APPENDIX A

Relation Between Penicillin and Siderophores Production in *Penicillium chrysogenum*

A.1 Methodology

A.1.1 Fungal Strains and Cultures Conditions

*Penicillium chrysogenum* strains analyzed in this study are described in chapter 5 and 6, and listed in Tables 5.1 and 6.1. Primers designed for the gene deletion constructs are listed in Tables S5.1 and 6.2. Media were prepared as described in chapter 5. The medium was supplemented with 18.4 mM phenylacetic acid (PAA), when indicated. Cells were growing for 7 days in shaking flasks at 220 rpm and 25°C.

A.1.2 qPCR Analysis and Gene Copy Number

The penicillin biosynthetic gene copy number was determined for all studied strains as described (Nijland et al. [2010]) using primers directed against the genes of penicillin cluster as listed in Tables S5.3 and S2.5.

A.1.3 Extracellular Metabolite Levels

Extracellular concentrations of phenylacetic acid and penicillin G were determined by high-pressure liquid chromatography (HPLC-UV) using an isocratic flow of acetonitrile at 245 g/l, 640 mg/l KH$_2$PO$_4$, and 340 mg/l H$_3$PO$_4$. Separation was performed on Shim-pack XR-ODS C18 column (3.0 × 75 mm, 2.2 µm, Shimadzu, Japan) at a flow rate of 0.5 ml/min at 40°C. Chromatograms were acquired at a wavelength
of 254 nm (Harris et al. [2006]). Levels were corrected for growth differences by dry weight measurements.

A.2 Results - Penicillin Production under Iron Limited Conditions

The DS54465 strain of *P. chrysogenum* shows a high β-lactam antibiotics production yield (Newbert et al. [1997]). Isopenicillin N synthetase, one of the key enzymes in β-lactam production, requires Fe$^{2+}$ for functioning. Therefore, the role of the *pss*, *psm*, *pst* genes that are involved in siderophore production (see Chapter 5 and 6) was analyzed in relation to penicillin production. Strain DS54465 contains eight copies of the penicillin biosynthetic gene clusters and these were maintained in the various *pss* deletion strains (Tab. 5.1) and the strain in which the putative transport genes *pst* and *psm* (Chapter 6) were deleted (data not shown). Strain DS54465 strain was grown in penicillin production medium (PPM) with phenylacetic acid (PAA) at: a) standard iron concentration (+Fe), b) at standard iron concentration with BPS (+Fe, +BPS), c) in the absence of iron (-Fe), and d) in the absence of iron with BPS (-Fe, +BPS). For the parental strain, there was no drastic change in β-lactam titer when cells were grown in the presence of a standard concentration of iron with or without the BPS chelator. However, under iron depletion (-Fe) and chelating conditions (-Fe, +BPS) the penicillin titers were reduced to 18 and nearly 0%, respectively (Fig. A.1A).

To examine the impact of the *pss* gene deletion on penicillin production, iron standard (+Fe) and depletion (-Fe) conditions were examined. However, there was no major additional effect of the deletion of the *pssA*, *pssB* and *pssC* genes on penicillin production even when cells were grown in the absence iron (Fig. A.1C). Also for the various putative siderophore transporters gene deletion, there was no major effect on penicillin production (Fig. A.1D). Finally, the expression of the *pss* genes was analyzed by qPCR in relation to penicillin production. The cultures were grown under standard penicillin producing conditions in the presence (+Fe, +PAA) or absence of iron (-Fe, +PAA), while control growth experiments were not supplemented with PAA. The expression of the *pss* genes was elevated in the iron depleted growth conditions, but was only marginally affected by the presence or absence of PAA (Fig. A.1B).

A.3 Discussion

The formation of penicillin (Fig. A.1A) (Leiter et al. [2001]) and other secondary metabolites (Yin et al. [2013]) can be affected by the iron content in the medium. The iron requirement for optimal penicillin production in *Penicillium chrysogenum*
A.3. Discussion

exceeds that for growth by a factor 10 (Nielsen and Jørgensen [1995]). In addition to many iron-dependent proteins involved in crucial intracellular processes (Oberegger et al. [2002]), the iron concentration has to be sufficient to allow for full activity of the key enzyme of β-lactam biosynthesis Isopenicillin N-synthase. In vitro, the enzyme requires 100 µmol/l of Fe^{2+} to reach its maximum activity (Ramos et al. [1985]). We have investigated the relation between the penicillin G production and iron, with a special emphasis on siderophores biosynthesis using a high β-lactam producing industrial strain of *P. chrysogenum* (Fig. A.1C,D). Previous observations have shown that the addition of iron and/or iron in combination with siderophores (ferrichrome and coprogen) did not influence the β-lactam production in low-iron minimal medium (Leiter et al. [2001]). Although, in contrast to our experiment where the cultures were grown under iron-devoid conditions, Leiter et al. [2001] grew the *P. chrysogenum* cells on a complex medium and the amount of iron was enough for antibiotic biosynthesis even after the transfer to low-iron media.

PssA is responsible for the production of coprogen (Chapter 5). The expression of *pssA* has been shown to be slightly up-regulated when cells are grown in the presence of phenylacetic acid (PAA) – a precursor of penicillin production (Berg et al. [2008]) contrasting with the marginal negative effect in iron depletion media (A.1B). PAA has been earlier reported to reduce the biomass and penicillin production in early stage cultures (Leiter et al. [2001]). Our data demonstrated a reduced penicillin concentration in the Δ*pssA* mutant when cells are grown in iron replete conditions which was also apparent but to lesser degree when cells were grown in iron depleted medium (A.1C). On the other hand, the double deletion mutant of Δ*pssA* and Δ*pssB* did not show this phenomenon. At high concentration, PAA might be oxidized to p-hydroxy-PAA (Hockenhull et al. [1952]; White et al. [1999]). Deletion strains of orthologs of PssA in *Magnaporthe grisea* and *Cochliobolus heterostrophus* display hypersensitivity towards oxidative stress (Hof et al. [2009]; Gillian Turgeon et al. [2008]). Therefore, the mutant lacking coprogen biosynthesis might be more sensitive to the toxic effect of PAA, which is often associated with increased cellular autolysis and decreased penicillin production White et al. [1999].
Figure A.1: Penicillin cluster copy number analyzed by qPCR as described by (Nijland et al. [2010]) in the strains used in this study. The DS54465 is the high penicillin producer derivative strain and carries eight copies of penicillin cluster. The deletion of pssA, pssB and double deletion (∆pssA, ∆pssB) was performed in DS54465 strain as a parental strain, which resulted in the mutants containing eight-nine penicillin cluster amplicons. Genomic DNA of DS47274 (one penicillin cluster) was used as a control in the procedure. Error bars presented standard error of the mean.
Figure A.2: Penicillin G production of *P. chrysogenum* DS54465 strain and deletion mutants of genes involved in siderophore biosynthesis under different iron availability conditions: +Fe (black), +Fe and +BPS (grey), -Fe (white).

(A) Error bars represent standard deviation from two biological replicates and three technical replicates of DS54465 strain.

(B) qPCR analysis of NRPS genes involved in siderophores biosynthesis: Pc16g03850 (*pssA*), Pc22g20400 (*pssB*), Pc13g05250 (*pssC*) in DS54465 strain. Bars represent fold changes of transcript level relative to penicillin non-producing conditions (no PAA in the medium).

(C,D) Penicillin G production in cultures growing at the presence of iron (black) and the absence of iron as trace element (white). Error bars represent standard deviation from three (C) and four biological replicates (D). The cultures were growing in the penicillin production medium (PPM) containing phenylacetic acid (PAA) for 7 days at rotary incubator at 200 rpm, at 25°C.
References


