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ESTABLISHMENT OF CRISPRI TOOLS IN ACINETOBACTER BAYLYI ADP1

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ABSTRACT

Holmén Olli: Establishment and calibration of CRISPRi tools in *Acinetobacter baylyi* ADP1
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Regulating genes synthetically is important for metabolic engineering purposes. However, many organisms still lack established and optimized gene regulation techniques. CRISPR interference has emerged in the previous years as a promising gene repression tool. It is reversible, specific and the repression level can be regulated in multiple ways. CRISPRi has been successfully applied to many prokaryotic hosts including *Escherichia coli*, *Pseudomonas putida* and *Pseudomonas aeruginosa*. In this thesis CRISPRi tools were established in *Acinetobacter baylyi* ADP1 -bacteria for the first time.

DCas9 gene was embedded into ADP1's genome and was expressed under a cyclohexanone inducible promoter while sgRNA was expressed from a plasmid under an arabinose inducible promoter. The repression levels were studied by silencing *GFP* gene which was integrated into the genome as well. Optimal inducer concentrations were 0.0002 mM of cyclohexanone and 1.0% of arabinose with the maximum repression of 3.8-fold compared to the uninduced strain containing only dCas9 but no sgRNA or 6.1-fold when compared to a strain without the CRISPRi machinery. Repression kinetics were investigated by silencing *luxC* gene from the *luxCDABE* operon (located in the genome): the repression started approximately 2 h after the induction of CRISPRi.

The burden caused by CRISPRi was investigated in terms of cell density and bioluminescence production, which functioned as a reporter of the cell's metabolism. Increasing dCas9 expression decreased the cell growth rate. On the contrary, increasing sgRNA expression increased the growth rate when the dCas9 expression was kept constant. With the optimal inducer concentrations the growth was not affected. On the other hand, the optimal CRISPRi machinery expression (and 10-fold smaller dCas9 expression) levels decreased bioluminescence production. Hence, the CRISPRi machinery caused a burden to the cell on a metabolic level that was not visible in the growth of the bacteria.

Expression of dCas9 without *GFP*-targeting sgRNA repressed GFP expression by 1.4-fold. Possibly dCas9 binds to off-target genes and represses them, at least in the absence of sgRNA. The off-targeting by sgRNA:dCas9 complex was not studied.

Keywords: CRISPRi, *Acinetobacter baylyi* ADP1, bioluminescence, gene silencing, burden monitoring

TIIVISTELMÄ

Holmén Olli: CRISPRi työkalujen käyttöönotto ja kalibrointi *Acinetobacter baylyi* ADP1 bakteerissa
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Geenien säätelyn kontrollointi on tärkeää monissa synteettistä biologiaa hyödyntävissä prosesseissa. Kaikille näissä prosesseissa käytetyille mikrobeille ei kuitenkaan ole vielä kehitetty toimivia ja optimoituja geenien säätelytekniikoita. Viime vuosien aikana CRISPRi (CRISPR häirintä) on kehittynyt lupaavaksi säätelytekniikaksi. Se on hyvin tarkka, sen hiljetämistasoa voidaan säätää monin eri keinoin ja geenin hiljentäminen on palautuvaa. Sitä onkin käytetty onnistuneesti jo monissa mikrobeissa, mukaan lukien *Escherichia coli*, *Pseudomonas putida* ja *Pseudomonas aeruginosa.* Tässä työssä CRISPRi työkalu otettiin ensi kerran käyttöön *Acinetobacter baylyi* ADP1 bakteerissa.

DCas9 geeni liitettiin ADP1:n genomiin ja sen ilmentymistä säädeltiin sykloheksanonilla indusoituvalla promoottorilla. SgRNA taas ilmennytettiin plasmidista arabinoosilla indusoituvan promoottorin avulla. Geenin hiljentämistä tutkittiin tukahduttamalla *GFP* geeni, joka myös sijaitsi genomissa. Ihanteellisiksi indusori pitoisuuksiksi löydettiin 0,0002 mM sykloheksanonia ja 1.0% arabinoosia, joilla saavutettiin maksimissaan 3,8-kertainen hiljentäminen verrattuna indusoimattomaan kontrollikantaan, jossa oli *dCas9* geeni, mutta ei sgRNA:ta. Toisaalta 6,1-kertainen hiljentäminen saavutettiin kun kontrollikantana toimi kanta ilman CRISPRi koneistoa. Hiljentämisen kinetiikkaa tutkittiin kohdistamalla CRISPRi *luxC* geeniin *luxCDABE* operonista (sijaitsi genomissa), toisin sanoen tukahduttamalla bioluminesenssin tuotantoa. Geenin hiljentäminen alkoi noin kaksi tuntia indusoimisen jälkeen.

CRISPRi:n aiheuttamaa taakkaa soluille tutkittiin solujen kasvun ja bioluminesenssin tuotannon avulla. Bioluminesenssi kertoi tarkemmin solun sisäisestä metaboliasta. DCas9 pitoisuuden kasvattaminen aiheutti hitaampaa kasvua. Toisaalta sgRNA:n pitoisuuden nostaminen paransi kasvua kun dCas9 pitoisuus pysyi samana. Optimoiduilla indusoripitoisuuksilla vaikutusta kasvuun ei huomattu kontrollikantaan verrattuna. Kuitenkin optimoitujen (ja 10-kertaa pienemmän sykloheksanonipitoisuuden) indusoripitoisuuksien käyttäminen vähensi bioluminenessin tuotantoa. CRISPRi siis aiheutti soluille taakan, joka ei näkynyt kasvussa vaan vain metabolisella tasolla.

DCas9:n ilmentäminen ilman *GFP* geeniä tunnistavaa sgRNA:ta hiljensi GFP:n tuotantoa maksimissaan 1,4-kertaisesti. Tämä mahdollisesti johtui dCas9:n sitoutumisesta geeneihin ilman kohdentamista. Muiden kuin kohdegeenien hiljentymistä tutkittiin ainoastaan dCas9:n avulla joten tulevaisuudessa tutkittavaksi jäi, miten sgRNA:dCas9 kompleksi toimii samassa tilanteessa.

Avainsanat: CRISPRi, *Acinetobacter baylyi* ADP1, bioluminesenssi, geenin hiljennys, kuormituksen tarkkailu

PREFACE

The experiments of this master's thesis were conducted in the Synthetic Biology group in the Laboratory of Chemistry and Bioengineering of Tampere University of Technology from July to December 2018.

I would like to express my deepest gratitude to my examiners and instructors, doctor Suvi Santala and assistant professor Ville Santala, for the opportunity to work with a very interesting topic in the field of metabolic engineering. Thank you for the guidance and support throughout the whole process. I highly appreciate Suvi's knowledge in the actual laboratory work with CRISPRi: her help was priceless in planning the experiments and analyzing the results. I am grateful of Ville's help with all the practical issues and in connecting my work to the big picture of bioengineering. I would also like to thank both of them for the valuable feedback and advises during the writing process.

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Olli Holmén

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
Cas	CRISPR associated proteins
Cas1	CRISPR associated endonuclease 1
Cas2	CRISPR associated endonuclease 2
Cas9	CRISPR associated protein 9 nuclease
Cas12a	A class 2 CRISPR effector working as a single RNA-guided endonuclease (also known as Cpf1)
CDS	Coding sequence
Chn	Cyclohexanone
Cpf1	A class 2 CRISPR effector working as a single RNA-guided endonuclease (also known as Cas12a)
CRISPR	Clustered regularly interspaced short palindromic repeats
CRISPRa	CRISPR activation
CRISPRi	CRISPR interference
CRISPR/Cas9	CRISPR system that utilizes the Cas9 nuclease
crRNA	CRISPR RNA
dCas9	Deactivted CRISPR associated protein 9 nuclease (lacks nuclease activity)
ED	Entner-Doudoroff pathway
EMP	Embden-Meyerhof-Parnas pathway
EPS	Exopolysacharide
GDA	Gene duplication and amplification
GFP	(Momeric superfolder) green fluorescent protein
gRNA	Guide RNA
HR	Homologous recombination
LA	Lysogeny broth with agar
LB	Lysogeny broth
NHEJ	Non-homologous end joining pathway
OD_{600}	Optical density at 600 nm
PAM	Protospacer adjacent motif
PCR	Polymerase chain reaction

RBS	Ribosome binding site
RNAi	RNA interference
RNAP	RNA polymerase
RNaseIII	Ribonuclease III
rpm	Rounds per minute
sgRNA	Single (or synthetic) guide RNA
sRNA	Small regulatory RNA
tracrRNA	Trans-activating crRNA
USER	Urasil-Specific Excision Reagent
WT	Wild type

1 INTRODUCTION

Gene editing has become part of everyday workflow in laboratories all around the world as the tools have developed from enhancing the natural rate of mutagenesis with chemical agents (Auerbach et al., 1947) or radiation (Muller, 1927) to accurate site-directed mutagenesis (Carrigan et al., 2011) and even to artificial gene synthesis (Villalobos et al., 2006). Using these techniques novel genetic pathways can be introduced into prokaryotic hosts for programming them to execute a desired function, for example to produce a product of interest such as insulin (Williams et al., 1982).

An important factor in the optimization of the production of a desired product in genetically engineered cells is controlling the gene expression. For example, Wu et al. (2017) metabolically engineered *Escherichia coli* to produce 1,4-butanediol (1,4-BDO) and then increased (1,4-BDO) titer for 100% and simultaneously reduced titers of unwanted byproducts by downregulating the genes diverting the flux from 1,4-BDO biosynthesis. Technologies allowing accurate gene regulation include zinc fingers (Klug, 1999), RNA interference (RNAi) (Hannon, 2002), transcription-activator-like effectors (TALEs) (Sanjana et al., 2012) and a novel technology called the clustered regularly interspaced short palindromic repeats interference (CRISPRi) which utilizes the deactivated Cas9 protein (dCas9) (Qi et al., 2013). CRISPRi tool has rapidly become a powerful gene regulation tool that has been used successfully in many hosts (Qi et al., 2013; Tan et al., 2018).

This thesis focuses on CRISPRi technology. More accurately the objective was to establish and optimize CRISPRi tools in *Acinetobacter baylyi* ADP1 which is a suitable bacteria for versatile biotechnological applications (Elliott and Neidle, 2011; Kannisto, 2018). Until now CRISPRi has not been implemented into ADP1 but it is highly important to adopt novel and promising technologies to enable more versatile metabolic engineering, for example in order to allow regulation of multiple genes simultaneously.

In this study, the establishment and optimization of CRISPRi tools were done by studying the repression of the *green fluorescen protein* (*GFP)* gene. DCas9 was expressed under a cyclohexanone promoter from the genome and sgRNA from a plasmid under an arabinose inducible promoter. Hence, the optimization of the CRISPRi macinery was studied in terms of inducer concentrations.

Possibly the most negative aspect of the CRISPRi machinery is the burden it causes to the cells (Cleto et al., 2016; Cui et al., 2018; Nielsen and Voigt, 2014). In this thesis the effect of CRISPRi machinery on the cell viability was studied (in addition to cell density) by using bioluminescence as the indicator of the cell's inner state. As bio-

luminescence requires many cofactors (e.g ATP, NADPH) which are important also to the normal metabolism of the cell (Close et al., 2009), following the changes in the bioluminescence production can provide an insight to the metabolic state of the cell in real time.

In addition to targeting the *GFP* gene, the *luxC* gene, which is the first gene in the *luxCDABE* gene cluster, was targeted by CRISPRi. *LuxC* gene codes for a protein responsible of a critical step in the production of an aldehyde substrate for bioluminescence production (Close et al., 2009). As a result, the effect of CRISPRi on the whole operon and on downstream genes of *luxC* was studied by adding external substrate (decanal) after a clear repression took place. Decanal functions as a substrate for bioluminescence production (Meighen, 1993) and thus it can be used to bypass the need of *luxC*. As the bioluminescence is produced fast and faints quickly (Meighen, 1993), it is rapid and dynamic tool for studying the efficiency and toxicity of CRISPRi. Thus, the repression of bioluminescence was used to study the repression kinetics as well.

The studies concerning the effects of CRISPRi on the cell metabolism in real time have not yet been performed. Thus, this study provides the first insight and novel information about how CRISPRi affects the cell in ways which are not visible in the growth. Hence, the results can also be used to take advantage of implementing and optimizing the CRISPRi machinery in other bacterial hosts.

In this study, the hypotheses were that CRISPRi can be implemented to ADP1 and that it successfully represses the targeted gene, however the repression level might have to be compromised to lessen the toxic effect of CRISPRi. The repression kinetics were hypothesized to be approx. the same as in recombinant protein production. Thus, the repression should be visible already 30 min after the induction (Huang et al., 2005). As bioluminescence production is proven to function as a sensitive indicator of cell's metabolic state (Falls et al., 2014), the burden of CRISPRi probably can be well seen as a change in the bioluminescence.

2 THEORETICAL BACKGROUND

2.1 CRISPRi tools in bacterial gene editing

CRISPRi system has been derived from a prokaryotic immune system called clustered regularly interspaced short palindromic repeats (CRISPR) that utilizes endonucelase activity of CRISPR associated protein 9 (Cas9) and complementarity of single guide RNA (sgRNA) to degrade invading DNA or RNA sequences (Qi et al., 2013). Originally CRISPR/Cas9 system produces a double strand break in the sequence of an alien DNA it recognizes using the complementary sgRNA sequence (Cho et al., 2018a). Hence, the alien DNA is degraded before it can incorporate into the genome and utilize the cell's protein producing machinery.

One of the CRISPR systems, type II, has been genetically modified to prevent the double strand break. Instead, this novel machinery, named CRISPRi (i stands for interference), binds to the gene of interest and inhibits its transcription. (Qi et al., 2013)

2.1.1 CRISPR/Cas9 - a natural defense system in prokaryotes

Most of the archeas and 40% of bacteria use CRISPR as part of their natural immunity system (Kunin et al., 2007). CRISPR systems are divided into two different classes (I and II), six types (I-VI) and 19 subtypes according to the Cas protein effectors' configurations (Makarova et al., 2015; Shmakov et al., 2015; Xu and Qi, 2018). All of the types have their distinctive characteristics even though in the end they perform a similar task. Class I systems (types I, III and IV) utilize multisubunit CRISPR RNA (crRNA)–effector complexes (consisting of several proteins) and class II systems (type II, putative types V and VI) use single endonucleases: either Cas9 (in type II) or Cas12a (earlier known as Cpf1, type V) (Makarova et al., 2015; Wang et al., 2016). Additionally, class II systems possess a single (type V-A) or double crRNA (most of the other class II CRISPR systems) (Xu and Qi, 2018). More detailed descriptions of classification of different CRISPR systems can be found from literature (Koonin and Krupovic, 2015; Maeder et al., 2013; Makarova et al., 2015; Rath et al., 2015; Wiedenheft et al., 2012).

The high efficiency (fast elimination of the invading DNA), high specificity and the DNA recognition by RNA-guiding make the class II systems superior choices as gene editing tools (Xu and Qi, 2018). Hence, in this thesis the emphasis is given to one of the simplest CRISPR systems, class II, type II system (also named as CRISPR/Cas9) which was isolated from *Streptococcus pyogenes* (Jinek et al., 2012). The functioning

and characteristics of this CRISPR system are fairly known which makes it easy tool to be implemented to a new host in which no CRISPR machinery has been utilized before.

The CRISPR immunity can be divided into two (A and B) or three (1-3) distinctive phases (Figure 2.1). (A), (1) Immunization where the part of the invaders DNA or RNA sequence is integrated into the CRISPR array as a novel spacer and (B) (2) immunity where the CRISPR array is transcribed into pre-CRISPR RNA (pre-crRNA) which is then maturated to crRNA. Then, Cas complex and crRNA bind together to form a complex, (3) which targets and interferes with the foreign DNA or RNA (complementary to the crRNA) intruding the cell. (Horvath and Barrangou, 2010) The CRISPR array consists of short direct repeats which are separated by short, variable DNA sequences (spacers). The Cas genes are situated next to the CRISPR array (Makarova et al., 2015).

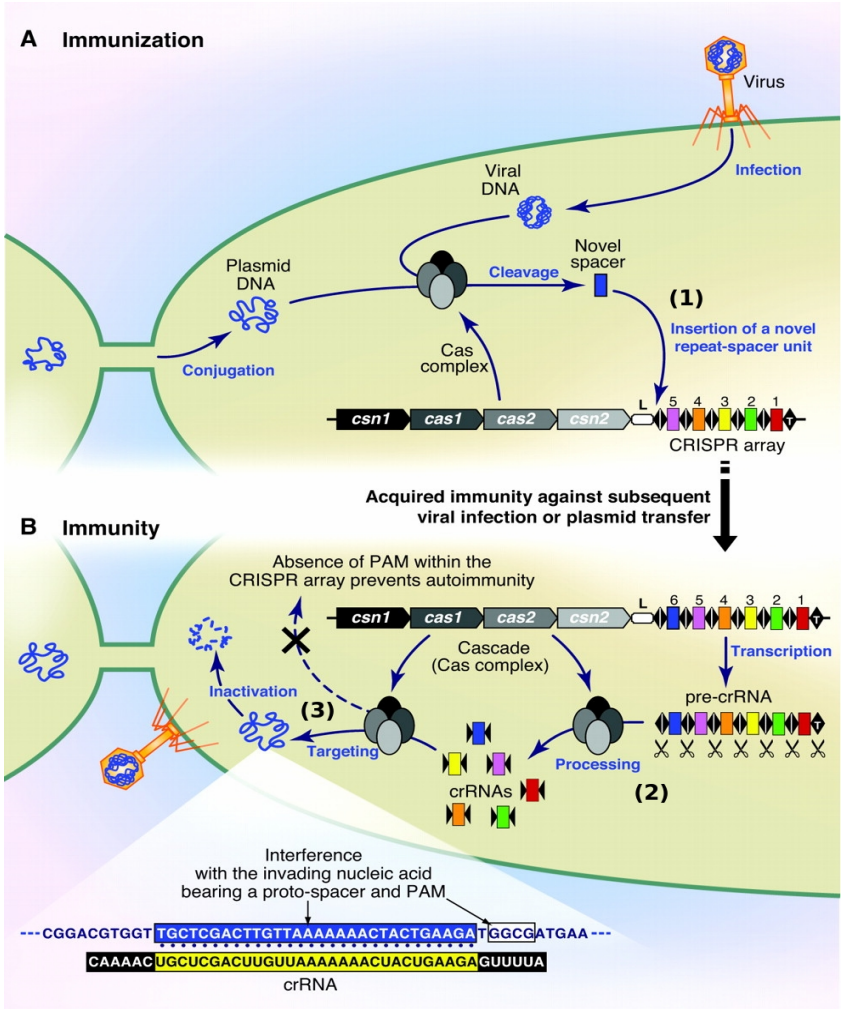

Figure 2.1 *Natural functioning of the CRISPR systems. The process of immunization (**A**): after exogenous DNA enters the cell, the Cas complex recognizes it and integrates part of the sequence into the CRISPR locus as a novel spacer (1). The process of immunity (**B**): the CRISPR array (repeats and spacers) is transcribed into pre-crRNA which matures into crRNA after processing (2). Mature crRNAs guide the Cas complex by complimentarity to an exogenous DNA or RNA sequence invading the cell to destroy it (3). Spacers are presented as rectangles, repeats as diamonds and L presents CRISPR leader which probably functions as a promoter for CRISPR array. (modified from Horvath and Barrangou, 2010)*

The only conserved proteins between every CRISPR type, Cas1 and Cas2, form a complex which mediates the addition of new spacer sequences in the initial stage of CRISPR immunity (Makarova et al., 2011; Nuñez et al., 2014). In CRISPR/Cas9 system, a single guide RNA (sgRNA) guides the Cas9 (~950–1400 amino acids (Makarova et al., 2015)) to the invading DNA or RNA (Qi et al., 2013). SgRNA is a complex of two preliminary RNAs: a mature CRISPR RNA (crRNA) and a partially complementary transacting RNA (tracrRNA) (Cui and Bikard, 2016; Qi et al., 2013). TracrRNA and RNaseIII are required for the maturation of the crRNA by cleaving the pre-crRNA, which is produced by the transcription of the whole CRISPR array, into single repeat-spacer-repeat units (Deltcheva et al., 2011; Pyne et al., 2016). Additionally to the complementarity of RNA and DNA sequences, protospacer adjacent motif (PAM) is used to recognize the alien DNA or RNA as it locates in the exogenous DNA or RNA but not in the CRISPR array of the prokaryotic genome (Shah et al., 2013). Hence, the CRISPR should not target endogenous genes. A PAM consist of a short (2–5 bp) signature sequence that differs between different CRISPR systems and organisms from the sequence and its location. (Shah et al., 2013) In the type II CRISPR system the PAM consists of NGG (N being any nucleotide) and it is situated one base pair (bp) downstream from the binding site of the sgRNA (Jinek et al., 2012; Shah et al., 2013).

However, CRISPR can lead to autoimmunity in the organisms utilizing it: approx. 18% of all organisms containing CRISPR have (genomic) self-targeting sequences even though the PAM sequence should prevent it. A self targeting CRISPR system is thought to be a prokaryotic autoimmunity disease which can lead to possible loss of CRISPR for cell survival. (Stern et al., 2010) This happens especially because prokaryotes lack efficient tools to repair a double strand break. Non-homologous end joining pathway (NHEJ) which is commonly used by eukaryotes (Cui and Bikard, 2016) is missing from most of the prokaryotes (Lu et al., 2018). Hence, these species rely on homologous recombination (HR) which is functional only if all the chromosomes are not cut simultaneously as a template is needed for the DNA repair (Bowater and Doherty, 2006). In the case of a double strand break in all of the chromosomes, the cell is not able to repair the double strand breaks and dies (Shuman and Glickman, 2007). This effect has already been exploited in the development of novel antimicrobials, for example (Pan et al., 2006).

2.1.2 CRISPRi

To enable gene regulation with the type II CRISPR system, endonuclease activity of Cas9 (from *S. pyogenes)* was eliminated by introducing two point mutations, D10A and H840A in genes RuvC1 and HNH, respectively. Hence, the deactivated Cas9 (dCas9) protein and a system called CRISPRi were established. As dCas9 lacks the ability to break double stranded DNA, it only binds to the DNA preventing the transcription up to 1000 fold in inducible and reversible manner. (Qi et al., 2013) As a result, the CRISPRi

system can be used to regulate genes by inducing its expression for example with a chemical agent or light and thus inducing the repression of the gene of interest.

After the sgRNA:dCas9 complex binds to a promoter region it attenuates or completely blocks transcription by preventing transcription factors or the RNA polymerase (RNAP) from binding to the DNA. If the target situates downstream from the promoter, the RNAP collides physically with the sgRNA:dCas9 complex while transcription is taking place (Figure 2.2). That prevents the movement of the RNAP and thus inhibits the elongation of the newly synthesized DNA strand. (Qi et al., 2013)

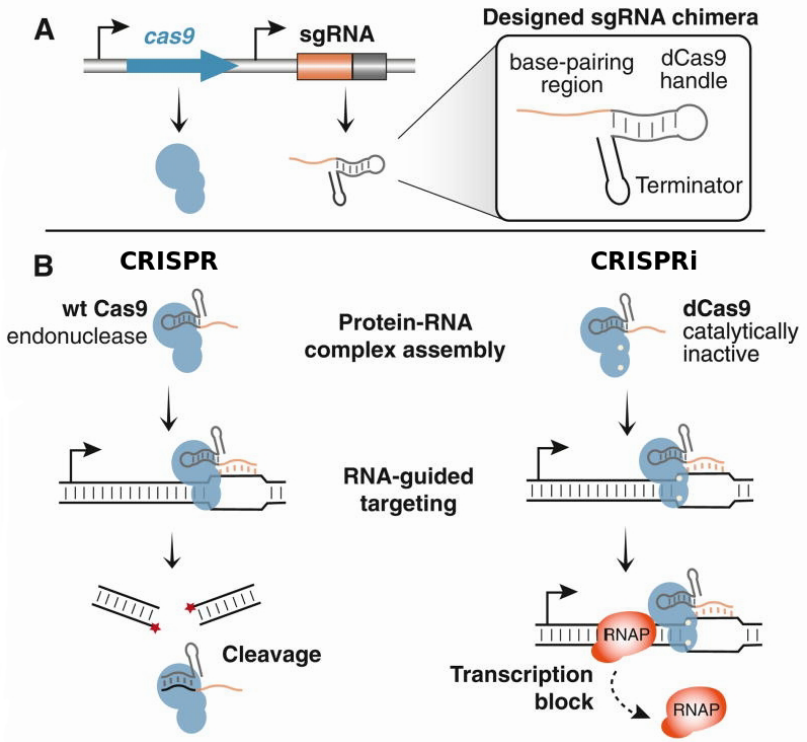

Figure 2.2 *The design and working principle of CRISPR interference systems. (**A**): CRISPRi system consists of two genes: one coding for Cas9 (or catalytically inactive Cas9) and one coding for designed sgRNA chimera. The sgRNA (boxed area) consists of three domains: the base-pairing region (complementary with the gene of interest), the dCas9 handle and the transcription terminator sequence. (**B**): Wild type Cas9 (on the left) forms a complex with sgRNA, then targets the DNA strand and produces a double strand break. Catalytically inactive dCas9 (on the right) also forms a complex with sgRNA but instead of producing a double strand break, it physically blocks binding or movement of the RNA polymerase thus preventing transcription. (modified from Qi et al., 2013)*

In CRISPRi the original crRNA-tracrRNA complex (or in other words sgRNA), is replaced with a designed synthetic guide RNA (also named sgRNA) (Figure 2.3). Hence, no processing of crRNA by tracrRNA and RNaseIII is needed for maturation because the synthetic guide RNA folds correctly by itself. SgRNA can be designed to target any DNA region and either (template or non-template) strand (Qi et al., 2013).

The sgRNA complex consists of a 20–25 nucleotide (nt) long base-pairing sequence, a 42 nt long hairpin for dCas9 binding (dCas9 handle) and and a 40 nt long transcription terminator from *S. pyogenes* (Figure 2.3 A). (Jinek et al., 2012; Larson et

al., 2013). The base-pairing region includes a 12 nt long seed region (Larson et al., 2013; Nielsen and Voigt, 2014) which targets the region next to the PAM and is thought to be highly important for binding to the target DNA (Jinek et al., 2012; Larson et al., 2013). Hence, the seed region should be fully complementary with the targeted sequence for strong binding. On the other hand, in the 5' terminal of the base-pairing region up to six contiguous non-complementary bases are tolerated (Jinek et al., 2012).

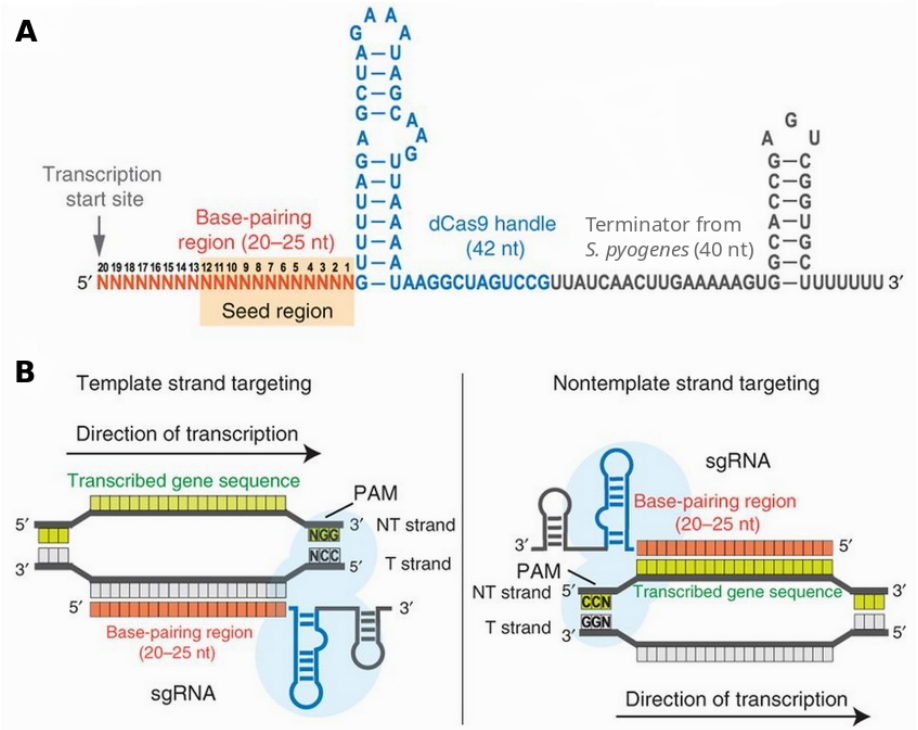

Figure 2.3 *The design of synthetic guide RNA (sgRNA). (**A**): The sgRNA chimera consists of three domains: the base-pairing region (20–25 nt) which guides the sgRNA to the complementary target, the dCas9 handle (42 nt) in which dCas9 protein binds and the terminator from S. pyogenes (40 nt). The seed region (12 nt, shaded in orange) is the most important part of the base-pairing region. It should be fully complementary with the target for strong binding. (**B**): The sgRNA:dCas9 complex can target both, template and non-template strands. The protospacer adjacent motif (PAM) sequence is recognized by the dCas9. When template strand is targeted, base-pairing region of sgRNA is the same as the targeted gene sequence. If nontemplate strand is targeted, the base-pairing region of sgRNA is complementary with the targeted gene. (modified from Larson et al., 2013)*

The sgRNA:dCas9 complex can have any orientation when it is bound to the DNA. However, the level of gene suppression differs between different targets inside a gene. (Cui et al., 2018) Higher repression has been achieved by targeting the non-template strand or promoter region (either strand) (Vigouroux et al., 2018). Thus, targeting for example -35 box leads to strong repression (100-fold). When sgRNA:dCas9 complex binds to the template strand, the helicase activity of RNAP could unzipp and release the sgRNA:dCas9 complex from the DNA, thus decreasing the transcription inhibition. Using multiple sgRNAs to target the same gene increases the repression. For example, two sgRNAs, each with 300 fold repression, repressed a gene by 1000 fold when they were used together. (Qi et al., 2013) Additionally, some sgRNA sequences

seem to have stronger effect than others. Variability was observed between different sgRNAs that targeted the same essential gene in the same orientation but different part of the gene. (Cui et al., 2018)

Additionally to targeting different parts of a gene, repression levels can be regulated by adjusting the expression levels of dCas9 or sgRNA (Fontana et al., 2018; Li et al., 2016) or by varying the complementarity between sgRNA base-pairing region and the gene of interest (Vigouroux et al., 2018). Changes in the sgRNA expression level are shown to have a stronger effect on the repression than changes in the dCas9 expression (Fontana et al., 2018). Additionally, the overexpression of dCas9 can be toxic to the cells (Cho et al., 2018b). Regulating the repression by altering expression levels of dCas9 leads also to high repression variability between individual cells, in other words to noise. On the other hand, when repression levels are regulated by the changing complementarity of sgRNA with the gene of interest, the repression levels are very similar between individual cells, thus providing a noiseless way to alter the repression. In addition, the repression level can be controlled in a linear manner by adjusting the complimentarity. (Vigouroux et al., 2018)

In conclusion, for accurate gene regulation without toxicity, *dCas9* and *sgRNA* genes should be expressed under tightly regulated promoters to achieve controlled expression of the CRISPRi machinery. Jang et al. (2018) showed that dCas9 is the limiting part in CRISPRi system: when the dCas9 concentration was under the threshold level, the repression of the gene of interest could not be controlled by regulating the sgRNA concentration. When sgRNA and dCas9 levels were regulated simultaneously, difference in the repression of the gene of interest was 30-fold. As varying the sgRNA expression level has a stronger effect on the inhibition than controlling the dCas9 concentration, it could be feasible to find out the concentration of dCas9 that no or only a slight toxic effect, express dCas9 continuously at this level and regulate the repression of the targeted gene by controlling the sgRNA level, changing the level of complementarity of sgRNA or the target or change the targets inside the gene of interest. Possibly all these methods could be used in combination to achieve more complex regulation of the repression.

On the other hand, Qi et al. (2013) targeted *red fluorescent protein* (*RFP*) and *green fluorescent protein* (*GFP*) genes simultaneously in *E. coli* using CRISPRi system and attained highly specific repression with no significant off-targets. Thus, sgRNA:dCas9 complex could be used to control multiple genes simultaneously. With careful sgRNA design other genes than the genes of interest would not be affected.

In addition to the type II system, also other CRISPR systems have been developed as gene modification tools (Chang et al., 2016; Pyne et al., 2016). Therefore, in the future, when different CRISPR systems are better known, a different system might prove more functional than the type II. For example, Pyne et al. (2016) found the type I (endogenous) machinery to have 100% efficiency in transformation. The same effi-

ciency was not achieved with type II (heterogenous) system. In another example, type V CRISPR utilized Cpf1 endonuclease instead of Cas9. Type V system has three distinctive features when compared to type II: (1) tracrRNA is not needed for maturation of CRISPR RNA, (2) protospacer-adjecent motif (PAM) is T-rich instead of G-rich and (3) Cpf1 cleaves DNA in a staggered way producing sticky end (4–5 nt overhang) in the 5' end (Zetsche et al., 2015). Therefore, it could be used in different applications, for example when sticky ends are preferred or when a gene with a T-rich PAM is the gene of interest.

As a real example of a functioning endogenous CRISPRi machinery, type I CRISPRi system was exploited successfully in *E. coli* by Chang et al. (2016). They deleted *Cas3 protein* gene from the genome and expressed crRNA (which targets the gene of interest) from a plasmid. The CRISPR cascade protein matured the crRNA and together they formed a complex that could bind to the gene of interest and inhibit its transcription. If the cascade protein was expressed from the genome and it was endogenous, the sequence would already be optimized by the natural selection and the cell burden would be lower. As a result, cell growth should be faster. In the same study of Chang et al. (2016) GFP was acting as a reporter protein and its expression could be repressed 6–82%. Additionally, the engineered strains produced from three to four-fold more poly-3-hydroxbutyrate (PHB) than the control strain. However, even though there are many positive aspects in using the endogenous CRISPR machinery, it must be included in the genome so that it can be used. As only 40% of bacteria have CRISPR machinery, it could not be exploited in every bacterial strain. Hence, type II CRISPR system is important tool as it can be incorporated virtually to any host strain.

Additionally to the gene repression, gene activation has been achieved with modified CRISPR machinery, called CRISPR activation (CRISPRa) (Bikard et al., 2013; Dong et al., 2018; Hilton et al., 2015; Konermann et al., 2015). In this machinery dCas9 fusion proteins are used to engage trancription activators which in turn enhance the expression of the targeted gene (Dominguez et al., 2016). For example, dCas9 has been fused with transcription factors p65 (Konermann et al., 2015), VP64 (Maeder et al., 2013), acetyltransferase p300 catalytic core (Hilton et al., 2015) and synergistic activation mediator (SAM) (Konermann et al., 2015) for successful activation of the targeted genes. CRISPRa has been used mostly in eukaryotes (Dominguez et al., 2016) but it has been established in *E. coli* as well (Bikard et al., 2013; Dong et al., 2018). Together CRISPRi and CRISPRa could provide a versatile tool to simultaneously down regulate and up regulated multiple genes. However, care must be taken so that CRISPRi machinery would not form a complex with sgRNA which is meant activate gene expression. Otherwise, the targets might be regulated to the opposite direction than planned. Additionally, as dCas9 can cause growth defects (Cho et al., 2018b), expression of dCas9 in CRISPRi and CRISPRa simultaneously could end up in too high concentration of dCas9, hence inhibiting the growth.

2.1.3 Toxicity and off-target repression

The CRISPRi machinery is shown to induce growth defects: overexpressing dCas9 seems cause more burden but also the overexpression of sgRNA can lead to toxicity. (Cleto et al., 2016; Cui et al., 2018; Dominguez et al., 2016; Ji et al., 2014; Nielsen and Voigt, 2014). Cui et al. (2018) noticed that five specific nucleotides in sgRNA induced strong fitness effects and even killed *E. coli*. This happened at high Cas9 concentrations, lowering dCas concentrations alleviated the toxicity. They named the effect as "bad seed -effect". The reason behind it is unclear: the researchers suggest that dCas9 might bind simultaneously to multiple targets or that it could be caused by some completely different phenomenon. However, off-target repression was thought not to be the cause. Seed sequences found causing the toxic effect were ACCCA (strong toxicity) and ATACT (intermediate toxicity). However, there might be still more sgRNA sequences which induce similar toxicity. Thus, in the case of growth defects when CRISPRi machinery is incorporated into the cell, the seed of the sgRNA base-pairing region must also be considered as one possible reason.

Additionally to the toxicity caused by the "bad seed -effect", also off-target repression can be a problem. It is caused by complementarity between the base-pairing region of sgRNA and a non-targeted gene. Homology of nine nucleotides can already result in strong repression. However, designing a sgRNA without any off-targets can be tricky as sgRNAs targeting the chromosome of *E. coli* (MG1655) have a median of four off-targets with perfect complementarity of nine nt or more and additionally the correct seed sequence and the PAM. (Cui et al., 2018) On the other hand, Nielsen and Voigt (2014) designed sgRNAs without off-targets that targeted a gene (*malT*) in the genome of *E. coli*. In that case, when dCas9 was expressed so that it induced only a small toxic effect, different expression levels of sgRNA did not enhance the toxicity. Therefore, the sgRNA toxicity is probably more connected to the base-pairing region sequence than to the concentration of sgRNA in the cells.

Nevertheless, its is important to design the sgRNA carefully and also align and compare sequence of the base-pairing region to the genome of the organism of interest to find out possible off-targets. In the case of off-targets, a new base-pairing region should be designed. If that is not possible, possible off-targets should fall away from regulatory regions but rather in neutral regions and on the template strand (Cui et al., 2018). Hence, the probability for growth defects decreases because the inhibition does not have an effect on regulatory networks and because of the lower repression when targeting the template strand, respectively.

Not only can a poor design of a sgRNA lead to an inadequate repression but a badly designed target can also lead to repression of other genes than the gene of interest. For example, in the study of Cui et al. (2018) targeting within approx. 100 bp after the stop codon repressed the upstream gene. On the other hand, sgRNAs targeting at 100-200 bp after the stop codon did not have a significant effect on the upstream genes.

When sgRNA targets a gene, the genes downstream might be repressed as well (Cui et al., 2018; Dominguez et al., 2016) which might cause a strong fitness defect (Cui et al., 2018).

The dCas9 alone (without sgRNA) has been shown to reduce RFP and GFP expression by 1.5-fold. Additionally, in the cells containing sgRNA:dCas9 complexes targeting *RFP*, the GFP expression was reduced by 1.2-fold. Reduction correlated with the dCas9 concentration. (Ji et al., 2014) Cleto et al. (2016) studied how placing the dCas9 gene downstream from a constitutive promoter (P_{tac}) affects the viability of *Corynebacterium glutamicum*. No colonies were formed after the transformation. Also Nielsen and Voigt (2014) found out that overexpressing dCas9 leads to toxicity in the cells: higher dCas9 expression increased the fold repression but additionally decreased the cell growth. The reason for the dCas9 toxicity is still unknown in the context of prokaryotic cells but expressing it could be a burden to the cells in the same way as expressing other recombinant proteins, for example (Gill et al., 2000). On the other hand, dCas9 could also target non-specifically and even without forming the sgRNA:dCas9 complex. In human cells high concentration of sgRNA:dCas9 complexes have been shown to result in off-target cleavage (Pattanayak et al., 2013). Cho et al. (2018b) showed that dCas9 in *E. coli* bound to off-targets (with or without sgRNA) when expressed in high levels. Expression of 574 genes which were involved many different functions was changed. From these genes 310 were upregulated and 217 were downregulated .

High expression level of dCas9 can also impact the cell morphology: Cho et al. (2018a) found out that process of the cell division of *E. coli* was severely affected because no septa was formed during the division and the chromosomes were bundled in the cells evenly without segregation. This resulted in an abnormal linear filamentous morphology in the stationary phase. The same result was observed when two inducers (anhydrotetracycline and doxycycline) were used, thus proving that this was not caused by the inducers. Additionally, they noticed that the morphology of cells remained filamentous no matter if sgRNA targeting a gene was expressed or not. However, basal levels (promoter leaked a bit) of dCas9 did not produce the abnormal morphology, though the gene of interest was silenced. They also found out that different *E. coli* strains behaved differently: cells of BL21 and W strains were not as large in the stationary phase as MG1655 and DH5α cells, suggesting that the morphological changes are strain dependent.

Additionally to the impacts on the cell growth, overexpressing of dCas9 can lead to its degradation (Cho et al., 2018b). Hence, the overexpression of dCas9 is not feasible as it uses the cell's resources only to produce and then immediately degrade the protein. This could be also one reason the cells experience decreased growth rates (Bentley et al., 1990).

To prevent the toxicity but to control the gene expression, it is important to find an optimal expression level of dCas9 for each host so that the gene of interest could be

repressed as strongly as possible but toxicity to the cells could be kept be as low as possible (preferably none). For example, Nielsen and Voigt (2014) made a compromise to use 0.625 ng/ml of anhydrotetracycline to induce dCas9. Thus, they achieved almost complete repression of the target gene and the cell growth was impacted <15% (OD_{600} of 0.44 against 0.51 (no induction), after 6 h of cultivation).

In conclusion, CRISPRi machinery affects in multiple ways to the whole life cycle of the cell. More research is needed to really understand the functioning of it. After better knowledge of the machinery and its effect on the host organisms is acquired, it can be applied to many more hosts and applications.

2.1.4 Comparison to other gene regulation technologies

Other technologies capable of controlling genomic gene expression include RNA interference (RNAi), zinc fingers, riboswitches and small regulatory RNAs (sRNAs), for example. When compared with CRISPRi they all have their positive and negative aspects.

RNAi is a naturally occurring defence mechanism against invading double stranded RNA (viral or cellular RNAs) mostly in eukaryotic cells (Makarova et al., 2006; Saurabh et al., 2014). Many prokaryotes lack the RNAi system (Xu and Qi, 2018). In RNAi defence small non-coding RNAs recognize the target (Saurabh et al., 2014) and a dicer-like enzyme produces a double strand break in the RNA (Pare and Hobman, 2007). Also other proteins, for example a RNA-induced silencing complex (RISC) (composed of multiple proteins) (Redfern et al., 2013) and Argonaute proteins (Riley et al., 2012), are required for functioning of the RNAi machinery. As RNAi requires many cofactors, it would be difficult to synthetically express all of the genes associated with RNAi machinery in prokaryotic cell. Hence, CRISPRi provides, with the need to express only sgRNA and dCas9, simpler approach to regulate genes even if the CRISPR machinery is lacking from the microbe of interest.

In prokaryotes small regulatory RNAs (sRNAs) can inhibit or activate gene expression by basepairing (with extended or limited complementarity) with mRNA or by modulating protein activities, sometimes by mimicking other nucleic acids. The action is often achieved either by blocking ribosomes from translating the mRNA (when sRNA binds to the ribosome binding site, RBS), interfering with ribosomes in other ways (when sRNA binds somewhere else than to RBS), increasing the ribosome binding (sRNA prevents formation of inhibitory secondary structures) or by decreasing or increasing mRNA stability. The RNA binding protein Hfq is often required for sRNA regulation to function in Gram-negative bacteria. (Storz et al., 2011) Utilization of synthetic sRNA regulation pathways in bacteria is relatively straight forward as it can be implemented easily into the cell. Hence, it provides possibility to regulate chromosomal genes without modifying them and thus no strain library construction is needed (Na et al., 2013). As a result, sRNAs have many similar positive aspects with CRISPRi technology when compared to gene-knockout strategies. However, as sRNA often inhibits

the translation phase, cells need to spend energy for producing mRNA that will not be translated. On the other hand, CRISPRi inhibits already the transcription phase so that the cell will not spend energy in transcribing genes that will not be translated. However, if a very strong repression is required, it might be suitable to combine sRNA and CRISPRi technologies. Thus, in case that CRISPRi leaks, sRNAs would prevent the translation of the mRNA.

Zinc fingers are small peptide motifs that recognize and bind to a sequence coding for three amino acids (Pavletich and Pabo, 1991). They can be used as a interchangeable building blocks for building proteins that can recognize and bind to a specific DNA sequences with various lengths (Klug, 1999). When the zinc finger is fused with transcription activating or inhibiting protein domains, genes of interest can be selectively turned off or on (Klug, 2010). For example, more than 10-fold repression of human *CHK2* gene was achieved by using zinc fingers (targeting a 18 nt long sequence) (Tan et al., 2003). However, constructing a zinc finger repression tool can be a long process because the zinc finger motifs need to be rationally designed – which can take a long time as nucleotide-protein interactions are complex – or they can be chosen from a library after enough zinc finger motifs have been found and characterized (Klug, 1999). As comprehensive library of zinc fingers has not been established, the latter option is still unavailable. On the other hand, CRISPRi provides very simple and fast workflow from finding the target in the gene of interest until expressing the machinery in the cell and repressing the gene of interest. For specific targeting with the sgRNA:dCas9 complex, up to a 20 nt long sequence of the gene of interest must be chosen and complementary base-pairing region of sgRNA can be designed easily based on that. Then, the base-pairing region can be synthetically manufactured and cloned into sgRNA expression vector.

Riboswitches are gene control systems that are based solely on RNA (Lynch et al., 2007). In nature riboswitches often locate in the 5'-untranslated region of some mRNAs where they control gene expression at the transcriptional or at the translational level (Wachsmuth et al., 2013). Riboswitches mostly activate but also in some cases also repress the gene expression by binding to a small molecule ligand with an aptamer part of the riboswitch, which in turn alters the conformation of the riboswitch leading to changes in the gene expression (Lynch et al., 2007). Riboswitches can also be constructed synthetically to control the gene expression of a desired gene. This has been successfully done in both, Gram-negative and Gram-positive bacteria, including *A. baylyi* (leading to maximum 40 fold activation) (Topp et al., 2010). As no mention of synthetic riboswitch that represses a gene after a ligand (inducer) addition could be found from literature, it is difficult to compare riboswitches to CRISPRi, which usually represses the gene expression in the presence of a ligand. However, riboswitches could be used in a opposite manner to CRISPRi for gene regulation. In other words, a gene could be repressed until a ligand is introduced to the cells. When the ligand is present

the gene of interest would be expressed. In this case, both systems, riboswitches and CRISPRi, could be compared. As riboswitch is a bit simpler (it lacks the need of proteins), it could cause less burden to the cells. Both can be easily programmed to target the gene of interest by complementary RNA sequences. However, as riboswitches can also target mRNAs, it could be feasible to control gene expression in transcriptional level with CRISPRi and in translational level with riboswitch to achieve stronger regulation of the microbial metabolism. Or CRISPRi machinery could be used to repress a gene and riboswitch could be used in the same construct to activate the same or some other gene. Thus, more complex regulation pathways could be designed.

2.1.5 CRISPRi in reality – examples for what it can be used

As already discussed, regulating the gene expression can increase production metrics such as titer, yield and productivity (Wu et al., 2017). Additionally, CRISPRi has been successfully used to control extracellular electron transfer (EET) pathway (Cao et al., 2017) and as a help in studying essential genes (Liu et al., 2017). CRISPRi has been demonstrated to function in both, Gram-negative and Gram-positive bacteria. It has been proven to be functional at least in *Actinomycetales* (Tong et al., 2015), cyanobacteria (Yao et al., 2016), *Shewanella oneidensis* (Cao et al., 2017), *E. coli* and *Corynebacterium glutamicun* (Cleto et al., 2016) to mention few examples. Hence, many real life applications have already been found for the CRISPRi technology in broad spectrum of hosts and there surely are many more to come.

To improve production metrics of a desired product, simultaneously regulation of several genes can be important. One way to achieve this by CRISPRi could be to individually design multiple sgRNAs targeting different genes. Expression of different sgRNAs could be regulated by promoters that are induced by different inputs. Different repression levels could be achieved also by designing sgRNAs with variable complementarities with the targeted genes. Fernandez-Rodriguez et al. (2017) did this by expressing three sgRNA sequences, targeting three different genes (*pta*, a*ckA* and *poxB*), under (red, green and blue) light sensing promoters. In other words, red light induced one promoter as well as green and blue light. Thus, three genes in *E. coli* involved in acetate production could be controlled simply by using light. As a result, maximum of 2.9-fold repression in acetate titer was observed. On the other hand, Vigouroux et al. (2018) used five sgRNAs with various complementarities to target both, *superfolder GFP* and *mCherry* genes. They managed to repress both of the genes independently, achieving 2–100% of the original expression levels.

One of the most useful aspects of CRISPRi system is that the repression is reversible (Qi et al., 2013). Thus, no final alterations are needed to be made in the genome for gene regulation. As a result, one of the most useful ways to exploit CRISPRi tools could be to regulate genes in the genome. It would provide means to regulate multiple genes individually during distinctive growth phases. One idea is to

program cells to recognize cell densities and then respond by expressing different enzymes which help the whole bacterial community in the reactor (Nielsen and Voigt, 2014). For example, this could be done by utilizing the CRISRPi in natural mixed bacterial population. The CRISPRi machinery could be delivered in a plasmid and by exploiting horizontal gene transfer between bacterial cells (Ji et al., 2014). Other way to transfer the system to the recipients with low transformation success could be bacteriophages (Ji et al., 2014; Westwater et al., 2002). With these mechanisms the CRISPRi could be constructed also to strains that are usually difficult to genetically modify in laboratory conditions or to mixed populations.

The ability to produce and associate into biofilms could be altered with CRISPRi. For example, Zuberi et al. (2017) manipulated *E. coli*'s ability to produce biofilms and attach to surfaces by silencing *fimH* gene. This resulted in approx. two fold decrease in the amount of adherent cells. In the microscopy pictures it was visible that treated cells did not have flagella what to use for adhering.

CRISPRi can also be used to temporarily knock-out genomic genes for studying essential genes, especially in prokaryotes (Liu et al., 2017). In eukaryotic cells normal CRISPR has been proven to outperform CRISPRi in this task (Evers et al., 2016). In prokaryotic cells CRISPR would be lethal as the cells do not have an efficient double strand break repairing mechanism. Silencing the genes using CRISPRi is also fast when compared to the traditional gene knockout: results that are comparable to gene deletion were achieved in three days when the CRISPRi technology was used in *C. glutamicum* (Cleto et al., 2016).

The CRISPRi system could be also used in combination with other gene regulation technologies to achieve more complex regulatory pathways. For example, *Shewanella oneidensis*' *mtrA* gene in the EET pathway was repressed more efficiently when both, CRISPRi and the Hfq-dependent sRNA system, were used in combination than when they were used individually. The maximal repressions achieved with CRISPRi, sRNA and combined CRISPRi-sRNA system were 46, 16 and 59% respectively. (Cao et al., 2017) By combining these two tools transcription and translation could be regulated simultaneously to achieve higher level of gene regulation. Wu et al. (2017) utilized CRISPRi together with CRISPR in *E. coli*. First they introduced point mutations, replacements, knock-outs and insertions with CRISPR to make it possible to produce 1,4-BDO in *E. coli* which normally lacks the production pathway. Then, they down regulated three genes (*gabD*, *ybgC* and *tesB*) that divert the flux away from 1,4-BDO production with CRISPRi and were able to enhance 1,4-BDO production by 100%.

To produce more complex regulative networks, genetic circuits, for example NOT, OR and AND gates, have been combined with CRISPRi (Nielsen and Voigt, 2014). One example is presented by Chappell et al. (2017): they combined CRISPRi with Transcription Activating RNAs (STARs). STAR and dCas9 were combined into

NIMBLY (A AND NOT B) logic gate. Using that gate, the expression of the gene of interest could be achieved only when STAR was expressed but dCas9 was not. In addition, an incoherrent type 1 feed-forward loop (I1-FFL) was designed based on STAR and CRISPRi system so that inducing STAR induced expression of both, the dCas9 and the gene of interest. As a result, the gene of interest was expressed faster in the terms of normalized fluorescence than with direct activation (when STAR induced only the expression of the gene of interest) and in a pulsing manner. Pulses were achieved because two hours after the induction of STAR in I1-FFL, CRISPRi reached repression threshold and started to repress the expression of the gene of interest. As a result, the total level of induction achieved with I1-FFL gate was lower than with direct activation.

In literature no studies concerning CRISPRi in ADP1 could be found even though there would be multiple ways to use a gene regulation tool in this strain. To name one example, the wax ester production of ADP1 could be enhanced by silencing genes diverting the flux away from the wax ester pathway.

2.2 *Acinetobacter baylyi* ADP1

Acinetobacter species are widespread in the nature and they can be found from versatile environments, including soil, water and other living organisms. They are strictly aerobic, usually non-motile and oxidase-negative. *Acinetobacters* often pair as non-motile cocci rather than as monoflagellate rods. One *Acinetobacter* strain often used in the laboratories, for example in biotechnical applications and for studying genomics, is *Acinetobacter baylyi* ADP1. It is relatively easy and fast to cultivate in laboratory environment and it is naturally competent. (Barbe et al., 2004)

Originally ADP1 is a soil bacteria, closely related to *Pseudomonas putida* and *Pseudomonas aeruginosa*. All of the members of *Acinetobacter* group are versatile chemoheterotrophs and ADP1 does not make an exception. As it can utilize variety of substrates, many different mediums can be used in laboratory cultivations. Additionally, ADP1 grows rapidly (doubling times < 1h), forming colonies in overnight incubations from a single cell. (Barbe et al., 2004)

Even though most of the *Acinetobacters* are non-motile, ADP1 is motile by twitching (Gohl et al., 2006). It extends a pili (type IV) to attach to a surface. Next, the bacteria retracts the pili, thus pulling itself toward the point of attachment. This pili plays also a important part in the natural transformation capability of ADP1 as it can attach it also to a DNA strand and pull it into the cell. (Leong et al., 2017) This motility is depended of light so that blue light inhibits the twitching (Bitrian et al., 2013).

In addition to *P. putida* and *P. aeruginosa*, ADP1 is also closely related to *E. coli*. Hence, the knowledge concerning *E. coli* can be applied almost directly to ADP1. They both are protorophic (requiring only inorganic compounds for growth), aerobic, grow overnight in both, rich and minimal salt medium. Optimal growth temperatures are be-

tween 30-37 ^{O}C. Laboratory strains of *A. baylyi*, for example ADP1, are harmless to humans. They are not pathogenic or do not carry virulence factors. However, there are two differences that make ADP1 a better organism for metabolic engineering and research than *E. coli*: ADP1's natural competence for linear and plasmid DNA and a strong natural tendency for homologous recombination. In other words, ADP1 is naturally 10-100 times as competent as *E. coli* treated with calsium chloride. (Metzgar et al., 2004)

2.2.1 Genetics of the naturally transformable bacteria

ADP1 has a small genome (3 598 621 bp) (compared to E. coli's 4.6 Mb genome (Blattner et al., 1997)) as a circular chromosome with the G-C content of 40.3% (Barbe et al., 2004). The genome has 3325 coding sequences (CDS) (Barbe et al., 2004) from which 499 genes have been proposed to be essential in minimal medium (de Berardinis et al., 2008). One fifth of the genome is thought to be involved in catabolism. These genes are situated in five clusters which are called "islands of catabolic diversity". (Barbe et al., 2004).

ADP1 lacks some genes that are involved in the central metabolism of many other organisms: *glucokinase* (and *glucose transporter phosphotransferase system* and *hexokinase*), *pyruvate kinase*, *6-phosphogluconolactonase*, *glucose-6-phosphate dehydrogenase* and *6-phosphofructokinase* (Barbe et al., 2004). These genes code for enzymes involved in the Embden-Meyerhoff-Parnass (EMP) glycolysis (Kannisto, 2018). However, ADP1 has genes for glucose catabolism through the Entner-Doudoroff (ED) pathway (Barbe et al., 2004). Nonetheless, as the EMP pathway is missing, it can be complicated to metabolically engineer ADP1 to utilize other sugars than glucose (Kannisto, 2018).

Some of the bacterial strains can obtain new genes by natural transformation in which the bacteria actively takes up foreign DNA and integrates it as a part of the bacteria's physiology. In other words, transfer of the DNA is initiated by the recipient cell and not by the donor cell as in other lateral gene transferring mechanisms: conjugation, lateral gene transfer and transduction. (Johnsborg et al., 2007) ADP1 is naturally transformable to high degree: 25% of the cells have been proven transform when a plasmid DNA was introduced into the culture (Palmen et al., 1993). On the other hand, the tendency for the natural transformation has been shown to decrease in evolution experiments in laboratory conditions (Bacher et al., 2006; Renda et al., 2015). One suggested reason was that the DNA released by ADP1 cells during cultivation (Palmen and Hellingwerf, 1995) is more inhibitory to competent cells than non-competent cells. Thus, transforming to non-competent provides a fitness improvement (Renda et al., 2015). This has also been proven: genomic DNA of ADP1 added to the culture is more inhibitory to the wild type (WT) (competent) ADP1 cells than non-competent mutant of ADP1. Another suggested reason for competence decrease is that competent cells can and will take up the DNA that mutates the cells to non-competent. On the other hand,

non-competent cells can not take up DNA that would mutate them to become competent. (Bacher et al., 2006)

ADP1 could be engineered to be a much more suitable host organism for metabolic engineering by removing six transposon *IS1236* sequences. This strain, which is called ADP1-ISx, accumulates reporter gene inactivating mutations 7–21 times less often than WT ADP1. Additionally, it has an increased natural competence, shows a shorter lag phase, grows to higher cell density and undergoes autolysis to a lesser degree. (Suárez et al., 2017) As ADP1-ISx has many positive aspects when compared to WT ADP1, it should be favored in the experiments in the future.

The natural competence of ADP1 is induced in the beginning of exponential growth phase and remains until few hours after the start of stationary phase (Palmen et al., 1993). The transformation frequency is affected by six variables: (1) acidity of the medium (competence is lowered at pH under 6.5), (2) the selection marker used, (3) the type of DNA used and its concentration (up to a certain limit), (4) the incubation time in the presence of the foreign DNA (plateau is achieved in few hours, after that the transformation frequency does not increase), (5) DNA homology and (6) the genomic location of the insert (Palmen et al., 1993; Ray et al., 2009). Additionally, the DNA uptake is Mn^{-2}, Ca^{2+} or Mg^{2+} dependent. Thus, divalent cations are required in the medium. On the other hand, transformation does not depend on the carbon source used. The genomic transformation happens after the cell takes up a plasmid (containing an insert cassette) by replacing a genomic DNA sequence that corresponds to the cassette's flanking regions (replacement recombination) or by integrating the whole plasmid into the chromosome. The former has been shown to happen more frequently. (Palmen et al., 1993)

ADP1 probably has an endogenous CRISPR system because *cas1* gene is predicted to be in the genome (UniProt: Q6F9L2). However, as cas1 is highly conserved protein in the different CRISPR systems (Makarova et al., 2015), the actual CRISPR system in ADP1 is not known. As endogenous type I CRISPRi systems has been successfully used in *E. coli* (GFP was repressed up to 82%) (Chang et al., 2016), it might be also feasible to utilize ADP1's own CRISPR. The large genes needed for CRISPRi machinery would be already in the genome and evolved through the natural selection towards perfection for this strain especially. Hence, they could cause less burden to the cells than CRISPRi that has been taken from another bacteria and transformed into ADP1.

2.2.2 Metabolism

ADP1 is known to be a nutritionally versatile strain (Barbe et al., 2004). Thus, it can catabolize many different plant derived carbon sources, for example organic acids, which are actually the preferred carbon source of *Acinetobacter* and *Pseudomonas* bacteria (Gerischer, 2002). As ADP1 lacks EMP pathway enzymes, glucose is the only sugar carbon source that is known to solely support the growth of the cells (Taylor and Juni,

1961). According to van Schie et al. (1987) ADP1 can also oxidize partially other sugars, for example xylose and galactose, to their corresponding lactones. Lactones can be then hydrolyzed to sugar acids if the pH is elevated. As a result, the growth might be enhanced in the mediums where carbon is the limiting substrate but which contains other sugars than glucose.

On the other hand, if glucose is used as the sole carbon source in an ADP1 cultivation, H^+ is formed in the ED pathway (when gluconate is produced from glucose) and thus pH should decrease (Taylor and Juni, 1961). However, if pH is 5 or lower, gluconate is not produced from glucose, at least in *A. calcoaceticus* LMD79.41 (van Schie et al., 1987). As *A. calcoaceticus* LMD79.41 and ADP1 are closely related, it is probable that the same happens with ADP1. Hence, the pH in the cultivations should be controlled to avoid the inhibition caused by the decrease in the pH following the glucose degradation.

One good example of an organic acid carbon source favored by ADP1 is acetate (or acetic acid), which for example *E. coli* TG1 accumulates to the medium when the growth rate exceeds 0.17 h^{-1} in a fed-batch reactor (Korz et al., 1995). Accumulation of acetate can inhibit the growth of *E. coli* cells (Korz et al., 1995) and also recombinant protein production (Eiteman and Altman, 2006). On the other hand, ADP1 does not produce acetate when grown on glucose, even at high growth rates. However, if ethanol (EtOH) is used as a substrate, acetate is produced as an intermediate. In that case acetate can accumulate in the growth medium. (Kannisto, 2018) Additionally to acetate, other acids often inhibit the growth of microbes in bioreactors (Palmqvist and Hahn-Hägerdal, 2000; van Zyl et al., 1991). ADP1 could provide a possible solution to decrease the acid levels in co-cultivations with other strains that more sensitive to acid inhibition. For example, ADP1 could be co-cultivativated with *E. coli* to utilize the produced acetate so that the level of acetate would not inhibit the growth or the recombinant protein production of *E. coli*.

Many carbon sources used during aromatic compound degradation by ADP1 can inhibit the aromatic compound catabolism. Acetate and succinate produce the strongest repression. On the other hand, glucose does not induce any inhibition of aromatic compound catabolism and gluconate represses catabolism of some aromatic compounds only slightly. Additionally, gluconate functions as an inducer of the *catA*, which is a gene in an aromatic compound degradation pathway. (Dal et al., 2002) In overall, the aromatic compound catabolism is under complex regulatory mechanisms (Bleichrodt et al., 2010; Dal et al., 2002). Hence, if ADP1 is used in an application that includes degradation of aromatic compounds, care should be taken so that only non-inhibitory additional carbon sources are used.

Exopolysacharides (EPS) are exerted by *A. baylyi* strains for protection against desiccation (Ophir and Gutnick, 1994) and to prevent cells from forming aggregates (Juni and Heym, 1964). The composition of EPS differs slightly according to the used

carbon source and the analysis method. However, regardless the carbon source or the analysis methods, the most abundant component of EPS is rhamnose, second abundant is glucose (and glucoronic acid) and the least abudant is mannose. (Kannisto, 2018) If an additional protein component is present, EPS exerted by ADP1 can also function as emulsifier (Kaplan et al., 1987). Additionally, an outer membrane protein OmpA that is secreted by ADP1 is shown to function as a strong emulsifier (Walzer et al., 2006). ADP1 has been proven to degrade crude oils (Lal and Khanna, 1996), which could be the reason ADP1 secretes emulsifiers. It possibly uses them to turn oil into a phase that is easier to degrade. In the laboratory environment when rich medium is used the exertion EPS or producing an EPS capsule does not probably give a selective advantage to the cells as no desiccation can happen. Hence, eliminating EPS production could be beneficial for enhancing the production metrics as then carbon and energy flux would be directed to desired product synthesis instead of the production of EPS. Additionally, EPS might make the product extraction and purification more laborious. On the other hand, EPS can protect the cells from inhibitory chemicals which might be present because of some substrates, for example which are formed during hydrolyzis of lignocellulosic biomass (Kannisto, 2018).

2.2.3 ADP1 in biotechnological applications

Acinetobacters are thought to be suitable for example for production of biochemicals, as biosensors and in bioremediation (Abdel-El-Haleem, 2003) and ADP1 does not does not differ from the other *Acinetobacter* strains in this case. Additionally, ADP1 is also flexible and versatile model organisms for genetic analysis. As it is easy to genetically manipulate and it can express many different foreign genes, it can be used with only little equipment and genetics expertise to study genes of interest, for example. (Metzgar et al., 2004)

Gene duplication and amplification (GDA) allows rapid adaption to environmental changes (Andersson and Hughes, 2009) and in bacteria GDA also affects virulence, antibiotic resistance and vaccine failures (Craven and Neidle, 2007). Even though GDA is important and common event, it is difficult to study (Elliott and Neidle, 2011). However, these difficulties have been overcame in ADP1 by utilizing its pathways for aromatic compound catabolism (Reams and Neidle, 2003). Additionally to these well characterized pathways, the junction sequences in GDA events could be identified by exploiting natural competence of ADP1 (Reams and Neidle, 2004). Another example of genetic studies performed with ADP1 is a study about persistence and dissemination of transgenes in soil. It was found out that a small portion of DNA molecules escaped degradation and persisted in the soil microcosmos at least for four years and they were still capable to transform *A. baylyi*. (Pontiroli et al., 2010)

One example of production of the product of interest by ADP1 is presented by Lehtinen et al. (2017a). They used two stage process, the first step involving microbial

electrosynthesis by *Sporomusa ovata* to reduce carbon dioxide to organic compounds, mostly acetate. In the second step ADP1 produced long chain alkyl esters from the acetate. This was the first proof-of-principle study for producing long alkyl esters with bacteria using carbon dioxide and electricity as only carbon and energy sources, respectively.

ADP1 has also been used in co-cultures to enhance the product titer of *E. coli* and subsequently genetically engineered to produce the same product as *E. coli*. Santala et al. (2014) used ADP1 to consume acetate in the co-cultivation with *E. coli* K12 to prevent acetate from inhibiting the *E. coli*. As a result, they managed to enhance the biomass and recombinant product titers in both rich and minimal growth mediums. Additionally, they further enhanced the product titer by genetically engineering ADP1 to produce the same product. In other study by Santala et al. (2011) APD1's natural triacylglycerol production was enhanced by 5.6-fold by deleting three genes. However, genetic engineering is not always needed for enhancing product titers as in a study performed by Elbahloul et al. (2005). Only optimizing the growth conditions increased cyanophycin production from 3.5% (wt/wt) of dry cell mass to 46.0% (wt/wt). Furthermore, the cyanophycin titer was increased still by 8.6-fold by genetically engineering ADP1. Additionally, the genetic engineering removed the need of arginin as the carbon source which reduced the overall costs of the cyanophycin production dramatically.

Lehtinen et al. (2017b) engineered two layer biosensor into ADP1. Hence, the real time monitoring of alkane biosynthesis or degradation became possible as alkanes induced the expression of GFP. This way alkane concentrations could be measured online as fluorescence. Additionally, they engineered into the same strain a system that sensed intermediates of the alkane biosynthesis/degradation pathway. This was achieved by introducing a bacterial luciferase which enzymatically recognized a specific intermediate in the alkane production/degradation pathway thus producing light. The group argued that this approach could provide the means to optimize and study the kinetics of a heterologous pathway hence helping to develop more efficient cell factories.

Even though there are already many studies concerning *Acinetobacters* and ADP1 especially, there is still work to be done in that field of research. For example, ADP1 could be used to produce EPS. They have emulsifying properties (Kaplan and Rosenberg, 1982) and thus they could possibly be used as emulsifiers in industrial or other applications. However, there are no extensive studies or this aspect of ADP1 has not been subjected to metabolic engineering yet (Kannisto, 2018). Furthermore, *Acinetobacters* are suggested as the model organism for environmental microbiological studies, industrial scale production of chemicals and pathogenicity tests due to their presence in diverse environments and their versatile metabolic characteristics. However, thorough studies in the areas of physiological characteristics, for example motility, stress responses and quorum sensing, are still to be made. (Jung and Park, 2015)

One way to achieve better control over ADP1 genetics and metabolism could be to introduce CRISPRi machinery into the strain. As a result, genes could be controlled in reversible manner. This could provide means, for example, to produce EPS in higher titers and possibly as a more suitable compound for a special requirement. Additionally, CRISPRi would make genetic research easier as it can be constructed into hosts fast and it could be used to silence and thus study functions of genes in ADP1 as it has been used in other hosts already.

2.2.4 Bioluminescence provides an insight to the inner state of a cell

The *lux* operon used in this study contains genes *luxCDABE*. *LuxD* produces a transferase which is the first protein to act in the aldehyde (substrate for bioluminescence) biosynthesis pathway (Figure 2.4). It forms a fatty acid by transferring an activated fatty acyl group to a water molecule and becomes acylated at the same time. Next, the product of *luxC* gene (reductase) attaches an AMP part from an ATP molecule to the acid to activate it. As a result, a fatty acyl-AMP, which is tightly bound to the enzyme, is formed. (Close et al., 2009) Then *luxE* gene product (synthetase (Close et al., 2012)) reduces fatty acyl-AMP to aldehyde by using NADPH as an energy source (Meighen, 1991). *LuxA* and *luxB* gene products form a heterodimer protein, called luciferase. It is in the end responsible of producing bioluminescence from the aldeyde substrate produced by genes *luxDCE*. (Close et al., 2012)

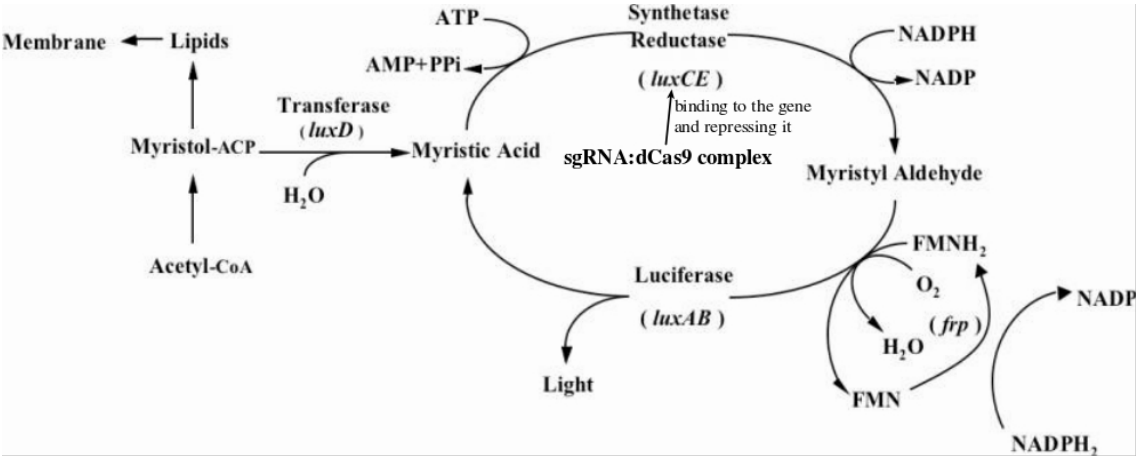

Figure 2.4 *Many different cofactors, which are important for the functioning of the cell, are needed in the biosynthesis pathway of bioluminescence by luxCDABE gene cluster. As a result, it provides a good way to observe changes in the cell vitality. It can show in real time the burden of CRISPRi on the cells. Additionally, as the changes is the bioluminescence production take place fast, it can be used to study the repression kinetics. Other objective of this study is to obtain information how repressing the luxC gene (by sgRNA:dCas9 complex) affects other genes in the luxCDABE gene cluster as the need of luxC in bioluminescence production can be bypassed by adding decanal. (modified from Close et al., 2009)*

Bioluminescence production pathway requires many cofactors which are essential for cell functioning: NADPH, FMNH2, ATP, acyl-CoA and oxygen, for example (Figure 2.4) (Close et al., 2009). Hence, disturbances in the concentrations of these compounds will show rapidly and dynamically in the bioluminescence production. As a result, if CRISPRi machinery did affect the cell metabolism and thus the concentration of

the compounds mentioned above changed, the amount of bioluminescence produced would change as well. In other words, by using the *lux* operon, the burden of the machinery can be quantified. This operon can be transformed into several versatile bacterial hosts and it does not need any special substrates (Meighen, 1993). As a result, it could be used to study many other pathways used in genetically engineered strains.

Bioluminescence is produced fast and it also fades fast (Meighen, 1993). As a result, it can be considered as rapid and dynamic tool for studying changes in the gene expression. Hence, it can be well used as the indicator of repression kinetics.

In addition, *luxC* is the first gene in the *luxCDABE* cluster. Subsequently, repression of downstream genes can be easily studied as the need of the *luxC* gene in bioluminescence production can be bypassed by using an external substrate (in this case decanal). If other genes in the cluster are also silenced, the bioluminescence is decreases or remains at the same level even after adding the external substrate. If only the *luxC* is silenced, adding decanal should rapidly increase the bioluminescence production.

3 MATERIALS AND METHODS

3.1 Reagents and instruments

Escherichia coli XL1-Blue cells were from Stratagene, USA. Plasmids (pBAV1G-ara-sgRNA and pp2-chn-dCas9/pIM1463) and primers (GFP_fwd, GFP_rev, Lux_fwd, Lux_rev, 1a-pBAV_for and 1b-pBAV_rev) were obtained from the laboratory supervisor. The sequences and plasmid map of pBAV1G-ara-sgRNA[null, GFP and Lux] plasmids are introduced in the Appendix A and the primers in the Appendix B.

GeneJet Plasmid Miniprep Kit, GeneJet PCR Purification Kit, d(C/A/T/G)TPs (which were used to make dNTP mix), 5x Phusion HF buffer, 100% DMSO, Phusion U HS, Fast Digest (FD) DnpI, 10x FD-buffer, T4 ligase, FD Eco31I, FD PstI, FD Green Buffer were from Thermo-Scientific, USA. T4 ligase buffer was from Fermentas, Lithuania. USER enzyme and OneTaq Master Mix were produced by New England Biolabs, USA. Arabinose and cyclohexanone were obtained from ACROS Organics, China and United Kingdom, respectively.

NanoDrop2000 (Thermo-Scientific) was used for DNA quantification. Electroporations were done with the electroporator BioRad Micropulser (Bio-Rad, USA). Cell mass (measured as optical density at 600 nm, OD_{600}) was determined using spectrophotometer Ultrospec 500pro (Amersham Biosciences; GE Healthcare Life Sciences, USA) when cultures were incubated in growth tubes. Microplate reader Spark (TECAN, Switzerland) was used to measure fluorescence, bioluminescence and OD_{600} when 96 well microplates (white μClear, Cellstar cell culture microplates with F-bottom) (Greiner bio-one, Germany) were used for incubations. Measurement of pH was done with pH papers with scale 1–10 or 4–7. Both were obtained from Merck, Germany.

The constructed sgRNAs were sequenced by Biocenter, Institute of Biotechnology, University of Helsinki, Finland, using S1 primer. The sequencing results were aligned with the designed sequence using Standard Nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) (Altschul et al., 1990).

3.2 Construction of the sgRNA plasmids

All the incubations were done at 30 ^{O}C unless otherwise stated. Additionally, when liquid medium was used the tubes were rotated at 300 rpm. All of the plasmid extractions were done with GeneJet MiniPrep Kit according to the kits instructions (except only 30 µl of the elute was used). Additionally, DNA concentration of every extracted plasmid was analyzed with a NanoDrop2000.

The plasmid pBAV1G-ara-sgRNA[null] (contained a base-pairing region which was designed not to target any gene) (Appendix A) was obtained from the laboratory supervisor. To amplify it, the plasmid was transformed by electroporation to XL1-Blue cells. Inoculation on LA-plates (50 µM glucose and 10 µg/ml gentamycin) was done with 100 µl of transformed cells. Negative control was done by inoculating a similar LA-plate with XL1-Blue strain containing *GFP* gene under a mphR promoter. It did not contain gentamycin resistance (obtained from a previous work, data unpublished).

For a subsequent cloning of GFP and Lux targeting base-pairing sequences into the plasmid pBAV1G-ara-sgRNA, a colony containing the plasmid was transferred from the LA-plate to LB-medium (0.4% glucose and 10 µg/ml gentamycin) and it was incubated overnight at 37 ^{O}C. Next, plasmid was extracted from 1.5 ml of culture broth.

Urasil-Specific Excision Reagent (USER) cloning method (Nour-Eldin et al., 2006) was used to construct pBAV1G-ara-sgRNA[GFP] (sgRNA targeting *GFP*) and pBAV1G-ara-sgRNA[Lux] (sgRNA targeting *luxC*) plasmids (Appendix A). Primers for the reactions were designed with the AMUSER web server (Genee et al., 2015). The cloning was performed according to a protocol of New England Biolabs: Cloning with USER enzyme (New England Biolabs, 2018). The target plasmids were amplified using touchdown PCR with GFP_for (or Lux_for) and GFP_rev (or Lux_rev) reverse primers and pBAV1G-ara-sgRNA[null] plasmid as a template. As exceptions to the protocol, the incubation with the USER enzyme was done in two steps, (1) 37 ^{O}C for 30 min, (2) 37 ^{O}C for 2 min. The second step was repeated for 27 times and the temperature was decreased by 1 ^{O}C during every cycle. As subsequent transformations were done by electroporation, USER reactions were ligated using T4 ligase.

The plasmids pBAV1G-ara-sgRNA[GFP] and pBAV1G-ara-sgRNA[Lux] were transformed by electroporation to XL1-Blue cells. Cells transformed with the sample and control USER reactions were plated on LA-plates (50 µM glucose and 10 µg/ml gentamycin) which were incubated approx. for two days (until the colonies were big enough to be counted and transferred to LB-medium medium).

Five differently sized colonies of each plasmid were transferred to LB-medium (0.4% glucose and 10 µg/ml gentamycin) and incubated until the medium was clearly turbid. Then, plasmids were extracted. The constructs were verified by restriction analysis and sequencing. One of each plasmid (pBAV1G-ara-sgRNA[GFP] and pBAV1G-ara-sgRNA[Lux]) was selected for the inbound transformations according to the correct

alignment with the original, designed plasmid. Vital was that the base-pairing region was exactly the same as in the designed plasmid. As a result, plasmids pBAV1G-ara-sgRNA[GFP]-2 and pBAV1G-ara-sgRNA[Lux]-3 were chosen for continuation.

3.3 Construction of the ADP1 strains

Control strains used in this study were ADP1-Lux, ADP1-Lux-GFP, ADP1-Lux-GFP-dCas9 and ADP1-Lux-dCas9. ADP1-Lux-GFP-sgRNA[GFP]-dCas9 (sgRNA targets the *GFP* gene) and ADP1-Lux-sgRNA[Lux]-dCas9 (sgRNA targets the *luxC* gene) were the studied strains (Table 3.1). A cyclohexane inducible promoter was used regulate dCas9 gene (which was located in genome), sgRNA was expressed from the plasmid pBAV1G-ara-sgRNA using an arabinose inducible promoter, monomeric superfolder green fluorescent protein (msfGFP – in this thesis abbreviated to only GFP) (Landgraf, 2012) was expressed from genome as well as Lux operon. Both of the latter genes were expressed under regulation of constitutive promoters.

The strain ADP1-Lux was obtained from the laboratory supervisor. It was constructed by transforming ADP1Δacr1::tdk/Kan^R strain with the plasmid pVKK81-T-lux (V. Santala and M. Karp, unpublished) which contains the bacterial luciferase operon *luxCDABE* from *Photorhadus luminescens*. The genomic locus of the lux operon is not known.

Additionally, the plasmid (iBG42/pAK400c) containing the *GFP* gene and a gene for chloramphenicol resistance was obtained from the laboratory supervisor. The *GFP* gene and its strong constitutive promoter were cloned from the construct pBG42 (Zobel et al., 2015) into the integrable cassette i_Cm^R/pAK400c (Santala et al., 2011). This novel construct was named iBG42/pAK400c (unpublished). The integrable cassette part (iBG42) replaces the gene *ACIAD3381* (*poxB*) in the ADP1's genome.

The plasmid iBG42/pAK400c was transformed into ADP1-Lux using the following protocol. First, preculture of ADP1-Lux was incubated overnight in LB-medium (0.4% glucose and 50 µg/ml kanamycin). Second, 50 µl of preculture was used to inoculate approx. 1 ml of fresh LB-medium (1% glucose) and the tube was incubated for 2 h 30 min (until exponential growth phase was achieved and the liquid became turbid). Third, the culture was divided into two 0.5 ml aliquots. Fourth, approx. 1 µg of the plasmid was added into one of the aliquots, the other aliquot functioned as a control. Fifth, the tubes were incubated for 2–4 h and then each aliquot was plated on two LA-plates (50 mM glucose and 25 µg/ml chloramphenicol) which were incubated overnight at 30 ^{O}C. If colonies did not appear after overnight incubation, plates were left at the room temperature for the second night.

Five colonies were transferred from the tranformation plates into LB-medium (0.4% glucose and 25 µg/ml chloramphenicol) and they were incubated overnight. To ensure successful transformation, absorbance (λ=600 nm) (OD_{600}), fluorescence (excita-

tion λ=485 nm and emission λ=535 nm) and bioluminescence were measured from 100 µl of cultures using Spark. The two strains with the highest fluorescence and bioluminescence were chosen to be used in the study.

The pBAV1G-ara-sgRNA[GFP]-2 and pBAV1G-ara-sgRNA[Lux]-3 plasmids were transformed into ADP1-Lux-GFP and ADP1-Lux, respectively, using the same protocol as described above (except the LA-plates contained also 10 µg/ml of gentamycin). Additionally, the pBAV1G-ara-sgRNA[null] plasmid was transformed into both of the strains using the same procedure. To verify successful transformation (in addition to gentamycin selection), two colonies of each transformation were transferred to LB-medium (0.4% glucose, 10 µg/ml gentamycin and 25 µg/ml chloramphenicol in the case of ADP1-Lux-GFP-sgRNA[null/GFP]) and incubated overnight. Plating was repeated from a single colony to ensure that only a single colony was selected for continuation. Next, plasmids were extracted, digested with PstI and run in an agarose gel for 60 min at 70 V to verify the successful transformations.

For transforming the *dCas9* gene to ADP1, XL1-Blue containing a plasmid with *dCas9* gene (pp2-chn-dCas9/pIM1463) was obtained from the laboratory supervisor on a LA-plate. Two colonies were transferred to LB-medium (0.4% glucose and 50 µg/ml spectinomycin) and incubated overnight. Then, plasmids were extracted from both cultures. The plasmid was transformed into ADP1-Lux-sgRNA[null], ADP1-Lux ADP1-Lux-sgRNA[Lux], ADP1-Lux-GFP, ADP1-Lux-GFP-sgRNA[null] and ADP1-Lux-GFP-sgRNA[GFP] strains following the same procedure as in earlier ADP1 transformations (except now LA-plates contained 10 µg/ml gentamycin and 50 µg/ml spectinomycin). Successful transformations were verified by colony PCR using dCas9_fwd and dCas9_rev primers in OneTaq Master Mix. Then the reactions were ran in an agarose gel at 70 V for 60 min. If *dCas9* was present in the genome, approx. a 500 bp long band was supposed to be visible.

Table 3.1 *Strains used in this study.*

Strain	Genotype	Antibiotic resistance
ADP1-Lux	ADP1Δacr1::tdk/Kan^R, [luxCDABE]	kanamycin
ADP1-Lux-dCas9	ADP1Δacr1::tdk/Kan^R, Δpp2::ChnR/pChnB-dCas9/Spec^R, [luxCDABE]	kanamycin, spectinomycin
ADP1-Lux-sgRNA[null]-dCas9	Δacr1::tdk/Kan^R, Δpp2::ChnR/pChnB-dCas9/Spec^R, [luxCDABE], pBAV1G-ara-sgRNA[null]	kanamycin, gentamycin, spectinomycin
ADP1-Lux-sgRNA[Lux]-dCas9	Δacr1::tdk/Kan^R, Δpp2::ChnR/pChnB-dCas9/Spec^R, [luxCDABE], pBAV1G-ara-sgRNA[Lux]	kanamycin, gentamycin, spectinomycin
ADP1-Lux-GFP	Δacr1::tdk/Kan^R, ΔpoxB::gfp/Cm^R, [luxCDABE]	kanamycin, chloramphenicol
ADP1-Lux-GFP-dCas9	Δacr1::tdk/Kan^R ΔpoxB::gfp/Cm^R Δpp2::ChnR/pChnB-dCas9/Spec^R, [luxCDABE]	kanamycin, chloramphenicol, spectinomycin
ADP1-Lux-GFP-sgRNA[null]-dCas9	Δacr1::tdk/Kan^R ΔpoxB::gfp/Cm^R Δpp2::ChnR/pChnB-dCas9/Spec^R, [luxCDABE], pBAV1G-ara-sgRNA[null]	kanamycin, chloramphenicol, gentamycin, spectinomycin
ADP1-Lux-GFP-sgRNA[GFP]-dCas9	Δacr1::tdk/Kan^R ΔpoxB::gfp/Cm^R Δpp2::ChnR/pChnB-dCas9/Spec^R, [luxCDABE], pBAV1G-ara-sgRNA[GFP]	kanamycin, chloramphenicol, gentamycin, spectinomycin

3.4 Proof-of-principle test and finding the optimal expression levels of dCas9 and sgRNA

The proof-of-principle test was performed in growth tubes using LB-medium (50 mM glucose, 7.5 µg/ml of gentamycin and 37.5 µg/ml) at 28 ^{o}C, 300 rpm. Overnight incubated preculture, which was inoculated from a single colony on a LA-plate, was diluted to OD_{600}=0.2 with fresh LB-medium. Cyclohexanone (the end concentration 0.2 mM) and arabionse (the end concentration 0.5%) for inducing dCas9 and sgRNA production, respectively, were added to the medium of selected tubes. One tube contained neither inducer, one tube only cyclohexanone, one tube only arabinose and one tube both. A parallel culture of each tube was prepared. Samples were taken at 0, 3, 6 and 20–24 h after the induction. Optical density at 600 nm (OD_{600}) was measured immediately. For the fluorescence measurement, 500 µl of the sample was centrifuged (20 000 g, 1 min), the supernant was removed and the cells were resuspended in buffer (10 mM Tris-Hcl, 150 mM NaCl, pH 8) to avoid the effect of pH on the GFP folding. Approx. 1 h 30 min

after preparation of the last sample, fluorescence was measured from two parallel samples (200 µl) using Spark with gain=50.

The optimal expression level of dCas9 was studied in the terms of cyclohexanone concentration. The experimental setup was identical to the one used in proof-of-principle test, except the end arabinose concentration was 0.5% and cyclohexanone concentrations were 0, 0.00002, 0.0002, 0.002, 0.02, 0.2 mM. Each cyclohexanone concentration was tested with and without arabinose. In the end of the experiment pH was measured using pH paper (with scale 1–10 or 4–7). Additionally to the sample strain (ADP1-Lux-GFP-sgRNA[GFP]-dCas9), the same experiment was repeated with the control strains (ADP1-Lux-GFP-sgRNA[null]-dCas9, ADP1-Lux-GFP-dCas9). With ADP1-Lux-dCas9 the antibiotic used was spectinomycin (37.5 µg/ml). To find out if the changes in the growth, fluorescence or pH were caused by cyclohexanone, arabinose or the CRISPRi machinery, the same experiment with 6 different cyclohexanone concentrations (with and without 0.5% of arabinose) was performed with a control strain without any component of the CRISPRi machinery (ADP1-Lux-GFP). The used antibiotic was kanamycin (50 µg/ml).

As the sgRNA expression was induced with arabinose, five different arabinose concentrations (0.0, 0.1, 0.2, 0.5 and 1.0%) were tested with (0.0002 mM) and without cyclohexanone to find out the optimal arabinose concentration for sgRNA induction. The experiment was performed in the exact same way as the finding the optimal dCas9 expression level (explained in detail above) except that the control strain (ADP1-Lux-GFP-dCas9) was included in the experiment with 1.0% of arabinose and with (0.0002 mM) or without cyclohexanone.

3.5 Studying the effects of CRISPRi machinery on the cell metabolism in terms of bioluminescence production

Precultures of each strain were started from a single colony on a LA-plate (with appropriate antibiotics). After an overnight incubation in a growth tube shaker at 28 ^{o}C and 300 rpm in LB-medium (50 mM glucose and antibiotics (50 µg/ml of kanamycin, 50 µg/ml spectinomycin or 7.5 µg/ml gentamycin and 37.5 µg/ml spectinomycin)) the precultures were diluted to OD_{600}=0.2 (when 0.00002 mM of cyclohexanone was used) or OD_{600}=0.1 (with 0.0 and 0.0002 mM cyclohexanone). Different OD_{600} was used in different experiments for discovering the optimal conditions for bioluminescence production.

The diluted precultures were incubated in the shaker for an additional 2 or 1 h, if the starting OD_{600} was 0.2 or 0.1, respectively. Next, 180 µl of the each culture was pipetted into wells of a 96 microplate, totaling six wells. Then, the plate was transferred into the microplate reader Spark for 15 h incubation (at 28 ^{o}C). Bioluminescence, OD_{600} and fluorescence (gain=50) were measured every 15 min. After the measure-

ments the plate was shaken until the start of a new cycle. Additionally, the plate was shaken for 10 s before each individual measurement.

To see the possible changes in the bioluminescence production, the strains were incubated in Spark until the bioluminescence started to increase. Then, the incubation was paused and the inducers were pipetted into the wells. All of the wells were induced with cyclohexanone and half of the wells with arabinose (1.0%) in each experiment. Different cyclohexanone concentrations were studied in individual experiments due to cylohexanone's volatile nature and low effective inducing concentrations. If cyclohexanone concentration was 0.0 mM, MQ water was used instead. The induction times were 4.25, 2 and 3 h, in the experiments with 0.0002, 0.00002 and 0.0 mM of cyclohexanone, respectively.

To study the effect of CRISPRi on downstream genes, similar experiment as described above was performed with ADP1-Lux-sgRNA[Lux]-dCas9. The differences to the experimental procedures mentioned were that after a clear repression of bioluminescence production took place (approx. 6 h after the induction) the run was stopped, the bioluminescence was measured, decanal diluted into 70% ethanol was added into the wells to the end concentration of 0.05% and the bioluminescence was measured once again.

3.6 Mathematical and statistical analysis

Average values, which are presented as the results in this study, were calculated from parallel samples. The background fluorescence of the medium was deducted from fluorescences of the actual results prior to further analysis as well as background optical density (at 600 nm). To cancel the effect of cell density to the fluorescence, fluorescence results were normalized. In other words, the fluorescence result of each time point was divided by the OD_{600} of the same culture at the same time point. The effectiveness of CRISPRi in silencing GFP expression is presented as relative fluorescence: the normalized fluorescence of the uninduced culture was designated as 1 and normalized fluorescences of other cultures were divided by the normalized fluorescence of the uninduced culture of the same time point. Additionally, relative bioluminescence was calculated by dividing the bioluminescence of a time point by the bioluminescence of the time point just before the induction.

Time point of 6 h was chosen to study the relative fluorescence and the effects of dCas9 and sgRNA expression on the cell growth because at that point repression should have already taken place but cells are still in exponential growth phase. As a result, OD_{600} results should be accurate as only a minor amount of the cells have died.

Standard deviation was calculated when there were at least three parallel samples and it was plotted as Y-axis error bars. In the case of only two parallel samples (mostly

in the OD_{600} analysis) the standard deviation was omitted due to the low sample amount. As a result, no error bars were plotted.

4 RESULTS

In this study CRISPRi machinery was established in *Acinetobacter baylyi* ADP1. Additionally, to find out the repression efficiency, the burden to the cells and the repression kinetics, the same strain was transformed with *GFP* and *luxCDABE* operons (under constitutive promoters) and the CRISPRi was designed to target *GFP* or *luxC* genes or nothing as controls (Table 3.1).

In addition, the bioluminescence production was used as an indicator of the cells' inner state and for changes in the metabolism which might not be visible from the cell density. This was done by targeting *GFP*, and following amount of bioluminescence in real time before and after induction of the CRISPRi machinery.

4.1 Construction of the strains

The plasmid pBAV1G-ara-sgRNA[null], containing pBAV1G (with gentamycin resistance) backbone and synthetic guide RNA (sgRNA) coding sequence under arabinose inducible promoter, was constructed and verified prior to this study (unpublished). The plasmids pBAV1G-ara-sgRNA[GFP] (containing *GFP* targeting sequence) and pBAV1G-ara-sgRNA[Lux] (containing *luxC* targeting sequence) were constructed from pBAV1G-ara-sgRNA[null] (does not contain a targeting sequence) by USER cloning in *E. coli* and verified by sequencing (starting approx. 100 bp upstream from base-pairing sequence of the sgRNA). The plasmids with correct sequences, pBAV1-ara-sgRNA[GFP]-2 and pBAV1-ara-sgRNA[Lux]-3 were selected to be continued with. All of the sequencing results can be found from the Appendix C.

The plasmid iBG42/pAK400c, containing the *GFP* gene under a constitutive promoter, chloramphenicol resistance and flanking regions for genomic integration into ADP1 genome to replace *poxB* gene, was transformed into ADP1-Lux (constructed prior to this study, unpublished) by natural transformation and selected on LA-plates using chloramphenicol (25 µg/ml). Additionally, a blue light table was used to confirm the fluorescence production. As a result, an ADP1 strain with a genotype [Δacr1::tdk/ Kan^R ΔpoxB::gfp/Cm^R, [luxCDABE] was constructed.

Next, ADP1-Lux-GFP and ADP1-Lux strains were transformed with pBAV1G-ara-sgRNA[null] (both strains), pBAV1G-ara-sgRNA[GFP] (ADP1-Lux-GFP) and pBAV1G-ara-sgRNA[Lux] (ADP1-Lux) plasmids separately using natural transformation and selected on LA-plates containing gentamycin (10 µg/ml). Selective pressure of gentamycin was not strong as colonies were growing on control plates as well. How-

ever, colonies on the control plates were smaller and there were approx. half of the colonies compared to the transformation plates. Next day, few colonies from each transformation plate were transferred into liquid medium, incubated overnight and the plasmids were extracted. The succession of the transformations was verified by restriction analysis.

The last transformations were done by transforming pp2-chn-dCas9/pIM1463 plasmid, containing *dCas9* gene under a cyclohexanone inducible promoter, spectinomycin resistance and flanking regions for genomic integration to replace *pp2* gene, by natural transformation into ADP1-Lux-sgRNA[null], ADP1-Lux-sgRNA[Lux], ADP1-Lux-GFP-sgRNA[null] and ADP1-Lux-GFP-sgRNA[GFP] strains. The selection was done on LA-plates containing gentamycin (10 µg/ml) and spectinomycin (50 µg/ml). Additionally, successful transformation was verified by colony PCR (using dCas9_fwd and dCas9_rev primers) and electrophoresis. As a result, ADP1-Lux-sgRNA[null]-dCas9, ADP1-Lux-sgRNA[Lux]-dCas9, ADP1-Lux-GFP-sgRNA[null]-dCas9 and ADP1-Lux-GFP-sgRNA[GFP]-dCas9 strains were obtained with genotypes presented in the Table 3.1. Lux means that the strain produces bioluminescence constantly; GFP that it produces green fluorescent protein and thus fluorescence constantly; sgRNA that the strain expresses sgRNA (under arabinose inducible promoter) that targets no gene in ADP1's genome (sgRNA[null]), targets *GFP* (sgRNA[GFP]) or *luxC* genes (sgRNA[lux]) and dCas9 means that the strain expresses dCas9 protein under the cyclohexanone inducible promoter.

However, as ADP1-Lux-sgRNA[null]-dCas9 and ADP1-Lux-GFP-sgRNA[null]-dCas9 strains did not grow in subsequent experiments after cyclohexanone induction, ADP1-Lux and ADP1-Lux-GFP were transformed with pp2-chn-dCas9/pIM1463 plasmid as well. The same procedure as explained above was used, except that the selection was done with 50 µg/ml of spectinomycin only. Successful transformations were verified by colony PCR. As a result, ADP1-Lux-dCas9 and ADP1-Lux-GFP-dCas9 with genotypes listed in the Table 3.1 were obtained.

4.2 Proof-of-principle test

After the strains were constructed the next step was to prove the functioning of the CRISPRi machinery. Proof-of-principle test was performed as a 22 h growth tube cultivation using strains ADP1-Lux-GFP-sgRNA[null]-dCas9 (control) and ADP1-Lux-GFP-sgRNA[GFP]-dCas9 (studied strain). Expression of sgRNA was induced by 1% arabinose and dCas9 expression by 0.2 mM cyclohexanone. To see the effect of inducing only the sgRNA, dCas9 or both of them at the same time, either arabinose, cyclohexanone or both were added in the medium of different growth tubes. Optical density (at 600 nm) (Figure 4.1 A) and fluorescence were measured at 0, 3, 6 and 22 h after the induction to see the effects of the CRISPRi on the cell growth and GFP production.

Normalized fluorescence (Figure 4.1 B) was calculated by dividing the fluorescence of a time point by the OD_{600} of the same time point.

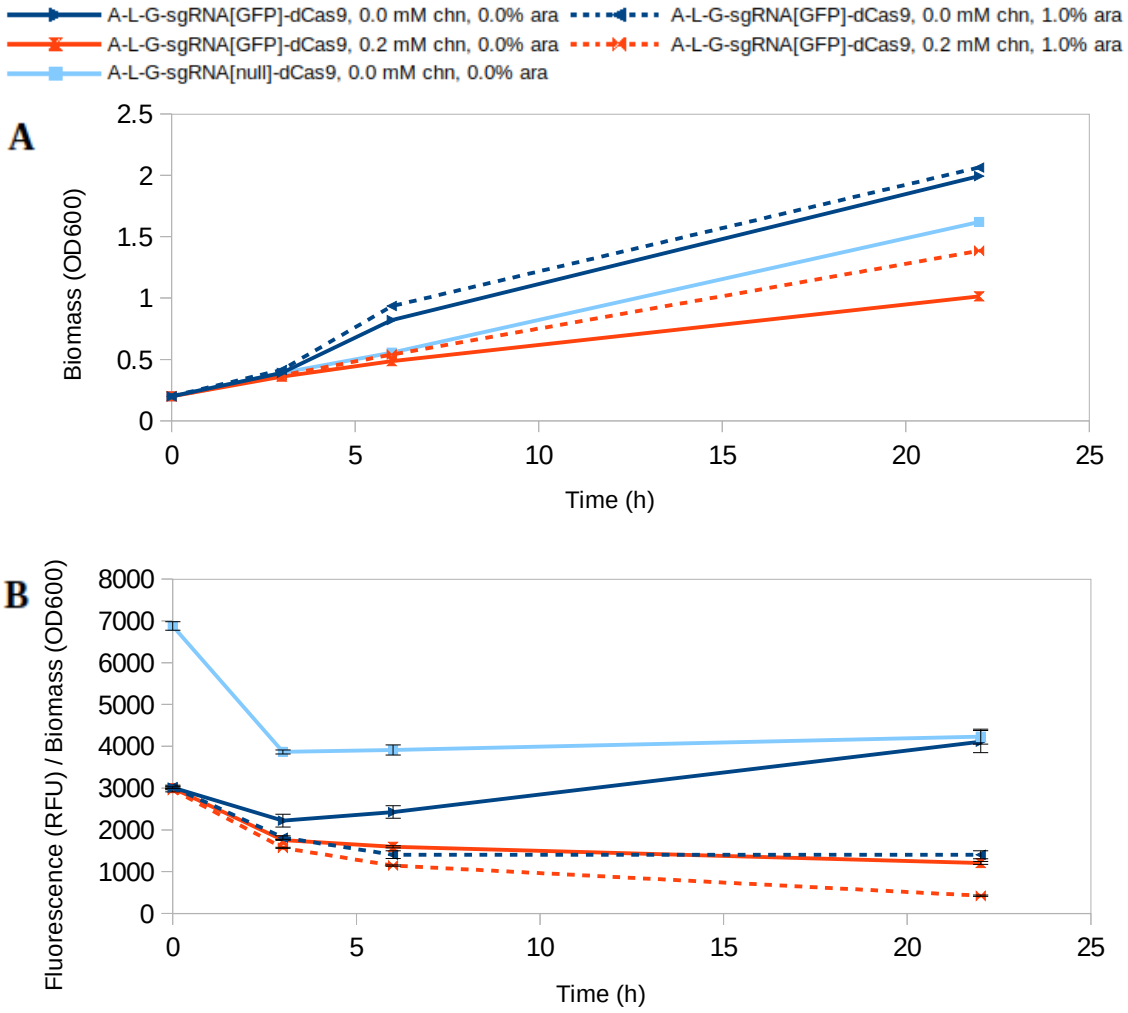

Figure 4.1 *Biomass in terms of optical density (λ=600 nm) (**A**) and normalized fluorescence (fluorescence divided by optical density) (**B**) of ADP1-Lux-GFP-sgRNA[GFP]-dCas9 and the control strain ADP1-Lux-GFP-sgRNA[null]-dCas9. The induction of CRISPRi machinery was achieved by 0.2 mM of cyclohexanone and 1.0% of arabinose. The effect of inducers were studied together and individually. The experiment was performed as a 22 h growth tube experiment. Results of induced ADP1-Lux-GFP-sgRNA[null]-dCas9 are not presented due to their poor growth. The error bars show standard deviation of four parallel samples. No error bars were plotted for biomass results due to low (2) amount of parallel cultures.*

All of the cultures grew similarly until 3 h after the induction (Figure 4.1 A). At 6 h differences in the growth can be noticed: uninduced ADP1-Lux-GFP-sgRNA[GFP]-dCas9 achieved the highest cell density and arabinose induced (1%) ADP1-Lux-GFP-sgRNA[GFP]-dCas9 the second highest. On the other hand, cyclohexanone (0.2 mM) induced ADP1-Lux-GFP-sgRNA[null] cultures showed no growth (Figure Appendix D 1 A) which suggest that either the dCas9 or the sgRNA[null]:dCas9 complex is toxic to the cells. Arabinose induced (with or without cyclohexanone) ADP1-Lux-GFP-sgRNA[GFP]-dCas9 strain (Figure 4.1 A) and uninduced or only arabinose induced ADP1-Lux-GFP-sgRNA[null]-dCas9 strain (Figure Appendix D 1 A) grew similarly

when compared to each other. Adding arabinose enhanced growth (11% higher cell density at 6 h) in the ADP1-Lux-GFP-sgRNA[GFP]-dCas9 strain when also cyclohexanone was present in the medium. When the same strain was induced with both inducers the cell density was 34% lower than without induction.

ADP1-Lux-GFP-sgRNA[GFP]-dCas9 produced approx. 4000 RFU/OD_{600} lower fluorescence in the beginning of the experiment than ADP1-Lux-GFP-sgRNA[null]-dCas9. Additionally, addition of only a single inducer repressed GFP production. These results suggest that the promoters are leaking.

Highest fluorescences were obtained with ADP1-Lux-GFP-sgRNA[null]-dCas9 with only arabinose added (Figure Appendix D 1 B). The uninduced ADP1-Lux-GFP-sgRNA[GFP]-dCas9 had the highest fluorescence of the cultures in which sgRNA targeted GFP (Figure 4.1 B). ADP1-Lux-GFP-sgRNA[GFP]-dCas9 cultures induced with only arabinose or cyclohexanone had approx. the same fluorescences. The culture induced with both inducers produced the lowest, continuously decreasing fluorescence. This suggest that CRISPRi silences GFP production.

To find out the actual repression levels, relative fluorescences were calculated and plotted (Figure 4.2). This was done by dividing the normalized fluorescence of a culture at 6 h by the average normalized fluorescence of the uninduced cultures at 6 h. ADP1-Lux-GFP-dCas9 functioned as a control strain.

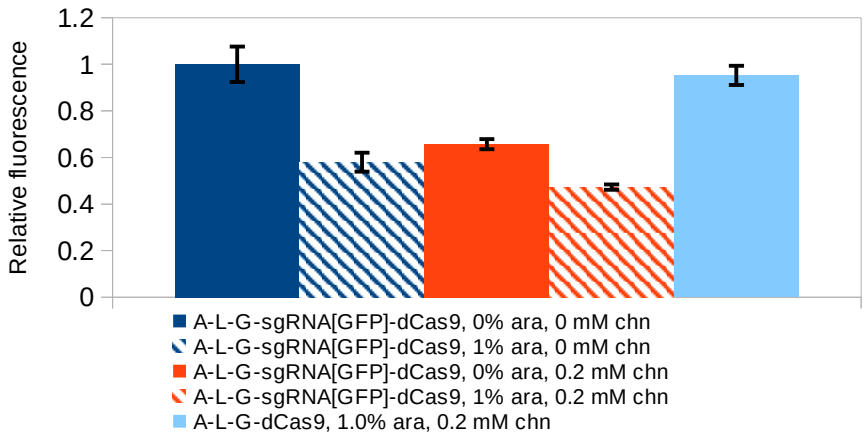

Figure 4.2 *Relative fluorescence at 6 h after the induction (normalized fluorescence of a culture at 6 h divided by the average normalized fluorescence of the uninduced cultures at 6 h) of ADP1-Lux-GFP-sgRNA[GFP]-dCas9 and ADP1-Lux-GFP-dCas9 strains with either 1% arabinose, 0.2 mM cyclohexanone or both of them. The error bars show standard deviation of four parallel samples.*

Lower relative fluorescence compared to the uninduced ADP1-Lux-GFP-sgRNA[GFP]-dCas9 (relative fluorescence 1) was achieved when either, dCas9 (relative fluorescence 0.66) or sgRNA[GFP] (relative fluorescence 0.58) expression was induced with 0.2 mM of cyclohexanone or 1.0% of arabinose, respectively (Figure 4.2). However, the lowest relative fluorescence (0.47) was achieved when both inducers were added to the medium. The fluorescence production of the control strain ADP1-Lux-GFP-dCas9 was insignificantly repressed (relative fluorescence 0.95).

4.3 Effect of cyclohexanone and arabinose on the cell growth

Increased expression of dCas9 decreased the cell growth of the strain ADP1-Lux-GFP-sgRNA[GFP]-dCas9. On the other hand, increasing sgRNA expression increased the growth. To find out if the effect was caused by the inducers, similar growth tube experiment as in the proof-of-principle test was performed with the control strains ADP1-Lux-GFP-dCas9 and ADP1-Lux-GFP. Six cyclohexanone concentrations (0.0, 0.00002, 0.0002, 0.002, 0.02 and 0.2 mM) were tested with (0.5%) and without arabinose.

The biomasses of the strains ADP1-Lux-GFP-dCas9 and ADP1-Lux-GFP at 6 h after induction were plotted against the concentration of cyclohexanone in Figures 4.3 A and B, respectively. The complete growth curves can be found in Figures Appendix D 2 A and D 3 A.

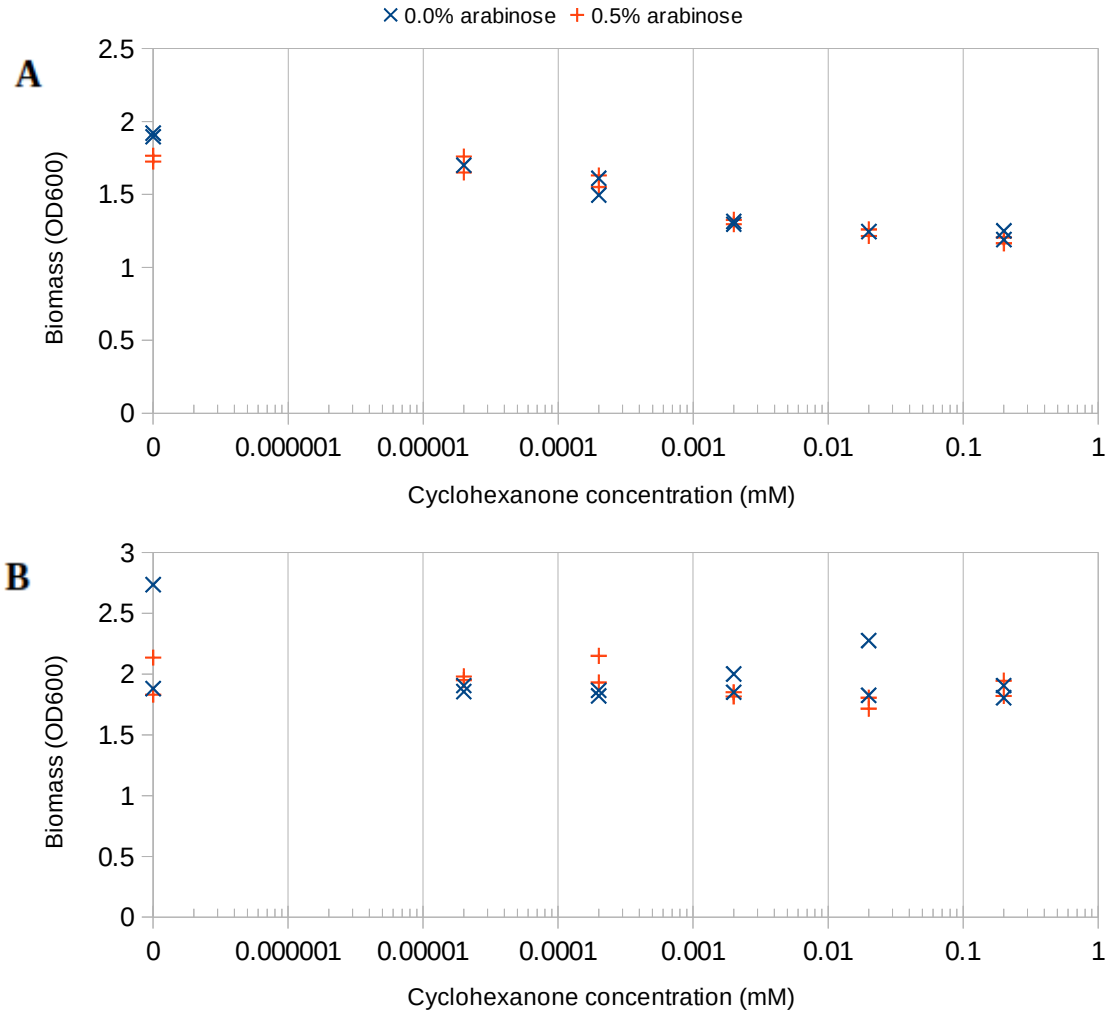

Figure 4.3 *The biomass in terms of optical density (λ=600 nm) of the control strains ADP1-Lux-GFP-dCas9 (**A**) and ADP1-Lux-GFP (**B**) 6 h after the addition of cyclohexanone (0.0, 0.00002, 0.0002, 0.002, 0.02 and 0.2 mM) and arabinose (0.0 or 0.5%). Each cyclohexanone concentration was tested with and without arabinose. Results of both of the parallel cultures are presented as individual data points.*

The cell density decreased when cyclohexanone concentration was increased if dCas9 gene was present in the strain (ADP1-Lux-GFP-dCas9) (Figure 4.3 A) which also was the case with ADP1-Lux-GFP-sgRNA[GFP]-dCas9 (Figure 4.1 A). However, addition of arabinose did not increase the cell density of ADP1-Lux-GFP-dCas9 as it increased it in ADP1-Lux-GFP-sgRNA[GFP]-dCas9. On the other hand, the growth of the control strain without dCas9 (ADP1-Lux-GFP) was not impacted by the cyclohexanone or arabinose (Figure 4.3 B). As a result, it can concluded that the used inducers do not affect the growth of ADP1.

4.4 Optimal expression level of dCas9

As 0.2 mM of cyclohexanone was found to be a burden to the cells detected as decreased cell growth, the next step was to find out the amount of cyclohexanone that can be used to induce dCas9 expression with acceptable effect on the cell growth but which would effectively repress *GFP*. Six different cyclohexanone concentrations (0, 0.00002, 0.0002, 0.002, 0.02, 0.2 mM) were tested with and without arabinose (0.5%) in the similar growth tube cultivation experiment as the proof-of-principle test. The studied strain was ADP1-Lux-GFP-sgRNA[GFP]-dCas9. The functioning and effects of CRISPRi targeting the *GFP* gene were studied by following OD_{600} (Figure Appendix D 4 A) and fluorescence (Figure Appendix D 4 B).

The biomass (OD_{600}) at 6 h was plotted against the cyclohexanone concentration (Figure 4.4 A). Relative fluorescence of each culture at 6 h was calculated by dividing the normalized fluorescence of the culture by the average normalized fluorescence of uninduced cultures of the same time point (Figure 4.4 B).

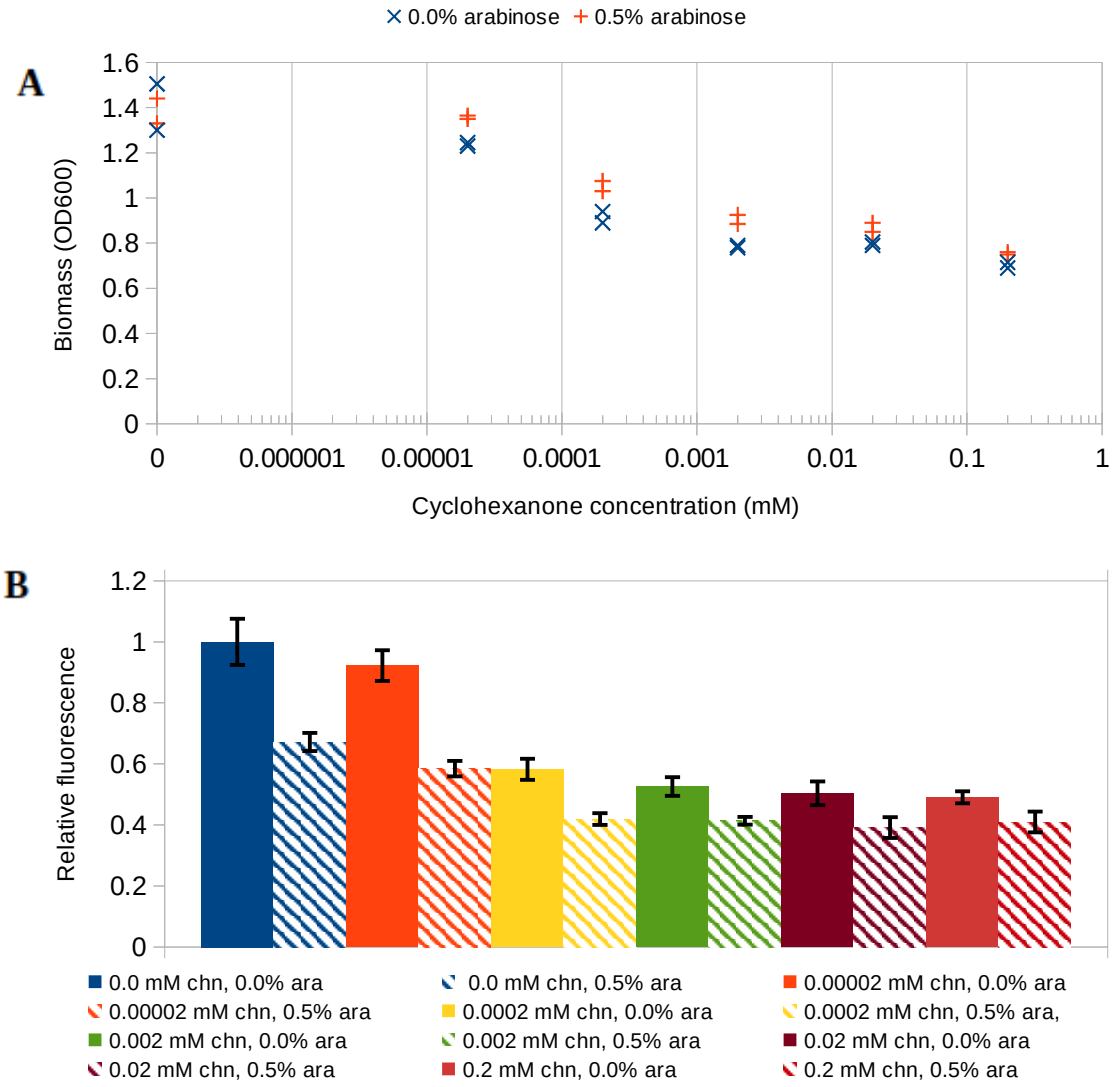

Figure 4.4 *ADP1-Lux-GFP-sgRNA[GFP]-dCas9 strain's biomass in terms of optical density (λ=600 nm) at 6 h (results of both of the parallel cultures are presented as individual data points) (**A**) and relative fluorescence (normalized fluorescence of a culture divided by the average normalized fluorescence of the uninduced cultures) at 6 h after induction (**B**) in the experiment to find out the optimal dCas9 expression level. Cyclohexanone (0.0, 0.00002, 0.0002, 0.002, 0.02 and 0.2 mM) was used to induce dCas9 production. Each cyclohexanone concentration was tested with (0.5%) and without arabinose which induced sgRNA production. The error bars show standard deviation of four parallel samples.*

The growth of the cells was affected by the dCas9 expression (Figure 4.4 A): the higher dCas9 concentration, the lower growth. For example, the biomass achieved with 0.00002 mM cyclohexanone and 0.5% arabinose was 1.29-fold of the biomass with inducer concentrations of 0.0002 mM cyclohexanone and 0.5% arabinose. On the other hand, adding arabinose seemed to have a slight positive effect on the cell growth, for example the biomass with 0.0002 mM cyclohexanone and 0.5% arabinose was 1.15-fold of the biomass with only 0.0002 mM of cyclohexanone. As inducers did not affect the growth of the control strain without CRISPRi machinery (Figure 4.3 B), dCas9 itself seems to be toxic to the cells instead of cyclohexanone and sgRNA seems increase the cell growth instead of arabinose.

The relative fluorescence was inversely proportional to the cyclohexanone concentration (Figure 4.4 B). In other words, the fluorescence (*GFP*) repression seems to be directly proportional to the cyclohexanone concentration. However, the maximum repression was not achieved with the highest cyclohexanone concentration tested but with 0.02 mM of cyclohexanone and 0.5% of arabinose. With arabinose added to the medium the repression was stronger than with cyclohexanone only.

Between the cultures induced with 0.0 mM, 0.00002 mM and 0.0002 mM of cyclohexanone and 0.5% arabinose, fluorescence decreased when the cyclohexanone concentration was increased: the relative fluorescence decreased 13% when cyclohexanone concentration was increased from 0.0 mM to 0.00002 mM and 28% when the concentration was further increased to 0.0002 mM (Figure 4.4 B). On the contrary, between the cultures with four highest cyclohexanone concentrations, 0.0002, 0.002, 0.02 and 0.2 mM, the change in the fluorescence was insignificant. As a result, almost the strongest repression was achieved with 0.0002 mM of cyclohexanone. In addition, with this concentration the biomass at 6 h was the highest (25% lower when compared to the biomass achieved without inducers added) when four concentrations with the strongest repressions are compared (Figure 4.4 A), indicating that this concentrations resulted in the best cell growth. Hence, 0.0002 mM is chosen to be the optimal cyclohexanone concentration.

4.5 Optimal expression level of sgRNA

To further optimize the functioning of the CRISPRi machinery, the next step after finding the optimal expression level of dCas9 was to do the same with sgRNA. The optimal sgRNA expression level was studied by inducing its expression with five different arabinose concentrations (0.0, 0.1, 0.2, 0.5 and 1.0%). Every arabinose concentration was studied with (0.0002 mM) and without cyclohexanone. The experiment was performed as a 23.5 h growth tube incubation in the same way as proof-of-principle and finding the optimal dCas9 expression tests. The studied strain was ADP1-Lux-GFP-sgRNA[GFP]-dCas9 and the control strain ADP1-Lux-GFP-dCas9, in which only one arabinose concentration (1.0%) was tested. The control strain was constructed after multiple failures in incubating ADP1-Lux-GFP-sgRNA[null]-dCas9 in the presence of cyclohexanone.

Optical density at 600 nm (Figure Appendix D 5 A**)** and fluorescence (Figure Appendix D 5 B) were measured at 0, 3, 6 and 23.5 h after the induction. Biomass (OD_{600}) of every culture at 6 h was plotted against the arabinose concentration (Figure 4.5 A) and relative fluorescences (Figure 4.5 B) were calculated by dividing the normalized fluorescence of a culture at 6 h by the normalized fluorescence of the uninduced control strain at 6 h.

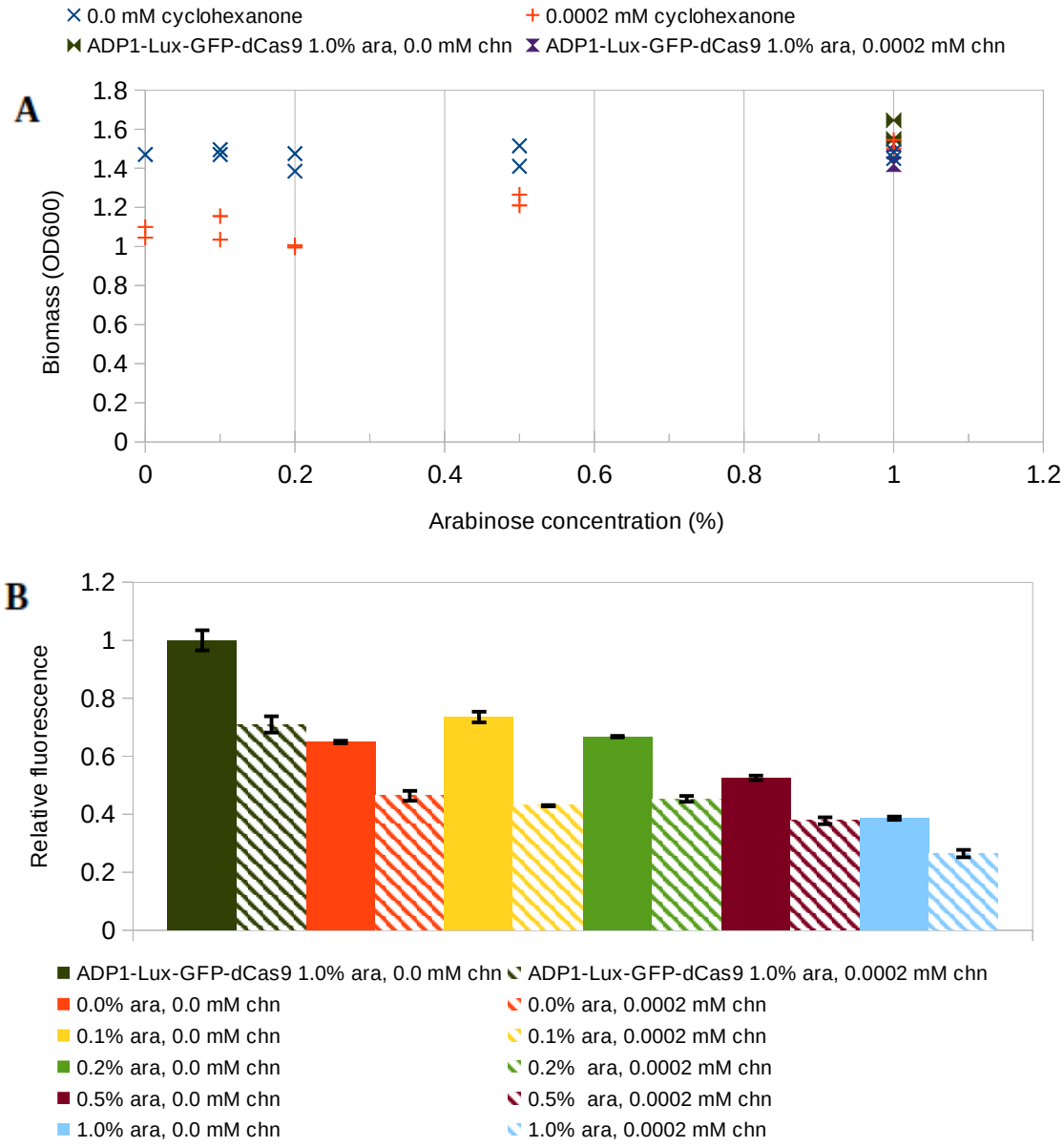

Figure 4.5 *ADP1-Lux-GFP-sgRNA[GFP]-dCas9 and ADP1-Lux-GFP-dCas9 strains' biomasses in terms of optical density (λ=600 nm) at 6 h (results of both of the parallel cultures are presented as individual data points) (**A**) and relative fluorescences (normalized fluorescence of a culture divided by the normalized fluorescence of the uninduced control strain) at 6 h after induction (**B**) in the experiment to find out the optimal sgRNA expression level. Arabinose (0.0, 0.1, 0.2, 0.5 and 1.0%) was used to induce sgRNA production and cyclohexanone (0.0 or 0.0002 mM) was used to induce dCas9 production. Each arabinose concentration was tested with and without cyclohexanone. The error bars show standard deviation of four parallel samples.*

Increasing the arabinose concentration enhanced the growth (Figure 4.5 A). The maximum growth at 6 h (OD_{600} = 1.6) was achieved with only arabinose induced ADP1-Lux-GFP-dCas9 (control) strain. The second highest cell density was obtained with 1.0% arabinose and 0.0002 mM cyclohexanone induced ADP1-Lux-GFP-sgRNA[GFP]-dCas9 strain. Otherwise, cell densities were higher (in average 25%) when cyclohexanone was not added to the medium but only arabinose was used.

In addition to the highest cell density, 1.0% of arabinose and 0.0002 mM of cyclohexanone together produced the strongest repression of *GFP* (relative fluorescence 0.26) (Figure 4.5 B). Adding 0.1 or 0.2% of arabinose to the medium did not result in lower relative fluorescences when compared to the non-induced ADP1-Lux-GFP-sgRNA[GFP]-dCas9 when cyclohexanone was not added into the medium (relative fluorescence 0.65). On the contrary, the arabinose concentrations of 0.5 and 1.0% resulted in increased repression: relative fluorescence were 0.53 and 0.39, respectively. When cyclohexanone was present in the medium, increasing the arabinose concentration increased repression, except when the concentration was increased from 0.1 to 0.2%. Repression of *GFP* was notable also in the control strain ADP1-Lux-GFP-dCas9 when 0.0002 mM of cyclohexanone was added to the medium: the relative fluorescence decreased 29% when compared to the same strain without cyclohexanone.

Altering the sgRNA expression level did not change the repression level greatly (maximum 43%) when 0.0002 mM of cyclohexanone was present in the medium (Figure 4.5A). When no cyclohexanone was used, the maximum change was 64%.

In conclusion, 1.0% of arabinose proved to be the optimal concentration of the tested concentrations. Thus, it was chosen to be used in the following experiments.

4.6 Repression kinetics by studying the repression of *lux* operon

After establishing and optimizing the CRISPRi machinery in ADP1 the functioning of it was studied in more detail. The kinetics of the CRISPRi repression were investigated by targeting *luxC* gene in the *luxCDABE* operon and following the bioluminescence production before and after the induction in real time (Figure Appendix D 8 A). Used inducer concentrations were 0.0002 mM of cyclohexanone and 1.0% of arabinose or 0.0 mM of cyclohexanone and 0.0% of arabinose. The relative bioluminescence curves are presented starting from 15 min before the induction (Figure 4.6). The relative bioluminescences were calculated by dividing the bioluminescence of each time point by the bioluminescence of the first time point.

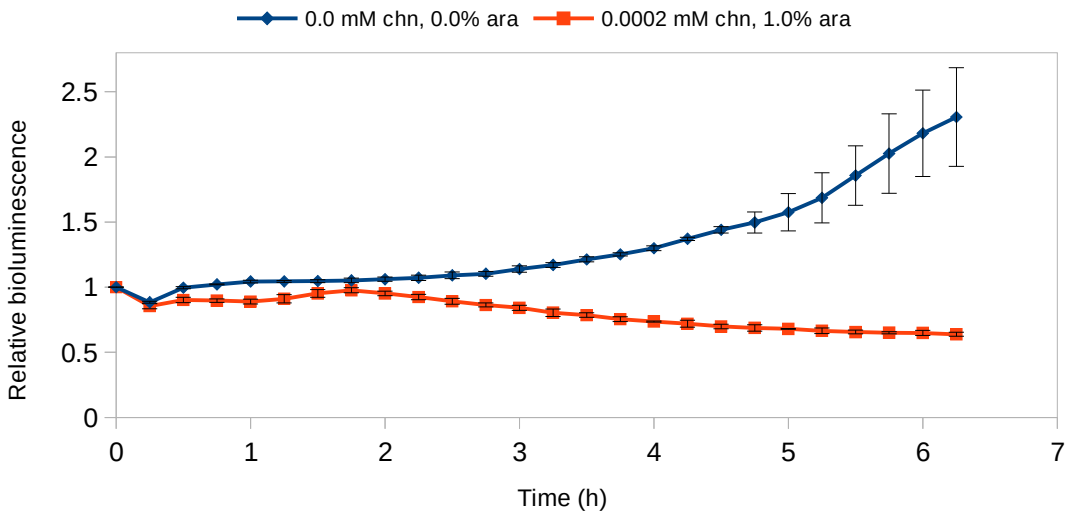

Figure 4.6 *The relative bioluminescence production starting 15 min before the induction. The studied strain was ADP1-lux-sgRNA[Lux]-dCas9 which targets luxC gene in the synthetic operon luxCDABE. Inducer concentrations were 0.0002 mM of cyclohexanone and 1.0% of arabinose (red) or 0.0 mM of cyclohexanone and 0.0% of arabinose (blue). The relative luminescence was calculated by dividing the amount of bioluminescence of each time point by the bioluminescence of the first time point. The error bars show standard deviation of three parallel cultures.*

A clear repression of *luxC* was achieved: the bioluminescence was 72% lower at 6.25 h with 0.0002 mM of cyclohexanone and 1.0% of arabinose when compared to the bioluminescence produced without inducers (Figure 4.6). Until 1.75 h roughly the same amount of bioluminescence was produced with both inducer concentrations. Then, at 2 h, the bioluminescence started to decrease when inducers were present in the medium. Approx. at 3 h after the induction a clear difference in the bioluminescences was achieved. The difference increased until the end of the experiment. This indicates that the repression continues at least up to 6 h after the induction. In conclusion, it took approx. 2 h from the induction for the repression to set in.

4.7 Effect of CRISPRi repression on downstream genes

To see how CRISPRi affects downstream genes, the *luxC* gene of the *luxCDABE* operon was targeted (in strain ADP1-Lux-sgRNA[Lux]-dCas9). The strain was incubated first until the bioluminescence started to increase, then the expression of the CRISPRi machinery was induced with 0.0002 mM of cyclohexanone and 1.0% of arabinose. When the repression was visible decanal, dissolved in 70% ethanol, was added to the end concentration of 0.05%. If only *luxC* was silenced, the luciferase (produced by *luxAB*) would have too little aldehyde substrate produced by genes *luxDCE* to produce the maximum bioluminescence. In this case addition of decanal, which functions as similar substrate as the aldehyde for luciferase (Kirchner et al., 1989), would increase the bioluminescence production. On the contrary, if also downstream genes of *luxC* were silenced, there would not be excess of luciferase compared to the aldehyde substrate. Thus, there would not be an increase in the bioluminescence.

The bioluminescence was measured every 15 min during the whole incubation. Relative bioluminescence was calculated by dividing the bioluminescence of a time point by the bioluminescence just before the induction (Figure Appendix D 6). When the repression was visible (5.3 h after the induction) the run was stopped, bioluminescence was measured, decanal was added and bioluminescence was measured once more. The relative bioluminescence was calculated by dividing the bioluminescence of both measurements by the bioluminescence of just before adding decanal (Figure 4.7).

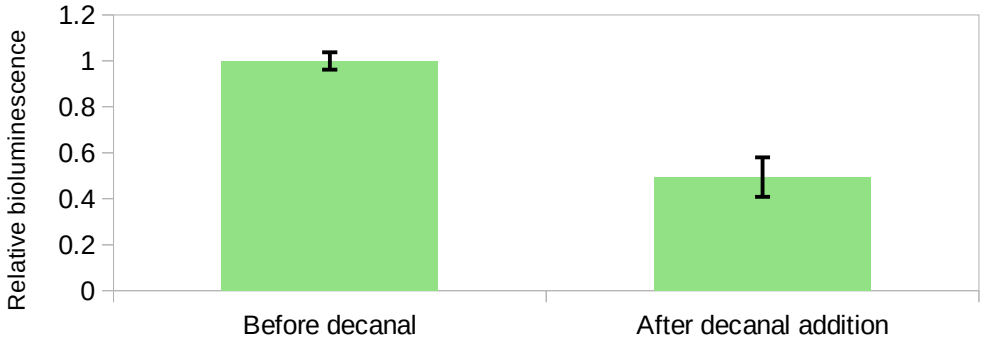

Figure 4.7 *The relative bioluminescence of ADP1-Lux-sgRNA[Lux]-dCas9 before and after decanal addition. CRISPRi in the strain targets luxC gene in the luxCDABE operon. The experiment was to investigate if also downstream genes are repressed by CRISPRi: decanal addition should increase the luminescence production in the case only luxC is silenced but not luxA and luxB. The inducer concentrations were 0.0002 mM of cyclohexanone (for dCas9 expression) and 1.0% arabinose (for sgRNA expression). The relative bioluminescence was calculated by dividing the bioluminescence of both measurements by the bioluminescence before decanal addition. The error bars show standard deviation of three parallel cultures.*

The bioluminescence production decreased 102% after decanal was added to the the medium (Figure 4.7). As a result, there was not surplus of luciferases. This implies that the whole *luxCDABE* operon was silenced by CRISPRi.

4.8 Metabolic burden caused by the CRISPRi

Bioluminescence production was used to reveal the burden CRISPRi causes to the cell that possibly would not be visible from changes in the cell growth. This was done by incubating the cells while the bioluminescence and OD_{600} were measured every 15 mins (Figures Appendix D 7–9). The incubation was continued until the bioluminescence started to increase, then the inducers were added to the medium: the studied cyclohexanone concentrations were 0.0, 0.00002 and 0.0002 mM which were all tested with (1.0%) and without arabinose. The relative bioluminescence and the relative biomass of each time point were calculated by dividing the bioluminescence or optical density (at 600 nm) of a time point by the bioluminescence or optical density of the time point just before the induction, respectively. Four different time points (0, 2, 4 and 6 h after the induction) were chosen for closer inspection (Figure Appendix D 10 and 11). From these the time point of 6 h was used to compare how different strains were affected (Figure 4.8).

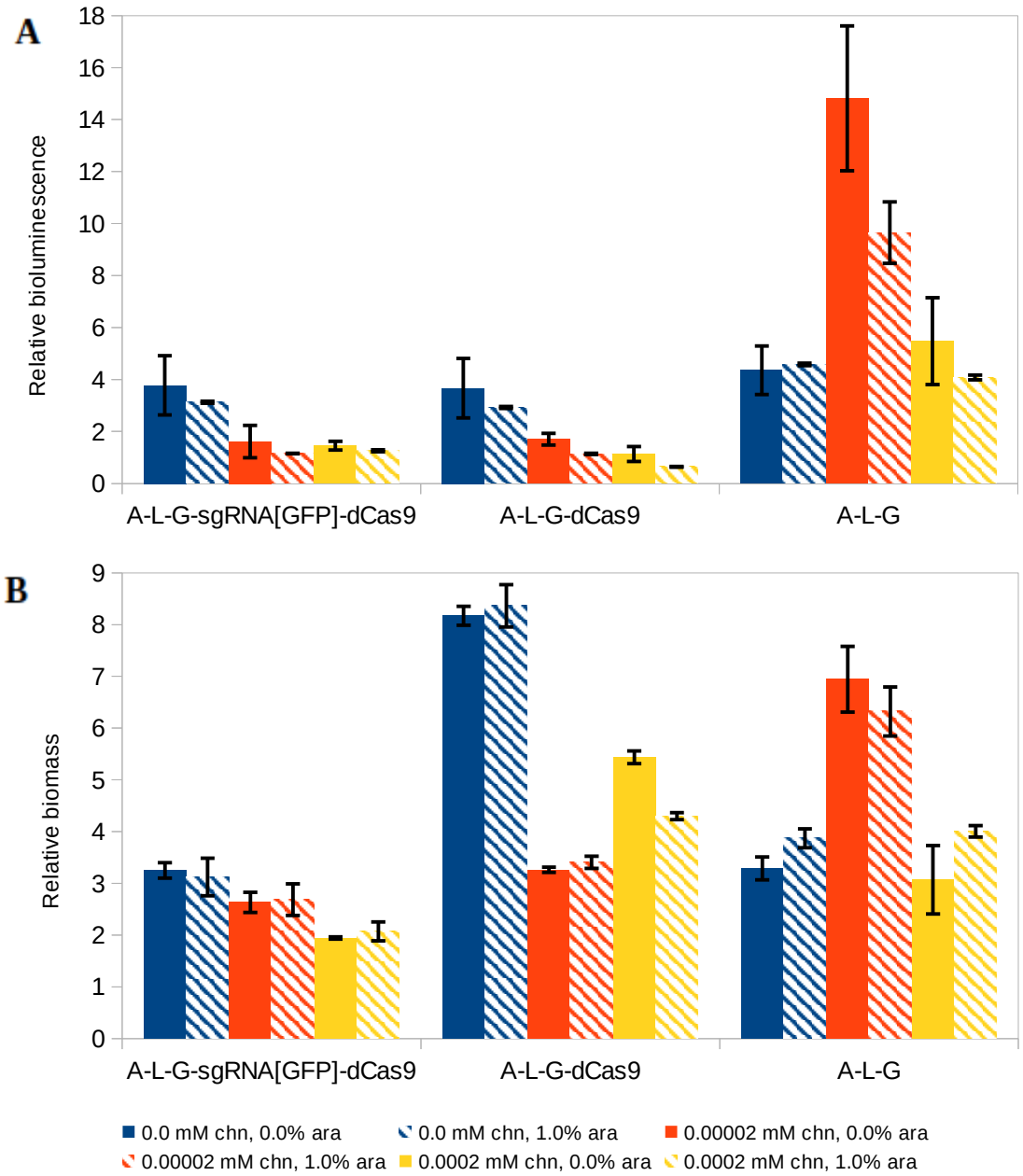

Figure 4.8 *Relative bioluminescence (**A**) and relative biomass (**B**) of ADP1-Lux-GFP-sgRNA[GFP]-dCas9, ADP1-Lux-GFP-dCas9 and ADP1-Lux-GFP strains at 6 h after the induction with 0.0, 0.00002 or 0.0002 mM of cyclohexanone. Each cyclohexanone concentration was tested with (1.0%) and without arabinose. In the strains ADP1-Lux-GFP-sgRNA[GFP]-dCas9 and ADP1-Lux-GFP-dCas9 cyclohexanone was used to induce dCas9 expression. Arabinose was used to induce sgRNA expression in the strain ADP1-Lux-GFP-sgRNA[GFP]-dCas9. ADP1-Lux-GFP did not contain any part of the CRISPRi machinery. Relative bioluminescence and relative biomass were calculated by dividing the bioluminescence or optical density (at 600 nm) of the culture at 6 h by the bioluminescence or optical density of the same culture at the time point just before induction, respectively. The error bars show standard deviations of three parallel cultures.*

At 6 h a clear decrease in bioluminescence was observed with ADP1-Lux-GFP-sgRNA[GFP]-dCas9 and ADP1-Lux-GFP-dCas9 strains when cyclohexanone concentration was increased (Figure 4.8 A). Additionally, the same held true at other time points except with ADP1-Lux-GFP-sgRNA[GFP]-dCas9 at 2 h the lowest relative bio-

luminescence was obtained with 0.00002 mM of cyclohexanone and not with 0.0002 mM (Figure Appendix D 10 A).

On the other hand, with the control strain lacking the CRISPRi machinery completely, ADP1-Lux-GFP, the lowest relative bioluminescence at 6 h was achieved without inducers (Figure 4.8 A). The highest relative bioluminescence was obtained with 0.00002 mM of cyclohexanone and 0.0% of arabinose. Addtionally, there was no clear connection between the relative bioluminescence and cyclohexanone concentration at any other time point either (Figure Appendix D 10 C). In conclusion, cyclohexanone does not affect the metabolism of this strain.

With ADP1-Lux-GFP-sgRNA[GFP]-dCas9 the relative biomass decreased in the same way as relative bioluminescence when the cyclohexanone concentration was increased (Figure 4.8 B). On the other hand, with the control strains ADP1-Lux-GFP-dCas9 and ADP1-Lux-GFP there was no clear connection between cyclohexanone concentration and the relative biomass. Thus, cyclohexanone affected the growth of ADP1-Lux-GFP-sgRNA[GFP]-dCas9 but not the growth of ADP1-Lux-GFP-dCas9 and ADP1-Lux-GFP.

Also the addition of arabinose (1.0%) decreased relative bioluminescence in all of the studied strains (Figure 4.8 A and Figure Appendix D 10) with few exception: the strain ADP1-Lux-GFP-sgRNA[GFP]-dCas9 produced 11 and 17% higher relative bioluminescence at 2 and 4 h, respectively, when 0.0 mM of cyclohexanone and 1.0% of arabinose were used than without arabinose (Figure Appendix 10 A) and at 2 h ADP1-Lux-GFP-dCas9 produced similar relative bioluminescence with 1.0% of arabinose (and 0.0 or 0.00002 mM of cyclohexanone) and without it (Figure Appendix D 10 B).

In conclusion, 1.0% of arabinose generally decreased the relative bioluminescence in all of the studied strains. Additionally, cyclohexanone (0.00002 or 0.0002 mM), which was used to induce dCas9 expression, decreased the relative bioluminescence in the strains with dCas9 expression system but not in the strain which lacked the expression system. However, the same effect was not visible from the relative biomass. Hence, the expression of dCas9 and addition of arabinose affected the metabolism in a way that is not visible from the growth but only from the bioluminescence production.

To assess the effect of the CRISPRi machinery and inducers on pH, it was measured after the 20–23 h incubation experiments (Figure 4.9). ADP1-Lux-GFP-sgRNA[GFP]-dCas9, ADP1-Lux-GFP-dCas9 and ADP1-Lux-GFP were the studied strains.

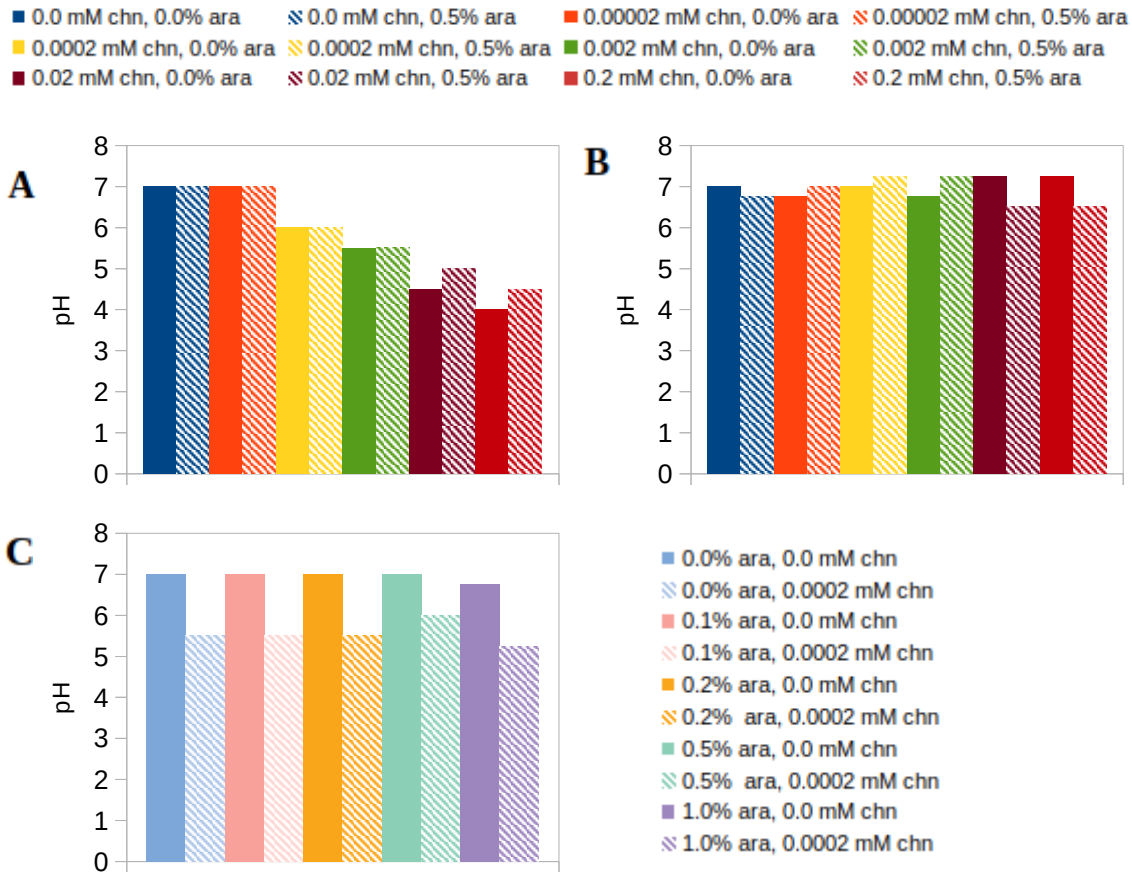

Figure 4.9 *The end pH of growth tube incubation experiments: finding the optimal dCas9 expression level with the strains ADP1-Lux-GFP-dCas9 (**A**) and ADP1-Lux-GFP (**B**) and finding the optimal sgRNA expression level with the strain ADP1-Lux-GFP-sgRNA[GFP]-dCas9 (**C**). Cyclohexanone (0.0, 0.00002, 0.0002, 0.002, 0.02 and 0.2 mM) was tested with (0.5%) or without arabinose in the finding the optimal dCas9 expression level experiment. In the finding optimal sgRNA expression level experiment arabinose (0.0, 0.1, 0.2, 0.5 and 1.0%) was tested with (0.0002 mM) and without cyclohexanone. Cyclohexanone induced dCas9 production in ADP1-Lux-GFP-dCas9 and ADP1-Lux-GFP-sgRNA[GFP]-dCas9 and arabinose induced sgRNA production in ADP1-Lux-GFP-sgRNA[GFP]-dCas9. The upper legend refers to the figures A and B and the lower legend to the figure C. A mistake with the pH paper was made while measuring pH in the experiment "Finding optimal dCas9 expression levels" with ADP1-Lux-GFP-sgRNA[GFP]-dCas9, thus those results are omitted. Due to low amount (2) of parallel cultures, no error bars were plotted.*

The overall trend was that pH decreased (to the minimum of 4) the more the cyclohexanone concentration was increased in the medium of the strain containing the *dCas9* gene with the cyclohexanone expression system (ADP1-Lux-GFP-dCas9) (Figures 4.9 A). In the control strain containing no *dCas9* gene (ADP1-Lux-GFP) was no connection between cyclohexanone concentration and pH (Figure 4.9 B). Increasing the arabinose concentration increased pH in some of the cultures (Figures 4.9 A, B and C), however the increase was insignificant.

4.9 Repression of non-targeted genes

The repression of fluorescence was used as the initial indicator of the off-targeting of CRISPRi. The same growth tube experiment was performed using control strains ADP1-Lux-GFP-dCas9 and ADP1-Lux-GFP as which was done with ADP1-Lux-GFP-

sgRNA[GFP]-dCas9 in the Chapter 4.3. Six different cyclohexanone concentrations (0, 0.00002, 0.0002, 0.002, 0.02, 0.2 mM) were tested with and without arabinose (1.0 or 0.5%). Relative fluorescences at 6 h were calculated by dividing the normalized fluorescence of a culture by the average normalized fluorescence of the uninduced cultures of the same time point (Figures 4.10 A and B).

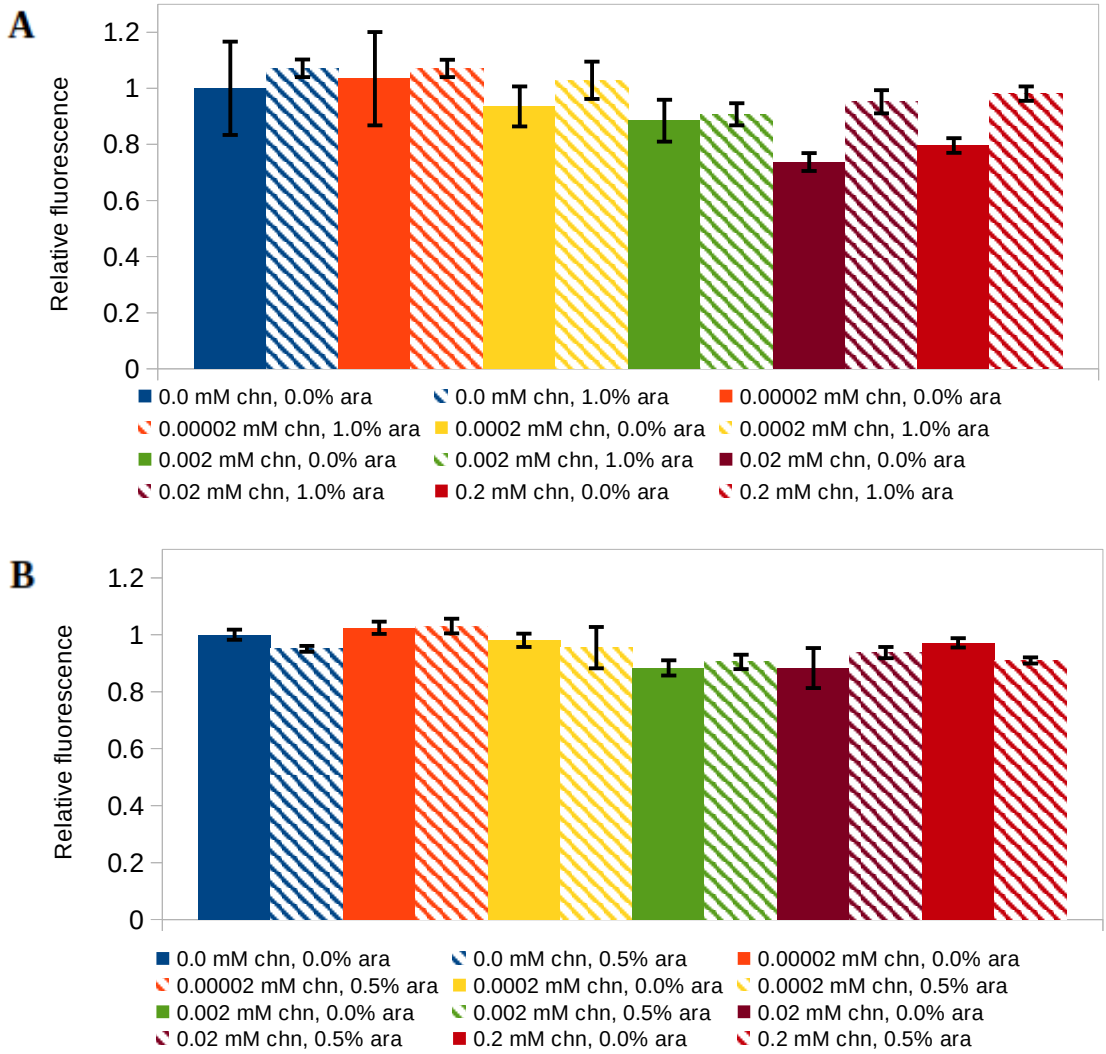

Figure 4.10 *Relative fluorescences (normalized fluorescence of a culture at 6 h divided by the average normalized fluorescence of the uninduced cultures of the same strain at 6 h) of the control strains ADP1-Lux-GFP-dCas9 (**A**) and ADP1-Lux-GFP (**B**). Cyclohexanone (0.0, 0.00002, 0.0002, 0.002, 0.02 and 0.2 mM), which was used to induce dCas9 production in ADP1-Lux-GFP-dCas9, was added in the medium of both of the strains. Each cyclohexanone concentration was tested with (1.0% with ADP1-Lux-GFP-dCas9 or 0.5% with ADP1-Lux-GFP) and without arabinose which would have induced sgRNA expression in ADP1-Lux-GFP-sgRNA[GFP]-dCas9. The arabinose concentration differs because the experiment for A figure was performed after the optimal arabinose concentration was found out and experiment for B figure was carried out before that. The error bars show standard deviation of 4 parallel samples.*

In overall, the relative fluorescence decreased with ADP1-Lux-GFP-dCas9 when cyclohexanone concentration was increased and when arabinose was not present (Figure 4.10 A). The minimum relative fluorescence (0.74) was obtained with 0.02 mM of cyclohexanone and without arabinose. When also arabinose was present the relative flu-

orescence decreased as the cyclohexanone concentration was increased, up to 0.002 mM. However, the decrease was insignificant (the lowest relative fluorescence = 0.91). With 0.02 and 0.2 mM of cyclohexanone and 1.0% of arabinose no decrease in the relative fluorescence was observed. Addition of arabinose increased the relative fluorescence in this strain, for example addition of 1.0% of arabinose resulted in 10% higher relative fluorescence when 0.0002 mM of cyclohexanone was used.

With the strain containing no CRISPRi (ADP1-Lux-GFP) the relative fluorescence decreased slightly from 0.00002 mM up to 0.02 mM of cyclohexanone when arabinose was not present in the medium (Figure 4.10 B). However, the minimum relative fluorescence was 0.88 which is 19% higher than the minimum relative fluorescence of ADP1-Lux-GFP-dCas9. When 0.5% of arabinose was added to the medium the decrease in the relative fluorescence was insignificant. As a result, it can be concluded that the change in the relative fluorescence was negligible. Thus, cyclohexanone did not affect the fluorescence production in this strain.

5 DISCUSSION

CRISPRi has been earlier applied to different bacterial hosts, including *Escherichia coli* (Li et al., 2016), *Pseudomnas putida* and *Pseudomonas aeruginosa* (Tan et al., 2018). In this thesis CRISPRi was established for the first time in *Acinetobacter baylyi* ADP1. Secondly, the optimal expression levels of the CRISPRi machinery parts, dCas9 and sgRNA, were determined. Thirdly, the repression kinetics were investigated. Fourthly, the burden caused by CRISPRi on the growth was measured. Additionally, the burden on the whole cell metabolism was studied by using bioluminescence production as the reporter of the cell's inner state. Lastly, the repression of non-targeted genes was investigated.

ADP1 strain containing CRISPRi was successfully constructed and up to 3.8-fold (or 74%) repression of *GFP* was achieved when compared to the strain with only *dCas9* gene but no sgRNA. The repression was 6.1-fold (or 84%) when compared to the control strain without any part of the CRISPRi machinery. However, up to 300-fold repression of *GFP* has been reported in *E. coli* (Qi et al., 2013) and 100-fold repression in β-galactosidase activity in *P. aeruginosa* (Tan et al., 2018). When compared to these results, the *GFP* repression in ADP1 was weak. The repression could be possibly enhanced by targeting a different loci of the gene (Larson et al., 2013).

The optimal expression levels of dCas9 and sgRNA were found in terms of cyclohexanone (0.0002 mM) and arabinose (1.0%), respectively. With the four highest dCas9 expression levels tested (0.0002–0.2 mM of cyclohexanone) the repression of *GFP* increased only 7%. On the other hand, the cell growth decreased 32% when the cyclohexanone concentration was increased from 0.0002 to 0.2 mM. This could imply that almost all of the copies of the *GFP* gene were saturated with sgRNA:dCas9 complexes already when 0.0002 mM of cyclohexanone was used to induce dCas9 expression, thus repressing the gene as strongly as possible. The unbound complexes then possibly bound to off-target genes, repressing them and thus resulting in decreased growth. However, the increments in which the cyclohexanone concentration (and dCas9 expression level) was increased were relatively large (10-fold). Hence, the actual optimal dCas9 expression level was probably not found. To investigate it more precisely, inducing the dCas9 expression using cyclohexanone concentrations near 0.0002 mM should be studied.

On the contrary, the strongest repression of *GFP* was achieved with the highest sgRNA expression level tested (1.0% of arabinose). The arabinose inducible promoter has been shown not to be very strong in APD1 (Santala et al., 2018). Hence, stronger

repression might be achievable with higher arabinose concentration, which would be feasible to study.

A clear repression (35%), compared to the strain with CRISPRi not targeting any known gene, was achieved without addition of either inducer. Additionally, as adding only one inducer to the medium repressed *GFP* expression, one (or both) of the components of the sgRNA:dCas9 complex has probably been present in the cell without induction. As a result, one or both of the used expression systems probably leak, which seems to be universal attribute of promoters (Huang et al., 2015). However, the leaking was not proved using a reporter protein. Thus, it remains unknown which promoter was leaking and how much. The leakiness might have affected the results as the control strain without CRISPRi was not always cultivated at the same time with the studied strains. As the strains grew differently in every experiment they might have also produced different amounts of fluorescence. Thus, the comparison of the strains is difficult and unreliable even though the effect of biomass was eliminated by normalizing the results.

The promoter leaking could however be taken advantage of by using this CRISPRi system (including the cyclohexanone and arabinose promoters, or other leaky promoters) for applications which need a low continuous repression of a gene. Additionally, if at some point of the application higher repression was desired, the promoters could be induced and stronger repression would be achieved.

CRISPRi started to repress a gene approx. 2 h after the induction. Also in *E. coli* CRISPRi has been shown to reach repression threshold 2 h after induction (Chappell et al., 2017). This is a slow action when compared to salicylate induced bioluminescence production in ADP1, in which a visible increase in bioluminescence was achieved already approx. 0.5 h after the induction and approx. 4 h after the induction the bioluminescence production reached its maximum (Huang et al., 2005). On the contrary, when bioluminescence production was repressed in this thesis, the minimum bioluminescence was not achieved even after 6 h. These results imply that it either took long time to produce dCas9 and sgRNA and for them to form a complex or it took long for the sgRNA:dCas9 complex to bind to its target. When sgRNA:dCas9 complexes are abundant in the cell it takes in average 2 min to find the target and bind to it (Jones et al., 2017). Thus, the latter case is not a probable cause. However, the results obtained might be affected by the leakiness of the promoters: if the gene is already repressed, it might be difficult to see small changes in the expression. Thus, the repression would be seen later than it would actually happen in an optimal system. The dynamics of the CRISPRi machinery expression could be studied for example by taking samples every 15 min after the induction and then analyzing the amount of dCas9 and sgRNA. Additionally, it could be feasible to change the expression systems to less leaky ones and repeat the experiment.

The change in the repression level was not large, maximum of 2.8-fold change was achieved (the relative fluorescence decreased maximum of 64%) when dCas9 and sgRNA concentrations were altered in the experiments to find out the optimal dCas9 and sgRNA expression levels. As a result, the repression level could not be greatly adjusted by altering dCas9 and sgRNA expression levels. Also this could have been caused by the leakiness of the promoters. If the gene was already repressed without the addition of inducers, the change in the repression level would not be as large after induction as in the case of inducing a non-leaky CRISPRi expressions system in which the gene of interest is fully expressed. In the study of Fontana et al. (2018) changing the sgRNA concentration lead to repression levels ranging from 5- to 300-fold when the dCas9 concentration was not limiting. In addition, altering dCas9 expression levels has been shown to repress the targeted gene up to 10-fold (Li et al., 2016). For better controllability of the repression system, the complementarity of the base-pairing region of the sgRNA with the targeted gene could be altered. This has been already done successfully by Vigouroux et al. (2018). They varied the complementarity from 10 to 20 base pairs which resulted to a repression of 8.3- to 50-fold, respectively. In addition, altering the amount of complementary base pairs lead to linear control of the repression level.

Even though CRISPRi was successfully constructed, it did not work fully as planned: a strain with sgRNA not targeting any known gene did not grow when dCas9 was expressed. On the contrary, a strain containing only dCas9 gene grew well after expressing dCas9. Was the sgRNA sequence targeting an essential gene in ADP1's genome? The base-pairing sequence of it was Blasted against ADP1's genome (Altschul et al., 1990). As a result, only ACIAD2025 (hypothetical protein; putative signal peptide) was found to align with the sequence with five nucleotides. Yet, Cui et al. (2018) argued that complementarity of nine nucleotides is needed for strong repression. Hence, that is not a probable cause. On the other hand, the base pairing region of the sgRNA contained four nucleotides (ACCC) that are the same as in a five nucleotide sequence (ACCCA) that is shown to induce strong toxic effects in *E. coli* (Cui et al., 2018). Another reason could be that the strain might have produced a mutation in the base pairing sequence of sgRNA, thus modifying it to target an essential gene in ADP1's genome. This could have been investigated by sequencing the sgRNA after transforming it to ADP1. Now the sgRNA was sequenced only before transformation so it can not be know for sure that the sequence was correct. Nevertheless, the phenomena was not studied more due to the time limitations and thus the reason for high toxicity remains unclear.

CRISPRi caused burden also in the strain containing dCas9 but not sgRNA. The more dCas9 was expressed the more growth was impaired. However, in every experiment the growth kinetics of the strains varied for unknown reason. As a result, different cell masses were obtained at same time points of the incubations even when the same strain was used. Additionally, the amount of parallel cultivations was low (2) which in-

creased the variation of the results. To obtain more reliable results, more parallel cultivations and preferably all the strains should be incubated in the same experiment to remove the effect of different environment conditions or slightly different experimental procedures.

Nevertheless, dCas9 has been shown to induce growth defects in *E. coli*, directly proportionally to the amount of dCas9 present in the cells (Zhang and Voigt, 2018). This could be caused because of repression of (off-target) native genes, which then causes abnormal cell morphology (Cho et al., 2018b). DCas9 alone, without sgRNA bound to it, is argued to induce higher toxicity than with sgRNA (Zhang and Voigt, 2018). Similar results were obtained in this thesis with the strain with a complete CRISPRi machinery (sgRNA targeting *GFP*): the growth decreased when the dCas9 expression level was increased, but the more sgRNA was expressed the less toxic was the same amount of dCas9. One reason for the toxicity could be that dCas9 proteins (unbound to the sgRNA) open the double stranded DNA and bind to the PAM sites, thus repressing genomic genes. This has been shown to be the case in *E. coli* (Jones et al., 2017). When sgRNA:dCas9 complex is formed, it requires also complementary (with the base-pairing region of sgRNA) sequence in the targeted gene for binding, not only PAM sites. Off-target repression lessens possibly because of this.

As GFP was repressed maximum 1.4-fold after expression of dCas9 without sgRNA (in the strain ADP1-Lux-GFP-dCas9), off-target repression could be also the case when dCas9 was expressed in high levels in ADP1. This could impair cell growth but stronger repression of the targeted gene would not be achieved. The inducers used in thesis were not the cause as the growth of a control strain without CRISPRi machinery was not affected by them. However, as the off-targeting was not studied in detail, it can not be said for sure what causes it in ADP1 and how much it affects. Additionally, when the repression of *GFP* was studied using the strain with dCas9 but without sgRNA, some of the relative fluorescence results showed large standard deviations. As the amount of parallel cultures was low (2), the differences in the results might be smaller in reality between the tested dCas9 expression levels than observed in this thesis. To obtain more reliable results, the experiment could be repeated with larger number of parallel cultures.

The repression of off-target genes could be caused for example by the off-targeting nature of dCas9, the observed decreases in pH or the burden caused on the cells by the expression of the dCas9. Hence, this phenomena should be studied more to understand the mechanism behind it. For example, burden caused to the cells could be studied in the terms of the cell morphology by inducing dCas9 and sgRNA in different quantities and using a microscope to visualize the cells. How CRISPRi targets a gene could also be studied by attaching fluorescent probes on dCas9 or sgRNA and then visualizing their movement in real time (Jones et al., 2017). The sgRNA not targeting any known gene could be redesigned and the amount and targets of sgRNA:dCas9 com-

plex's off-targeting could be compared to dCas9's off-targeting. Additionally, the effect of pH could be studied by controlling the pH of the medium and analyzing the amount of fluorescence produced.

Even though the cell density did not decrease with the optimal dCas9 and sgRNA expression levels, the bioluminescence production decreased. This was also the case when 10-fold lower cyclohexanone concentration was used to induce dCas9 expression. Hence, the expression of dCas9 or the repression of non-targeted genes affect the cell metabolism in way that is not visible from the growth. However, it can not be ruled out that the cyclohexanone expression system did not cause the effect. This could be studied by repeating the experiment after replacing *dCas9* gene with a reporter protein. It could be also feasible to study how expression of dCas9 with different expression system affects the cell metabolism.

Instead of sgRNA expression, decreased bioluminescence production after the addition of arabinose could have resulted from decreased amount of oxygen molecules. Oxidation of arabinose by the glucose dehydrogenase *gcd* requires oxygen (Santala et al., 2018) and thus if there was arabinose in the medium, there would be less oxygen molecules that are important for the bioluminescence production (Close et al., 2009).

In overall, bioluminescence worked as a sensitive reporter of the cell's metabolic state. However, it was a possibly even too sensitive reporter. It was difficult to cultivate the cells in every experiment the same way in terms of bioluminescence production. This can be seen from the large standard deviation in many of the results. To obtain more reliable results, the experiments should be repeated many times to achieve larger amount of parallel cultures. Nevertheless, bioluminescence could be used as a reporter in many bioengineering applications, for example to compare the burden caused by different gene regulation techniques or virtually any synthetic pathways.

Low pH (approx. 4) was measured after the incubation periods when dCas9 was highly expressed. The same effect was not observed with the control strain lacking CRISPRi. Hence, dCas9 expression seems to cause a decrease in the pH. Utilizing glucose as a carbon source by ADP1 produces H^+ which should lower the pH of the medium (Taylor and Juni, 1961). However, as no significant decrease in pH of the control strain without CRISPRi was observed, dCas9 could repress genes that are responsible of pH buffering. Higher concentration of sgRNA would have allowed more sgRNA:dCas9 complexes to be formed and thus guided it to *GFP* gene instead of genes responsible for pH control. This effect could be further studied by changing the carbon source for example to acetate which has not been shown to cause H^+ production in ADP1.

Additionally, the growth seemed to be connected with the end pH so that higher pH lead to higher cell density. However, which one is the cause and which one the consequence remains unknown. Unfortunately the experimental procedure was not optimal for comparing pH with the growth as the pH was only measured after 21 h of incuba-

tion. At that point of the growth the cells might be already in death phase and thus the cell density might have already decreased from the maximum (Barbe et al., 2004). To investigate the phenomena better, growth tube experiments could be repeated so that pH would be measured at least during every sampling time. Hence, the connection between pH and growth and between pH and and dCas9 expression (and possibly sgRNA expression) could be studied better. Also the pH measuring technique was not very accurate as only pH paper with visual inspection was used. If subsequent experiments were to be performed, it would be beneficial to use an accurate pH meter.

On the other hand, expressing GFP in high quantities with the same cyclohexanone expression system (which was used for dCas9 expression in this thesis) has been shown to decrease pH in ADP1 (data unpublished). Hence, the low pH might be actually caused by the expression system and not by dCas9 expression. To further investigate if this is the cause, dCas9 could be expressed using an another expression system while pH would be measured during the incubation. Additionally, effect of the cyclohexanone expression system on pH could be studied more by using different reporter proteins with similar experimental procedures as in this thesis.

The repression of downstream genes in the same operon was expected result as it has been reported also in earlier studies (Cui et al., 2018; Dominguez et al., 2016). As sgRNA:dCas9 complex physically blocks the movement of RNA polymerase (Qi et al., 2013), a separate promoters would be needed for each gene to allow polymerase binding after a silenced gene.

6 CONCLUSIONS

The CRISPRi machinery was successfully established in *Acinetobacter baylyi* ADP1 for the first time. The gene coding for dCas9 protein was embedded into ADP1's genome and sgRNA was expressed from the pBAV1G plasmid. DCas9 and sgRNA were under regulation of cyclohexanone and arabinose inducible promoters, respectively.

The optimal inducer concentrations according the repression efficiency and the cell growth were 0.0002 mM of cyclohexanone and 1.0% of arabinose. With these concentrations maximum of 3.8-fold (or 74%) repression was achieved compared to the strain with dCas9 but without sgRNA. However, if compared to the strain lacking the CRISPRi machinery completely, 6.1-fold (or 84%) repression was achieved. Both promoters appeared to be leaky, thus repression was achieved already without addition of inducers. However, after the induction it took 2 h for stronger repression to take place.

Expression of dCas9 was toxic to the cells, for example the cells induced with 0.0002 mM of cyclohexanone had 28% lower cell density than uninduced culture after 6 hours of cultivation. On the other hand, high expression level of sgRNA increased the cell growth when also dCas9 was expressed, for example addition of 1.0% arabinose increased cell density for 42% when 0.0002 mM of cyclohexanone was used. Hence, it is possible that the toxicity of dCas9 is decreased when its bound to sgRNA. The optimal inducers concentrations did not affect the growth when compared to the uninduced control.

Additionally to the cell density, the bioluminescence production was used as an indicator of the cell's metabolic state. It was utilized for studying the effects of the CRISPRi machinery on the cell metabolism for the first time. Even though the cell density was not affected, bioluminescence production decreased when the optimal or 10-fold lower dCas9 expression level were used. Additionally, either expression of sgRNA at the optimal level or arabinose oxidation decreased the bioluminescence production. In conclusion, dCas9 or its expression causes a burden to the cells that can not be seen from the cell density. Hence, bioluminescence functions well as an indicator of cell's metabolic state.

Fluorescence (GFP) production was repressed maximum of 1.4-fold when dCas9 without sgRNA was expressed. However, the cause for the repression of non-targeted genes remains unknown. No off-target repression of sgRNA:dCas9 complex was studied due to the toxicity of the sgRNA which was designed not to target any known gene.

Cyclohexanone (or dCas9) concentration, pH and growth seemed to be connected so that the higher cyclohexanone concentration, the lower pH and the lower cell density. It was not found out if the decrease in pH and growth were caused by the cyclohexanone expression system or increased concentration of dCas9. The expression of GFP with the same cyclohexanone expression system caused similar decrease in pH in an other study performed by the same group.

The hypotheses mostly held true. The CRISPRi was functional in ADP1, high concentrations of dCas9 were toxic to the cells and expression of the CRISPRi machinery decreased bioluminescence production. On the other hand, approx. the maximum repression level was achieved with dCas9 and sgRNA expression levels which did not affect the growth. Thus, no compromise between repression level and the cell burden was needed to be made. Additionally, the start of the repression was slower than hypothesized: repression was visible 2 h after the induction instead of 30 min.

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APPENDIX A: PLASMIDS

In this appendix the plasmid sequences as they were designed and the plasmid map of pBAV1G-ara-sgRNA[null] can be found. As the plasmids pBAV1G-ara-sgRNA[null], pBAV1G-ara-sgRNA[GFP] and pBAV1G-ara-sgRNA[Lux] differed only from the base-pairing region (20 nt sequence) in the sgRNA, only one map is presented. The base-pairing regions are written in bold in the plasmid sequences.

pBAV1G-ara-sgRNA[null]

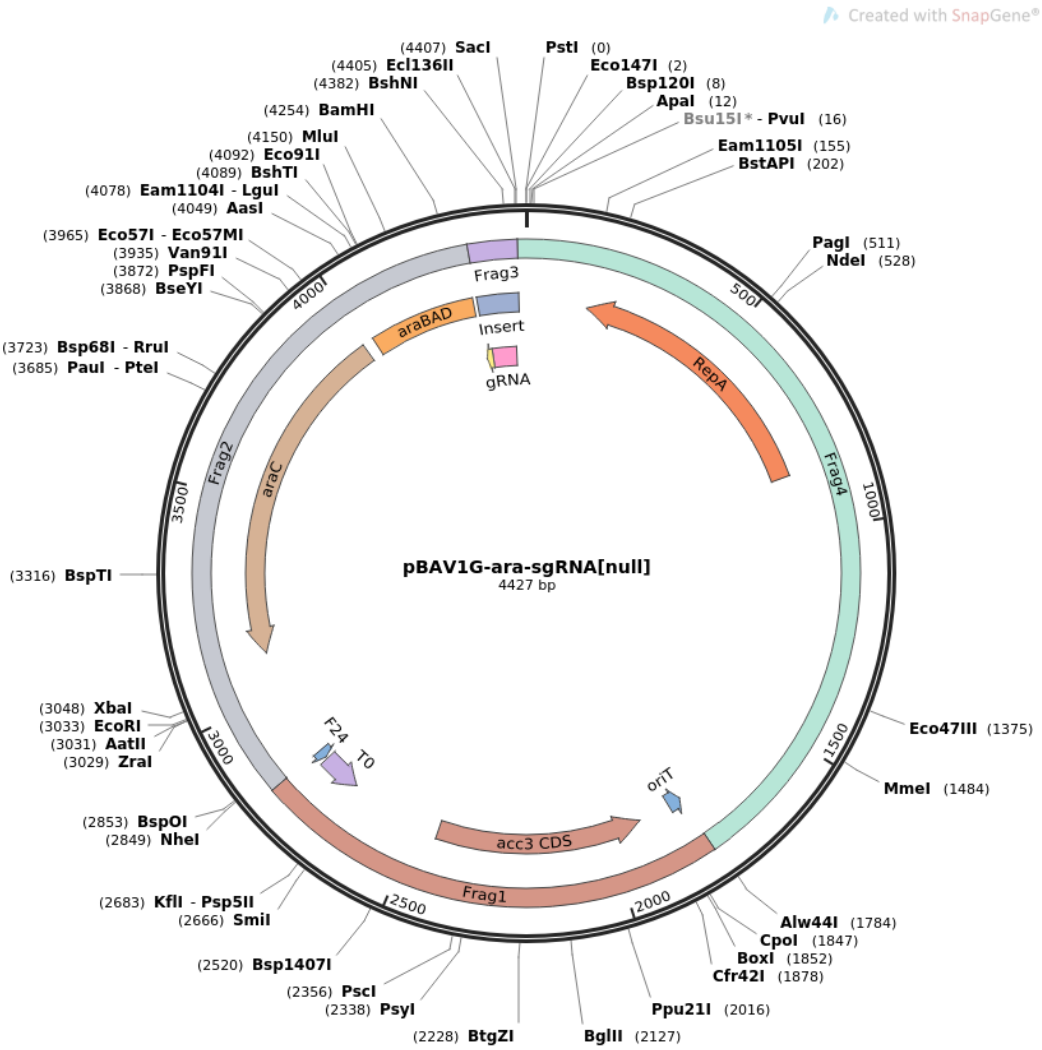

Figure Appendix A 1 *The map of plasmid pBAV1G-ara-sgRNA[null]. The only region that differs between the pBAV1G-ara-sgRNA[null] and pBAV1G-ara-sgRNA[GFP] and pBAV1G-ara-sgRNA[Lux] plasmids is 20 bp long sequence (the yellow arrow), left from gRNA coding sequence.*

>pBAV1G-ara-sgRNA[null] ggcctcagggcccgatcgatgccgccgcttaattaattaatccagaggcat-
caaataaaacgaaaggctcagtcgaaagactgggcctttcgttttatctgttgtttgtcggtgaacgctctcctgagtagga-
caaatccgccgccctagacctagtgtcattttatttcccccgtttcagcatcaagaacctttgcataacttgctctatatccacact-
gataattgccctcaaaccataatctaaaggcgctagagtttgttgaaacaatatcttttacatcattcgtatttaaaattccaaactc-
cgctcccctaaggcgaataaaagccattaaatcttttgtatttaccaaattatagtcatccactatatctaagag-
taaattcttcaattctctttttggctttcatcaagtgttatatagcggtcaatatcaaaatcattaatgttcaaaatatctttttgtcg-
tatatatgtttattcttagcaatagcgtcctttgattcatgagtcaaatattcatatgaaccttgatataatcaagtatctcaacat-
gagcaactgaactattccccaattttcgcttaatcttgttcctaacgctttctattgttacaggatttcgtgcaatatatataacgt-
gatagtgtggtttttatagtgctttccatttcgtataacatcactactattccatgtatctttatctttttttcgtccatatcgtgtaaag-
gactgacagccatagatacgcccaaactctctaatttttccttccaatcattaggaattgagtcaggatataataaaaatc-
caaaatttctagctttagtatttttaatagccatgatataattaccttatcaaaaacaagtagcgaaaactcgtatccttc-
taaaaacgcgagctttcgcttatttttttgttctgattcctttcttgcatattcttctatagctaacgccgcaaccgca-
gattttgaaaaaccttttgtttcgccatatctgttaatttttatcttgctcttttgtcagagaaatcataactctttttttcgattct-
gaaatcaccatttaaaaaactccaatcaaataatttttataaagttagtgtatcactttgtaatcataaaaacaacaataaagctact-
taaatatagatttataaaaaacgttggcgaaaacgttggcgattcgttggcgattgaaaaacccctttaaacc-
cttgagccagttgggatagagcgtttttggcacaaaaattggcactcggcacttaatgggggtcgtagtacggaag-
caaaattcgcttcctttcccccccatttttttccaaattccaaatttttttcaaaaattttccagcgctaccgctcggcaaaattgcaag-
caatttttaaaatcaaacccatgagggaatttcattccctcatactcccttgagcctcctccaaccgaaatagaagggcgct-
gcgcttattatttcattcagtcatcggctttcataatctaacagacaacatcttcgctgcaaagccacgctacgctcaagggctttt-
tacgctacgataacgcctgttttaacgattatgccgataactaaacgaaataaacgctaaaacgtctcagaaacgattttga-
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APPENDIX B: PRIMER LIST

Name	Sequence	Target
GFP_for	ACCAGGAUCGGAACAACACGTTTTAG AGCTAGAAATAG	Base pairing region of sgRNA
GFP_rev	ATCCTGGUTTATGGAGAAACAGTAGAG	Base pairing region of sgRNA
Lux_for	AAAGATUTCAACCGTTTTAGAGCTAGA AATAG	Base pairing region of sgRNA
Lux_rev	AATCTTUCCCGAAATATGGAGAAACAG TAGAG	Base pairing region of sgRNA
1b-pBAV_for	ATTTTAAGUGCACTCGCTTGGACTCCT GTTG	4 bp on oriT and upstream
1a-pBAV_rev	AGCTCACUAGTAGCGGCCGCTGCAGG	6 bp downstream from sgRNA
dCas9_for	Not known – primer was acquired from Technical University of Denmark	Not known
dCas9_rev	Not known – primer was acquired from Technical University of Denmark	Not known
S1	ATCACGGCAGAAAAGTCC	112 bp upstream of the base pairing region of sgRNA

APPENDIX C: SEQUENSING RESULTS

The pBAV1-ara-sgRNA[null] plasmid was verified by sequencing prior to this study (data unpublished). The plasmids pBAV1-ara-sgRNA[Lux] and pBAV1-ara-sgRNA[GFP] were sequenced to verify the correct sgRNA guiding sequence. The primer used in sequencing bound 112 bp upstream from the base pairing sequence of sgRNA.

The 100% correct and correctly situated guiding sequences in the sgRNA are highlighted and bold. If the sequence was not 100% similar with the designed sequence or it was wrongly located, its only bold.

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APPENDIX D: KINETIC DATA OF THE EXPERIMENTS

The results presented in the Chapter 4 were mostly acquired only from single time point during a 23 or 15 h long experiment. As a result, a big amount of data was excluded from the mentioned chapter. In this appendix the results of each experiment are presented as continuous curves.

Additionally, the results of control strains, ADP1-Lux-GFP-sgRNA[null], ADP1-Lux-dCas9 and ADP1-Lux, are presented. All of the error bars were omitted from the results due to the large amount of curves in each figure.

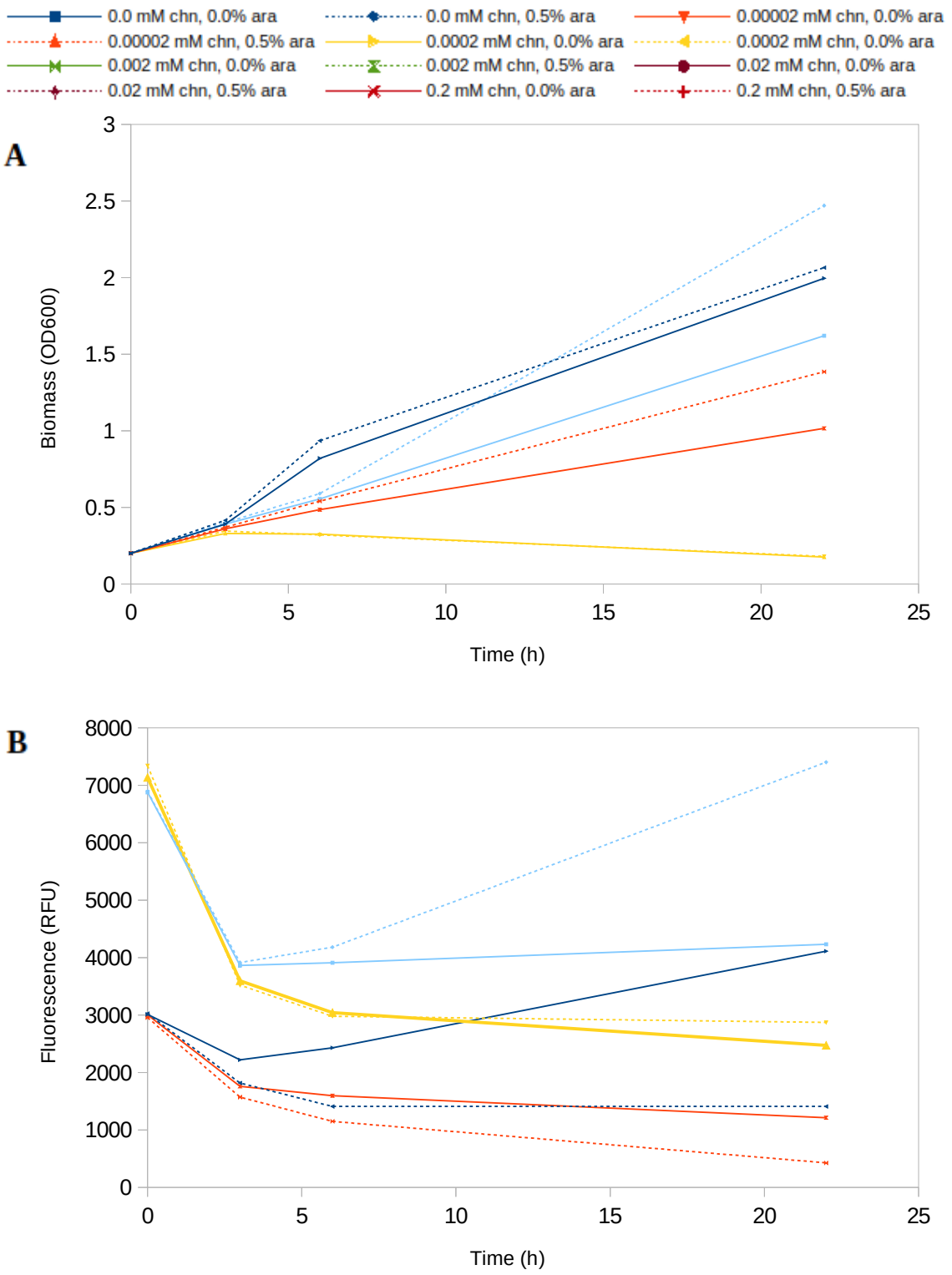

Figure Appendix D 1 *The biomass (optical density at 600 nm) (**A**) and normalized fluorescence (fluorescence divided by optical density) (**B**) of ADP1-Lux-GFP-sgRNA[GFP]-dCas9 and ADP1-Lux-GFP-sgRNA[null]-dCas9 in the proof-of-principle experiment. The inducer concentrations were 0.0 and 0.2 mM of cyclohexanone and 0.0 or 1.0% of arabinose which were tested alone and together with both strains. The experiment was performed as a 23 h growth tube incubation in which the inducers were added at 0 h.*

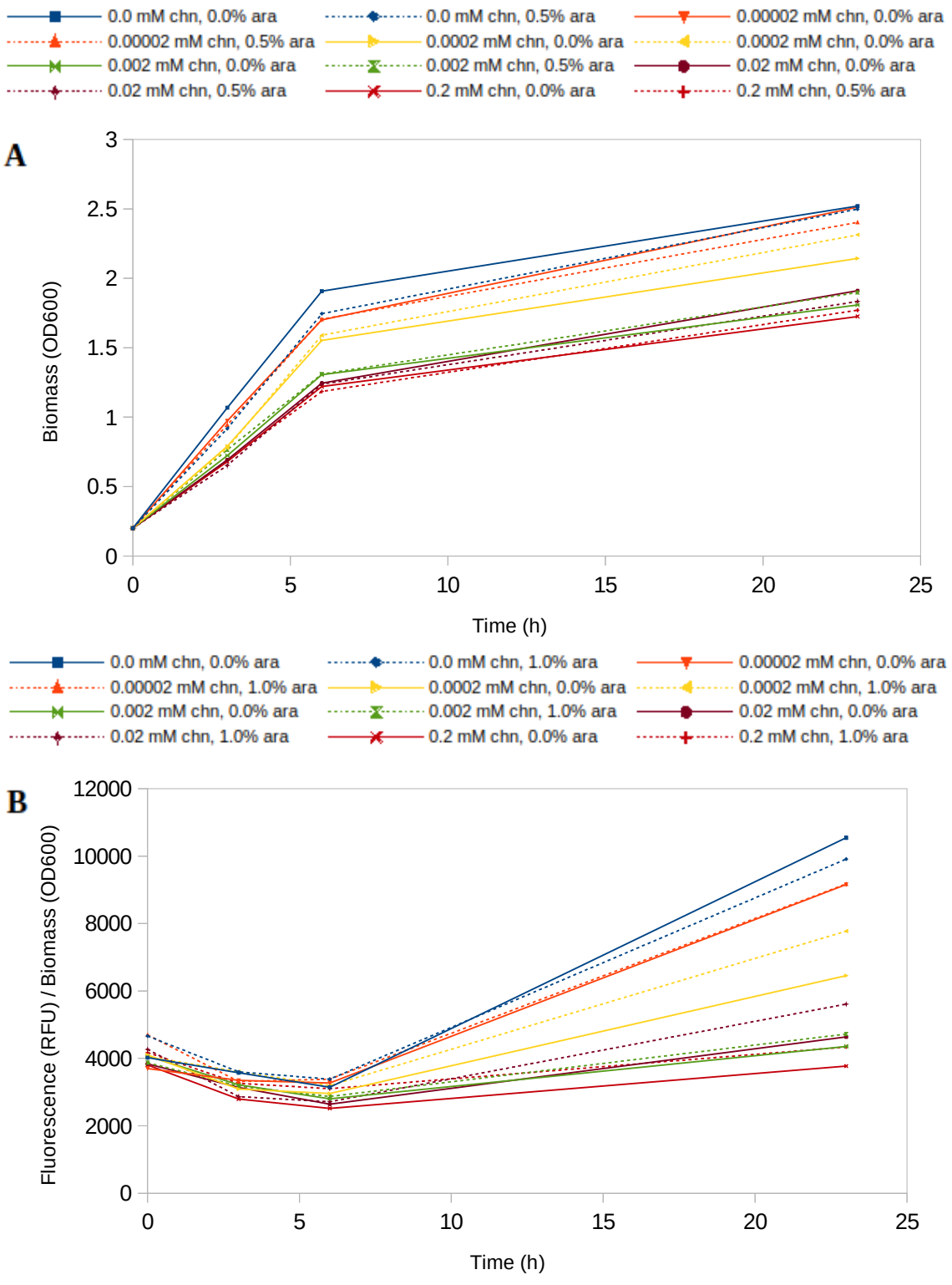

Figure Appendix D 2 *The biomass (optical density at 600 nm) (**A**) and normalized fluorescence (fluorescence divided by optical density) (**B**) of control strain ADP1-Lux-GFP-dCas9 in the experiment where off-targeting of CRISPRi was studied. The inducer concentrations were 0.0, 0.00002, 0.0002, 0.002, 0.02 and 0.2 mM of cyclohexanone and 0.0 and 0.5 or 1.0% of arabinose. Each cyclohexanone concentration was tested with and without arabinose. The experiment was performed as a 23 h growth tube incubation in which the inducers were added at 0 h. The figure (A) and (B) were obtained from different experiments due to the mistake done in fluorescence measurements in the first experiment.*

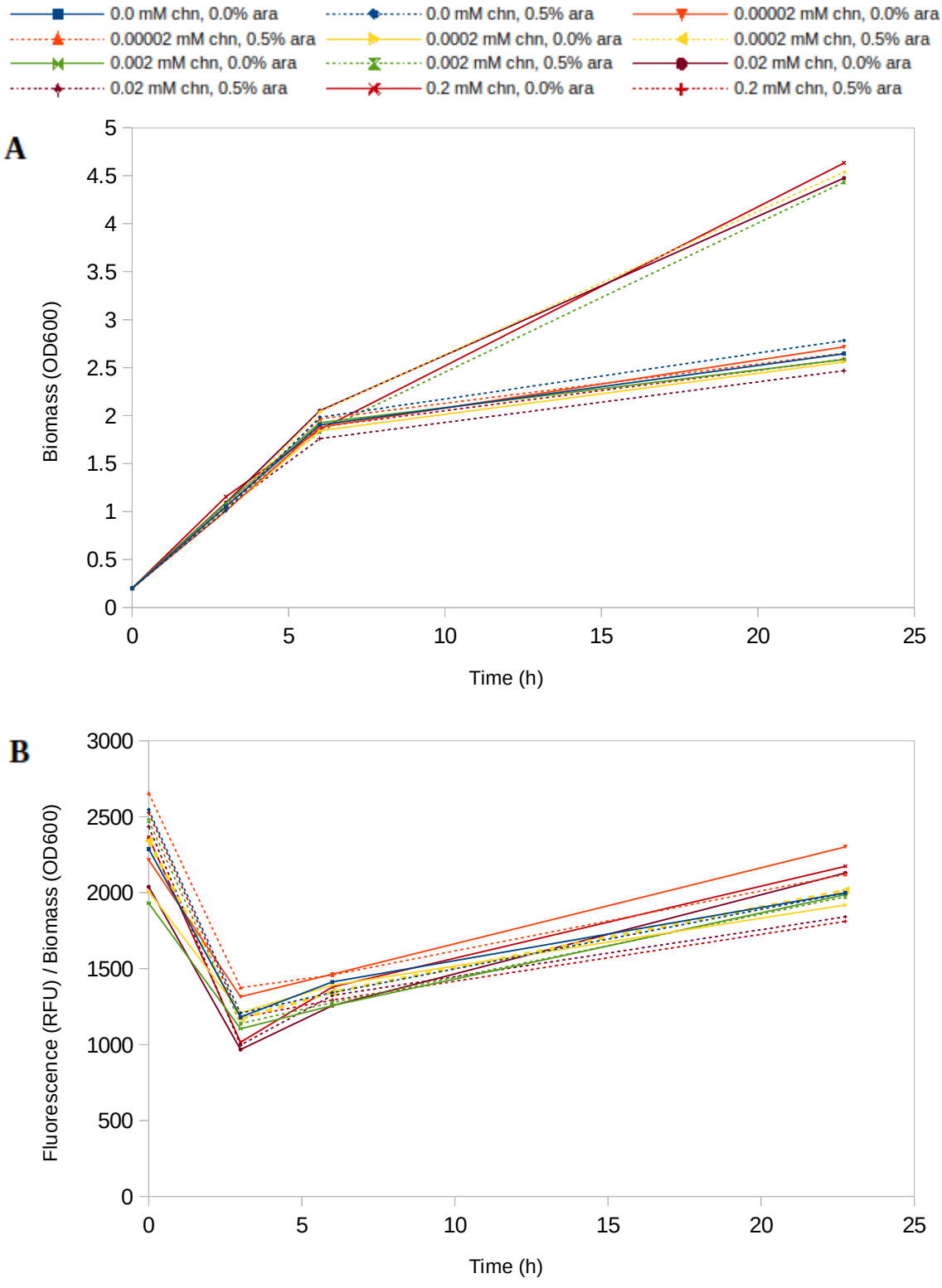

Figure Appendix D 3 *The biomass (optical density at 600 nm)* ***(A)*** *and normalized fluorescence (fluorescence divided by optical density)* ***(B)*** *of control strain ADP1-Lux-GFP in the experiment where off-targeting of CRISPRi was studied. The inducer concentrations were 0.0, 0.00002, 0.0002, 0.002, 0.02 and 0.2 mM of cyclohexanone and 0.0 and 0.5% of arabinose. Each cyclohexanone concentration was tested with and without arabinose. The experiment was performed as a 23 h growth tube incubation in which the inducers were added at 0 h.*

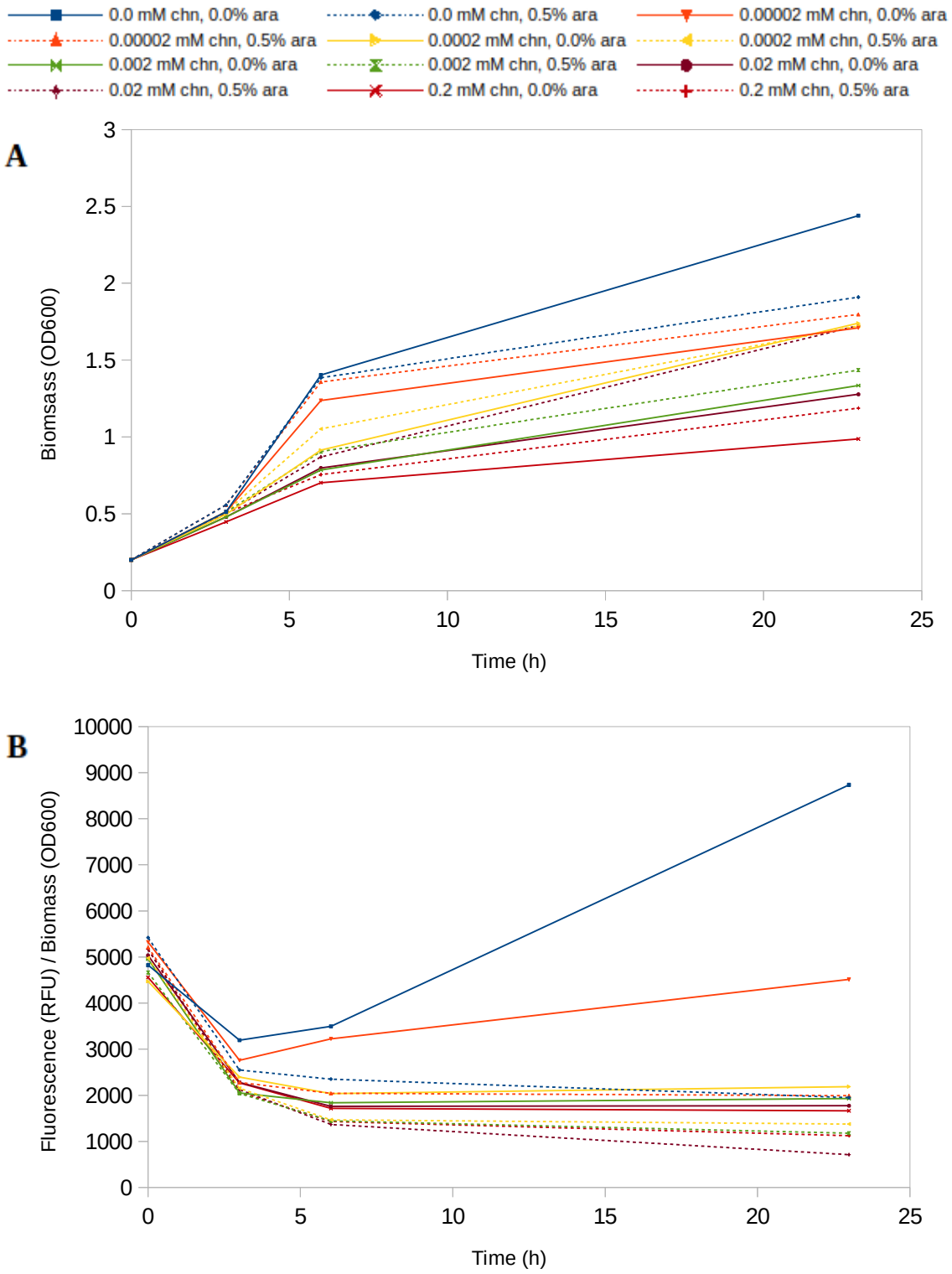

Figure Appendix D 4 *The biomass (optical density at 600 nm) (**A**) and normalized fluorescence (fluorescence divided by optical density) (**B**) of ADP1-Lux-GFP-sgRNA[GFP]-dCas9 in the experiment where optimal dCas9 expression level was searched. The inducer concentrations were 0.0, 0.00002, 0.0002, 0.002, 0.02 and 0.2 mM of cyclohexanone and 0.0 and 1.0% of arabinose. Each cyclohexanone concentration was tested with and without arabinose. The experiment was performed as a 23 h growth tube incubation in which the inducers were added at 0 h.*

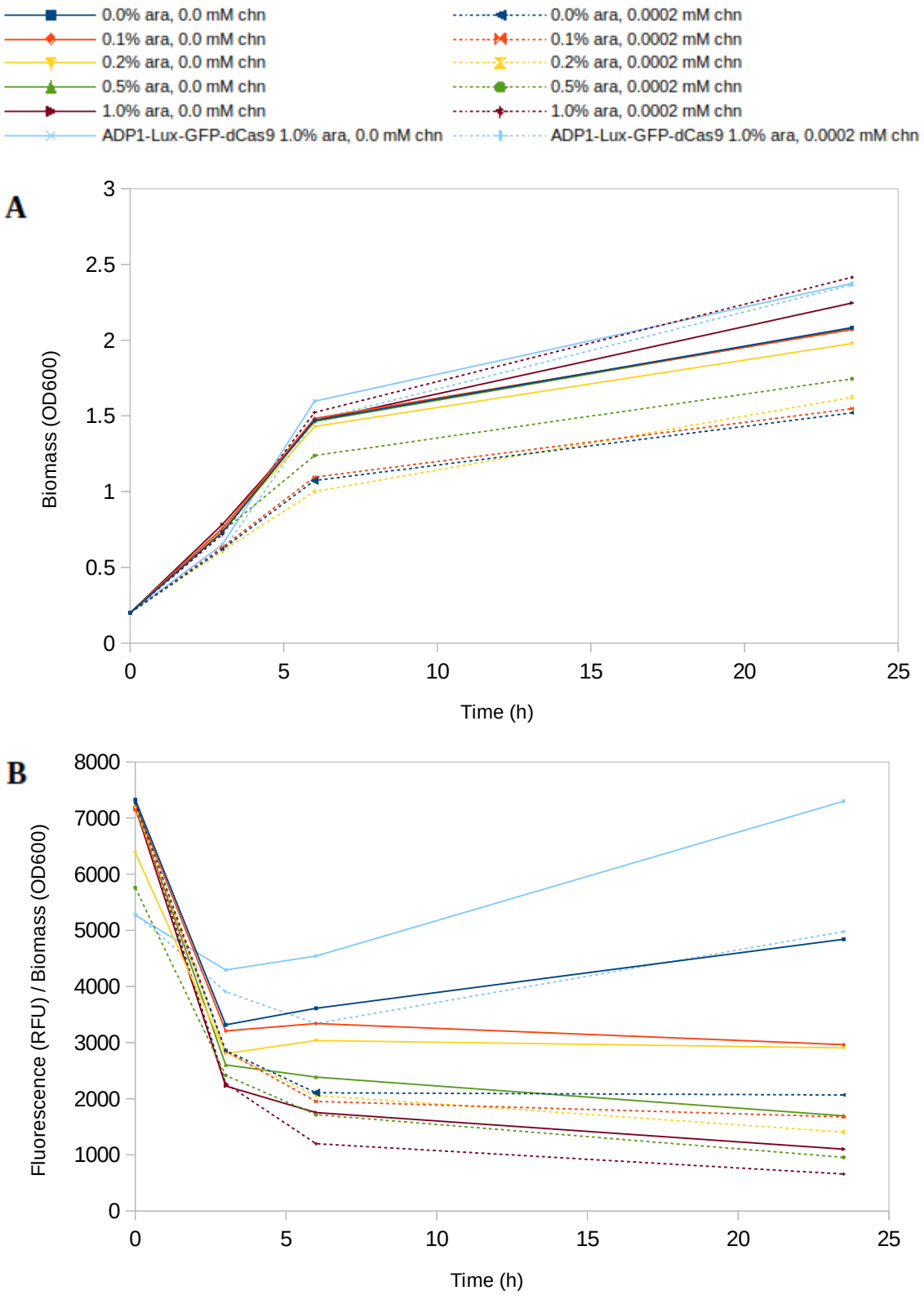

Figure Appendix D 5 *The biomass (optical density at 600 nm) (**A**) and normalized fluorescence (fluorescence divided by optical density) (**B**) of ADP1-Lux-GFP-sgRNA[GFP]-dCas9 and control strain ADP1-Lux-GFP-dCas9 in the experiment where optimal sgRNA expression level was searched. The inducer concentrations were 0.0 and 0.0002 mM of cyclohexanone and 0.0, 0.1, 0.2, 0.5 and 1.0% of arabinose. Each arabinose concentration was tested with and without cyclohexanone. The experiment was performed as a 23 h growth tube incubation in which the inducers were added at 0 h.*

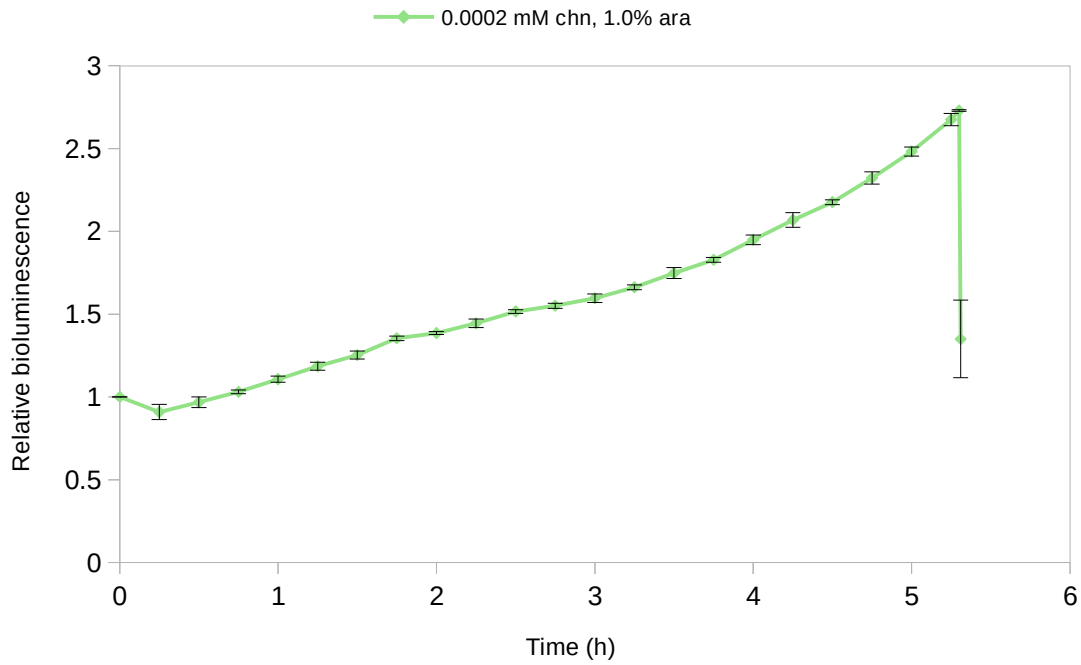

Figure Appendix D 6 *The relative bioluminescence (calculated by dividing bioluminescence of a sample by the bioluminescence of the sample at t=0) of ADP1-Lux-sgRNA[Lux]-dCas9. Decanal was added at 5.25 h. Cyclohexanone (0.0002 mM) and arabinose (1.0%) were used to induce dCas9 and sgRNA production, respectively. The error bars show standard deviation of three parallel cultures.*

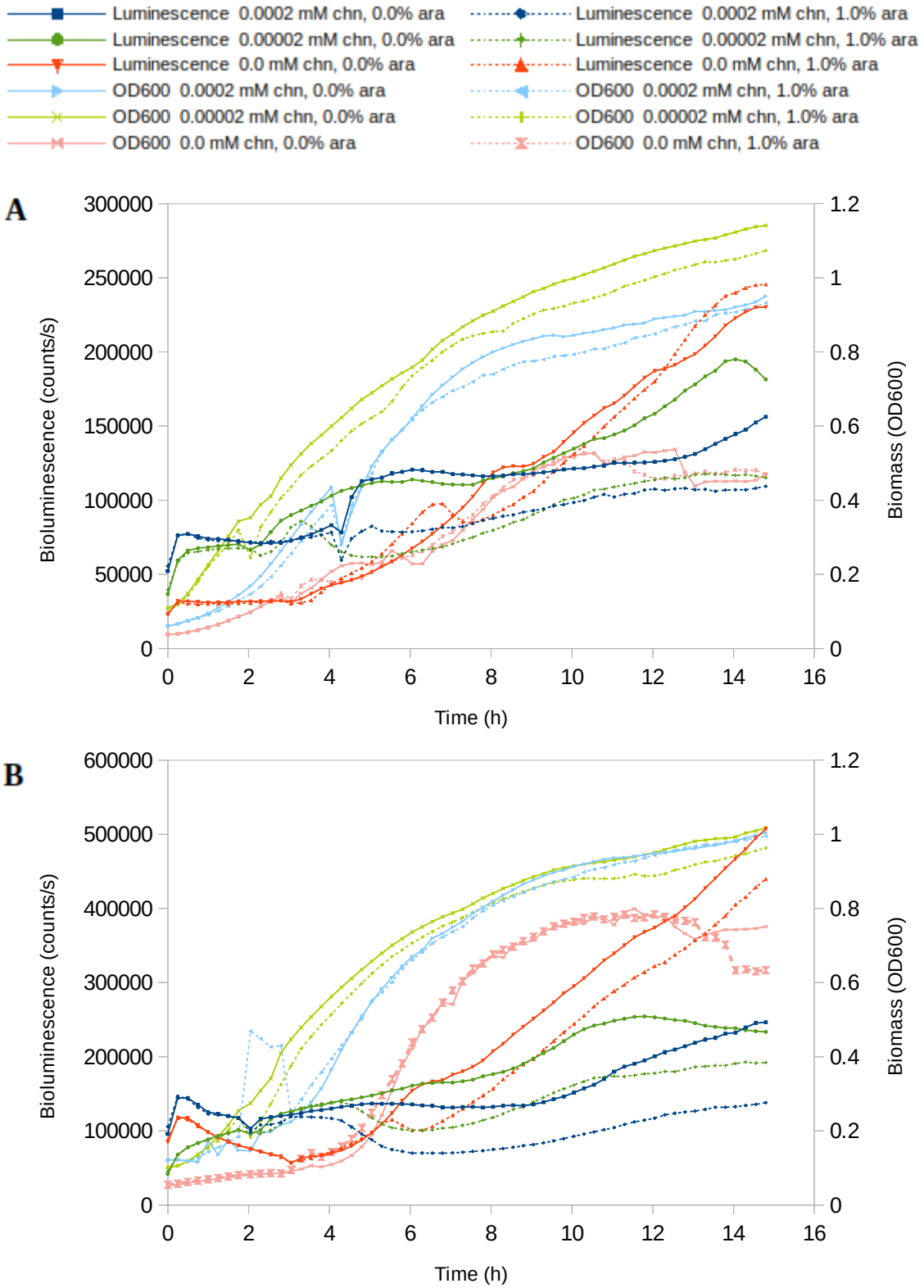

Figure Appendix D 7 *Bioluminescence (primary x-axel) and biomass (optical density at 600nm) (secondary y-axel) of ADP1-Lux-GFP-sgRNA[GFP]-dCas9 (**A**) and ADP1-Lux-GFP-dCas9 (**B**). The results are combined from three different experiments (with different cyclohexanone concentrations). Inducers were added at 4.25, 3 and 2 h after the start of the incubation when 0.0, 0.00002 and 0.0002 mM of cyclohexanone was used, respectively. All of the cyclohexanone concentrations were tested with (1.0%) and without (0.0%) arabinose.*

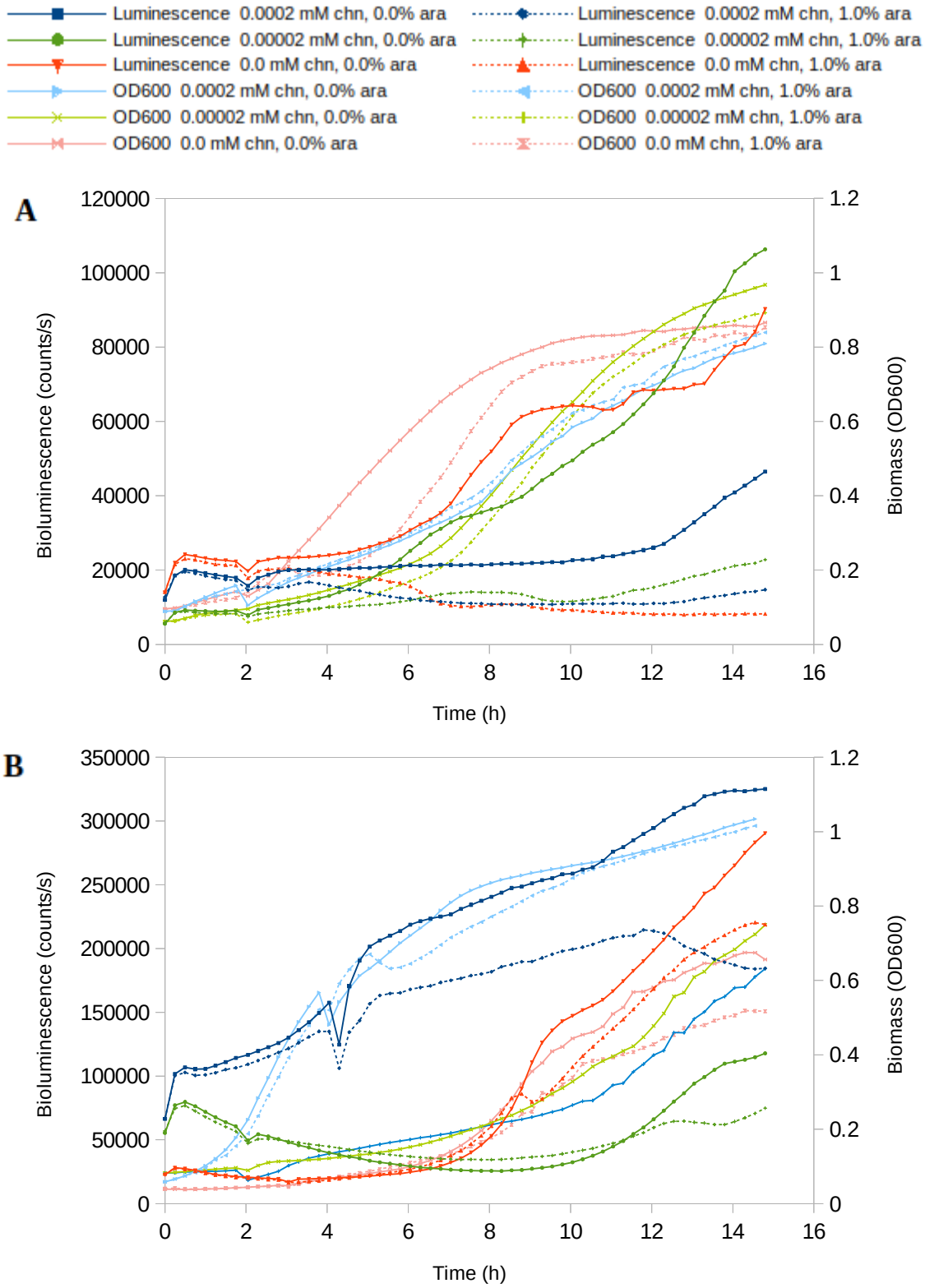

Figure Appendix D 8 *Biouminescence (primary x-axel) and biomass (optical density at 600nm) (secondary y-axel) of ADP1-Lux-GFP-sgRNA[Lux]-dCas9 (**A**) and ADP1-Lux-dCas9 (**B**). The results are combined from three different experiments (with different cyclohexanone concentrations). Inducers were added at 4.25, 3 and 2 h after the start of the incubation when 0.0, 0.00002 and 0.0002 mM of cyclohexanone was used, respectively. All of the cyclohexanone concentrations were tested with (1.0%) and without (0.0%) arabinose.*

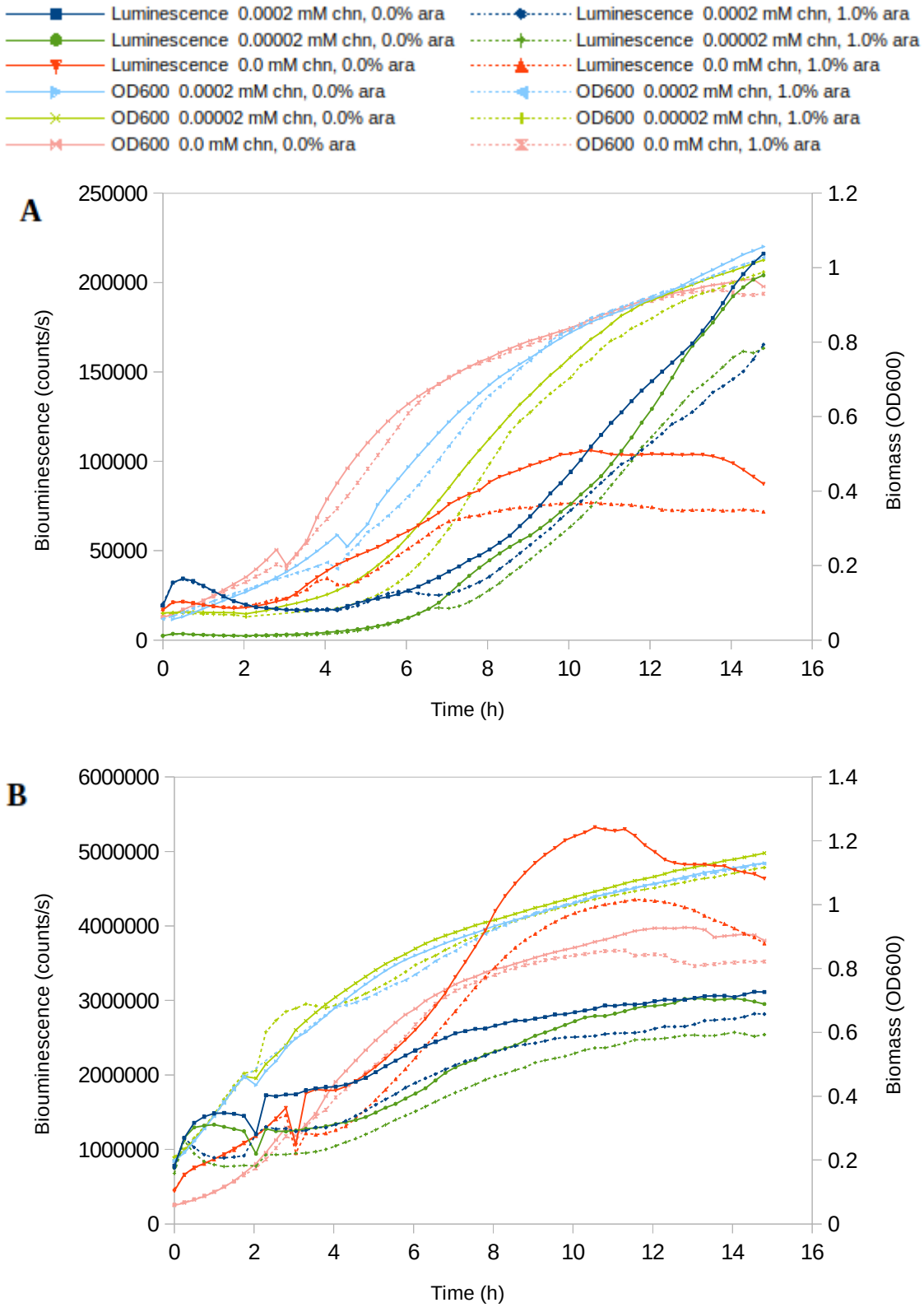

Figure Appendix D 9 *Biouminescence (primary x-axel) and biomass (optical density at 600nm) (secondary y-axel) of ADP1-Lux-GFP (**A**) and ADP1-Lux (**B**). The results are combined from three different experiments (with different cyclohexanone concentrations). Inducers were added at 4.25, 3 and 2 h after the start of the incubation when 0.0, 0.00002 and 0.0002 mM of cyclohexanone was used, respectively. All of the cyclohexanone concentrations were tested with (1.0%) and without (0.0%) arabinose.*

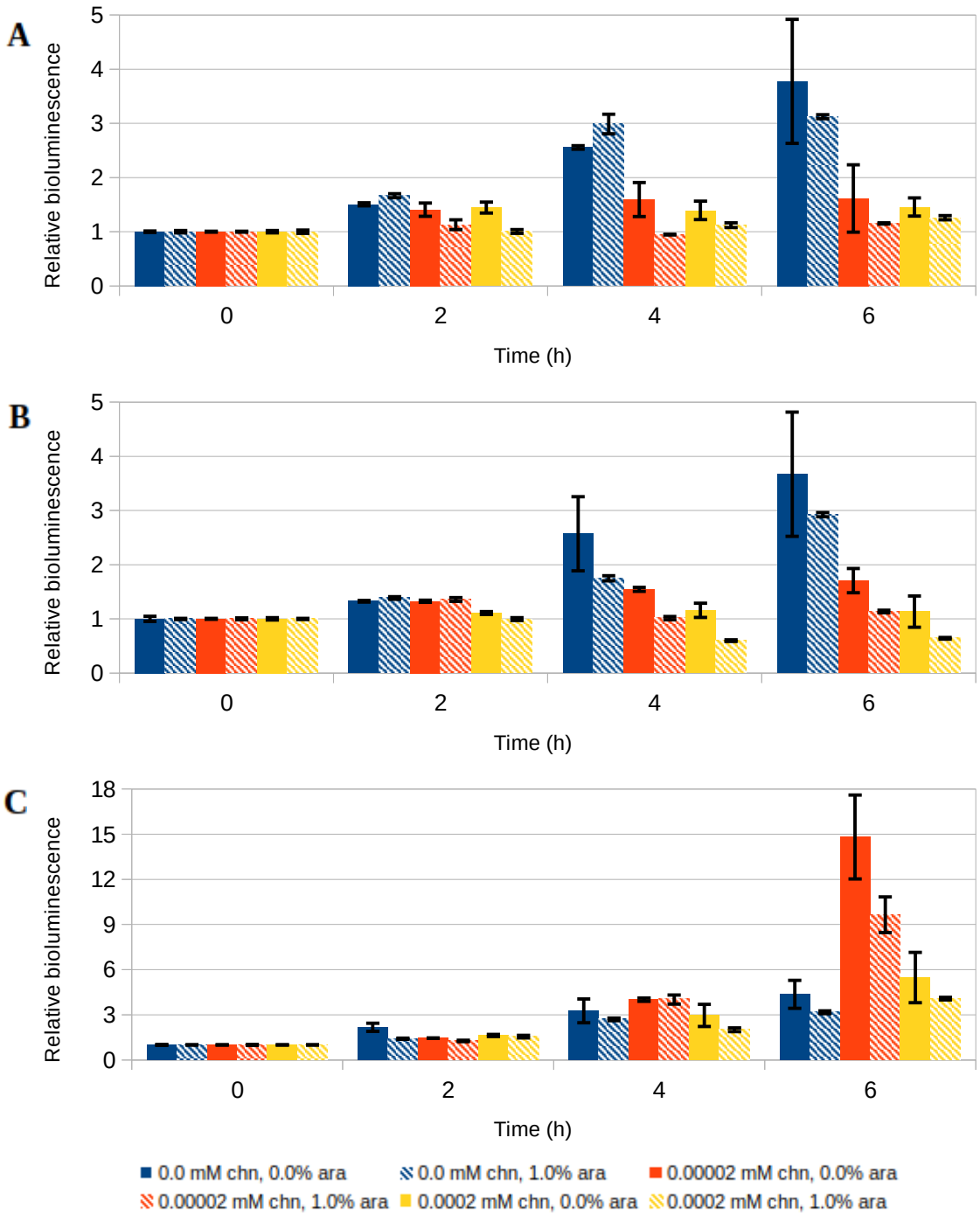

Figure Appendix D 10 *Relative bioluminescences (calculated by dividing the bioluminescence of a time point by the lbiouminescence of the time point just before the induction) at 0, 2, 4 and 6 h after the induction of ADP1-Lux-GFP-sgRNA[GFP]-dCas9 (**A**), ADP1-Lux-GFP-dCas9 (**B**) and ADP1-Lux-GFP (**C**). Inducer concentrations were 0.0, 0.00002 mM and 0.0002 mM of cyclohexanone and 0.0 and 1.0% of arabinose. Each cyclohexanone concentration was tested with and without arabinose. In the strains ADP1-Lux-GFP-sgRNA[GFP]-dCas9 and ADP1-Lux-GFP-dCas9 cyclohexanone was used to induce dCas9 expression and arabinose was used to induce sgRNA expression in the strain ADP1-Lux-GFP-sgRNA[GFP]-dCas9. ADP1-Lux-GFP did not contain any part of the CRISPRi machinery. The error bars are standard deviations of three parallel cultures.*

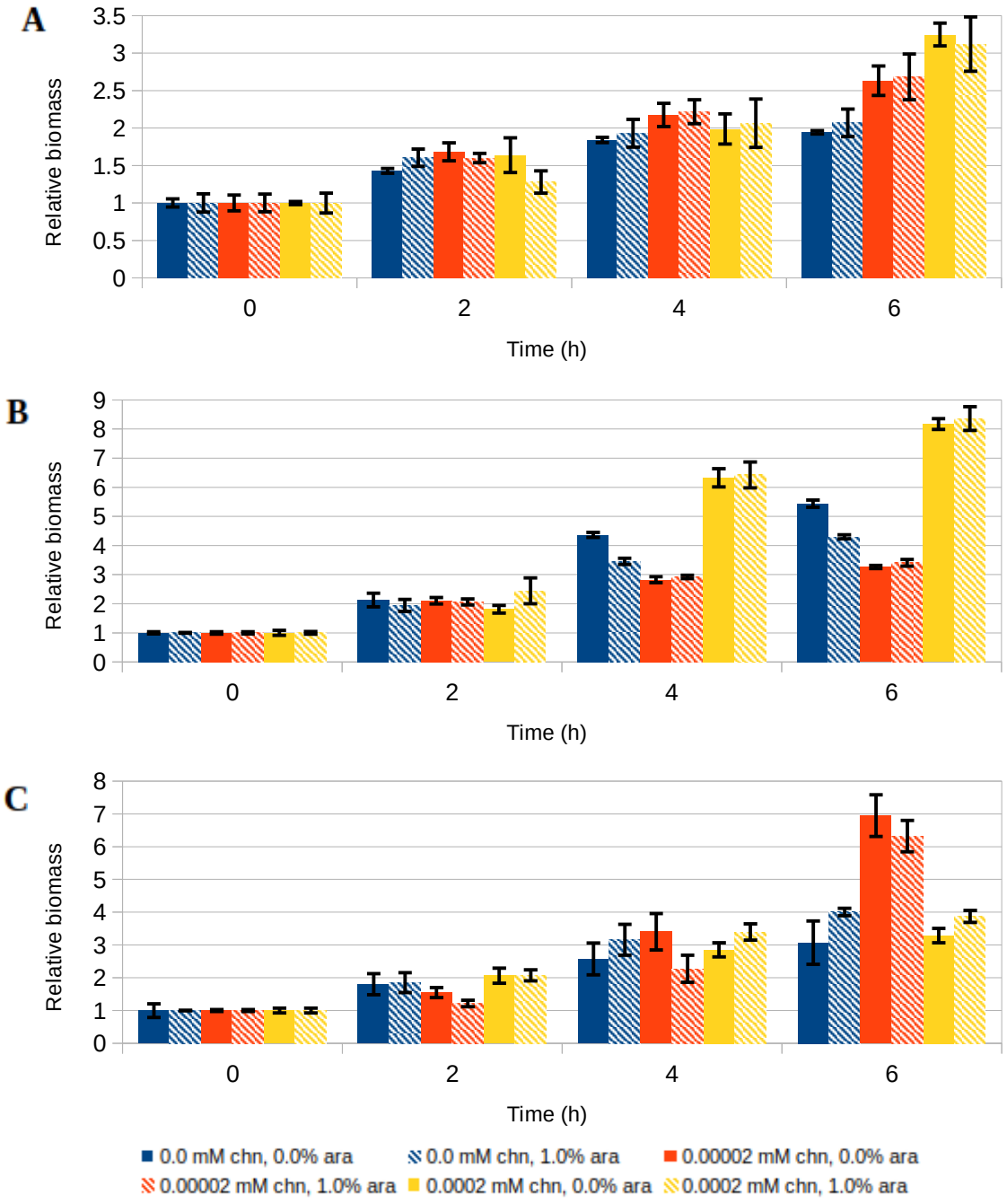

Figure Appendix D 11 *Relative biomasses (calculated by dividing the optical density at 600 nm of a time point by the optical density at 600 nm of the time point just before the induction) at 0, 2, 4 and 6 h after the induction of ADP1-Lux-GFP-sgRNA[GFP]-dCas9 (**A**), ADP1-Lux-GFP-dCas9 (**B**) and ADP1-Lux-GFP (**C**). Inducer concentrations were 0.0, 0.00002 mM and 0.0002 mM of cyclohexanone and 0.0 and 1.0% of arabinose. Each cyclohexanone concentration was tested with and without arabinose. In the strains ADP1-Lux-GFP-sgRNA[GFP]-dCas9 and ADP1-Lux-GFP-dCas9 cyclohexanone was used to induce dCas9 expression and arabinose was used to induce sgRNA expression in the strain ADP1-Lux-GFP-sgRNA[GFP]-dCas9. ADP1-Lux-GFP did not contain any part of the CRISPRi machinery. The error bars are standard deviations of three parallel cultures.*