Characterizing the RpoN transcriptome in *Escherichia coli*

Sarwat Tahsin Khan[1], Mark Horsman[2], Mark Dornan[2] & Christopher Boddy [2,3]

[1]Department of Biochemistry, University of Ottawa, Ottawa,
[2]Department of Chemistry, University of Ottawa, Ottawa,
[3]Department of Biology, University of Ottawa, Ottawa.

Abstract

The Boddy Lab has developed an *Escherichia coli* based heterologous expression (HE) system for polyketide biosynthetic gene clusters. HE allows us to take a pathway producing a natural product of interest from one bacterial species and to put it into a rapidly replicating and inexpensive strain, so we can produce significant amounts of the desired product and study the nuances regulating the biochemical pathway.

Regulation of Oxytetracycline Pathway by RpoN Overexpression

This project attempts to characterize the RpoN transcriptome. RpoN is a sigma factor, the overexpression of which has been found to positively regulate the oxytetracycline (oxytet) biosynthetic pathway. The pathway is native to the strain *Streptomyces rimosus*, but has been introduced into *E.coli*. RpoN overexpression induces oxytet production in the transformed *E.coli*. To study this regulation, RNA produced during RpoN overexpression and oxytet production was isolated. Quantifying and characterizing the transcripts produced will allow us to identify new regulators of the pathway.

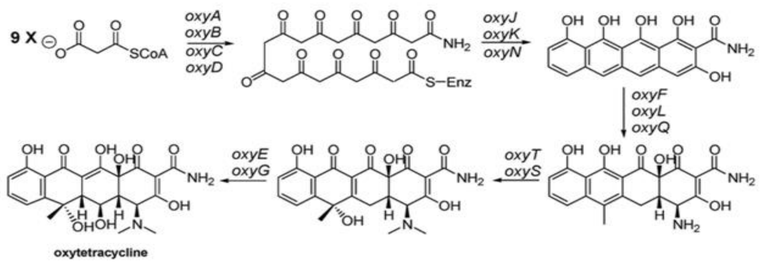
Importance

HE is an important tool for analyzing the intrinsic sufficiency of biosynthetic pathways. Better understanding these biochemical pathways and their regulation as well as maximizing efficiency of the HE system will be immensely beneficial for natural product research and its applications to medicine and industry.

Introduction

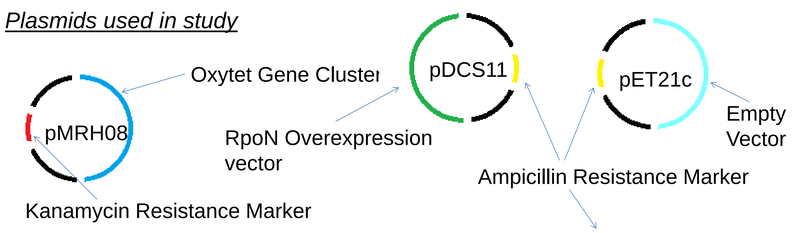
Efforts to heterologously express oxytet in *E. coli* under T7 promoter regulation failed to generate oxytet. Curiously, growth in nitrogen limitation conditions allowed oxytet synthesis. Ultimately, it was shown that the nitrogen starvation response factor (RpoN) was sufficient to facilitate oxytet production.

Enzymatic Pathway for Biosynthesis of Oxytetracycline

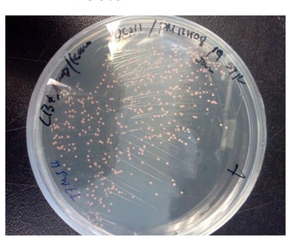


Importantly, overexpression of the RpoN sigma factor allows for oxytet production in the absence of nitrogen starvation. Sigma factors enable specific binding of RNA polymerases to gene promoters, thus regulating RNA synthesis and subsequently protein expression. Therefore, it became imperative to study RpoN regulation of the pathway by characterizing the transcripts produced during RpoN overexpression.

Plasmids used in study



Here, we characterize the transcriptome produced during RpoN overexpression in *E. coli* to identify additional genes regulating the oxytet pathway that are under direct control of RpoN overexpression. For comparison, we also characterize the transcriptome produced during no overexpression and RpoS overexpression. RpoN and RpoS have exhibited unique cross-play interactions in previous studies, therefore we have included RpoS in our study.



Escherichia coli BAP 1 cells with pDCS11 and pMRH08 inserted.

Electroporation and Cell Growth

Time Constants for Electroporations

Plasmids inserted	Time Constant (mSec)
pDCS11 + pMRH08	5.6
pET21c + pMRH08	3.2
pNDP06 + pMRH08	5.4

E.coli BAP1 cells were transformed using electroporation at 1800V. The transformed cells were then allowed to recover in LB for 3 hours.

The cells were then grown in plates, seed cultures and subsequently in 30 mL cultures at 37ºC. For all steps after recovery, cells were grown in LB with antibiotics (ampicillin and kanamycin) for which the inserted plasmids have resistance markers.

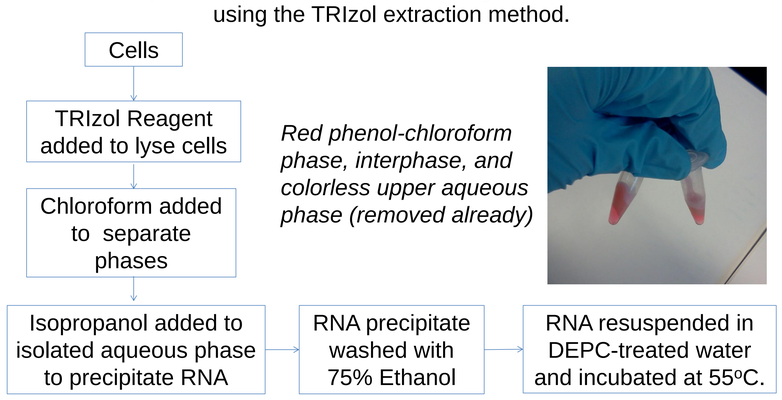
IPTG induction was done when OD of 30 mL cultures reached 0.4-0.6. Post-induction, cells were grown at 20ºC for 24 hours for RNA isolation and 48 hours for oxytet production.

Isolation of RNA

Concentration and Quality of Isolated RNA Determined by Measuring Absorbances using a Nanodrop

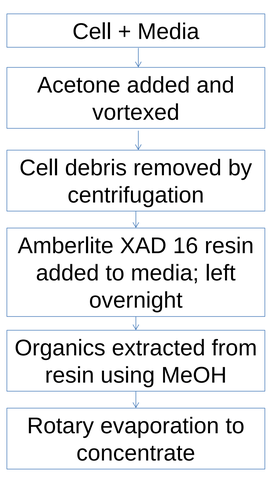
Overexpression	Plasmids	Concentration (ng/uL)	A260/ A280	A260/ A230
RpoN	pDCS11 pMRH08	447.7	2.01	1.84
Nil	pET21c pMRH08	342.0	2.00	1.89
RpoS	pNDP06 pMRH08	530.4	1.97	1.45

Cells were separated from media after centrifugation and RNA was isolated using the TRIzol extraction method.

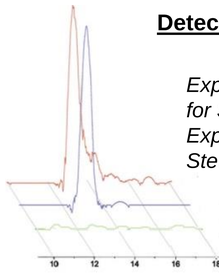


Red phenol-chloroform phase, interphase, and colorless upper aqueous phase (removed already)

Extraction of Oxytetracycline

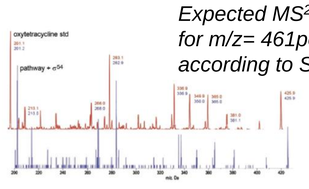


Detection using LC/MS



Expected Retention Times for Standard and Experimental according to Stevens et al.

Observed



Expected MS² spectrum for m/z= 461peak according to Stevens et al.

Discussion

Cell Transformation After transformations, the derived cell lines grew in selective media, even after multiple transfers suggesting both plasmids (overexpression vector and oxytet gene cluster) were inserted successfully into the respective cells. Notably, the growth rate under plasmid retention conditions slowed significantly; for example, it took almost 30 hours to reach an OD of 0.4-0.6.

RNA isolation Our RNA samples gave A260/A280 ratios of 2, which is generally taken to indicate a pure RNA sample Furthermore, our samples indicated A230:A260:A280 ratios close to 1:2:1. This ratio is a further indicator of pure RNA sample void of protein/DNA contamination. We also obtained a sufficiently high concentration of RNA, which (given the volume of RNA extracted) is sufficient for subsequent RNASeq analysis and further quality checks.

Oxytetracycline isolation The established extraction protocol was used to isolate oxytet from developed strains, but oxytet was not detected at sufficiently high levels using LC/MS and derived Multiple Reaction Methods to claim oxytet was produced. Oxytet may have degraded during the isolation and storage process since it is not very stable. Also, oxytet may not have been produced in large amounts in our 30 mL cultures.

Future Directions

Short term Goals

- Repeated isolation and detection of oxytetracycline from the prepared strains.
- Verification of developed LC/MS method using oxytetracycline standards.
- Further analysis of isolated RNA quality using a bioanalyzer to obtain RNA integrity score (preferably greater than 8).
- Preparation of RNA samples for off-site Mi-Seq analysis.

Long term Goal

Analysis of transcriptome data from Mi-Seq using RNAseq analysis pipeline software will allow detailed analysis of transcript regulation by RpoN overexpression and allow identification of transcriptional start sites consistent with direct RpoN regulation. This data set will be the starting point for discovery of additional regulators of oxytetracycline biosynthesis.

Reference

Stevens DC, Conway KR, Pearce N, Villegas-Penaranda LR, Garza AG, et al. (2013) Alternative Sigma Factor Over-Expression Enables Heterologous Expression of a Type II Polyketide Biosynthetic Pathway in Escherichia coli. PLoS ONE 8(5): e64858.

Acknowledgements

In addition to Dr. Chris Boddy, Mark Horsman and Mark Dornan, I would like to thank Luis R Villegas-Penaranda and Kofi Gyan for helpful discussions, the rest of the Boddy Lab for their support during my project and, finally, UROP for this exciting opportunity.