (representing 2% of the *P. chrysogenum* genome) that were differentially expressed in at least one of the comparisons according to the statistical criteria applied (|fold difference| ≥ 2 with a false discovery rate of 1%). The majority of the genome (9304 transcripts) did not show significantly different transcript levels between the two conditions and 4154 ORFs (30% of the genome) yielded transcript levels that were below the detection limit in all situations (Figure 3).

All cephalosporin-biosynthesis genes (Table 3) were expressed at high levels (2000-5000), independent of the presence of adipic acid. Both heterologous genes, *cefEF* (Pc90d00700) and *cmcH* (Pc90d01150), were transcribed at comparable levels to the native ß-lactam biosynthesis genes, *pcbAB* (Pc21g21390); *pcbC* (Pc21g21380) and *penDE* (Pc21g21370). Similar to what was found for the production of penicillin-G [71], transcript levels of the PAA-CoA ligase (*phl*, Pc22g14900) [41] were substantially lower (240-350) than those of the genes involved in the synthesis of isopenicillin-N. Interestingly, a second CoA-ligase, Pc22g20270, with strong similarity to 4-coumarate-CoA ligase in *Arabidopsis thaliana* was strongly induced in the presence of adipic acid (7-11 fold in DS49834 and DS50661 respectively), suggesting

that this gene might be involved in activation of adipic acid for adACCA biosynthesis or catabolism (Table 3).

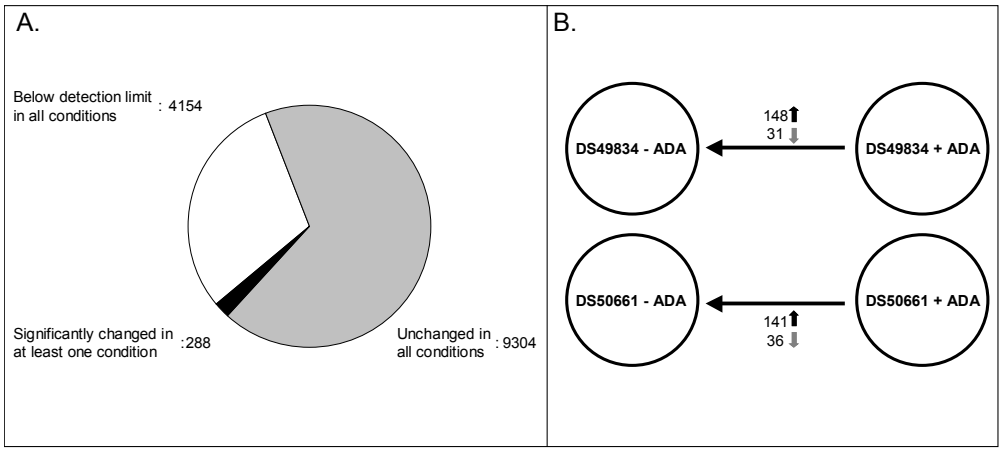


Figure 3: Global transcriptional response of DS49834 and DS50661 strains to the presence and absence of ADA. Total RNA was obtained from *P. chrysogenum* strains DS49834 and DS50661, grown in the presence and absence of adipic acid (ADA) in independent glucose-limited chemostat cultures at D=0.03 h^{-1} and hybridised to Affymetrix GeneChip® microarrays. Panel A, Pie chart of overall transcript changes of the DS49834 and DS50661 strains cultivated in the absence and presence of ADA. Panel B, Results of the pairwise comparisons of the two strains under the two conditions.==

In the final step of adACCA biosynthesis, adAHCA is carbamoylated by carbamoyl transferase. The required carbamoyl phosphate can be synthesised by carbamoyl phosphate synthase (CPS) that acts in arginine and pyrimidine biosynthesis. Based on published pyrimidine and protein contents of *P. chrysogenum* we estimated that even at the low specific rates of adACCA production observed in the chemostat cultures, adACCA production increases the carbamoyl phosphate demand by 15% (Table 4A). In most eukaryotes, two different enzymes catalyse carbamoyl phosphate synthesis, an arginine (CPSI) and a pyrimidine (CPSII) specific enzyme. CPSI, which uses NH_3 as a nitrogen source, consists of two subunits encoded by *cpa1* and *cpa2* [14,46]. CPSII, which uses glutamine as its nitrogen source, consists of 1 unit encoded by *ura2* [5,77]. Homologs of each of these genes were identified in the *P. chrysogenum* genome (Table 4B) and, irrespective of the conditions or strain tested, were transcribed at high levels. No transcriptional responses of genes involved in arginine and pyrimidine biosynthesis were observed that might be indicative for draining of carbamoyl phosphate pools. Nevertheless, we cannot exclude the possibility that the low specific productivity of adACCA in the slowly growing chemostat cultures (as compared with the higher productivity in shake flasks) may be caused by low steady-state pools of carbamoyl phosphate. As, moreover, increased productivity of adACCA will have a strong impact on carbamoyl-phosphate requirements, future metabolic engineering strategies may require overexpression of carbamoyl phosphate synthase.

Gene ID	Descriptions	DS49834 -ADA	DS49834 +ADA	DS50661 -ADA	DS50661 +ADA
Pc21g21390	alpha-aminoadipyl-cysteinyl-valine synthetase pcbAB *acvA* – *P. chrysogenum*	4340 ± 840	3630 ± 340	12 ± 0	12 ± 0
Pc21g21380	isopenicillin N synthase ips *PcbC* – *P. chrysogenum*	5160 ± 800	4060 ± 280	12 ± 0	12 ± 0
Pc21g21370	acyl-coenzyme A:isopenicillin N acyltransferase AAT *penDE* – *P. chrysogenum*	2290 ± 340	2040 ± 500	12 ± 0	12 ± 0
Pc90d00700	*AccefEF*_ExpandaseHydroxylase- *A. chrysogenum*	4810 ± 970	3310 ± 220	12 ± 0	12 ± 0
Pc90d01150	*cmcH*_carbamoyl transferase- *S. clavuligerus*	3520 ± 1160	2430 ± 220	12 ± 0	12 ± 0
Pc22g14900	phenylacetyl-CoA ligase *pclA* – *P. chrysogenum*	240 ± 10	350 ± 30	180 ± 20	360 ± 50
Pc22g20270	strong similarity to 4-coumarate-CoA ligase 4CL – *A. thaliana*	180 ± 10	1360 ± 170	160 ± 40	1910 ± 190

Table 3: Transcript levels of the adACCA biosynthesis genes. Total RNA was obtained from *P. chrysogenum* strains DS49834 and DS50661, grown in the presence and absence of adipic acid (ADA) in independent glucose-limited chemostat cultures at D=0.03 h^{-1} and hybridised to Affymetrix GeneChip® microarrays. Results are the averages ± S.D. (σ_{n-1}).

Catabolism of adipic acid

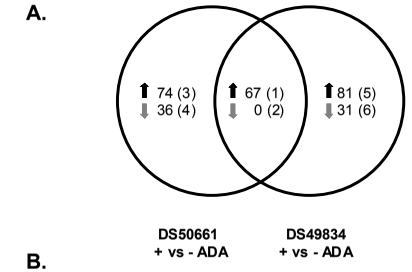
Consumption of adipic acid resulted in significantly higher transcript levels of 67 genes, independent of the production of adACCA (group 1, Figure 4). No genes were identified that showed significantly lower transcript levels under these conditions. Of the genes that were upregulated in cultures fed with adipic acid, almost all showed higher transcript levels in the cluster-free DS50661 strain (which consumed more adipic acid than the adACCA-producing DS49834 strain). Hypergeometric distribution analysis showed that genes involved in lipid, fatty-acid and isoprenoid metabolisms were highly overrepresented among the adipic-acid-upregulated genes. More than half of these genes (34 genes) were present in one of these overrepresented categories (Figure 4). The remaining 33 genes often carried annotations with similarity to hypothetical proteins thus precluding biological interpretation. Among the differentially expressed transcripts, genes with similarity to each of reactions in the ß-oxidation pathway were identified (Figure 5). Whereas ß-oxidation in yeasts exclusively occurs in the peroxisomes [32], both mitochondrial and peroxisomal ß-oxidation have been reported in *Aspergillus* species [25,45]. Also in *P. chrysogenum*, sequence characteristics suggest a dual localization of this pathway, with the responsible genes showing a similar transcriptional response to adipic acid (Figure 5).

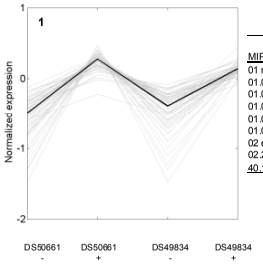
Calculated data	**μ = 0.022 h^{-1}**		**μ = 0.039 h^{-1}**	
Arginine content biomass	6.79	-	8.34	$\mu mol \cdot g^{-1} \cdot h^{-1}$
Pyrimidine content biomass	2.28	-	2.45	$\mu mol \cdot g^{-1} \cdot h^{-1}$
Arginine for adACCA	0.09		0.10	$\mu mol \cdot g^{-1} \cdot h^{-1}$
CP for adACCA	1.40		1.40	$\mu mol \cdot g^{-1} \cdot h^{-1}$
Total CP requirements				
Non producing conditions	9.07		10.80	$\mu mol \cdot g^{-1} \cdot h^{-1}$
Producing conditions	10.47		12.30	$\mu mol \cdot g^{-1} \cdot h^{-1}$

Table 4: Carbamoyl phosphate in *P. chrysogenum*. **Panel A:** Calculated carbamoyl phosphate demand in producing and non-producing conditions. Calculations were based on experimental data for biomass concentrations and specific adACCA production rates and published values for RNA, DNA, UMP, CMP. dCMP, cTMP. protein and arginine. RNA content 0.043^{a}-0.046^{b} $g \cdot g_{Cx}^{-1}$ [31,47], DNA content, 0.009^{a}-0.010^{b} $g \cdot g_{Cx}^{-1}$ [31,47], UMP content RNA 0.81 $mmol \cdot g_{RNA}^{-1}$ [47,58,63], CMP content RNA 0.61 $mmol \cdot g_{RNA}^{-1}$ [47,58,63], dCMP content DNA 0.86 $mmol \cdot g_{RNA}^{-1}$ [47,58,63], dTMP content 0.79 $mmol \cdot g_{RNA}^{-1}$ [47,58,63], protein content 0.390^{a}-0.445^{b} $g \cdot g_{Cx}^{-1}$ [31,47], arginine content in protein, 0.065^{a}-0.070^{b} $mol \cdot mol_{prot}^{-1}$ [34,35]. [a] reported for μ = 0.022 h^{-1}, [b] reported for μ = 0.039 h^{-1}, CP, carbamoyl phosphate. Panel B: next page

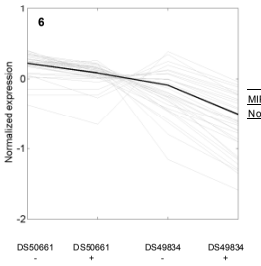
Table 4, Panel B: Transcript levels of genes involved in carbamoyl phosphate metabolism in *P. chrysogenum* Total RNA was obtained from *P. chrysogenum* strains DS49834 and DS50661, grown in the presence and absence of adipic acid (ADA) in independent glucose-limited chemostat cultures at D=0.03 h^{-1} and hybridised to Affymetrix GeneChip® microarrays. Results are the averages ± S.D. (σ_{n-1}).

Gene ID	Description	DS49834 - ADA	DS49834 + ADA	DS50661 - ADA	DS50661 + ADA
Arginine metabolism					
Pc21g17970	~ arginine-specific carbamoyl phosphate synthase small subunit like protein An17g00820 – *A. niger*	1240 ± 120	1250 ± 170	920 ± 130	1290 ± 240
Pc22g20150	~ arginine-specific carbamoyl-phosphate synthase subunit Cpa2 – *S. cerevisiae*	680 ± 80	700 ± 110	630 ± 30	690 ± 40
Pc22g18390	~ ornithine carbamoyltransferase argB – *A. niger*	420 ± 20	400 ± 30	530 ± 40	510 ± 10
Pc12g11190	~ argininosuccinate synthase Arg1 – *S. cerevisiae*	1290 ± 220	1130 ± 140	1270 ± 20	1280 ± 110
Pc20g06010	~ argininosuccinate lyase ASAL – *C. albicans*	460 ± 60	440 ± 70	300 ± 50	330 ± 70
Pyrimidine metabolism					
Pc21g21940	~ arginine-specific carbamoyl-phosphate synthase subunit Cpa2 – *S. cerevisiae*	910 ± 100	860 ± 140	860 ± 270	850 ± 80
Pc21g21940	~ arginine-specific carbamoyl-phosphate synthase subunit Cpa2 – *S. cerevisiae*	910 ± 100	860 ± 140	860 ± 270	850 ± 80
Pc06g00380	~ dihydroorotase Ura4 – *S. cerevisiae*	340 ± 30	290 ± 50	260 ± 50	250 ± 20
Pc21g21940	~ arginine-specific carbamoyl-phosphate synthase subunit Cpa2 – *S. cerevisiae*	910 ± 100	860 ± 140	860 ± 270	850 ± 80
Pc22g21410	~ orotate reductase pyrE – *A. nidulans*	190 ± 20	190 ± 50	190 ± 10	200 ± 10
Pc15g00490	~ orotidine-5-monophosphate pyrophosphorylase - *Ajellomyces capsulatus*	450 ± 60	390 ± 60	360 ± 50	350 ± 40
Pc13g04420	orotidine 5-phosphate decarboxylase pyrG – *P. chrysogenum*	90 ± 10	70 ± 10	80 ± 10	70 ± 0

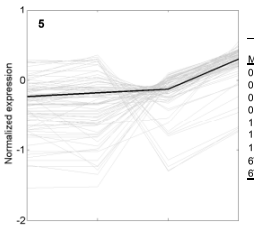




MIPS category	Frequency of occurence	p-value
01 metabolism	32 out of 2472	$2.18 \cdot 10^{-08}$
01.06 lipid, fatty-acid and isoprenoid metabolism	17 out of 410	$7.21 \cdot 10^{-12}$
01.06.01 lipid, fatty-acid and isoprenoid biosynthesis	8 out of 252	$2.92 \cdot 10^{-05}$
01.06.01.05 fatty acid biosynthesis	5 out of 44	$2.21 \cdot 10^{-06}$
01.06.04 breakdown of lipids, fatty acids and isoprenoids	8 out of 91	$1.28 \cdot 10^{-08}$
01.06.07 lipid, fatty-acid and isoprenoid utilization	8 out of 52	$1.31 \cdot 10^{-10}$
02 energy	9 out of 312	$1.91 \cdot 10^{-05}$
02.25 oxidation of fatty acids	4 out of 13	$3.56 \cdot 10^{-07}$
40.19 peroxisome	7 out of 68	$3.63 \cdot 10^{-08}$



MIPS category
No functional categories signficantly overrepresented



MIPS category	Frequency of occurence	p-value
01 metabolism	29 out of 2472	$1.00 \cdot 10^{-04}$
01.20 secondary metabolism	12 out of 379	$2.06 \cdot 10^{-06}$
01.20.05 biosynthesis of acetic acid derivatives	5 out of 97	$2.64 \cdot 10^{-04}$
01.20.05.11 biosynthesis of polyketides	5 out of 88	$1.67 \cdot 10^{-04}$
11 cell rescue, defense and virulence	22 out of 809	$8.76 \cdot 10^{-10}$
11.07 detoxification	14 out of 379	$3.93 \cdot 10^{-08}$
11.07.05 detoxification by export	9 out of 165	$5.17 \cdot 10^{-07}$
67 transport facilitation	16 out of 819	$1.89 \cdot 10^{-05}$
67.28 drug transporters	11 out of 183	$9.28 \cdot 10^{-09}$

Figure 4: Cross-sections, profiles and overrepresented functional categories of the pairwise comparisons. Transcript data from independent chemostat cultures of *P. chrysogenum* strains DS49834 and DS50661 grown at D=0.03 h^{-1} in the presence and absence of adipic acid (ADA) were compared in two pairwise comparisons (DS49834 + ADA versus DS49834 – ADA and DS50661 + ADA versus DS50661 – ADA. Genes significantly changed in at least one of the two pairwise comparisons were overlapped as shown panel A, this resulted in 6 different groups of genes comparing the response to ADA in DS49834 and DS50661 1. genes with a higher expression in the presence of ADA both in DS49834 and DS50661; 2. genes with a lower expression in the presence of ADA both in DS49834 and DS50661; 3. genes with higher expression in the presence of ADA in DS50661 and not in DS49834; 4. genes with lower expression in the presence of ADA in DS50661 and not in DS49834; 5. genes with higher expression in the presence of ADA in DS49834 and not in DS50661; 6. genes with lower expression in the presence of ADA in DS49834 and not in DS50661. Panel B shows the gene-expression profiles of the groups of specific interest with the results of the hypergeometric distribution analysis for enrichment of functional categories. The thick line represents the average of the mean normalized expression data of the genes comprising the cluster. The y-axis represents ^{10}log expression values. Functional categories are mentioned together with their P-value and the number of genes in the respective functional category in the group of genes with a higher expression compared to the prevalence of this functional category in the whole genome. Due to a large redundancy in the functional categories some categories might appear without having a significant biological relevance.

In agreement with the overrepresentation of genes participating to adipic acid catabolism via ß-oxidation, genes putatively involved in the transport of acetyl-CoA over the mitochondrial and/or the peroxisomal membrane showed co-regulated profiles. Although a gene with similarity to carnitine o-acetyltransferase of *C. tropicalis* (Pc20g15010), catalysing the coupling of acetyl-CoA to carnitine [21], could not be retrieved from group 1 (Figure 4), it was found in group 3 (only significantly higher in DS50661 in the presence of adipic acid, Figure 4). In both strains transcript levels of this gene increased when adipic acid was consumed, but in the adACCA producing strain the increase was below the threshold of 2-fold (1.4x). The gene with similarity to the transporter that exchanges acylcarnitine for carnitine in *A. nidulans* (Pc22g00520, [50,54]) showed an over 2-fold difference in transcript level in response to adipic acid in both strains. Acetyl-CoA can perform a biosynthetic role if its production can be coupled to the glyoxylate shunt (Figure 5). Indeed, genes with similarity to both glyoxylate-cycle enzymes, malate synthase (Pc20g13550) and isocitrate lyase (Pc22g03660) showed elevated transcript levels in the adipic acid consuming cultures. However, transcription of the putative isocitrate lyase gene was only increased by 1.9 fold in the adACCA producing strain and was thus retrieved in group 3. Increased production of acetyl-CoA from β-oxidation of adipic acid is also consistent with the higher transcript levels of a putative pyruvate carboxylase-encoding gene (Pc22g15180), as increased availability of acetyl-CoA will divert more glucose into anaplerosis [53,60].

Group 1 (Figure 4), which consists of genes with a higher transcript level in adipic acid-fed cultures of both strains, contained several additional genes that can be related to adipic acid metabolism via ß-oxidation (Figure 5). The glyoxylate cycle, which is involved in the assimilation of acetyl-CoA, is generally assumed to be located in the peroxisomes in fungi. A functional glyoxylate cycle requires the continuous oxidation of succinate via the mitochondrial succinate dehydrogenase. In *S. cerevisiae,* this is made possible by a mitochondrial succinate-fumarate antiporter [6]. This system probably also operates in *P. chrysogenum* as a gene with strong similarity to the yeast succinate-fumarate transporter (Pc21g19470) was specifically upregulated in the adipic acid consuming cultures (Figure 5). Group 1 also harboured a gene with a strong similarity to an *A. niger* mitochondrial dicarboxylic acid transporter (Pc12g07430) that has been reported to exchange succinate or fumarate for phosphate [48]. Given the structural similarities of succinate and adipate, it is conceivable that the encoded transporter also catalyses import of adipic acid into the mitochondria. Finally, this set of adipate-upregulated genes contained a gene with strong similarity to the yeast peroxisomal transporter (Pc13g03380) that has been reported in *S. cerevisiae* to replenish the ATP required for the activation of the fatty acids by acyl-CoA synthase [49,75].

Cephalosporin specific response

Groups 5 and 6 (Figure 4) contain genes that show a specific transcriptional response under conditions that enable adACCA production. 81 genes showed a higher transcript level under adACCA producing conditions and 31 genes showed a lower expression. Among the 31 genes with a lower expression when adACCA was produced, no functional categories were significantly enriched. Moreover, the function of a large fraction of these genes was unknown and could not be derived from comparisons with other organisms.

Among the genes that showed significantly elevated transcript levels under adACCA-producing conditions, a clear overrepresentation was observed for genes related to secondary metabolism, detoxification and transport (Figure 4, group 5). Remarkably, the gene with the highest fold difference compared to non-producing

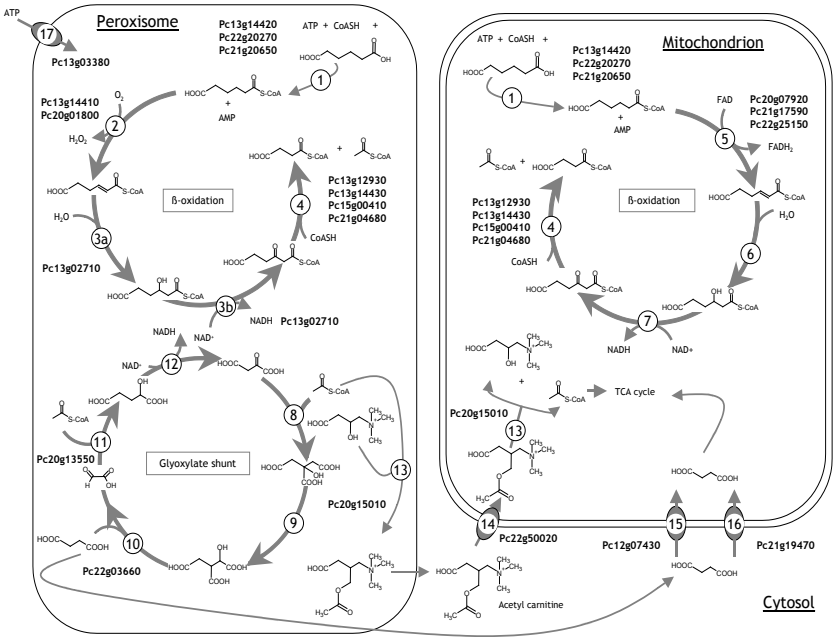


Figure 5: Genes involved in adipic acid catabolism and their putative placement in the metabolism of *P. chrysogenum*. Genes of which the transcript levels were significantly changed in the presence of adipic acid only in the DS50661 or in both the DS50661 and DS49834 strains (group 1 and 3) are shown in the figure. Genes named in the figure were annotated to be similar to genes with the following functions: 1, acyl:CoA synthetase; 2, acyl:CoA oxidase; 3a, enoyl:CoA hydratase activity of multifunctional enzyme; 3b, 3-hydroxacyl:CoA dehydrogenase activity of multifunctional enzyme; 4, 3-oxoacyl:CoA thiolase; 5, acyl:CoA dehydrogenase; 6, enoyl:CoA hydratase; 7, 3-hydroxyacyl: CoA dehydrogenase; 8, citrate synthase; 9, aconitase; 10, isocitrate lyase; 11, malate synthase; 12, malate dehydrogenase; 13, carnitine o-acetyltransferase; 14, carnitine acyl carnitine carrier; 15, dicarboxylic acid transporter; 16, succinate fumarate transporter; 17, peroxisomal transporter. Total RNA was obtained from *P. chrysogenum* strains DS49834 and DS50661, grown in the presence and absence of adipic acid (ADA) in independent glucose-limited chemostat cultures at D=0.03 h^{-1} and hybridised to Affymetrix GeneChip® microarrays.

conditions shows similarity to the 7α-cephem methoxylase subunit in *Streptomyces lactamdurans* (Pc13g04180). 7α-cephem methoxylase in *S. lactamdurans* consists of two subunits encoded by *cmcI* and *cmcJ* and it converts cephalosporin C to 7-methoxycephalosporin C [12]. Whereas the *P. chrysogenum* genome contains four genes with similarity to *cmcJ,* none show similarity to *cmcI*, which makes it difficult to speculate on any role in the production or modification of cephalosporins.

Group 5 (Figure 4) also contains numerous genes related to the production of other secondary metabolites. For instance four genes were found were found that showed sequence similarity to genes involved in biosynthesis of the secondary metabolite lovastatin. Three of these (Pc15g00520; Pc20g05830 and Pc12g14200) show similarity to *lovC* encoding for enoylreductase in *A. terreus* and one has similarity with *lovF* encoding for the polyketide synthase lovastatin diketide from *A. terreus*. This putative polyketide synthase gene (Pc21g05080) is located in a small cluster [71] together with a gene with strong similarity to membrane protein Tpo2 of *S. cerevisiae* (Pc21g05100) and Pc21g5110 of unknown function, with all three genes exhibiting the same transcript profile. The fourth member of this cluster was expressed at the highest levels, but not regulated upon adACCA biosynthesis. Within group 5 only one other gene was located in a cluster, Pc21g05060, with similarity to salicylate hydroxylase from *Pseudomonas stutzeri*. However, none of the other genes in this cluster showed coregulation. In addition to the putative lovastatin biosynthesis genes, three genes putatively encoding for the major facilitator superfamily cercosporin transporter were found in this group. This gene has been reported to be involved in export and resistance against the perylenequinone toxin cercosporin, produced by the plant pathogens *Cercospora nicotinae* and *C. kikuchii* [9,69].

The induction of four genes with homology to glutathione S-transferases (Pc22g23120; Pc21g23640; Pc12g06200 and Pc12g14390) would suggest an oxidative stress response under these conditions [23,52]. However, other characteristic genes involved in oxidative stress responses, such as those encoding superoxide dismutase and catalase, did not show increased expression following adACCA production. The genome of *P. chrysogenum* contains at least 12 putative catalase genes, of which nine were not expressed, one exhibited low transcript levels, and two showed very high transcript levels irrespective of the culture conditions. Similarly, the five putative superoxide dismutase genes in the genome were all transcribed one showing very high transcript levels under all four conditions tested. The transcriptional responses to adACCA production in *P. chrysogenum* showed a remarkable overlap with transcription responses in rats upon administration of aflatoxin B1. This response in rats involved transcriptional upregulation of an aflatoxin B1 aldehyde reductase, two glutathione S-transferases, a cytochrome p450 monooxygenase and a NADPH quinone oxidoreductase with each of the responsible genes containing oxidative

stress response elements in their promoter region [22]. Homologs to each of these genes are present within the group of genes with a higher transcript level under adACCA producing conditions. Of the three genes in the *P. chrysogenum* genome with strong similarity to 'old yellow enzyme' (NADPH oxidoreductase) genes, two were specifically upregulated in adACCA producing cultures (Pc16g11690, Pc18g06290). Although old yellow enzyme has been known since 1933, its precise function remains unknown [78]. In yeasts, it was shown to mediate resistance to the reactive aldehyde acrolein and to protect the actin cytoskeleton from oxidative stress [28,67]. In addition, in *S. cerevisiae* it is co-regulated with genes related to oxidative stress when the cells are switched from glucose to oleate as a carbon source [39]. Similarly, after exposure to H_2O_2 its protein abundance was also increased [27]. Although peroxisomal ß-oxidation is known to induce an oxidative stress response due to the H_2O_2 formed in the acyl:CoA oxidase step, the genes involved in the oxidative stress response identified here were not induced in the cluster-free strain in the presence of adipic acid, suggesting that the response is specifically the result of adACCA production. Several cephalosporins have been implicated in causing oxidative stress in bacterial and mammalian cells, probably via generation of hydrogen peroxide [4,10,36]. The exact mechanism for cephalosporin-mediated oxidative stress remains unclear and warrants further research in order to successfully address possible negative effects of high-level adACCA production by *P. chrysogenum*. adACCA-mediated oxidative stress may possibly have contributed to the different productivities in shake-flask and chemostat cultures as, due to the poor oxygen transfer characteristics of shake flask cultures, these may be less susceptible to oxidative stress.

Conclusions

Functional expression in a β-lactam-high-producing strain of *Penicillium chrysogenum* of the *Acremonium chrysogenum cefEF* gene and the *Streptomyces clavuligerus cmcH* gene, encoding an expandase/hydroxylase and a 3' hydroxymethylcephem-O-carbamoyltransferase, respectively, resulted in production of extracellular adACCA (adipoyl-7-amino-3-carbamoyloxy methyl-3-cephem-4-carboxylic acid) in cultures that were provided with the side-chain precursor adipic acid. AdACCA, a carbamoylated cephalosporin, forms an interesting synthon for several semi-synthetic cephalosporins. Although the first-generation adACCA producing strain DS49834 described in this paper was produced in a penicillin-G-high-producing background, DS17690, the adACCA titre was 5-10 times lower than the penicillin-G titre in the parent strain. Based on determinations of the intermediate metabolites, rate-controlling steps might reside in the last three steps of the biosynthesis pathway. While this study represents a proof of principle

for the production of carbamoylated cephalosporins by engineered *P. chrysogenum*, it is clear that the kinetics of the product formation pathway require further research. In particular, it will be necessary to investigate the *in vivo* kinetics of the expressed heterologous enzymes. Especially for the bacterial *cmcH* gene, it may prove beneficial to improve the *in vivo* activity of its protein product via adaptation of its codon usage and, when required, by further protein engineering.

Hitherto, strain improvement programmes for ß-lactam production have predominantly relied on mutagenesis and selection strategies [1,42]. Indeed, such approaches are likely to make significant contributions to the further improvement of adACCA production by engineered *P. chrysogenum* strains. The transcriptome analysis described in the present study indicates how, additionally, genomics- based approaches can guide strain improvement via targeted metabolic engineering. A simple combinatorial approach, involving chemostat experiments to reduce experimental variation, two strains and two growth conditions, was used to dissect transcriptional effects of side-chain precursor availability and ß-lactam production. By comparing transcriptomes of the adACCA producing *P. chrysogenum* strain, DS49834, and of a strain devoid of the capacity to produce ß-lactams (DS50661), both grown in the presence and absence of the side-chain precursor adipic acid, the transcriptional responses resulting from the availability of adipic acid per se and those related to adACCA production could be clearly distinguished.

A large fraction of the adipic acid fed to the chemostat cultures was used as an additional carbon source for biomass formation rather than as a side-chain precursor. As the side-chain precursor is an important cost factor in industrial fermentations, reduction of this catabolism forms a relevant target for strain improvement. The transcriptional responses to adipic acid strongly supported an earlier proposal, based on ^{13}C labelling experiments, that adipate metabolism in *P. chrysogenum* occurs via β-oxidation. Moreover, the transcriptome analysis identified a clear set of candidate genes for this pathway. After further analysis of the substrate specificity of the encoded proteins, these genes will provide clear targets for metabolic engineering to reduce or even eliminate adipate consumption. Based on the annotation of the adipate-induced genes it seems probable that ß-oxidation in *P. chrysogenum* resembles that in various *Aspergillus* species and occurs both in peroxisomes and in mitochondria. Complete elimination of adipate degradation via β-oxidation may therefore require deletion of genes for enzymes active in each of these compartments.

Among the genes whose expression profile followed that of adACCA production, many showed homology to genes involved in oxidative-stress-related responses. Several cephalosporins have been shown to induce oxidative stress via H_2O_2. Further research into the mechanism by which adACCA synthesis induces these responses, some of which exhibited at striking similarity to the transcriptional responses of rat

cells to aflatoxin B1, seems especially relevant for strategies to minimize product inhibition as the productivity and titres of adACCA in engineered *P. chrysogenum* are further increased.

The present study illustrates that integration of metabolic engineering, established procedures for classical strain improvement and newly implemented genomics approaches may contribute to the development of *P. chrysogenum* into a multi-purpose platform for fermentative production of non-native antibiotics.

Acknowledgements

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Supplementary material

Supplemental Material I: Construction of plasmid pISEWAn

The expandase gene (cefE) was PCR amplified from *Streptomyces clavuligerus*-ATCC 27064 chromosomal DNA [30] using the EXP-FWD and EXP-REV primers (based on [33,40], Supplemental Table 1), introducing NdeI and XbaI sites upstream and downstream of the gene respectively for cloning. The fragment was then cloned into the plasmid pMcTNdeI [44,62], digested with the same restriction enzymes, resulting in the plasmid pMcTSE.

The promoter of penDE (P_{penDE}) was PCR amplified from *P. chrysogenum* (based on [3], Supplemental Table 1) using the PromAT-FWD and PromAT-REV primers, introducing EcoRI and NdeI sites. The terminator of penDE (T_{penDE}) was PCR amplified from *P. chrysogenum* (based on [3]) using the TermAT-FWD and TermAT-REV primers (Supplemental Table 1), introducing BglI and SpeI sites. cefE, P_{penDE} and T_{penDE} were cloned into pBluescript (Stratagene, Cedar Creek, USA) digested with EcoRI-SpeI, resulting in the plasmid pASEWA (EcoRI-PpenDE-NdeI-cefE- BglI-TpenDE-SpeI).

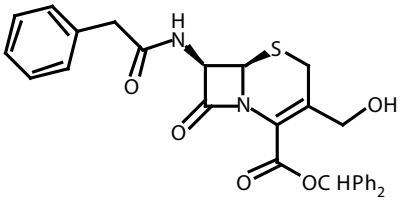
The IPNS promoter was amplified from *P. chrysogenum* (strain) chromosomal DNA using the PromIPNS-FWD [18] and PromIPNS-REV primers [8] (Supplemental Table 1) introducing EcoRI and NdeI sites. The fragment was then cloned into the plasmid pASEWA digested with the same restriction enzymes, exchanging the promoter of penDE. A second NotI site was introduced in front of the expression cassette via replacing the unique KpnI-HindIII fragment by 5'-GGT ACC GGG CCC CCC CTC GAG TGC GGC CGC AAG CTT-3' yielding pISEWAn.

Primer	Sequence (5' - 3')
EXP-FWD	GAT CAG TGA CAG TTG CAT ATG GAC ACG ACG GTG CCC ACC TTC AGC CTG
EXP-REV	CCC GGG TCT AGA TCT AGA CTA TGC CTT GGA TGT GCG GCG GAT GTT
PromAT-FWD	AGA ACG GAT TAG TTA GTC TGA ATT CAA CAA GAA CGG CCA GAC
PromAT-REV	GAC AGA GGA TGT GAA GCA TAT GTG CTG CGG GTC GGA AGA TGG
	TTC GAT GTC AGC CTG GAC GGC GAG ACC GCC ACG TTC CAG GAT TGG ATC GGG
	GGC AAC TAC GTG AAC ATC CGC CGC ACA TCC AAG GCA TGA AGG CTC TTC ATG
TermAT-FWD	ACG
TermAT-REV	GGA CTA GTG TCG ACC CTG TCC ATC CTG AAA GAG TTG
PromIPNS-FWD	CGA GGG GAA TTC CTT ATA CTG GGC TG CTG CAT TGG TCT G
PromIPNS-REV	CCC GGG CAT ATG CAT ATG GGT GTC TAG AAA AAT AAT GGT GAA AAC

Supplemental Table 1: Primers used for the construction of plasmid pISEWAn

Supplemental Material II: Chemical synthesis of adipoyl-7-amino-3-carbamoyloxymethyl-3-cephem-4-carboxylic acid (adACCA)

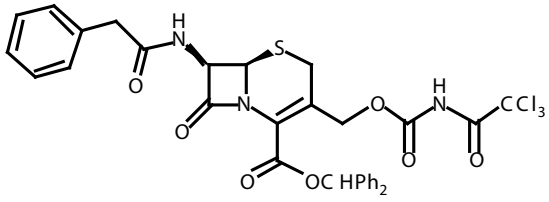
Preparation of phenylacetyl-7-amino-3-hydroxymethyl-3-cephem-4-carboxylic acid -benzhydryl ester [IV]



[IV]

8.5 ml of 20% NaOH solution was added dropwise to a stirred solution of 7-amino-cephalosporanic acid (5 g, 18.3 mmol) in water (20 ml) at 0°C. After stirring for 5 min the pH was adjusted to 8.5 with acetic acid and the solution was diluted with acetone (20 ml). Phenylacetyl chloride (2.9 ml, 22 mmol) in acetone (3 ml) was then added dropwise with the pH kept between 7.5 and 8.5 by addition of aqueous NaOH. The solution was then stirred for 1 h at 0°C. The acetone was then removed *in vacuo* before addition of ethyl acetate (70 ml) and acidification of the aqueous phase to pH 3 with dilute HCl. The organic phase was separated and the aqueous phase re-extracted with another portion of ethyl acetate. The organic phases were combined, washed with brine ($MgSO_4$) and filtered. To this solution was then added a solution of diphenyldiazomethane (5 g, 25.8 mmol) in ethyl acetate (5 ml) with stirring. The solution was concentrated to ca. 40ml *in vacuo* and left overnight at 4°C. The resultant precipitate was collected by filtration and washed with ethyl acetate giving the product as a white powder (3.83 g, 40%). δ_H ($(CD_3)_2SO$, 300 MHz) 3.54 & 3.63 (2H, ABq, J 13.9, $PhC\underline{H}_2$), 3.65 (2H, s, SCH_2), 4.25 (2H, s, $C\underline{H}_2OH$), 5.15 (1H, d, J 4.7, $CHC\underline{H}S$), 5.76 (1H, dd, J 4.7, 8.2, $NHC\underline{H}$), 6.94 (1H, s, $C\underline{H}Ph_2$) 7.2-7.6 (15H, m, $\underline{Ph}_2CH$, $\underline{Ph}CH_2$), 9.17 (1H, d, J 8.2, $N\underline{H}CH$). M/z (ES+) 537 (M+Na, 100%).

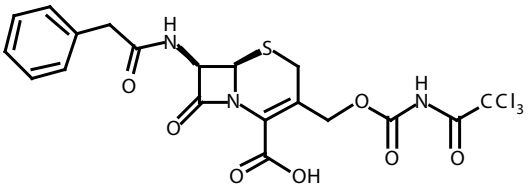
Preparation of trichloroacetyl- phenylacetyl-7-amino-3-carbamoyloxymethyl-3-cephem-4-carboxylic acid -benzhydryl ester [V]



[V]

Trichloroacetyl isocyanate (0.23 ml, 1.9 mmol) was added to a stirred solution of [IV] (600 mg, 1.2 mmol) in acetone (20 ml). After stirring for 2 h a white precipitate was collected by filtration, washed with acetone and dried (811 mg, 99%). δ_H ($(CD_3)_2SO$, 300 MHz) 3.54 & 3.62 (2H, ABq, J 13.9, PhC<u>H</u>$_2$), 3.66 & 3.76 (2H, ABq, J 18.5, SC<u>H</u>$_2$), 4.90 & 5.02 (2H, ABq, J 12.8, C<u>H</u>$_2$O), 5.20 (1H, d, J 4.8, CHC<u>H</u>S), 5.82 (1H, dd, J 4.8, 8.1, NHC<u>H</u>CH), 6.96 (1H, s, C<u>H</u>Ph$_2$), 7.2-7.6 (15H, m, CH<u>Ph</u>$_2$, <u>Ph</u>CH$_2$), 9.17 (1H, d, J 8.1, N<u>H</u>CH), 12.0 (1H, s, CON<u>H</u>CO). M/z (ES-) 702 (M-1,100%).

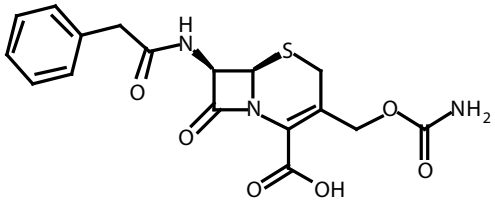
Preparation of trichloroacetyl-go phenylacetyl-7-amino-3-carbamoyloxymethyl-3-cephem-4-carboxylic acid rco [VI]



[VI]

[V] (5.15 g, 7.33 mmol) was dissolved in a cooled (0°C) mixture of trifluoroacetic acid (35 ml) and anisole (4 ml). After stirring for 2 h the solution was concentrated *in vacuo* to an oil which was triturated with petroleum ether (40-60°C fraction) then dissolved in ethyl acetate (30 ml) and decolourised with charcoal. After filtering, the solution was concentrated *in vacuo* to a yellow oil which was used for the next step without further purification. δ_H ($(CD_3)_2SO$, 300 MHz) 3.50-3.75 (4H, m, PhC<u>H</u>$_2$, SC<u>H</u>$_2$), 4.94 (1H, part ABq, J 12.5, CH$_2$O), 5.14 (2H, m, part ABq C<u>H</u>$_2$O, CHC<u>H</u>S), 5.72 (1H, m, NHC<u>H</u>CH), 7.0-7.4 (5H, m, <u>Ph</u>CH$_2$), 9.12 (1H, d, J 8.2, N<u>H</u>CH), 11.95 (1H, s, CONHCO). M/z (ES-) 536 (M-1, 67%).

Preparation of phenylacetyl-7-amino-3-carbamoyloxymethyl-3-cephem-4-carboxylic acid [VII]

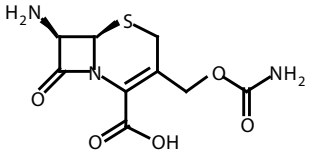


[VII]

The yellow oil from the previous step was carefully dissolved by addition of 10% $NaHCO_3$ solution until a pH of ca. 9 was reached. The solution was then stirred overnight. The pH was then lowered to 2 using dilute HCl at which point precipitation

occurred. The precipitate was removed by filtration and washed with ether to give a pale yellow solid (1.81 g, 63.3% over 2 steps). δ_H ($(CD_3)_2SO$, 300 MHz) 3.4-3.65 (peaks masked by H_2O Peak), 4.63 & 4.91 (2H, ABq, J 12.8, CH_2O), 5.10 (1H, d, J 4.5, CHC<u>H</u>S), 5.68 (1H, dd, J 4.5, 8.2, NHC<u>H</u>CH), 7.28 (5H, m, <u>Ph</u>CH_2), 9.11 (1H, d, J 8.2, N<u>H</u>CH). δ_H($(CD_3)_2SO$ + D_2O, 300 MHz) 3.52 (4H, m, PhC<u>H</u>$_2$, SC<u>H</u>$_2$), 4.63 & 4.88 (2H, ABq, J 12.9, C<u>H</u>$_2$O), 5.05 (1H, d, J 4.8, CHC<u>H</u>S), 5.65 (1H, d, J 4.7, NHC<u>H</u>CH), 7.28 (5H, m, <u>Ph</u>CH_2). M/z (ES-) 390 (M-1, 20%).

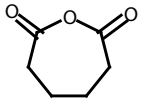
Preparation of 7-amino-3-carbamoyloxymethyl-3-cephem-4-carboxylic acid [VIII]



[VIII]

[VII] (760 mg, 1.95 mmol) was stirred in potassium phosphate buffer (20 ml, 0.5 M, pH 7) and the pH was raised to pH 7.8 by addition of aqueous NaOH. Penicillin amidase on acrylic beads (ca. 375 mg after washing to remove glucose stabilizing agent) was added and the resultant suspension was stirred for 2.5 h. The beads were then removed by filtration and the pH of the solution was lowered to 3 by addition of dilute HCl. The solution was then cooled overnight at 4ºC and then filtered to yield the product as a pale yellow powder (334 mg, 63%). δ_H ($(CD_3)_2SO$ + D_2O, 300 MHz) 3.36 & 3.53 (2H, ABq, J 18.1, SCH_2), 4.60 & 4.82 (2H, ABq, J 12.8, CH_2O), 4.74 (1H, d, J 4.9, CHC<u>H</u>S), 4.94 (1H, d, J 4.9, H_2NC<u>H</u>CH).

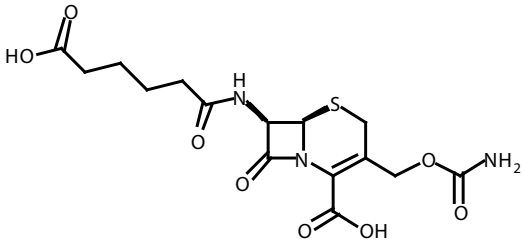
Preparation of cyclic adipic anhydride [IX]



[IX]

A mixture of adipic acid (5 g, 34 mmol) and acetic anhydride (15 ml) was heated at reflux for 4 h. The solution was then concentrated *in vacuo* and the remaining residue was vacuum distilled to give a colourless oil (exposure to atmospheric moisture causes polymerisation). δ_H ($CDCl_3$, 300 MHz) 2.0 (4H, m, $CH_2C\underline{H}_2C\underline{H}_2CH_2$), 2.76 (4H, t, J 6.6, $OC\underline{H}_2CH_2CH_2C\underline{H}_2O$).

Preparation of adipoyl-7-amino-3-carbamoyloxymethyl-3-cephem-4-carboxylic acid [X]



[X]

A solution of [VIII] (100 mg, 0.37 mmol) in aqueous acetone (10 ml, 1:1 v/v) was adjusted to pH 8.5 by careful addition of aqueous NaOH. To this solution a solution of [IX] (80 mg, 0.625 mmol) in acetone (2 ml) with the pH kept between 7.5 and 8.5 by addition of aqueous NaOH was added dropwise at 0°C. The resultant solution was stirred at 0°C for 2 h before removal of the acetone *in vacuo* and adjustment of the pH to 2 by addition of aqueous HCl. The solution was extracted with cyclohexanone (2 x 20 ml) and the combined organic phases concentrated to a few ml. The concentrated cyclohexanone solution was poured into cyclohexane (200 ml) giving a precipitate that was collected by filtration. (61 mg, 41%). δ_H ($(CD_3)_2SO$, 300 MHz) 1.50 (4H, m, $CH_2C\underline{H}_2C\underline{H}_2CH_2$), 2.22 (4H, m, $C\underline{H}_2CH_2CH_2C\underline{H}_2$), 3.45 & 3.59 (2H, ABq, J 18.1, SCH_2), 4.62 & 4.90 (2H, ABq, J 12.9, CH_2O), 5.10 (1H, d, J 4.8, CHC<u>H</u>S), 5.67 (1H, dd, J 4.8, 8.2, NHC<u>H</u>CH), 8.82 (1H, d, J 8.2, N<u>H</u>CH). M/z (ES-) 801 (2M-1, 17%), 400 (M-1, 35%).

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Summary

of the thesis: Transcriptomics and quantitative physiology of ß-lactam-producing Penicillium chrysogenum

Since the discovery of penicillin in 1928 by Alexander Fleming, and its subsequent commercial development in World War II, the use of antibiotics has increased tremendously. It has been estimated that antibiotics have added almost 10 years to our life expectancy. Soon after the implementation of penicillins in clinical use, the first resistant strains started to emerge, which has resulted in a still ongoing search for new and better antibiotics. The most important class of antibiotics are the so-called ß-lactam antibiotics, which among others comprise the penicillins and cephalosporins. These antibiotics are primarily produced by the filamentous fungi *Penicillium chrysogenum* and *Acremonium chrysogenum*, respectively. In essence, the cephalosporin production pathway is an extension of the penicillin biosynthesis route as they share the first biosynthetic reactions. Both antibiotics are produced from three amino acids, α-aminoadipic acid, cysteine and valine, which are condensed to form the tripeptide L-α-aminoadipyl-L-cysteinyl-D-valine. The concerted action of several enzyme-catalysed reactions, including ring closure and ring expansion, is required for the formation of these antibiotics. Although ß-lactams are considered as secondary metabolites, the requirement of three amino acids for their biosynthesis requires a constant interplay between central metabolism and product pathways. Especially in high-producing strains, one might expect that an equally high drain of these amino acids from central metabolism can have a severe impact on the metabolic network of the microorganism. Indeed, based on metabolic modelling studies, it was shown that high production of penicillin-G, one of the model penicillins, imposed a significant burden on the cells in terms of redox equivalents (in the form of NADPH) and energy requirement. These models were largely based on assumptions of the topology of the metabolic network, derived from better-studied microorganisms such as the yeast *Saccharomyces cerevisiae*. Therefore, before embarking on metabolic engineering strategies, it was a first priority to characterise the NADPH metabolism of *P. chrysogenum*. NADPH is a cofactor for many enzymes, including some involved in cysteine biosynthesis. As a conserved moiety, it can exist in two forms, an oxidized and reduced form ($NADP^+$ and NADPH, respectively). In many microorganisms, the reduction of $NADP^+$ to NAPDH occurs via the oxidative reactions of the pentose-phosphate pathway, while it is mainly oxidized in anabolic reactions, for instance in the biosynthesis of amino acids. Although this is the general picture of NADPH metabolism in microorganisms, some differences can occur, depending on microorganism and growth condition. Chapter 2 therefore investigates the most important reactions involved in oxidation

and reduction of NADPH in glucose-limited cultures of *P. chrysogenum*. It was shown that the first two enzymes in the pentose-phosphate pathway, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, were indeed the most important sources of NADPH. In addition, also isocitrate dehydrogenase was important in the provision of NADPH. In terms of competing reactions for NADPH, glutamate dehydrogenase was confirmed to be an important sink of NADPH. Interestingly, we also identified the presence of an external mitochondrial NADPH dehydrogenase coupled to the respiratory chain. This opens the possibility that, in addition to its well-established assimilatory role, NADPH can also fulfil a dissimilatory role in *P. chrysogenum*. This NADPH dehydrogenase forms an interesting target for metabolic engineering strategies, as it is a possible competitor for cytosolic NADPH of ß-lactam biosynthesis.

In addition to the importance of NADPH for ß-lactam biosynthesis, previous metabolic modelling studies also indicated an unexpectedly high additional energy dissipation associated with the production of these antibiotics. In Chapter 3 it was therefore investigated if the use of an auxiliary energy source could result in increased penicillin-G production levels. In organisms such as *P. chrysogenum* the carbon source, in this thesis glucose, is not only used as a carbon donor it also fulfils a dissimilatory role. Part of the glucose is required for the generation of ATP equivalents in dissimilation. As the carbon source is an important cost factor in industrial fermentations, it could be interesting if the required metabolic energy were provided by a cheaper source. Such an auxiliary substrate can be dissimilated to provide free energy equivalents, but cannot be used as a carbon source for growth. Formate is a suitable model auxiliary energy substrate as it is oxidized in one step to CO_2 by formate dehydrogenase with the reduction of NAD^+ to NADH. The formed NADH can be coupled to the mitochondrial respiratory chain and lead to ATP production. To test whether use of auxiliary substrates can indeed lead to an improved production of penicillin-G from glucose, a high-producing *P. chrysogenum* was cultivated in glucose-limited chemostat cultivations with increasing concentrations of formate. In theory, the glucose carbon freed by the provision of ATP by formate oxidation, could be used for both biomass formation and penicillin-G formation or a combination of both. Indeed, formate consumption resulted in increased biomass yields on glucose at a constant specific rate of penicillin production, thus resulting in increased volumetric productivity and penicillin yield on glucose. Based on metabolic modelling studies it could be concluded that transport of formate did not require the input of free energy. However, above a formate-to-glucose ratio of 4.5, formate concentrations in the broth increased, probably due to constraints in the formate-oxidizing system. This accumulation coincided with a loss of the coupling between formate oxidation and the production of biomass and penicillin-G. Nevertheless, these results demonstrate that, in principle, mixed-substrate feeding

can be used to increase the yield on carbon source of 'assimilatory' products such as ß-lactams.

The second part of this thesis benefited strongly from the availability of the complete genome sequence of *P. chrysogenum* and of custom-made microarrays. These developments, which were driven by our industrial partner DSM, allowed full-scale studies of the transcriptome of this organism. By comparing the transcriptome under various conditions, we sought to analyse which cellular functions were likely to be specifically important for each of these conditions. Penicillin-G biosynthesis requires the exchange of the α-aminoadipic acid side chain for a Coenzyme A-activated phenylacetic acid side chain. Until recently, the impact of penicillin-G production on the metabolism of *P. chrysogenum* was mainly studied by comparing the same strain cultivated in the presence and the absence of phenylacetic acid. However, this approach has two main disadvantages. 1. By addition of phenylacetic acid to the medium two parameters are altered at once, penicillin-G production and the presence of the weak acid phenylacetic acid. 2. Even in the absence of phenylacetic acid, *P. chrysogenum* produces some intermediates in the ß-lactam biosynthesis pathway. In order to circumvent this complication, a second *P. chrysogenum* strain was added to the analysis. This second strain was a direct derivative of the high-producing strain, but it lacked the three penicillin-biosynthesis (*pcbAB*, *pcbC*, *penDE*) genes and thereby could not produce intermediates, even in the absence of phenylacetic acid. By cultivating both strains in the absence and in the presence of phenylacetic acid it was possible to dissect the two effects: the presence of phenylacetic acid and the production of penicillin-G. Chapter 4 describes this combinatorial approach and shows that a small but significant fraction of the consumed phenylacetic acid is not retrieved in penicillin-G, but catabolised. Even in the penicillin-biosynthesis gene-clusterfree strain, some phenylacetic acid was consumed and its addition resulted in a small reduction of the biomass yields, which might be indicative for uncoupling by this weak acid. From the transcriptome studies it could be derived that catabolism of phenylacetic acid occurs via the homogentisate pathway. Genes in this pathway are among the most highly induced genes in the presence of phenylacetic acid and form interesting targets for metabolic engineering. Removal of the biosynthesis cluster resulted in pleiotropic effects. Interestingly, a group of genes physically related were increased in expression upon removal of the cluster. Based on the annotation of these genes it could be hypothesised that they are involved in the production of another secondary metabolite. The final, and possibly most interesting set of genes, were those with an expression profile that matched penicillin-G production. The response suggested involvement of genes involved in sulfur and nitrogen assimilation. The transporter for penicillin-G is still unknown. Although it cannot be excluded that the penicillin transporter is among the 700 constitutively transcribed

transporters, the 40 putative transporters with an expression profile that matches that of penicillin-G production form an interesting group for initial analysis. From these initial transcriptome studies many targets for metabolic engineering were identified. Future studies will need to show the importance of each of these genes for increased production levels or reduced oxidation of the side chain precursor.

Whereas the first chapters of this thesis all dealt with the model penicillin, penicillin-G, the final chapter studies the production of a cephalosporin intermediate, adACCA. Cephalosporins are naturally produced by *A. chrysogenum*. All cephalosporins in clinical use are semi-synthetic cephalosporins, being produced from fermentatively produced building blocks. As cephalosporin production levels in *A. chrysogenum* are relatively low, the superior ß-lactam-producing *P. chrysogenum* was modified to produce cephalosporin precursors. By expression of two heterologous genes from fungal and bacterial origin in *P. chrysogenum* it was possible to produce adACCA biologically upon the addition of the side chain precursor adipic acid. Production of adACCA was characterised in shake flasks and chemostat cultures. Except for adACCA, all intermediates in the pathway were also excreted in substantial amounts, which should require improvement in the future. In addition to producing adACCA, the engineered strains catabolised adipic acid in considerable amounts. By employing a similar strategy to that described in chapter 4, it was possible to specifically dissect the responses as a result of adipic acid consumption and of adACCA production. Based on the transcriptional response the hypothesis that adipic acid catabolism occurs via ß-oxidation was substantiated and target genes for future genetic intervention were identified.

The studies described in this thesis almost all form part of the important analysis phase of the metabolic engineering cycle. Using the newly available genome sequence of *P. chrysogenum* it was possible to explore transcriptional responses towards ß-lactam production and side chain catabolism at a genome-wide scale. In addition, the investigation of NADPH metabolism has addressed a targeted, important aspect of ß-lactam production in a classical manner. Each of these studies has provided putative targets for metabolic engineering, future work will now need to show if these targets have the desired effect.

Diana Harris

Samenvatting

van het proefschrift: Transcriptomics en kwantitatieve fysiologie van ß-lactam producerende Penicillium chrysogenum

Sinds de ontdekking van penicilline in 1928 door Alexander Fleming en de daaropvolgende commerciële ontwikkeling tijdens de Tweede Wereldoorlog, heeft het gebruik van antibiotica een enorme vlucht genomen. Het wordt geschat dat de antibiotica onze levensverwachting met bijna 10 jaar hebben verlengd. Snel na het implementeren van penicilline in de kliniek ontwikkelden de eerste resistente stammen zich. Dit heeft geleid tot een voortdurende zoektocht naar nieuwe en betere antibiotica. De belangrijkste groep van antibiotica wordt gevormd door de ß-lactam-antibiotica, waartoe onder andere de penicillines en cephalosporines behoren. Deze antibiotica worden voornamelijk geproduceerd door, respectievelijk, de filamenteuze schimmels *Penicillium chrysogenum* en *Acremonium chrysogenum*. In essentie is de cephalosporine productie-route een uitbreiding van de penicillinebiosynthese-route. De eerste biosynthetische reacties worden gedeeld en beide antibiotica worden geproduceerd uit dezelfde drie aminozuren, α-aminoadipaat, cysteine en valine. Deze worden gecondenseerd om zo de tripeptide L-α-aminoadipyl-L-cysteinyl-D-valine te vormen. Om ß-lactam-antibiotica te maken is de gelijktijdige activiteit van verschillende enzymgekatalyseerde reacties nodig, waaronder het vormen van de karakteristieke ring en het expanderen hiervan door het inbouwen van een extra koolstofatoom. Hoewel ß-lactam- antibiotica als secundaire metabolieten beschouwd worden, is een constante interactie tussen het centrale metabolisme en de productvormingsroute nodig om de benodigde aminozuren aan te leveren. Vooral in hoogproducerende stammen kan verwacht worden dat de hoge vraag naar de drie aminozuren uit het centrale metabolisme een flinke invloed op het metabole netwerk kan hebben. Inderdaad is op basis van metabole modelleringsstudies aangetoond dat hoge productie van penicilline-G, één van de modelpenicillines, een significante last vormde voor de cellen door een sterk toegenomen gebruik van redoxequivalenten (in de vorm van NADPH) en van vrije energie (ATP-equivalenten). De voor deze voorspellingen gebruikte modellen waren voornamelijk gebaseerd op aannames omtrent de samenstelling van het metabole netwerk, afgeleid van beter bestudeerde micro-organismen zoals de gist *Saccharomyces cerevisiae*. Voordat metabolic engineering van NADPH-metabolisme kon worden overwogen, was het daarom allereerst noodzakelijk om het NADPH-metabolisme van *P. chrysogenum* in kaart te brengen. NADPH is een cofactor voor verschillende enzymen, waavan een aantal betrokken is bij de cysteinebiosynthese. Als een conserved moiety kan deze cofactor in twee vormen voorkomen, een geoxideerde en een gereduceerde vorm (respectievelijk $NADP^+$ en NADPH). In veel micro-organismen wordt $NADP^+$

gereduceerd to NADPH via de oxidatieve reacties van de pentose-fosfaatroute. Oxidatie gebeurt voornamelijk in anabole reacties, bijvoorbeeld bij de biosynthese van aminozuren. Afhankelijk van het micro-organisme en de kweekcondities kunnen echter afwijkingen van dit algemene patroon optreden. In Hoofdstuk 2 werden daarom de belangrijkste reacties betrokken bij de oxidatie en reductie van NADPH in glucosegelimiteerde chemostaatcultures van *P. chrysogenum* in kaart gebracht. De eerste twee enzymen van de pentose-fosfaatroute, glucose-6-fosfaatdehydrogenase en 6-fosfogluconaatdehydrogenase, bleken inderdaad belangrijke bronnen van NADPH te zijn. Isocitraatdehydrogenase was een derde belangrijke bron van NADPH. Wat betreft reacties die concurreren met de penicillinebiosynthese voor NADPH is bevestigd dat glutamaatdehydrogenase een belangrijke rol speelt in NADPH-oxidatie. Behalve bovenstaande reacties werd ook een NADPH-dehydrogenase aan de buitenkant van de mitochondriën aangetoond. Door dit enzym kan NADPH in *P. chrysogenum*, naast een rol in assimilatie, mogelijk ook belangrijk zijn voor het leveren van energie. Aangezien deze NADPH-dehydrogenase zou kunnen concurreren voor het cytosolisch NADPH met de ß-lactam-biosynthese, vormt dit enzym een interessant doelwit voor metabolic engineering- studies.

Naast het belang van NADPH voor ß-lactambiosynthese, lieten eerdere metabole modelleringstudies ook een onverwacht hoge, aan antibioticaproductie gekoppelde investering van vrije energie zien. In Hoofdstuk 3 is daarom onderzocht of het gebruik van een tweede energiebron de productie van penicilline-G kon verbeteren. In micro-organismen zoals *P. chrysogenum* wordt de koolstofbron, in dit proefschrift glucose, niet alleen gebruikt als koolstofbron. Een deel van de glucose is namelijk nodig voor levering van ATP equivalenten via dissimilatie. Aangezien de koolstofbron een belangrijke factor is in de kosten van industriële fermentaties, zou het interessant kunnen zijn als de benodigde metabole energie geleverd kan worden door een goedkoper substraat. Een dergelijk “hulpsubstraat” kan wel gedissimileerd worden om energie-equivalenten te leveren maar kan niet gebruikt worden als koolstofbron voor groei. Formiaat is een interessant model voor een hulpsubstraat aangezien het in een stap tot CO_2 geoxideerd kan worden door het enzym formiaatdehydrogenase. Oxidatie van de hierbij gevormde NADH via de mitochondriële ademhalingsketen kan vervolgens ATP leveren. Om te onderzoeken of het gebruik van een hulpsubstraat inderdaad tot verbeterde productie van penicilline-G uit glucose kan leiden, is een hoogproducerende stam van *P. chrysogenum* in glucosegelimiteerde chemostaatcultures gegroeid. Aan deze cultures werden verschillende concentraties formiaat toegevoegd. In theorie zou vervangen van glucosedissimilatie door formiaatoxidatie kunnen leiden tot een verhoogde productie van de biomassa- en/of penicilline-G-opbrengst op glucose. Formiaatconsumptie leidde inderdaad tot verhoogde opbrengst van biomassa op

glucose. Omdat de specifieke penicillineproductiesnelheid van de biomassa niet werd beïnvloed namen bovendien zowel de volumetrieke productiviteit als de opbrengst van penicilline op glucose toe. Met behulp van metabole modellen kon afgeleid worden dat transport van formiaat in *P. chrysogenum* geen ATP kost. Boven een molaire formiaat-glucoseverhouding van 4.5 nam de formiaatconcentratie in het fermentatiebeslag echter toe. Waarschijnlijk was dit het resultaat van kinetische beperkingen in het formiaatoxiderende systeem. Gelijktijdig met deze ophoping werd de hierboven genoemde koppeling tussen formiaatoxidatie en de productie van biomassa en penicilline-G verloren. Desalniettemin tonen deze resultaten aan dat de opbrengst van assimilatoire producten zoals ß-lactamen verhoogd kan worden door het gebruik van een hulpsubstraat.

Het tweede deel van dit proefschrift heeft veel profijt gehad van de beschikbaarheid van de volledige DNA-volgorde van het genoom van *P. chrysogenum* en de daarbij behorende microarrays. Dankzij onze industriële partner DSM was het mogelijk om genoombreed het transcriptoom van *P. chrysogenum* te bestuderen. Door het transcriptoom onder verschillende condities te vergelijken, werd onderzocht welke cellulaire functies belangrijk waren voor ieder van die condities. Voor de biosynthese van penicilline-G moet de α-aminoadipaatzijketen omgewisseld worden voor een Coenzym-A-geactiveerde fenylazijnzuurzijketen. Tot voor kort werd de invloed van de productie van penicilline-G op het metabolisme van *P. chrysogenum* voornamelijk bestudeerd door dezelfde penicilline-G-producerende stam in aan- en afwezigheid van fenylazijnzuur te kweken. Deze aanpak heeft echter twee belangrijke nadelen: 1. Door de toevoeging van fenylazijnzuur aan het medium worden twee parameters tegelijkertijd veranderd, namelijk de productie van penicilline-G en de aanwezigheid van fenylazijnzuur, een zwak zuur. 2. Zelfs in de afwezigheid van fenylazijnzuur kan *P. chrysogenum* een aantal van de intermediairen in de ß-lactamroute produceren. Om meer precieze uitspraken te kunnen doen over processen die bij de penicilline-G-productie betrokken zijn, werd een tweede stam van *P. chrysogenum* onderzocht. Deze tweede stam is een directe afgeleide van de hoogproducerende stam, maar mist de drie penicilline-biosynthesegenen (*pcbAB, pcbC* en *penDE*), waardoor deze stam zelfs in de afwezigheid van fenylazijnzuur geen ß-lactamen meer kan produceren. Door beide stammen in de aan- en afwezigheid van fenylazijnzuur te kweken was het mogelijk om effecten van de aanwezigheid van fenylazijnzuur en van de productie van penicilline-G te onderscheiden. In Hoofdstuk 4 wordt deze combinatoriële aanpak beschreven. Hierbij bleek dat een klein, maar significant deel van de geconsumeerde fenylazijnzuur niet in penicilline-G terechtkomt maar gekataboliseerd wordt. Zelfs in de penicilline-biosynthese gen-clustervrije stam werd een deel van de fenylazijnzuur geconsumeerd. Toevoeging van fenylazijnzuur leidde in beide stammen tot een kleine verlaging van de

biomassaopbrengst op glucose. Dit zou kunnen wijzen op ontkoppeling door dit zwakke zuur. Uit de transcriptoomstudies kon worden afgeleid dat katabolisme van fenylazijnzuur hoogstwaarschijnlijk via de homogentisaatroute plaatsvindt. Genen voor de enzymen die betrokken zijn bij deze route vertoonden een zeer hoog boodschapper-RNA-niveau (mRNA) in aanwezigheid van fenylazijnzuur en zijn daarom interessante kandidaten voor metabolic engineering studies. Het verwijderen van de drie biosynthesegenen leidde tot wijdverspreide effecten op de transcriptie. Zo werd waargenomen dat een groep genen die naast elkaar gelokaliseerd is op het genoom, een verhoogd mRNA-niveau vertoonde ten opzichte van de hoogproducerende stam. Gebaseerd op de annotatie van deze genen lijken ze betrokken te zijn bij de productie van een ander secundair metaboliet. De derde, en mogelijk meest interessante groep genen, vertoonde een expressieprofiel dat direct gekoppeld was aan de productie van penicilline-G. Gebaseerd op de annotatie waren vooral genen betrokken bij de assimilatie van zwavel en stikstof significant in expressie veranderd. De transporter voor penicilline-G is nog steeds onbekend. Er kan niet uitgesloten worden dat de transporter van penicilline-G wordt gecodeerd door één van de 700 transporter-genen waarvan het mRNA-niveau onafhankelijk is van de penicilline-G-productiesnelheid. Echter een groep van 36 transporter-genen waarvan het mRNA-profiel precies het profiel van de penicilline-G-productie volgt, vormt een interessante groep van genen om in eerste instantie verder te bestuderen. In deze eerste transcriptoomstudies in *P. chrysogenum* zijn veel verschillende kandidaten voor functionele analyse en metabolic engineering geïdentificeerd. Verder onderzoek zal moeten uitwijzen hoe belangrijk elk van deze genen is voor verhoogde productie van penicilline-G of verminderde oxidatie van de zijketenprecursor.

Daar waar de eerste hoofdstukken van dit proefschrift de productie van penicilline-G bestudeerden, wordt in het laatste hoofdstuk de productie van het cephalosporine- intermediair adACCA bestudeerd. Cephalosporines worden in de natuur geproduceerd door *A. chrysogenum*. Alle cephalosporines die klinisch gebruikt worden zijn semisynthetische cephalosporines, die gemaakt worden van fermentatief geproduceerde bouwstenen. Aangezien de productie van cephalosporines in *A. chrysogenum* betrekkelijk laag is, is de betere ß-lactamproducent *P. chrysogenum* aangepast om cephalosporineprecursors te maken. Door twee genen, afkomstig uit een schimmel en uit een bacterie, in *P. chrysogenum* tot expressie te brengen werd het mogelijk om adACCA biologisch te produceren in *P. chrysogenum* culturen waaraan de zijketenprecursor adipaat was toegevoegd. De productie van adACCA is in schudkolven en chemostaten gekarakteriseerd. Behalve adACCA werden ook alle intermediairen van de syntheseroute in substantiële hoeveelheden uitgescheiden. Dit zal in de toekomst verbeterd moeten worden. Naast adACCA productie waren de

nieuwe stammen in staat tot de consumptie van flinke hoeveelheden adipaat. Via een vergelijkbare strategie als beschreven in hoofdstuk 4, was het mogelijk om de responsen als gevolg van adipaatconsumptie en adACCA-productie te onderscheiden. Gebaseerd op de transcriptionele responsen was het mogelijk om de hypothese dat adipaatkatabolisme via ß-oxidatie plaatsvindt te versterken. Daarnaast zijn genen geïdentificeerd die een sleutelrol kunnen spelen bij metabolic engineering voor het voorkomen van deze ongewenste afbraak van de zijketenprecursor.

Het onderzoek dat is beschreven in dit proefschrift vormt een onderdeel van de belangrijke analysefase van de "metabolic engineering-cyclus". Met de nieuw beschikbare genoomsequentie van *P. chrysogenum* was het mogelijk om genoomwijd responsen gerelateerd aan ß-lactamproductie en zijketenmetabolisme te bestuderen. Daarnaast is in het onderzoek naar het NADPH-metabolisme op een meer klassieke manier een belangrijk deelproces in de microbiële ß-lactamproductie bestudeerd. Elk van deze studies heeft mogelijke kandidaten voor metabolic engineering opgeleverd. Toekomstig onderzoek zal moeten uitwijzen of veranderingen in deze doelen het beoogde effect zullen hebben.

Diana Harris

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Nu dit boekje bijna voltooid is wordt het de hoogste tijd om alle mensen te bedanken die op de een of andere manier betrokken zijn geweest bij de tot standkoming hiervan (en dat zijn er veel!).

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Diana, Juni 2007

Curriculum Vitae

Diana Marianne Harris was born on December 8th 1977 in Leiden, The Netherlands. In 1996 she passed her pre-university exams (VWO) at the Rijnlands Lyceum Oegstgeest and started her academic career with the study Biomedical Sciences at Leiden University. During these studies she conducted three practical training periods of 3, 6 and 9 months. After a three-month internship at the Centre for Electron Microscopy at Leiden University in which the effect of TiO_2 containing sunscreens on the skin was examined, Diana went for half a year to Edinburgh, Scotland to work on HIV resistance against inhibitor medication at the Centre for HIV Research of the University of Edinburgh. Her final graduation project was performed at the Leiden University Medical Centre (LUMC) in which the T-cell response of celiac disease patients against gluten was studied. After graduation in November 2000, she started a 2-year postgraduate training in Biotechnology at the Delft University of Technology. The second year of this education was spent at Centocor BV in Leiden, where she worked in the Fermentation Development group under supervision of Nienke Vriezen and Ben Bulthuis on biomass control in large-scale mammalian cell cultures. In March 2003 she graduated *cum laude* and started her PhD research project at the Delft University of Technology in the section Industrial Microbiology of professor Jack Pronk in July of the same year. The subject of this research was the physiological and transcriptional characterisation of the filamentous fungus *Penicillium chrysogenum*. The results of this work are described in the current thesis. In August 2007 Diana moved back to Centocor BV to work on the production of monoclonal antibodies by mammalian cells.

List of publications

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