Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biotechnology and Food Science

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Development of a chassis organism based on CRISPR-Cas engineering in *Paenibacillus polymyxa*

Master's thesis in Chemical Engineering and Biotechnology
Supervisor: Jochen Schmid
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Abstract

Paenibacillus polymyxa DSM 365 is a bacterium that has shown great potential as a producer of exopolysaccharides, antimicrobial compounds and chemicals such as 2,3-butanediol. Chassis development of this organism can make it more suitable for industrial production by improving product yield and purity. The aim of the thesis is to perform CRISPR-Cas mediated deletions of target genes in *P. polymyxa* to develop it towards a chassis organism. Seven novel mutant strains with deletions of genes for Pectin lyases, endospore formation proteins and part of the paenan biosynthetic gene cluster were developed. Additional CRISPR-Cas systems were assembled for the deletion of UDP-glucose dehydrogenases, the second part of the paenan cluster, and the entire paenan cluster. The transfer of plasmids to *P. polymyxa* were improved by optimizing the procedure for conjugation and demonstrating successful electroporation. Future work includes the verification of the deletions of the paenan gene cluster, deletion of the identified antimicrobial biosynthetic gene clusters, and further characterization of the mutant strains.

Sammendrag

Paenibacillus polymyxa DSM 365 er en bakterie som har vist lovende potensial som produksjonsvert for eksopolysakkarider, antimikrobielle forbindelser og kjemikalier som 2,3-butandiol. Ved å utvikle denne bakterien i retning av en modell organisme, kan den gjøres bedre rustet for industriell produksjon, samtidig øke utbyttet og renheten av produktene. Målet med denne avhandlingen er å gjøre genmodifiseringer i *P. polymyxa* ved hjelp av CRISPR-Cas systemer. Disse modifikasjonene skal bidra til å utvikle bakterien i retning av en modell organisme. Syv nye mutasjonsstammer med slettinger av gener for Pektin lyaser, endospore dannelse, og deler av paenan genklyngen har blitt utviklet. I tillegg har CRISPR-Cas systemer for sletting av UDP-glukose dehydrogenaser, andre del av paenan genklyngen, samt alle genene for paenan biosyntese, blitt konstruert og verifisert. Prosedyrene for overføring av plasmid til *P. polymyxa* stammer via konjugasjon har blitt optimalisert ved å studere effekten mineraler i næringskildene har på rekombineringen. Suksessfull elektroporering har åpnet opp for en alternativ overføringsmekanisme for plasmid. Videre arbeid inkluderer verifisering av sletting av paenan genklyngen og UDP-glukose dehydrogenasene. I tillegg gjenstår sletting av antimikrobielle biosyntese genklynger, og videre karakterisering av mutasjonsstammene.

Preface

This master's thesis concludes my degree in M.Sc. in Chemical Engineering and Biotechnology at the Norwegian University of Science and Technology. The thesis is conducted at the Department of Biotechnology and Food Science during the spring semester 2020. The work is a continuation of the specialization project which was completed during the fall of 2019.

Acknowledgments

I would like to extend my sincere thanks to my supervisor, Associate Professor Jochen Schmid, for excellent guidance and supervision during this work. His enthusiasm, dedication and extensive knowledge was a great inspiration to me. I also had the pleasure of working with PhD. candidate Meliawati Meliawati, and would like to extend my gratitude for her helpful advice and assistance in the lab. I am also grateful to Hege Hetland Pedersen for her insightful suggestions and for helping me with the BRIG software.

Special thanks to Matias, my friends and family for their support, and for making these last five years such a joyful time.

Declaration

I hereby declare that the work in this study has been conducted honestly and independently, and all used references are denoted.

Trondheim, July 27, 2020,

Anne Kristin Haraldsvik

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Abbreviations

2.3-BD 2,3-butanediol.

AFB American foulbrood.

amp Ampicillin.

antiSMASH Antibiotics and secondary metabolites analysis shell.

BLAST Basic local alignment search tool.

BRIG BLAST ring image generator.

Cas CRISPR associated protein.

clm Chloramphenicol.

CRISPR Clustered regularly interspaced palindromic repeats.

crRNA CRISPR RNA.

D-Leu D-leucine.

D-Phe D-phenylalanine.

DS Downstream.

EPS Exopolysaccharides.

G α-L-guluronic acid.

genta Gentamycin.

GRAS Generally regarded as safe.

GT Glycosyltransferase.

HDR Homology-directed repair.

HPLC High performance liquid chromatography.

IAA Indole-3-acetic acid.

ICA Indole-3-carboxylic acid.

ISO Isothermal.

ISR Induced systemic resistance.

kan Kanamycin.

L-Dab L-diaminobutyric acid.

L-Ile L-isoleucine.

L-Leu L-leucine.

L-Thr L-threonine.

LPS Lipopolysaccharide.

M β-D-mannuronic acid.

neo Neomycin.

NHEJ Non-homologous end-joining.

NRPS Non-ribosomal peptide synthetases.

OE-PCR Overlap-extension PCR.

OPX Outer membrane export protein.

PAM Protospacer adjacent motif.

PCP Polysaccharide co-polymerase protein.

PCR Polymerase chain reaction.

PGPR Plant growth-promoting rhizobacteria.

PKS Polyketide synthases.

poly Polymyxin.

RAST Rapid annotation using subsystem technology.

RiPP Ribosomally synthesized and post-translationally modified peptides.

TOL Indole-3-ethanol.

tracrRNA Trans-activating crRNA.

UndPP Undercaprenol pyrophosphate.

US Upstream.

1 | Introduction

1.1 *Paenibacillus polymyxa*

A genus of bacteria that exists in environments from the ice cold Antarctica to the tropical soil of Brazil is the *Paenibacillus*[1]. The resilient genus include strains with a considerable potential for applications within agriculture, medical technology and process manufacturing[1][2]. Species of *Paenibacillus* are known to cause the honeybee disease American Foulbrood (AFB), and represents opportunistic pathogens in humans[1].
Paenibacillus is a Gram-positive genus that belongs to the *Firmicutes* phylum[3]. They were previously classified as *Bacillus* because of their physical properties, among them the ability to form endospores. Studies of the 16S rRNA sequences lead to the phylogenetic distinction of *Paenibacillus* from other *Bacillus* groups[4][5]. *Paenibacillus* is mostly found in soil, often in relation to plant roots[1]. There they act as a plant growth-promoting rhizobacteria (PGPR), gaining nutrients from the plant while fixating nitrogen and protecting the plant against pathogenic microbes[2].

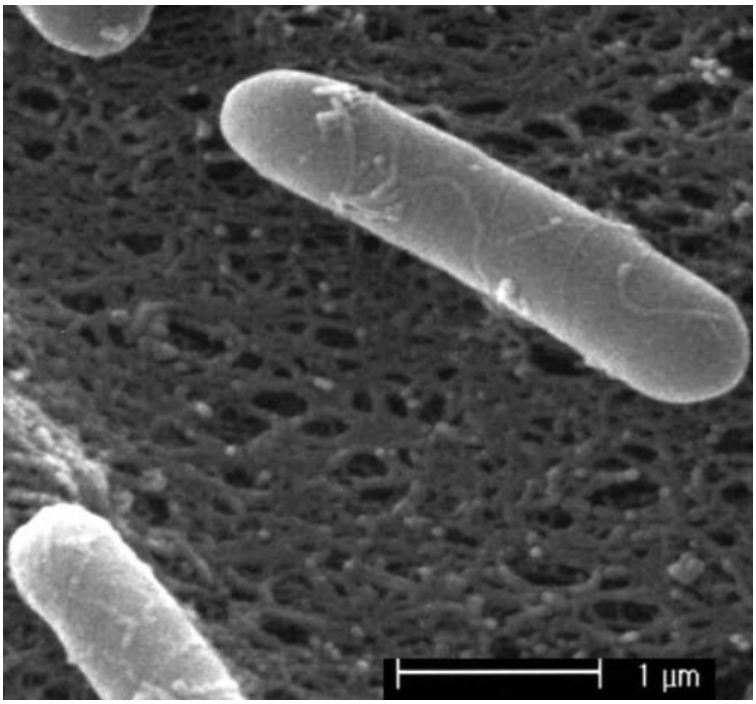


Figure 1.1.1: *Paenibacillus polymyxa* OSY-DF cells. Photo is taken with scanning electron microscopy by He et al. [6].

Paenibacillus polymyxa (*P. polymyxa*) has been isolated from a number of habitats, such as the roots of barley[7], corn[8], peppers[9], lodgepole pine[10], and from the cow rumen[11]. *P. polymyxa* strains have been investigated as potential biofertilizers. There the role of the bacterium is to improve plant growth by two different mechanisms, either directly or indirectly[2]. The first mechanism, direct plant growth promotion, includes nitrogen fixation, phosphate solubilization, iron acquisition, and phytohormone production. Nitrogen and phosphorus are highly central compounds in chemical fertilizer[12]. The production of these compounds require a high amount of energy, release of toxic hydrogen fluoride gas, and the finite resource which is rock phosphate[1]. Nitrogen and phosphorus supply to plants via *P. polymyxa* would be beneficial for the plant and could at the same time avoid the harmful effects of chemical fertilizer production. *P. polymyxa* can additionally produce auxin metabolites such as Indole-3-acetic acid (IAA), indole-3-ethanol (TOL), and indole-3-carboxylic acid (ICA)[13]. The auxin metabolites can be taken up by the plant and are highly influential for stimulation of plant growth[14].

Indirect PGPR mechanisms include secretion of antimicrobial substances, insecticides, and activation of induced systemic resistance (ISR)[1]. Antimicrobial substances produced by *P. polymyxa* include lipopeptides and polyketides. These are complex molecules made by non-ribosomal peptide synthetases (NRPS) and polyketide synthases (PKS)[15].

Well known lipopeptides produced by *P. polymyxa* are polymyxins [16]. Polymyxins are a family of antibiotics that were discovered already in 1947 and were at the time exceptional in their ability to target Gram-negative bacteria. They were named polymyxin after the first organism recognized to produce the substance, *P. polymyxa*[17]. The chemical structure of the different polymyxins are in general a decapeptide with a fatty acyl group at the N-terminal end. The decapeptide always includes a cyclic heptapeptide loop and specific amino acids at positions 1, 2, 4, 5, 8 and 9. Figure 1.1.2 shows the general structure of polymyxins, where black residues are conserved while the residues at the red positions can vary between the polymyxin types. L-diaminobutyric acid (L-Dab) is always found in five of the positions. L-threonine (L-Thr), D-leucine (D-Leu), L-leucine (L-Leu), D-phenylalanine (D-Phe), and L-isoleucine (L-Ile) are the most common residues at the variable positions[17]. In total, 37 different polymyxins have been identified up to now. These are categorized into 10 distinct groups, named alphabetically. Figure 1.1.3 shows the chemical structure of polymyxin B sulphate.

The primary target for polymyxin molecules are the lipopolysaccharides (LPS) bound to the outer membrane of Gram-negative bacteria. A combination of electrostatic and hydrophobic interactions between polymyxin and lipidA, a component in the LPS, lead to the disruption of the outer membrane. Resistance to polymyxin can occur by several mechanisms. A bacterium can modify it's LPS layer or it can get rid of the LPS layer entirely and thus prevent the polymyxin from binding to it. It can also secrete capsular exopolysaccharides that prevent the polymyxin from binding to the LPS. Lastly, efflux pumps are also known to increase the resistance to polymyxin[17]. Polymyxins have not been used much in humans the last decades because of their nephro-and neurotoxicity. However, they are sill used to treat multi-drug resistant bacterial infections[18][17]. Researchers are currently working on developing novel antimicrobial lipopeptides inspired by polymyxin to

Figure 1.1.2: General chemical structure of polymyxins. Polymyxins consist of a fatty acyl group and a decapeptide which includes a cyclic heptapeptide. L-diaminobutyric acid (L-Dab) are always present in position 1, 4, 5 , 8 and 9. L-Threonine (L-Thr) is present in position 2, while the other peptide positions (marked with red writing) can vary from one polymyxin type to another[17].

target resistant Gram-negative bacteria[17].

Other antibiotic substances produced by *P. polymyxa* are fusaricidin, tridecaptin, paenilipoheptin, and paenilan[2][19]. Insecticide components includes proteins that degrade the exoskeleton of insects and toxic crystal proteins[20][21]. Induced systemic resistance refer to the state, triggered by an inducer molecule, in which an entire plant becomes resistant to insect herbivores and pathogenic microbes[22]. One of these inducers is 2,3-butanediol (2,3-BD), produced by *P.polymyxa* DSM 365[23].

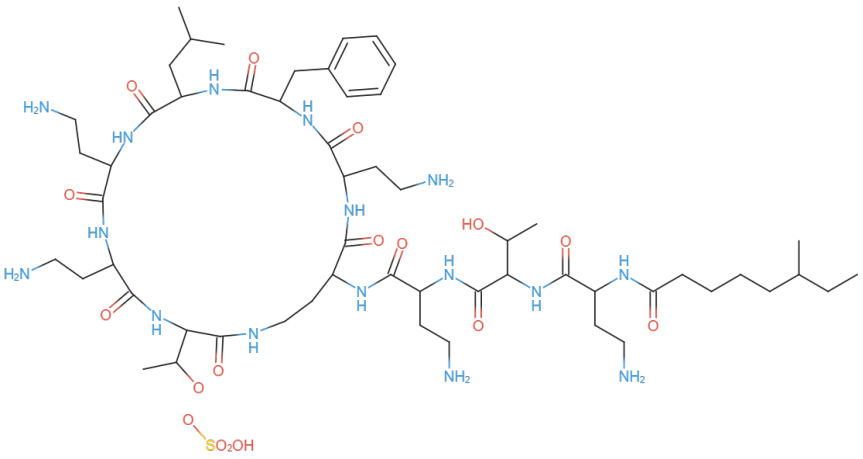


Figure 1.1.3: Chemical structure of polymyxin B sulphate[24].

P. polymyxa strains have been investigated as producers of industrially and medically important substances, such as enzymes, solvents and exopolysaccharides[1]. Enzymes like amylases, cellulases, lipases, and pectinases has been documented as products by *Paenibacillus*[1]. 14 different enzymes with endoglucanase activity have been identified for one single *P. polymyxa* strain[25]. These hydrolyzing enzymes are important for processing of cellulose and biomass derived plant fibers to produce valuable chemicals and biofuels[25]. *P. polymyxa* DSM 365 has been shown to be an efficient producer of 2,3-BD[26]. 2,3-BD and its derivatives are used in a variety of applications, from food flavoring to cosmetics or anti-freezing agents. The conversion of 2,3-BD into 1,3-butadiene is important for synthetic rubber industry while

methyl ethylketone, dehydrated 2,3-BD, is used as an effective additive in fuels[27]. Generally, important objectives for the process design and process engineering are product yield, economic sustainability, safety, product purity, regulatory considerations and to minimize the environmental impact.[28]. Production of 2,3-BD by *P. polymyxa* DSM 365 ensures a higher purity and production yield by only producing the relevant (2R,3R) isomer compared to other 2,3-BD producing organisms[27]. *P. polymyxa* is able to grow on a variety of biomass derived carbon sources, which makes it possible to circumvent fossil based production of 2,3-BD. The production can also be made economically sustainable by using cheap substrates and bio-waste as nutrient sources[26]. A safe production host is advantageous because it simplifies the production in industrial scale. Exemplary, organisms such as *Klebsilla* sp and *Serratia* sp are also good 2,3-BD producers. However, they are risk class 2 organisms what complicates industrial production as they would have to follow strict regulatory guidelines[26]. *P. polymyxa* is generally regarded as safe (GRAS) and is a risk class 1 organism. The high product yield and purity, the safety and the nutrient requirements, show how *P. polymyxa* possess a high potential for the sustainable production of 2,3-BD in the context of a growing bio-based industry[29][26].

Paenibacillus spp. are known producers of a wide range of different exopolysaccharides. *P. polymyxa* strains are described to produce levan, curdlan and paenan[30][31]. Levans are used for pharmaceutical and medical purposes, as well as in food and cosmetic products[32]. Curdlan is widely used as a thickening agent in the food industry, while paenan has shown promising physicochemical properties in surfactant systems[31].

1.2 Exopolysaccharides

Biopolymers produced by microorganisms are gaining more attention as alternatives to synthetic polymers and biopolymers derived from other bio-based sources such as plants or animals. Although synthetic polymers are more established in both production and application, there are certain advantages that are in favor of microbial biopolymers. The microbially produced biopolymers are more sustainable as they are renewable and biodegradable. Their biocompatibility also make them better suited for many applications within life science technology. Unlike biopolymers produced from plant-based sources they are not competing with food production industry for agricultural resources[33]. Microbial polymers can be produced from marine and agricultural waste. One example is the production of polysaccharides by *P. macerans* TKU029 growing on squid pen powder[30]. The development of novel tools for genomic engineering opens the opportunity to make tailor-made biopolymers[34]. Some challenges with microbial biopolymers are to optimize the fermentative production for an increased yield. As well as to make the production commercially competitive to synthetic polymers.

Microorganisms can produce polysaccharides with large variation in structure according to their biological function. Glycogen is produced for intracellular storage of energy. Capsular polysaccharides are closely associated with the cell surface and help protect the microbe. Exopolysaccharides (EPS) are microbial polysaccharides that are secreted towards the environments[35]. All these polysacharides have in common, that they are are biological macromolecules, composed of monosaccharide

units bound together by glycosidic linkages into high molecular weight chains[36]. They can consist of only one type of monosaccharide, in which case they are called homopolysaccharides, or they can be composed of several different monomer types. They are then called heteropolysaccharides. Levan is an example of an EPS consisting of only fructose units[30]. Paenan is another EPS built up by units of glucose, mannose, galactose, and glucuronic acid, as produced by *P. polymyxa*[31].

The structural and rheological properties of a biopolymer is not only determined by the types of monomer it includes, but also its macroscopic structure. Alginate is an example of a biopolymer where the properties largely depend on the intramolecular organization. Alginate consist of β-D-mannuronic acid (M) and α-L-guluronic acid (G). MG-blocks, where M and G are alternating, increase the solubility of the polymer. Long GG-blocks, regions containing only G, can lead to intramolecular cross-linking and gel formation[37]. The properties of alginate shows how important it is to understand both the monomer structure, ratio and distribution in the polymer.

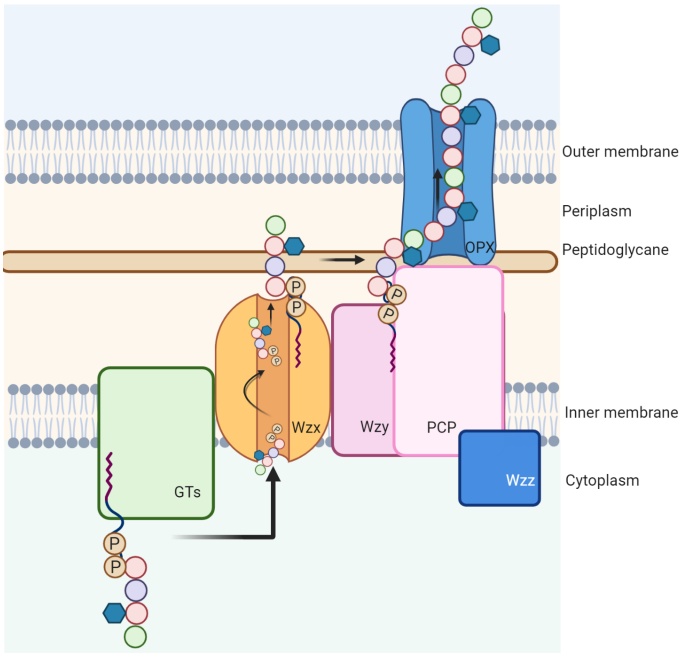


Figure 1.2.1: Overview of the Wzx/wzy-dependent pathway. The mechanism for biosynthesis of paenan in *P. polymyxa* DSM 365 is believed to follow the the Wzx/Wzy-dependent pathway. Repeating units of sugar monomers are attached to a lipid linker by Glycosyltransferases (GTs). Then the Wzx protein, also called flippase, translocate the repeating units. The Wzy protein polymerize the units and channel them towards the outer membrane polysaccharide export proteins (OPX) which transports them through the outer membrane. The polysaccharide co-polymerase protein (PCP) supports the Wzy and OPX, while the Wzz is important for chain length regulation.

The production of paenan by *P. polymyxa* is proposed to occur through the Wzx/Wzy-dependent pathway[34]. An illustration of this pathway is shown in Figure 1.2.1. In this pathway sugar monomers are attached to a lipid carrier on the cytoplasmic side of the inner cell membrane. This lead to the formation of an undercaprenol

pyrophosphate (UndPP). Different glycosyltransferases (GTs) add different sugar monomers until a polysaccharide repeat unit is attached to the UndPP[38]. Then a Wzx-protein, also called flippase, transpose the polymer units across the inner membrane and into the periplasmic space. The repeating units are then polymerised by the Wzy-protein. They are subsequently transported through the outer membrane by polysaccharide co-polymerase and outer membrane polysaccharide export proteins[35]. The Wzz protein determines the length of the polymer[38].

The gene cluster responsible for biosynthesis of paenan in *P. polymyxa* was identified by Rütering et al.[34]. This gene cluster spans 34.2 kb and encodes all the proteins needed in the EPS biosynthesis through the Wzx/Wzy dependent pathway. It encodes proteins involved in transport, EPS length regulation, GTs, polymerases, flippases, and glycosyl hydrolases. Several of the genes for a typical EPS biosynthesis are present in duplicates in the gene cluster. This is believed to either support a more reliable EPS production, allow for the assembly of two different repeating units, or to enable the production of two different EPS types, what is not clarified up to now. Figure 1.2.2 shows a schematic overview of the EPS gene cluster in *P. polymyxa*. Based on the duplicate genes for the flippase and the polymerase, the operon encoding paenan biosynthesis was assigned and named *Cluster1* and *Cluster 2* by Rütering et al.

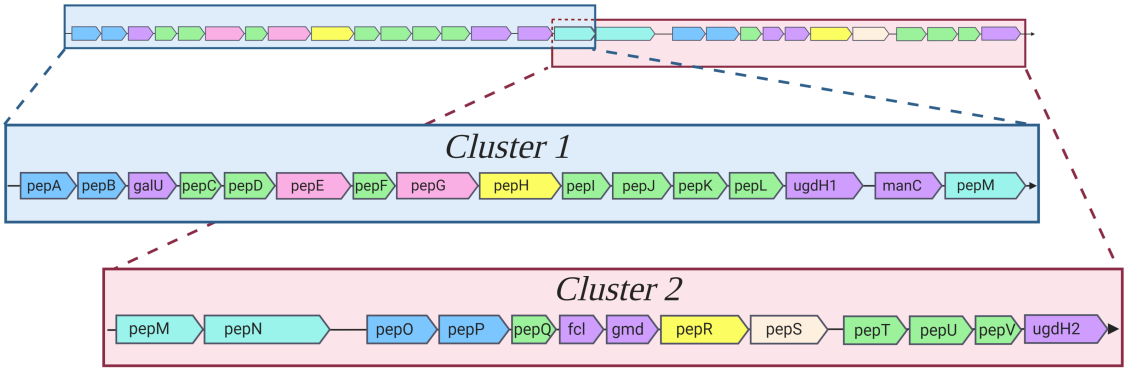


Figure 1.2.2: Gene cluster for production of paenan in *P. polymyxa* DSM 365. The operon was assigned *Cluster 1* and *Cluster 2* by Rütering et al. Proteins encoding chain length regulation and transport through the outer membrane are colored in dark blue. Purple arrows symbolize genes related to signaling and nucleotide sugar biosynthesis. Glycosyltransferase genes are marked in green, while pink arrows indicate polysaccharide polymerases. Translocation proteins (flippases) are encoded by the yellow genes. The bright blue arrows indicate glycosyl hydrolases. The beige arrow shows the *pepS* gene, which is still uncharacterized. [34]

1.3 Chassis development

During the recent years a drastic increase in microbial organisms holding promising potential for metabolic engineering and industrial production has been observed[39]. Previously, only a few "model" microbes were used to produce most of the biotechnologically produced chemical compounds. The lack of efficient and robust tools for

genomic engineering was blamed for the small assortment and low complexity of the microbially produced compounds[39]. With the emergence of novel genome modification technologies such as CRISPR/Cas9 and DNA assembly methods such as Golden Gate cloning and Gibson Assembly, new possibilities rose within metabolic engineering[40]. These innovations enable the successful design and assembly of microbial cell factories. The new technologies can be exploited to optimize conditions for microbial production or enable the production of new compounds from the same organism. Synthetic biology strives to engineer and produce biological systems with predictable functions in an automated and standardized matter[39]. By cross-linking knowledge found through systems biology, synthetic biology and metabolic engineering, the prospect of developing microbial strains with specific properties is highly promising[40].

A chassis organism can be described as *an organism that houses and supports genetic components by providing the resources that allow them to function*[40]. The development of a chassis organism can occur either by the top-down, or by the bottom-up approach. Completely synthetic organisms such as *JCVI-syn3.0*, which has a genome of only 531,560 bp, are likely to be close to the minimal set of genes required for cell viability[39]. The top-down approach involves the deletion of genes and nucleotide sequences to reduce the size of the genome. The top-down chassis developments do not require the same in-depth understanding of all essential metabolic pathways, as the bottom-up approach. The top-down approach can therefore be a well suited method when developing a chassis from novel, or less familiar, microbial strain. Based on research by Nikel and Calero, Nora et al. and Xu et al. the key points in the process of chassis development are the following[39][41][42]: Selection of host organism, development of molecular tools and characterization, and optimization of production. Figure 1.3.1 illustrates the main points of chassis development.

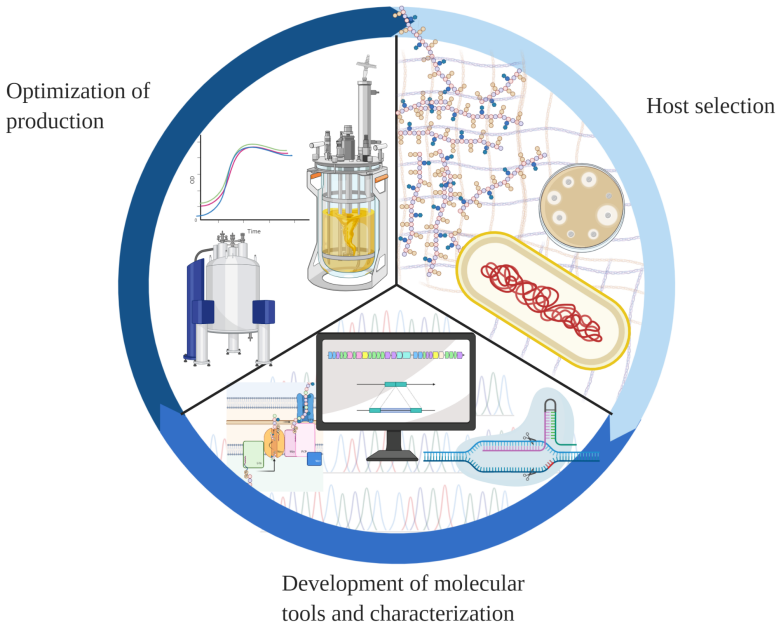


Figure 1.3.1: Three key points in the development of a chassis organism: Selection of host organism, development of molecular tools and characterization, and optimization of production.

Selection of host organism

The natural first step in the process of developing a microbial chassis is to determine the purpose of the chassis. Suitable candidates are selected based on the characteristics of the desired product. *Bacillus subtilis* is an example of a platform organism that is used for protein production because of its protein secretion capacity[39]. A second example is *Vibrio natriegens*, which is known for it's rapid growth rate and has therefore been investigated as a host for molecular cloning procedures[39]. The two examples show how some of the native properties of the strain can lay the foundation for their fitness as chassis organisms. This chapter will generally focus on the development of chassis organisms for production purposes. Nikel and Calero proposed six features that an ideal chassis used in metabolic engineering should possess[39]:

1. Knowledge about the strain. This includes the genome sequence, growth conditions and product properties.
2. Simple nutrient requirements. It should be able to grow on cheap and accessible carbon and nitrogen sources.
3. Tolerance to harsh conditions. The strain should be able to keep a stable growth and production while exposed to tough physical condition, such as high temperatures.
4. Rapid growth. A fast growth can lead to higher product yields.
5. Available and suitable molecular tools. This includes tools for targeted genome modifications and for metabolic regulation.
6. Inherent machinery for efficient secretion of the product. This is especially important for the secretion of larger molecules such as proteins and polysaccharides.

The list of features can be a useful guideline when searching for the best suited host organism. Another feature that can be important is whether the strain is generally recognized as safe (GRAS). Xu et al. highlights some of the features as more important when selecting a host organism. The strain should naturally be en efficient producer of the product. Then knowledge about the genome and metabolic network involved in production mechanism, in addition to access to functional genome editing tools are emphasized. Toxicity of products or intermediates should also be considered and evaluated. Finally, Xu et al. recommend that the organism have the ability to grow quickly on affordable substrates, and enable cheap purification processes[42].

Development of molecular tools and characterization

After selection of host organism for chassis development comes the phase where molecular tools have to be developed and the strain must be further characterized. Genome modification tools are essential for the investigation of genes, gene clusters and products secreted by the organism. If none of the already existing tools are functional in the organism, new ones need to be developed. Protocols and procedures for modification and characterization also have to be tested and established. Bioinformatics tools such as RAST, NCBI BLAST, CELLO 2.5, KEGG and an-

tiSMASH can be used to annotate genes and increase the understanding of the biosynthetic pathways involved[42][34]. Predictions of gene functions and pathway mechanisms via genetic models can be tested experimentally and provide more information about the strain. Synergy between molecular genetics and systems biology can be used to improve the accuracy of genetic models which in turn can lead to a better characterized chassis organism[42].

Optimization of production
A sufficiently characterized chassis organism with well established modification procedures lay the basis for further optimization of the microbial cell factory. A library of mutants can be established by doing genome deletions, insertions or replacements. The properties of the product can be altered by inserting heterologous genes that are predicted to promote certain characteristics. The new products should then be analyzed, both to learn about the properties of the new product, and to get an understanding of the role of these genes in the biosynthetic pathway. When the cellular production mechanism is well understood it could potentially be possible to engineer products with specific properties according to the area of application.

The genome can also be reduced to increase growth rate, purity of product, production yield as well as robustness. Genes coding for toxic substances and non-essential products, as well as non-coding DNA are typically targets for genome reduction. Methods such as dynamic regulation and directed evolution can be used to optimize the strains production capacity[42].

1.3.1 Current state for the *P. polymyxa* DSM 365 chassis

Rühmann et al. developed a method for fast analysis of the monomer composition of microbial EPS. This method enabled screening of large numbers of novel polysaccharides with specific properties[43][44][45]. Rütering et al. used this method to screen for EPS secreting microorganisms, and found that *Paenibacillus* sp. 2H2 was an effective producer. It was also discovered that the strain was able to produce both levan and another heteropolysaccharide (paenan), depending on the nutrient composition[29]. The heteropolymer produced by *Paenibacillus* sp. 2H2 and it's close phylogenetic relative *Paenibacillus polymyxa* DSM 365, was further characterized. The polymer was found to have exceptional properties, such as shear thinning behavior with a high viscosity at low-shear rate, stable gels and compatibility with a number of surfactants[31]. To get a better understanding of the biological mechanism for the production of EPS in *P. polymyxa* DSM 365, the genome was annotated and the gene cluster for paenan synthesis was identified[34]. A CRISPR-Cas tool for genome editing in *P. polymyxa* was designed and constructed. This tool was further used to investigate the mechanism for biosynthesis of paenan, which follows the Wzx/Wzy pathway. Deletion of certain genes in the gene cluster resulted in EPS with unique physicochemical properties[34]. Schilling et al. has made mutant strains for the individual deletion of all the glycosyl transferases, the *pep*-genes and a combination of the *pep* knock outs in the paenan cluster, to analyze the chemical structure of this polysaccharide (Unpublished data).

To summarize the current state of the chassis development of *Paenibacillus polymyxa* DSM 365: A host organism has been selected on the basis of the characteristics of one of it's products. The genes responsible for biosynthesis of exopolysaccharides

have been identified, and the mechanism for production has been proposed. A CRISPR-Cas system for the host organism has been constructed. This will allow further genome modifications in the strain and, in principle, for the production of custom-made exopolysaccharides. The process of developing a library of knock out mutants has begun and will potentially give valuable information about the biosynthetic machinery in *P. polymyxa* DSM 365.

1.3.2 Future potential and perspectives

P. polymyxa DSM 365 hold promising potential as a chassis organism for microbial production of polysaccharides, 2,3-BD and antimicrobial compounds. Still, many challenges lies ahead before the microbial cell factory is ready for the industry. The understanding of how the different genes impact the final properties of the polymer need to be increased. It should be clearly established how the knock out of any of the genes in the cluster affect the final EPS produced. Methods for the insertion of heterologous genes and assembly of synthetic gene clusters should be determined. By establishing a library of mutants with variation in gene cluster composition and characterize their products, it could be possible to predict how the combination of genes can be altered to obtain products with specific properties.

To ensure that the products are safe and have a high purity, one can remove genes encoding proteins that are toxic themselves or contribute to the synthesis of compounds that are unsafe. For *Paenibacillus polymyxa* DSM 365 some natural targets are genes responsible for synthesis of antimicrobial compounds, chemicals like 2,3-butanediol, and spore formation. When heterologous genes are to be expressed, native EPS production can be eliminated by removing genes for levan and paenan synthesis. To prevent the degradation of the produced polymers one could try to remove hydrolases and lyases from the organism. The same principle follows for the production of antimicrobial compounds and 2,3-BD. The ambition could be to engineer a streamlined chassis organism that keep the full (or extended) functionality of the original strain while having removed all non-essential and harmful traits. Research should focus on which carbon-sources give the best production with the lowest substrate costs. The strains ability to kill other microorgansims and be used for production in non-sterile conditions should also be assessed to further reduce costs.

1.4 Genome editing tools in *P. polymyxa*

Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR associated (Cas) proteins are an important part of the antiviral defence mechanism in bacteria and archaea[46]. If a bacterium is attacked by a bacteriophage then a protospacer sequence is copied from the viral DNA and integrated into the CRISPR loci in the genome. Every protospacer sequence has a short palindromic repeating sequence on both sides. The protospacer sequences in the CRISPR loci are then used to recognize bacteriophages and let the Cas complex make a cut in the viral DNA[46]. The bacterial CRISPR-Cas system has been used to make targeted genomic modifications effectively in organisms of all domains of life[47][48][49][50].

Three types of CRISPR-Cas systems has been identified. Type I and III are similar in mechanism and involves several Cas proteins in one complex. CRISPR RNA (crRNA) is made by transcribing the genomic region containing the protospacer sequence, this gives the pre-crRNA, then the pre-crRNA matures into crRNA. The crRNA forms a complex with several Cas proteins and these perform the targeted double strand cut[51]. Type II CRISPR systems are more commonly used and only depend on one Cas enzyme. The first step in the mechanism is to transcribe the CRISPR loci where the protospacers and the repeating sequences are located, the transcript is called pre-CRISPR RNA (pre-crRNA). A trans-activating crRNA (tracrRNA) is a sequence that is complementary to regions in the repeating sequences that are naturally found between protospacers in the CRISPR loci[46]. The tracrRNA binds to repeating units in the pre-crRNA. Then RNase III activity lead to the maturation of pre-crRNA into crRNA while still being associated with the tracrRNA. This structure forms a complex with the Cas9 protein[52]. To avoid self-targeting, the system is searching for both the target sequence and a protospacer adjacent motif (PAM) that varies between CRISPR-Cas systems[52][53].

Cas9 is a DNA endonuclease that make a double-stranded cut in the DNA. While associated to the crRNA and tracrRNA, the Cas9 enzyme will use the crRNA and the PAM to search the genome until it finds the target sequence. Then it cleaves the DNA at the specific site[47]. The crRNA and the tracrRNA can be fused by a loop linker into a single guide RNA (sgRNA) unit[51]. Figure 1.4.1 shows the CRISPR-Cas system locating and making a cut in the target sequence.

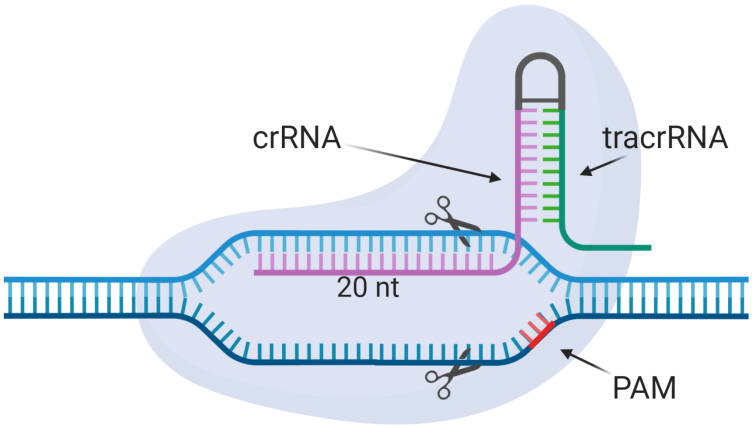


Figure 1.4.1: Schematic overview of the CRISPR-Cas mechanism. CRISPR RNA (crRNA) associated with trans-activating crRNA (tracrRNA) forms a complex with the Cas9 endonuclease. The protospacer-adjacent motif (PAM) is a sequence of three bases that is specific for the Cas protein and helps prevent self-targeting. The CRISPR-Cas sequence uses the PAM sequence and the 20 nucleotide sequence, complementary to the target sequence, to make a double strand break in the DNA at the correct location.

After the double strand cut in the target DNA sequence, the organism is dependent on being able to repair the break to survive. In general the DNA repair can be done by either non-homologous end-joining (NHEJ) or by homology-directed repair

(HDR). During NHEJ the double strand breaks are religated while HDR lead to homologous recombination by the use of a homologous template[54]. DNA repair by NHEJ can lead to mutations and insertion or removal of nucleotides[55]. This can be useful for gene knock outs because the DNA scar can prevent transcription or lead to non-functional product[55].

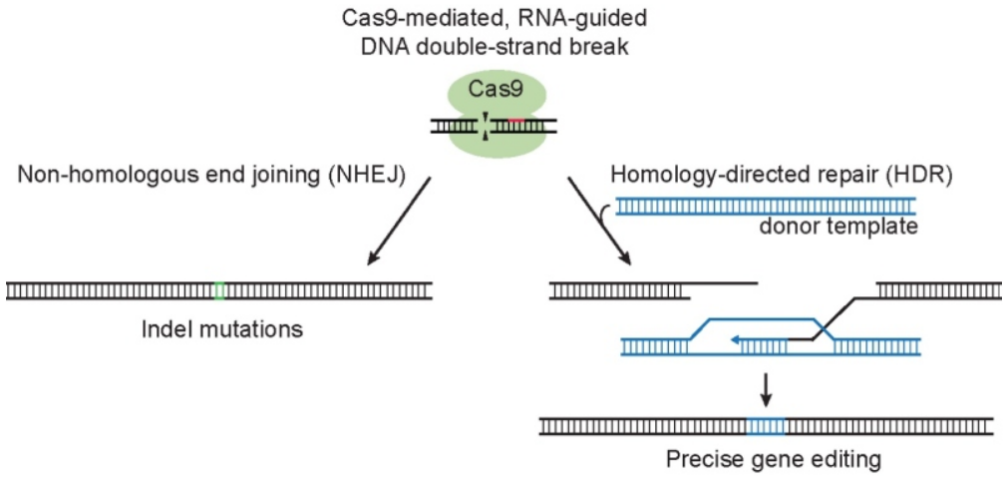


Figure 1.4.2: Repair mechanisms after double-stranded DNA break. After a CRISPR-Cas system has made a double stranded break in the DNA it can be repaired by either non-homologous end joining (NHEJ) or homology-directed repair (HDR). During NHEJ the DNA strands are repaired directly and this can induce mutations in the gene. Bacteria are not able to do NHEJ and repair DNA breaks by HDR. A HDR-template is then used to facilitate homologous recombination and correct ligation of the DNA strands. Figure by Marraffini et al.[56].

Most bacteria are not able to repair double strand DNA breaks by non-homologous end joining. They therefore use the HDR mechanism after Cas9 endonuclease activity. This mechanism depends on the presence of a HDR-template. For gene deletions the HDR-template is built up by homology arms from upstream and downstream regions of the gene. When a sequence should be replaced by a different one, it needs to contain the new sequence between the two homology arms[55]. When a double strand break by the Cas9 was performed, the bacteria is able to detect the HDR-template and use it for HDR-repair[56].

A CRISPR-Cas system has been designed and established by Rütering et al. for genome modifications in *P. polymyxa*. The system has been constructed as a plasmid called pCasPP. This plasmid contains genes for the Cas9 endonuclease, a *gapdh* promoter, loop linker and tracrRNA, neomycine selection marker and *repU* for plasmid replication. The unspecified system contains a *lacZ* cassette surrounded by BbsI restriction enzyme cut sites. This allows for the insertion of the 20 nucleotide spacer (referred to as sg) by Golden Gate Cloning. A SpeI restriction site allows for the insertion of HDR-template by restriction and insertion cloning[34]. Figure 1.4.3 shows the unspecified CRISPR-Cas system.

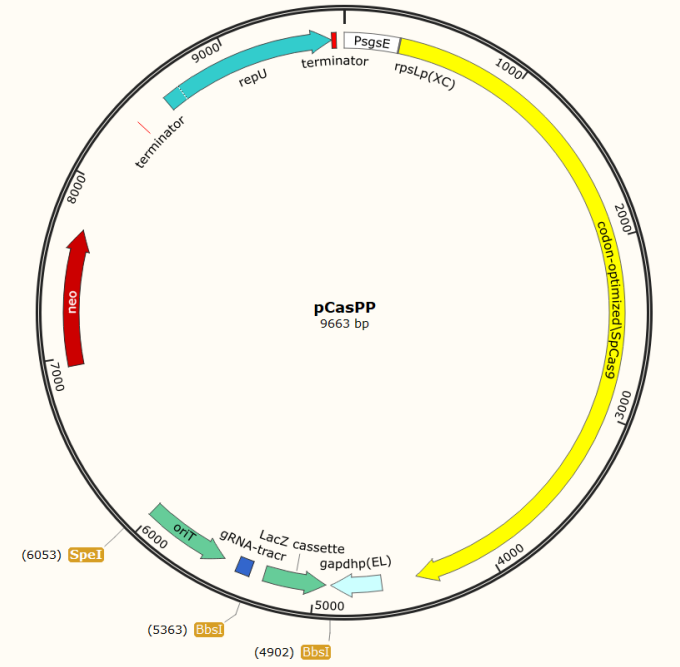


Figure 1.4.3: pCasPP: a CRISPR-Cas system designed for genome editing in *P. polymyxa*. The pCasPP contains genes for Cas9, repU for plasmid replication, *gapdh* promoter and neomycin resistance gene. The tracrRNA (gRNA-tracr) is located next to the BbsI cut site. This allows for simple insertion of the 20 nt guide sequence via Golden Gate Cloning. A SpeI restriction enzyme cut site facilitates insertion of HDR-template. The CRISPR-Cas system was made by Rütering et al.[34].

1.5 Aim of the thesis

1.5.1 Theoretical background

Pectin lyases are enzymes that break the glycosidic bond between the α-D-galacturonic acid monomers in pectin[57][58]. Two genes for Pectin lyases are found in the genome of *P. polymyxa* DSM 365 by RAST annotation: pectin lyase (EC 4.2.2.10) in contig 01 and Pectin lyase like protein in contig 19. The first is referred to as *PL01* while the second is referred to as *PLLP19* in this thesis. These are targets for gene deletion because it is desired to avoid degradation of the EPS product. CRISPR-Cas system for the deletion of *PL01* was designed and constructed during the previous specialization project by the author[59]. Both plasmids will be used to make deletions of pectin lyase genes in *P. polymyxa*.

Endospore formation is a property that is very useful for *P. polymyxa* in natural conditions as it makes the strain able to survive though conditions [3]. However, from an industrial production perspective this ability is not advantageous. The spores can contaminate the end product and it is therefore desired to remove this property from the strain. The cellular mechanism for endospore formation in *P. polymyxa* is thought to have similar characteristics to that of *Bacillus subtilis*. During stressful conditions signaling molecules are released by sensor proteins. This lead to a series of reactions involving transfer of phosphorus. This will result in a highly phosphorylated sporulation factor protein, called Spo0A. SpoIIE is a protein that is very important for transferring the signal from Spo0A to SpoIIAA and thus activating the latter protein. This result in the release of σ factor from the SpoIIAB which lead to the formation of endospores[3]. The endospore formation will stagnate without the activation of SpoIIAA. Schmid et al. has previously been able to eliminate the endospore formation by deleting the gene for SpoIIE. This deletion strain, *P. polymyxa* ΔSpoIIE, will therefore be used as a starting strain for further gene deletions.

To facilitate the introduction of heterologous or synthetic EPS clusters it is necessary to first remove genes and gene clusters responsible for native production of EPS. This is to prevent impure products or interference from native products. The levansucrase gene, *sacB*, is responsible for the biosynthesis of levan, while the paenan EPS cluster is responsible for the paenan production. The whole paenan EPS cluster is referred to as *Clu1Clu2*. *Clu1* refers to the first part from *pepA* to right after *pepM* and *Clu2* refers to the part just before *pepM* to after *ugdH2*. See Figure 1.2.2 for a detailed look at the paenan EPS cluster.

During previous work it was discovered that *P. polymyxa* Δ*Clu1* had reduced it's resistance to the antibiotic polymyxin[59]. It is desired to learn more about how polymyxin is produced and understand the mechanism behind polymyxin resistance. To be able to do gene deletions with strains that are sensitive to polymyxin, it will therefore be necessary to find an alternative antibiotic as a selection agent.

Loutet et al. found that there was a connection between UDP-glucose dehydrogenase activity and polymyxin B sensitivity in *Burkholderia cenocepacia*[60]. In the paenan gene cluster there are two genes for UDP-glucose dehydrogenase: *ugdH1*

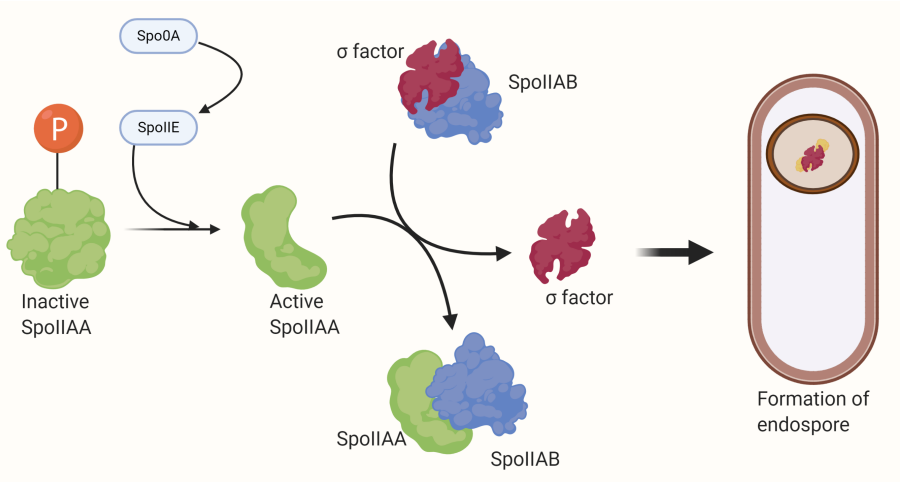


Figure 1.5.1: Role of SpoIIE in endospore formation. The sporulation factor Spo0A becomes highly phosphorylated during stressful conditions. The SpoIIE protein transfer the signal from Spo0A and activates the SpoIIAA protein. This lead to the release of a σ factor from the SpoIIAB protein, which in turn starts the process of endospore formation.

and *ugdH2*. CRISPR-Cas systems for the individual deletion of both of these genes will be constructed. The production and sensitivity to polymyxin will be analyzed after deletion of the two genes.

Bioinformatic analysis will be used to identify and annotate the genes responsible for polymyxin production. They will also be used to detect genes for other antimicrobials in *P. polymyxia* DSM 365. These could potentially be targets for future gene deletions. Comparison of genomes of other related strains will also be used to increase the understanding of the production and sensitivity to relevant antimicrobials. After the identification of the polymyxin gene cluster it is also desired to remove this cluster from the genome.

Previously, issues were experienced with transferring CRISPR-Cas plasmids to *P. polymyxa* strains by conjugation. It was discovered that presence of calcium chloride in the nutrient source could have a significant impact on the amount of clones obtained from conjugations. A goal for this thesis is therefore to investigate further how the mineral addition affects the recombination efficiency during a conjugation.

1.5.2 Aim

The aim of this thesis is to develop *P. polymyxa* DSM 365 towards a chassis organism through CRISPR-Cas based genome modifications. For this, ten genes and gene clusters in *P. polymyxa* DSM 365 were determined as targets which should be deleted. The goal of the deletions were to make the strain more suitable for industrial production and to get a better understanding of certain mechanisms of this strain. Methods for transfer of the CRISPR-Cas system into the chassis candidate will also be tested and optimized to simplify further engineering of the strain.

Finally, the deletion of paenan EPS cluster, levansucrase, polymyxin cluster, SpoIIE, and pectin lyases genes will be combined in the same strain. This strain will then be characterized. The aim of this thesis can be summarized as:

- Targeted gene deletions to optimize production yield and purity.
- Targeted gene deletions to facilitate heterologous expression of EPS.
- Investigation of genes involved in production of antimicrobial compounds, especially polymyxin.
- Combine the gene deletions in a target chassis strain.
- Characterization of the modified strains.
- Optimize procedures for future genome modifications.

Table 1.5.1: Targets for gene deletions

Target gene	Function
PL01	Degradation of pectins
PLLP19	Degradation of pectins
SpoIIE	Sporulation
Clu1	EPS production
Clu2	EPS production
Clu1Clu2	EPS production
ugdH1	Signalling/regulation and nucleotide sugar biosynthesis
ugdH2	Signalling/regulation and nucleotide sugar biosynthesis
pmx	Polymyxin production
sacB	Production of levan

2 | Materials and methods

This chapter contains all the procedures and protocols used during this thesis. Section 2.1 describes digital tools, section 2.2 contains protocols for media and solutions, and section 2.3 gives an overview of the bacterial strains and plasmids used. Sections 2.4 - 2.6 describes methods used for DNA isolation, analysis and molecular cloning. Protocols developed by professional providers of tools and equipment are described in their original formats, given in Appendix C. Sections marked with the citation [59]* originates from the TBT4500 Biotechnology, specialization project.

2.1 *In silico* design and bioinformatic analysis

Primers for the construction of plasmids, amplification of genes and positive controls, as well as analysis of successful gene knock outs were designed mainly by two softwares: Benchling and SnapGene.[59]*

2.1.1 www.benchling.com

Benchling is an online tool that was mainly used for the design of primers and guide sequences for the deletion of specific genes using the pCasPP CRISPR-Cas system. The Benchling tool "CRISPR" was used to design oligos for the guide sequences of the pCasPP constructs. This tool let the user define which sequence that is going to be deleted and which CRISPR-Cas system that is going to be used. The guide sequences with highest on-target score and lowest off-target score are given as the top rated results. To design primes for verification of successful gene knock outs, the "run Primer3" function was used. This gives a list of the best suited primers within the given requirements, and primers are chosen from this list[61] [59]*.

2.1.2 SnapGene

SnapGene is a software that allows for in-silico cloning by visualization, planning and documentation of processes related to molecular biology. Through this project it has been used to design primers, insert specific restriction enzyme cut sites, visualize gel electrophoresis, PCR and assembly reactions. It has also been used to analyze sequencing results and document cloning steps. [62][59]*.

2.1.3 BLAST

The *Basic Local Alignment Search Tool* (BLAST) developed by NCBI can be used to find similarities between DNA or protein sequences. BLAST compares a query sequence with sequences stored in the BLAST database and uses this to provide information about the sequence. Local alignment, used by BLAST, align short segments or domains that are similar, unlike the global alignment where the sequences are aligned in their full length. The tool is then able to detect more similarities not only in strains that are closely related [63][64].

BLAST can be used through NCBIs website, or as a standalone application. During this project the web site (`http://blast.ncbi.nlm.nih.gov`) has been used to do BLAST analysis of DNA regions. The standalone application BLAST+ was used indirectly when the BRIG program was utilized. In the NCBI BLAST website several types of alignment searches can be performed. Table 2.1.1 shows the five most common search tools on NCBIs website.

Search page	Query vs database
blastn	nucleotide vs nucleotide
blastp	protein vs protein
blastx	nucleotide (translated) vs protein
tblastn	protein vs nucleotide (translated)
tblastx	nucleotide (translated) vs nucleotide (translated)

Table 2.1.1: An overview of some of the functions available at the NCBI BLAST website. (`http://blast.ncbi.nlm.nih.gov`)

A BLAST analysis of a query sequence gives the following output values: Max score, total score, query cover, e-value, and percent identity. The number of characters that match for the longest fragment in a result is given by the max score. While the total number of aligned characters is given by the total score. The query cover tells how large part of the query that is similar enough to align with the database sequence. While the percent identity shows the percentage of aligned bases that are the same as the query. The e-value estimates the number of alignments that would happen by random at a given score[64][65].

2.1.4 antiSMASH

The *Antibiotics and Secondary Metabolites Analysis Shell* (antiSMASH) is a bioinformatics tool that can be used to detect gene clusters encoding antimicrobial peptides and metabolites. AntiSMASH is able to detect 52 different types of biosynthetic gene clusters based on identification of conserved core enzymes co-occurring in the genome. The tool takes advantage of a number of available tools such as NCBI BLAST+, HMMer 3, MUSCLE 3, FastTree, PySVG and Jquery SVG to do a time efficient analysis of genome sequences. AntiSMASH is the most established tool for biosynthetic gene cluster analysis in genomes of bacteria and fungi. During this project it has been used to detect gene clusters encoding antimicrobial peptides, and to analyze the polymyxin gene cluster[66].

2.1.5 BRIG

BLAST Ring Image Generator (BRIG) software tool that allows for circular visualization of BLAST comparisons. The program uses CGview to create the image and BLAST to do the DNA analysis. It has a graphical user interface and is therefore easier to learn and use, than the BLAST+ applications. BRIG can be used to show the similarity between a reference sequence and an unlimited number of query sequences. DNA files in FASTA, MULIT-FASTA and GenBank formats can be used as input arguments according to the set purpose. During the work of this thesis BRIG has been used to investigate and visualize the similarities in sequences related to polymyxin synthesis and resistance in *Paenibacillus* species [67].

2.2 Media and solution

LB
10 g/L Tryptone
5 g/L Yeast extract
5 g/L NaCl
LB agar plates
15 g/L Agar extract was added to the ingredients in LB before autoclavation. [59]*

LB plate variations

Table 2.2.1: Variations of the LB agar plates. Additives are added to 500 mL autoclaved LB agar in sterile conditions.

Plate name	Additives
LB with trace elements	500 μL trace elements solution
LB $CaCl_2$ [60 mM]	22 mL $CaCl_2{\cdot}2H_2O$ [200 g/L]
LB $CaCl_2$ [30 mM]	11 mL $CaCl_2{\cdot}2H_2O$ [200 g/L]

SOB
20 g/L Tryptone
5 g/L Yeast extract
0.5 g/L NaCl [59]*

0.8 % GelRed and 0.8 % GelGreen
3.2 g Seakem agarose
400 mL 1 x TAE-buffer
Microwaved solution until it boiled and dissolved completely. Then added 20 μL GelRed or GelGreen dye solution according to desired product.

Vitamins solution (RPMI 1640)
Vitamins solution was used to prepare EPS media and EPS agar. The ingredients are as follows:
0.02 g/L D-Biotin

0.3 g/L Choline chlorid
0.1 g/L Folic acid
3.5 g/L Myo-inositol
0.1 g/L Niacinamide
0.1 g/L p-Amino benzoic acid
0.025 g/L D-Pantothenic acid · 0.5 Ca
0.1 g/L Pyridoxal · HCl
0.02 g/L Riboflavin
0.1 g/L Thiamine · HCl
0.0005 g/L Vitamin B12
0.2 g/L KCl
0.2 g/L KH_2PO_4 (Anhydrous)
8.0 g/L NaCl
1.15 g/L Na_2HPO_4
[59]*

Trace elements solution
1.80 g/L $MnCl_2 \cdot 4H_2O$
2.50 g/L $FeSO_4 \cdot 7H_2O$
258 mg/L Boric acid
31.0 mg/L $CuSO_4 \cdot 7H_2O$
21.0 mg/L $ZnCl_2$
75.0 mg/L $CoCl_2 \cdot 6H_2O$
23.0 mg/L $MgMoO_4$
2.10 g/L sodium tartrate · $2H_2O$
Dissolved all elements and sterile filtered the solution. Trace elements solution was prepared by Jochen Schmid during the project. [59]*

EPS-medium
The final EPS media consist of the following ingredients:
30.0 g/L Glucose
5.0 g/L Casein peptone
1.33 g/L $MgSO_4 \cdot H_2O$
20.0 mL/L NaOH [2.0 M] (added to adjust pH in the at last)
20.0 mL/L KH_2PO_4 [83.5 g/L]
1.0 mL/L $CaCl_2 \cdot 2H_2O$ [50 g/L]
2.0 mL/L Vitamins solution (Sigma Aldrich)
1.0 mL/L Trace elements solution

Glucose and peptone were autoclaved separately at the given concentrations. Stock-solutions of magnesium sulphate, potassium phosphate and calcium chloride were autoclaved separately. All the autoclaved solutions, the vitamins solution and the trace elements solution were mixed together under sterile conditions after cooling down to room temperature. pH was adjusted by taking an aliquote of the medium, measure the pH and the amount of sodium hydroxide necessary to get a neutral pH. Then the amount of base necessary to get a pH of seven in the entire medium was calculated and added under sterile conditions.

To prepare plates with EPS, 1.5 % agar extract was added to the peptone solution before autoclavation. [59]*

Pectin broth
11 g/L K_2HPO_4
5.5 g/L KH_2PO_4
1.2 g/L $(NH_4)_2SO_4$
0.4 g/L $MgSO_4$
0.15 g/L $CaCl_2 \cdot 2H_2O$
4.0 g/L Pectin
Slowly added pectin to warm water while stirring. Then autoclaved pectin and salts separately. Finally ingredients were mixed under sterile conditions.

Electroporation buffer
250 mM Sucrose
1 mM HEPES
1 mM MgCl
10 % Glycerol
Autoclave and check that pH=7.0. Store at 4°C.

Neomycin stock solution [50mg/mL]
0.50 g Neomycin
10 mL Sterile water
Let the neomycin dissolve completely before filtering it through a 0.22 μm syringe filter. [59]*

Polymyxin stock solution [20mg/mL]
0.20 g Polymyxin
10 mL Sterile water
Let the polymyxin dissolve completely before filtering it through a 0.22 μm syringe filter. [59]*

Glycerol stocks
400 μL Glycerol [60%]
600 μL Bacterial culture
Mix by pipetting. Freeze at -80°C. [59]*

5x isothermal (ISO) reaction buffer
Prepared 6 mL 5x ISO reaction buffer by mixing the components listed in Table 2.2.2 in a 15 mL tube.

Table 2.2.2: Concentrations of each component in the 5x isothermal (ISO) reaction buffer. Concentration of stock solutions and amount used to get the correct concentrations.

Component	Stock solution	Volume/amount
25 (% w/v) PEG-8000	PEG-8000	1.5 g
500 mM Tris/Cl pH 7.5	1 M Tris/Cl pH 7.5	3000 μL
50 mM $MgCl_2$	2 M $MgCl_2$	150 μL
50 mM DTT	1 M DTT	300 μL
1 mM dATP	100 mM dATP	60 μL
1 mM dCTP	100 mM dCTP	60 μL
1 mM dGTP	100 mM dGTP	60 μL
1 mM dTTP	100 mM dTTP	60 μL
5 mM NAD	100 mM NAD	300 μL
	sterile ddH_2O	up to 6000 μL

1. Weigh out the PEG-8000.
2. Add the remaining ingredients from stock solutions.
3. Add sterile ddH_2O to a final volume of 6 mL.
4. Mix by vortexing until solution is homogenous.
5. Sterile filter the solution and store in 100 μL aliquots at -20°C.

5x ISO reaction buffer was provided by M. Meliawati during the project period.

Gibson Assembly master mix
Prepared 1.2 mL Gibson Assembly master mix by mixing the ingredients listed in Table 2.2.3 by pipetting up and down.

Table 2.2.3: Amount of each component in the Gibson Assembly master mix.

Component	Volume/amount
5x ISO reaction buffer	320 μL
T5 exonuclease (10 U/μL)	0.64 μL
Phusion High-Fidelity DNA Polymerase (2 U/μL)	20 μL
Taq DNA ligase (40 U/μL)	160 μL
Sterile ddH_2O	699.36 μL

The Gibson Assembly master mix was aliquoted with 15 μL in each, and stored at -20°C.

2.3 Bacterial stocks and plasmids

Table 2.3.1: Overview of the bacterial strains used and made during the project. NF=not finished.

Beginning of Table 2.3.1

Bacterial strains	**Description**	**Source**
P. polymyxa DSM 365	Wild type	DSMZ
P. polymyxa ΔSpoIIE	The SpoE2 gene is deleted, removing the strain's ability to form spores.	Jochen Schmid
P. polymyxa ΔClu1	The cluster 1 region is deleted, removing the strains ability to produce native exopolysaccharides.	Jochen Schmid
P. polymyxa ΔPL01	The pectin lyase gene is deleted, possibly removing the strains ability to degrade pectin.	This work
P. polymyxa ΔSpoIIEΔPL01	Strain without SpoIIE and pectin lyase genes	This work
P. polymyxa ΔPLLP19	Strain without the pectin lyase like protein.	This work
P. polymyxa ΔSpoIIEΔPLLP19	Strain without SpoIIE, and the pectin lyase like protein genes.	This work
P. polymyxa ΔSpoIIEΔPL01ΔPLLP19	Strain without SpoIIE, and the pectin lyase like protein and pectin lyase genes.	This work
P. polymyxa ΔPL01ΔPLLP19	Strain without PL01 and PLLP19 genes.	This work
P. polymyxa ΔClu1ΔPLLP19	Strain without Cluster 1 and PLLP19 genes.	This work
P. polymyxa ΔSacB	Strain without levansucrase	This work. NF
P. polymyxa ΔSpoIIEΔPL01ΔSacB	Strain without genes for sporulation, pectin lyase and levansucrase	This work. NF
P. polymyxa ΔPL01ΔPLLP19ΔSacB	Strain without genes for pectin lyase, pectin lyase like protein and levansucrase	This work. NF
P. polymyxa ΔClu2	Strain without the Cluster 2 part of the EPS gene cluster.	This work. NF
P. polymyxa ΔClu1Clu2	Strain without the two parts of the EPS cluster.	This work. NF
P. polymyxa ΔugdH1	Strain without the ugdH1 gene.	This work. NF
P. polymyxa ΔugdH2	Strain without the ugdH2 gene.	This work. NF
P. polymyxa ΔugdH1ΔugdH2	Strain without the ugdH1 and ugdH2 genes.	This work. NF

Continuation of Table 2.3.1

Bacterial strains	**Description**	**Source**
P. polymyxa ΔClu1Clu2 ΔpmxΔSpoIIEΔPL01ΔPL LP19ΔSacB	Target chassis strain	This work. NF
E. coli S17-1	Conjugation strain. recA pro hsdR RP42Tc::Mu-Km::Tn7	ATCC 47055
E. coli DH5α	Chemically competent, rapid growth. F– endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20 ϕ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17(rK–mK+), λ–	NEB
E. coli turbo	Chemically competent cells, rapid growth. glnV44 thi-1 Δ(lac-proAB) galE15 galK16 R(zgb-210::Tn10)TetS endA1 fhuA2 Δ(mcrB-hsdSM)5, (rK–mK–) F′[traD36 proAB+ lacIq lacZΔM15]	NEB

End of Table 2.3.1

Table 2.3.2: Overview of the plasmids that were used and made during the project. NF=not finished.

Plasmids	**Description**	**Source**
pCasPP	CRISPR-Cas system designed for gene editing in *P. polymyxa*	[34]
pCasPP Clu1	CRISPR-Cas system for deletion of Cluster 1 in *P. polymyxa*	[34]
pCasPP SacB	CRISPR-Cas system for deletion of the SacB gene in *P. polymyxa*	[34]
pCasPP PL01	CRISPR-Cas system for deletion of pectin lyase (E.C 4.2.2.10) in *P. polymyxa*	[59]
pCasPP PLLP19	CRISPR-Cas system for deletion of pectin lyase like protein in *P. polymyxa*	This work
pCasPP Clu2	CRISPR-Cas system for deletion of Cluster 2 in *P. polymyxa*	This work
pCasPP Clu1Clu2	CRISPR-Cas system for deletion of entire EPS cluster in *P. polymyxa*	This work
pCasPP ugdH1	CRISPR-Cas system for deletion of ugdH1 in *P. polymyxa*	This work
pCasPP ugdH2	CRISPR-Cas system for deletion of ugdH2 in *P. polymyxa*	This work
pCasPP pmx	CRISPR-Cas system for deletion of polymyxin gene cluster in *P. polymyxa*	This work. NF

Primers are listed in Appendix A and referred to by number.

2.4 DNA isolation

2.4.1 Genome extraction

Genomic DNA of *P. polymyxa* was extracted using*DNeasy blood and tissue kit* from Qiagen. In order to use the "Purification of Total DNA from Animal Tissues (spin-column protocol)" in this kit on *P. polymyxa* it was necessary to first do the "Pre-treatment for Gram-Positive Bacteria". During the pretreatment process, cells were harvested via centrifugation and subsequently lysed with lysozyme. Proteins were digested by adding proteinase A. The DNA was then precipitated by adding ethanol and the solution was transferred to a spin-column. The DNA was then bound to the membrane of the spin column and excess enzymes and contaminants were washed away. The DNA was then eluted from the membrane and collected in a microcentrifuge tube. Detailed protocol can be found in Section C.1.

2.4.2 Plasmid extraction

Plasmids were extracted from *E. coli* using the *GeneJET Plasmid Miniprep Kit*. Cells were first harvested by centrifugation, resuspended in resuspension solution and treated with an alkaline lysis solution. The solution was then neutralized to improve conditions for binding of DNA to the silica membrane in the spin-column. The solution was centrifuged to separate the solubilized DNA from the cell and protein debris. The supernatant were transferred to the spin column and DNA was bound to the membrane. Contaminants were washed away with an ethanol base washing buffer and the plasmid DNA was eluted into a microcentrifuge tube with an elution buffer. Section C.2 shows detailed protocol for plasmid extraction using this kit.

2.5 DNA analysis

2.5.1 Nanodrop spectrophotometer

A Nanodrop spectrophotometer was used to measure DNA concentration for samples of linear, plasmid and genomic DNA. Concentration is given in nano grams per micro liters. Two ratios regarding the quality of the sample are given as well, A260/280 and A260/230. A260/280 describes the purity of DNA and RNA. When DNA is analyzed, a value around 1.8 indicates high purity. The A260/230 ratio describes how pure the nucleic acids are. Values of A260/230 should be 2.0-2.2. Proteins, carbohydrates, phenols, EDTA and other contaminants in the samples can give a higher or lower value for the A260/280 and A260/230 ratios[68]. [59]*.

2.5.2 Gel electrophoresis

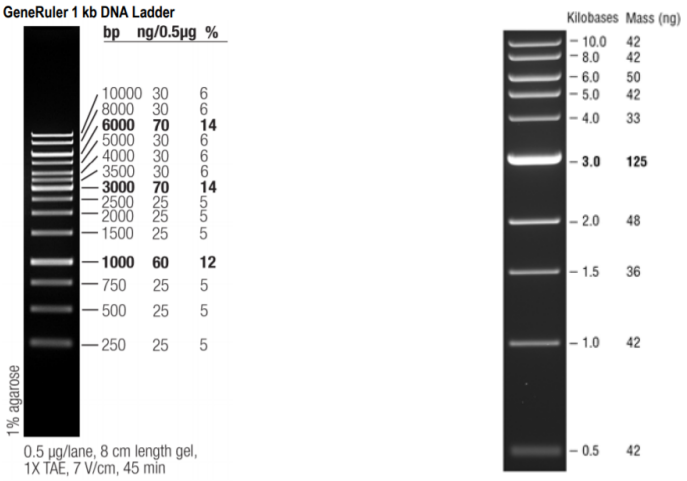
Gel electrophoresis was done using GelRed, 0.8 % agarose, for visualization of DNA lengths and GelGreen, 0.8 % agarose, when DNA was purified from the gel. DNA fragments were dyed using the following amounts:
Ladder:

- 2 μL Ladder
- 2 μL Loading dye
- 8 μL distilled water

Samples on GelRed:

- 5 μL sample
- 2 μL Loading dye
- 5 μL distilled water

Added dye to 20 % of sample amount when GelGreen was used. [59]*
In this project, the following *1 kb DNA Ladder* and *1 kb Plus DNA Ladder* from NEB was used:



(a) GeneRuler 1kb DNALadder from Thermo Fisher Scientific. **(b)** 1 kb DNA ladder from New England Biolabs

Figure 2.5.1: DNA ladders used during this project.

2.5.3 Gel purification

DNA fragments separated on GelGreen by gel electrophoresis was purified from the gel by the use of*E.Z.N.A. Gel Extraction Kit*. First, the DNA was excised from the gel with a scalpel. Then the agarose gel was solubilized by incubating it in binding buffer at 60°C. Then the DNA was bound to the membrane of the spin column and contaminants were washed away with an ethanol based washing buffer. At last, the DNA from the gel was recovered by elution into a new microcentrifuge tube. The protocol used is described in the appendix, section C.3.

2.6 Molecular cloning

2.6.1 DNA amplification by PCR

Polymerase chain reaction (PCR) was used to amplify specific DNA sequences. The Phusion High-Fidelity DNA polymerase (Phusion polymerase) and the Q5 High-Fidelity DNA Polymerase (Q5 polymerase) were used when amplifying sequences for cloning purposes. The Standard Taq DNA polymerase was used to amplify sequences that were used for verification of correct cloning or recombination. Exemplified, this means that for the Overlap-Extension PCR, the Phusion polymerase was used, while the fragment amplified to verify correct insertion of HDR-template was produced using Standard Taq polymerase. When the Phusion polymerase was used, the *PCR Protocol for Phusion High-Fidelity DNA Polymerase (M0530)* provided by NEB, was used. The protocol *PCR using Q5 High-Fidelity DNA Polymerase (M0491)* was followed when Q5 polymerase was used. While the *PCR Protocol for Taq DNA Polymerase with Standard Taq Buffer (M0273).* All protocols are given in the appendix, Sections C.4, C.5 and C.6. [59]*

2.6.2 PCR purification

PCR products were purified using the *GeneJET PCR Purification Kit #K0702* to remove enzymes, primers and buffers used during the PCR. First, the PCR products were mixed with a binding buffer. This binding buffer denatures proteins and assists in binding of DNA to the silica membrane in the spin columns. Impurities were washed away with an ethanol based buffer. Then the purified PCR products were eluted into a new microcentrifuge tube. Detailed protocol is found in the appendix, Section C.7.

2.6.3 Overlap-extension PCR

Amplify the upstream fragment (US) and the downstream fragment (DS) by PCR with genomic DNA from *P. polymyxa* DSM 365 as template. Phusion High-Fidelity DNA Polymerase and associated protocol was used. Overlap-extension PCR (OE-PCR) fuses the US and DS fragment and amplify it by the following procedure:
First PCR: Fusion of US and DS
Mix the following components in a PCR reaction tube. The polymerase is the component that is added to the mixture when the rest of the components are already added.

Component	**Amount**
Phusion HF-Buffer	6 μL
Phusion DNA Polymerase	0.3 μL
dNTPs	0.6 μL
DMSO	0.6 μL
DS and US fragment	50 ng
H_2O	add to 30 μL

The PCR is set to the following program: 1 min at 98°C then 10 cycles of 10 sec-

onds at 98°C, 30 seconds at 55°C and 15 seconds of 72°C. Finally the temperature is decreased to 12°C. [59]*

Second PCR: Amplification of the fused fragments
After the first PCR is done, the following components are added to the PCR reaction tubes:

Component	**Amount**
Phusion HF-Buffer	4 μL
Phusion DNA Polymerase	0.2 μL
dNTPs	0.4 μL
DMSO	0.4 μL
Forward US primer	5 μL
Reverse DS primer	5 μL
H_2O	5 μL

The mixtures are then run by a new PCR program. 20 cycles of 10 seconds at 98°C, 30 seconds at 57°C and 15 seconds at 72°C. Then 10 minutes at 72°C and cooling to 12°C. [59]*

2.6.4 Enzyme digestion

Type II restriction enzymes were used to cut the pCasPP and the HDR-templates before ligating the fragment and vector. The HDR-template was cut with XbaI, while the pCasPP was cut with SpeI, which has a corresponding cut site. The NEBcloner was used to find correct digestion protocol for a specific enzyme. [59]*

2.6.5 Ligation

Ligation of insert fragment into a vector was done by following the *Ligation Protocol with T4 DNA Ligase (M0202)*, shown in section C.8. The molar ratios were calculated using the NeBioCalculator[69]. Molar ratios of 3:1, 1:1 and 5:1 were used. [59]*

2.6.6 Golden Gate cloning

The Golden Gate cloning procedure was done according to the protocol described in supporting information to [34]. Linearized background fragments were not reduced as it was not necessary in this case. The protocol was then as follows:
Phosphorylation and annealing of oligonuclotides by mixing the following:

Component	**Amount**
Forward primer [100μM]	1 μL
Reverse primer [100μM]	1 μL
Polynucleotide kinase	1 μL
T4 ligase buffer	1 μL
Distilled water	6 μL

The mixture was incubated at 37°C for 30 min, then heated up to 95°C for 5 min. The mixture was then cooled down to 25°C by decreasing the temperature by a half degree every sixth seconds. After the annealing process, the mixture was diluted 1:100, and 1 μL was utilized for guide insertion via Golden Gate assembly. [70] [59]*

Component	**Amount**
BbsI-HF	0.75 μL
T7-Ligase	0.25 μL
10 x Cutsmart Buffer	1 μL
ATP [10 mM]	1 μL
DTT [10 mM]	1 μL
Plasmid backbone	100 ng
Annealed gRNA, diluted 1:100	1 μL
Distilled water	up to 10 μL

2.6.7 Gibson Assembly

The DNA segments to be assembled were produced and amplified by PCR reactions using the Phusion High-Fidelity DNA polymerase. Successfully amplified fragments were visualized by gel electrophoresis on GelGreen and purified from the gel. Molar ratios of the fragments were determined based on fragment length. For 10 μL Gibson Assembly reactions there were added a total amount of DNA of around 0,3-0,4 pmol. The DNA mixture were added to the prepared Gibson Assembly master mix and mixed by pipetting. The reaction was then incubated at 50°C for one hour. A 1:4 dilution with nuclease free water was made from the reaction mixture. Transformed two parallels with 4 μL of the dilution and of the reaction mixture into *E. coli* DH5α chemically competent cells. A sample from the reaction mixture was also visualized by gel electrophoresis to test for the presence of the correctly assembled product. [71]

2.6.8 Chemical transformation

Thawed competent cells on ice for 10 minutes. Then 1 pg - 100 ng plasmid DNA was added to the cells, and the tubes were flicked 4-6 times. Placed the mixture on ice for 30 minutes, heat shocked at 42°C for 45 seconds, and placed on ice for 5 minutes. Then 950 μL SOB media was pipetted into the mixture. Let the cell mixture incubate at 37°C, 225 rpm for 60 minutes. 100 μL cell solution was added to room tempered plates and incubated at 37°C for 8-12 hours. [59]*

2.6.9 Conjugation

Conjugation was used to transfer plasmids from *E. coli* S17-1 (donor strain) into *P. polymyxa* (recipient strain). Cultures of donor strain were grown in selective LB-media made with a sodium chloride concentration of 1 g/L over night. The recipient strain was grown in non-selective LB-media of the same type as used for the donor strain over night. Both cultures were diluted 1:100 the next day. The recipient strain was then grown for five hours and the donor strain for four hours. 900 μL recipient cultures was heat shock for 15 min at 42°C. Then the recipient culture

was mixed with 300 μL donor strain culture, and centrifuged for 3 min at 8000 x g. Discarded most of the supernatant, resuspended the cells and dropped the solution on non-selective plates with a $CaCl_2 \cdot 2H_2O$ concentration of 60 mM. The next day the colony was scraped from the agar and resuspended in 500 μL 0.9% NaCl. Plated the solution on selective LB agar and incubated at 30°C for 48 hrs. [59]*

2.6.10 Preparation of electrocompetent cells

Procedure for preparation of electrocompetent *Paenicabillus polymyxa* cells were adapted from procedure published by Zarschler et al. [72].
Preparations:
Electroporation buffer consisting of sucrose, HEPES, $MgCl_2$ and glycerol was prepared and stored at 4°C.
The *P. polymyxa* strains that were desired to make electrocompetent were streaked out on LB agar plates from glycerol stocks. The plate was incubated at 30°C over night. The next day a single colony was isolated and inoculated into 3 mL LB media in a 13 mL tube. The culture was grown at 30°C, 225 rpm over night. Then 1 mL preculture was inoculated into 100 mL LB in a 250 mL side-baffled Erlenmeyer flask. Then the cultures were grown until they reached an OD of 0.2-0.4. Kept electroporation buffer, 1.5 mL eppendorf tubes, falcon tubes and pipette tips in the refrigerator (4°C). Centrifuges were cooled to 4°C.

Preparation of electrocompetent cells:

1. Put cells on ice for 10 min.
2. Transfer 25 mL cell culture to a 50 mL falcon tube and centrifuge for 20 min at 4200 x g and 4°C.
3. Discard supernatant and wash cell pellet with 25 mL electroporation buffer. Centrifuge for 10 min at 4200 x g, 4°C.
4. Repeat the previous step two more times.
5. Discard supernatant and resuspend cells in 1 mL electroporation buffer.
6. Make 50 μL aliquots of the cell suspension in 1.5 mL cool eppendorftubes.
7. Shock frost in liquid N_2.
8. Store at -80°C

2.6.11 Electroporation

Thaw 50 μL aliquots of electrocompetent *P. polymyxa* cells on ice for ca 10 min. Add 100-500 ng plasmid DNA and mix by inverting tube carefully. Let the mixture sit on ice for 5 min. Then add 50 μL sterile dH_2O and mix by pipetting. Transfer the mixture to an ice-cold electroporation cuvette with either 1 mm or 2 mm gap width. Pulse with preprogrammed *Escherichia coli* program, EC1 for the 1 mm gap cuvette and EC2 for the 2 mm gap cuvette. Immediately add 1 mL LB or SOB media to the cuvette, then transfer the mixture to a 1.5 mL eppendorftube. Keep

the tubes on ice for 15 min. After cooling, the suspensions were transferred to 15 mL falcon tubes and diluted to 10 mL with LB media. Incubate the tubes at 30°C, 170 rpm for four hours. Plate 150 μL culture to selective plates (LBneo). Pellet cells by centrifuging for 10 min at 4700 rpm, then discard most of the supernatant. Resuspend the cells and plate 150 μL to selective plates. Incubate both plates at 30°C for 48 hours.

2.6.12 Curing

Plasmids transferred by conjugation were cured by picking colonies from the selective plates and transferred to non-selective LB-media. The cultures were diluted and tested on LB plates with neomycin to see if they still kept the plasmid. When the plasmids were cured, the strain was no longer be resistant to neomycin.

2.7 Characterization

2.7.1 Pectin assay

The pectin assay protocol was inspired by a pectin agar assay protocol by Laurent et al. and adapted to a pectin assay for liquid cultures during this project[73]. The ability to degrade pectin by different strains of *P. polymyxa* was assessed by observing the growth properties in pectin broth. Made preculture of the strains in question by inoculating 3 mL LB with a single colony. Let incubate at 30°C and 225 rpm over night. The next day, the precultures were diluted 1:100 by adding 1 mL preculture to 100 mL media in 250 mL side-baffled Erlenmeyer flask. Two parallels were made for each strain, one in LB and one in pectin broth. Cultures were incubated at 30° and either 170 or 225 rpm shaking. Measured the OD at 600 nm after ca 0, 2, 4, 6, 8, 12, 24, 30, 36, 50 and 60 hours. For each measurement three samples of 1 mL were measured, and the average value was used further.

2.7.2 Antibiogram

Labeled LB agar plates with strain name and number where the paper discs were going to be placed. Covered the LB agar with 100 μL overnight culture. Placed 6 mm paper discs onto marked space on the agar plates. Slowly added 20 μL antibiotic solution onto the disc. Let the plates incubate at 30°C for 24 hours when growth has become confluent. Measured the diameter of clear growth inhibition zone around the discs. Reported the result as either resistant (R), intermediate (I), or susceptible (S).

2.7.3 Antibiotic susceptibility test

Prepared LB agar with the desired concentration of antibiotic. Added 10 μL overnight culture to marked space on plate. Streaked out a zigzag pattern of the same overnight culture on the plate as well. Determined the antibiotic susceptibility by evaluating the amount of growth.

3 | Results

During the course of this thesis CRISPR-Cas systems were assembled for targeted gene deletions in *P. polymyxa*. Deletion mutant strains were constructed and certain properties were characterized. Protocol for conjugation was optimized based on the effect of calcium chloride in nutrient sources. Antimicrobial biosynthetic gene clusters in *P. polymyxa* DSM 365 were identified based on bioinformatic analysis.

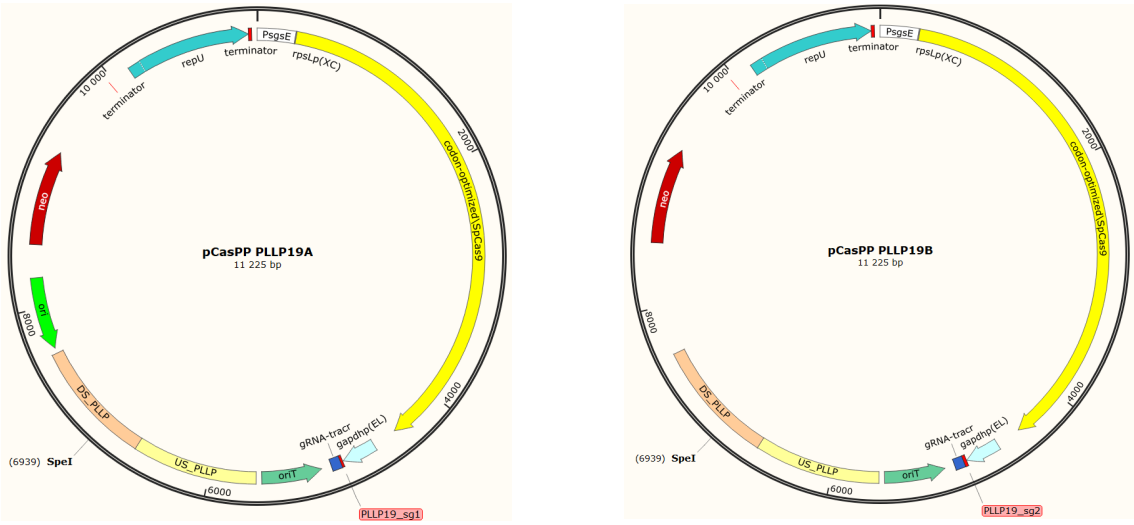
3.1 Assembly of plasmids for gene deletions in *P. polymyxa*

Primers are referred to with numbers, see Table A.0.1 in Appendix A for name, purpose and primer sequence. Genomic DNA from *P. polymyxa* DSM 365 (gDNA) and primers 7+8 were the most commonly used positive control for the PCR reactions.

3.1.1 Assembly of pCasPP PLLP19A and pCasPP PLLP19B

Primers for assembly of pCasPP *PLLP19* were designed during the previous specialization project [59]. The plasmids was assembled by inserting the guide sequences with Golden Gate Cloning and the HDR-template by restriction and digestion cloning into pCasPP. Two guide sequences were tested and the two plamids were denoted pCasPP PLLP19A, containing PLLP19_sg1 and pCasPP PLLP19B with PLLP19_sg2. Figure 3.1.1 shows the *in silico* designed plasmids for deletion of *PLLP19*.

Primers 13+14 were annealed to form PLLP19_sg1 and primers 15+16 were annealed to form PLLP19_sg2. Annealed oligos were inserted into pCasPP by Golden Gate Cloning. PCR with primers 3+5 were used to verify correct cloning product. Correct insertion of guide sequences would give fragment length fof 1280 bp, while no insertion would give fragment lengths of 1717 bp. Positive was genomic DNA from *P. polymyxa* DSM 365 (gDNA) and primers 7+8, which gives a fragment of 1119 bp. Figure 3.1.2 shows the correct insertion of both guide sequences.



(a) pCasPP PLLP19A **(b)** pCasPP PLLP19B

Figure 3.1.1: *In silico* design of plasmids for deletion of *PLLP19*. pCasPP PLLP19A includes PLLP19_sg1, while pCasPP *PLLP19B includes PLLP19_ sg2*

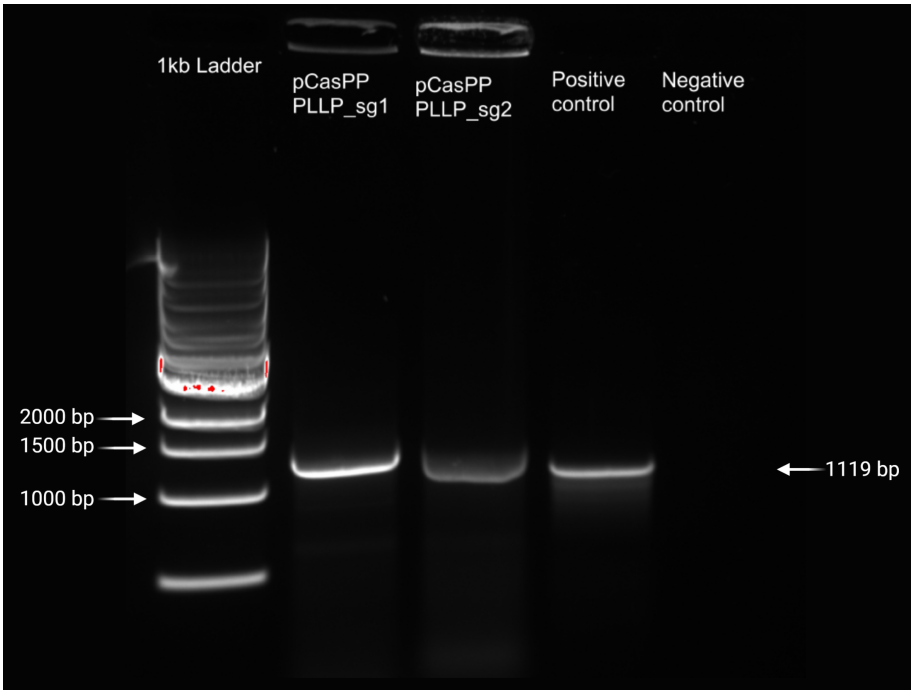


Figure 3.1.2: Insertion of guide sequences for *PLLP19* into pCasPP. Positive control: gDNA from *P. polymyxa* DSM 365 and primers 7+8.

HDR-template was assembled in two steps. First, ca 1000 bp upstream and downstream of the *PLLP19* gene were amplified. Then the two fragments were fused by overlap-extension PCR (OE-PCR). Figure 3.1.3 shows the fragment upstream of *PLLP19*, called PLLP19_US, and downstream of *PLLP19*, called PLLP19_DS. PLLP19_US was amplified by PCR with primers 17+18, while PLLP19_DS was amplified with primers 19+20. The two fragments were expected to have a fragment length of 1017 bp for PLLP19_US and 1010 bp for PLLP19_DS. Both fragments appeared to be of the correct size.

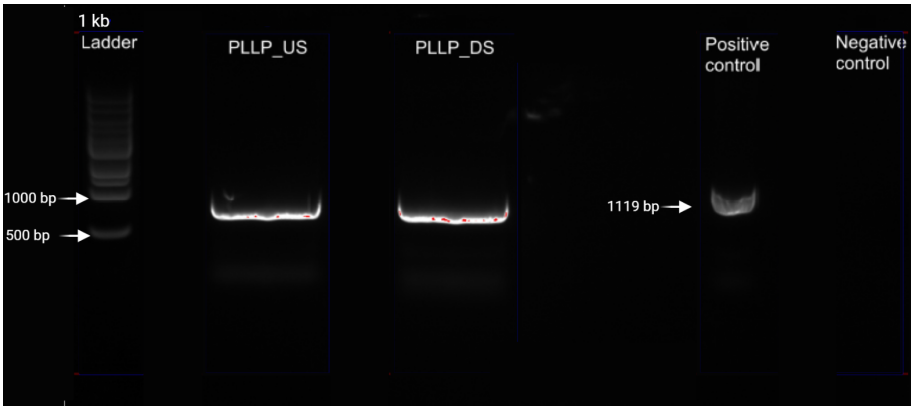


Figure 3.1.3: Amplicons of upstream (PLLP19_US) and downstream (PLLP19_DS) fragments of PLLP19 HDR-template. Positive control was gDNA and primers 7+8, giving amplicon of 1119 bp.

Figure 3.1.4 shows the products from the OE-PCR. The two parallels contain the same amplicons, both amplified with primers 17+20 and with a length of 1993 bp. Parallel 2 was purified from the gel and used further.

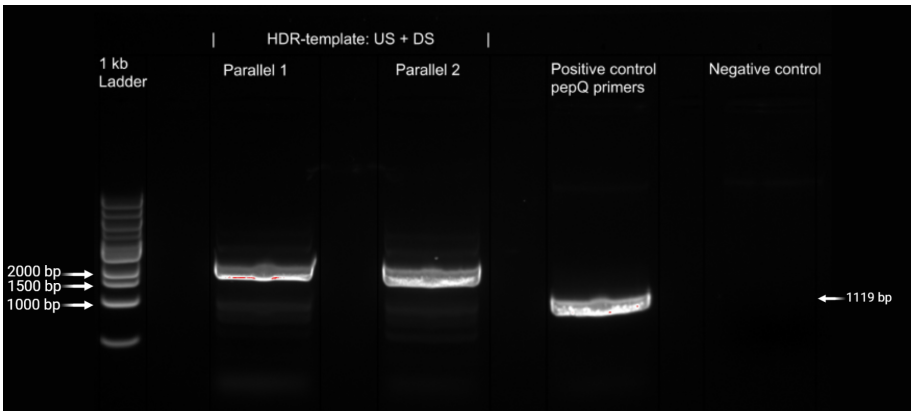


Figure 3.1.4: Products from OE-PCR. Parallel 1 and parallel 2 are identical and both contain the correctly fused HDR-template. Positive control: gDNA and primers 7+8 giving 1119 bp amplicon.

HDR-template was inserted into pCasPP plasmids containing the guide sequences, pCasPP PLLP19_sg1 and pCasPP PLLP19_sg2, by enzyme digestion and ligation. Plasmids were digested with SpeI-HF, while HDR-template was digested with XbaI. Then the vector and insert were ligated with molar ratios (insert:vector) of 1:1, 3:1 and 5:1. After transformation of the ligation products, colonies were tested by PCR using primers 3+4. Correct assembly would give a band at 2165 bp while no insertion would give 319 bp. Figure 3.1.5 shows the amplicons from the colony PCR. Correct assembly of pCasPP PLLP19A was found for the ligation with molar ratio of 5:1. pCasPP PLLP19B was correctly assembled for the ligations with molar ratio of 3:1 and 5:1. Plasmids from the parallels with 5:1 molar ratio were purified. Sequencing confirmed the correct assembly of both pCasPP PLLP19A and pCasPP PLLP19B.

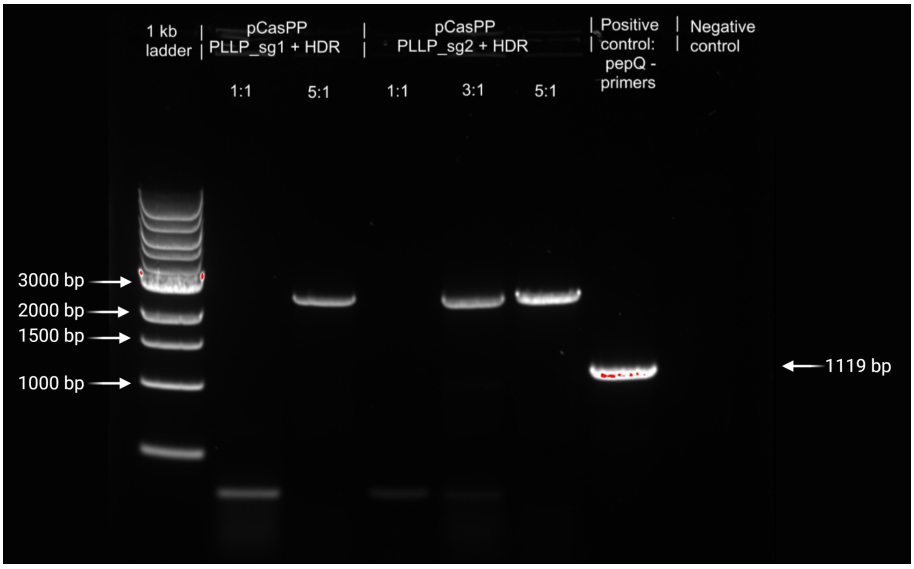


Figure 3.1.5: Ligation of HDR template and pCasPP PLLP_sg1 with molar ratio of 5:1 resulted in correctly assembled pCasPP PLLP19A. Two ligation parallels with molar ratios of 3:1 and 5:1 showed correct assembly for pCasPP PLLP19B. Positive control: gDNA and primers 7+8 giving 1119 bp amplicon.

3.1.2 Assembly of pCasPP Clu1Clu2, pCasPP Clu2, pCasPP ugdH1, and pCasPP ugdH2

CRISPR-Cas systems for the deletion of *ugdH1*, *ugdH2*, *Clu1Clu2*, and *Clu2* were designed *in silico* using Benchling and SnapGene. These plasmids were constructed by a different procedure than that used for pCasPP PLLP19A and pCasPP PLLP19B to see if the assembly process could be made more efficient. Figure 3.1.6 shows how the plasmids were assembled. One single Gibson Assembly reaction with five fragments was used to assemble pCasPP ugdH1 and pCasPP ugdH2. The *Clu1Clu2* gene cluster has a size of 34.2 kbp and *Clu2* has a size of 17.3 kbp. Because of the large sequences it was determined that it was necessary to have two guide sequences to delete the these targets. Sequences of 500 bp containing two sets of *gapdh* promoter, gRNA, and tracrRNA were designed and ordered as gBlocks. For pCasPP Clu1Clu2 and pCasPP Clu2 Gibson Assembly was first used to assemble pCasPP with HDR-templates. Then the gBlocks were inserted into the plasmids by Golden Gate Cloning.

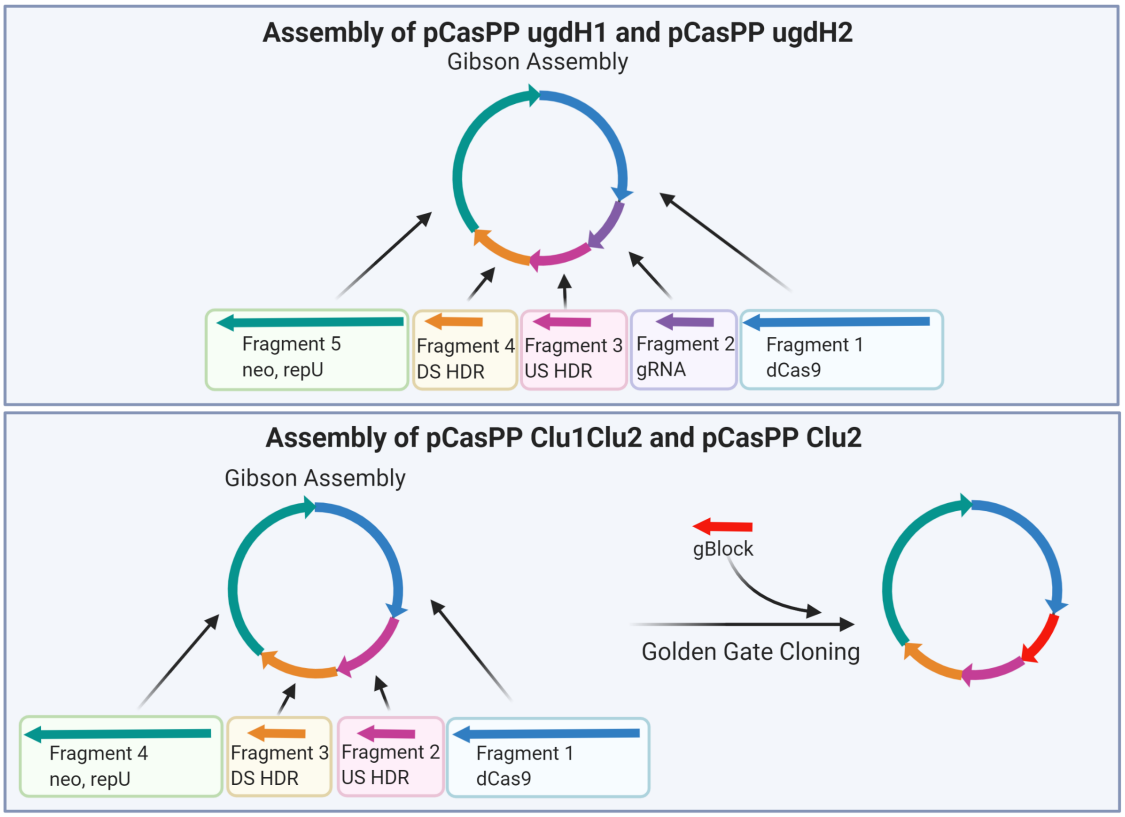
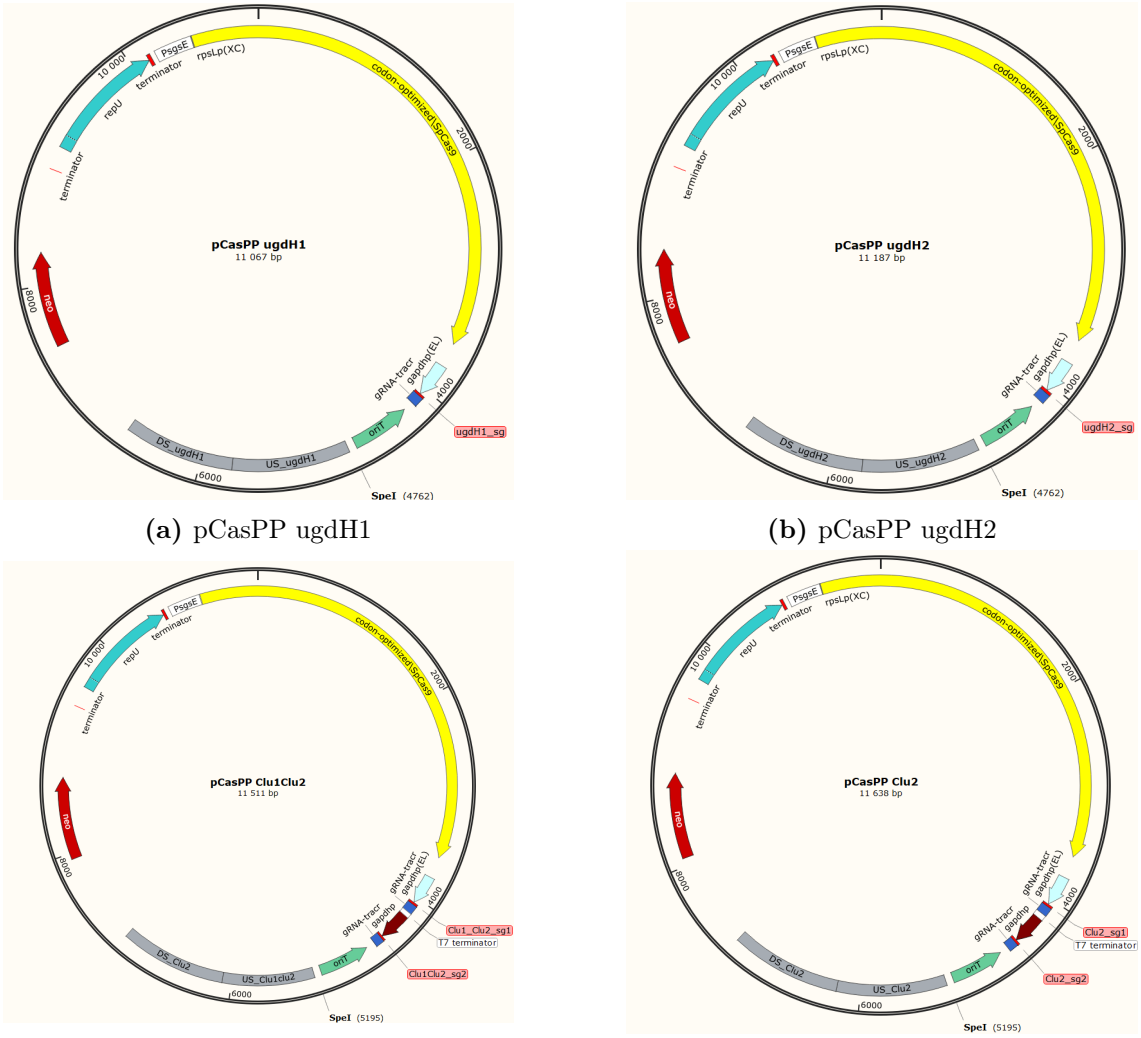


Figure 3.1.6: pCasPP ugdH1 and pCasPP ugdH2 were constructed by Gibson Assembly of five fragments. For pCasPP Clu1Clu2 and pCasPP Clu2 the construction was divided into two steps. First, Gibson Assembly of four fragments. Secondly, Golden Gate Cloning for insertion of gBlocks containing gRNAs.

Figure 3.1.7 show the four plasmids for deletion of *ugdH1*, *ugdH2*, *Clu1Clu2*, and *Clu2*. pCasPP Clu1Clu2 and pCasPP Clu2 have the same downstream part of the HDR-template and the same second gRNA. Table 3.1.1 shows the primers and

templates used to amplify the fragments used for Gibson Assembly. The table shows how fragment 4 for pCasPP Clu1Clu2 and pCasPP Clu2 are the same. Fragment 3, the downstream part of the HDR-template, for the same plasmids are almost the same with some minor differences at the end closes to the upstream part of the HDR-template.



(a) pCasPP ugdH1

(b) pCasPP ugdH2

(c) pCasPP Clu1Clu2

(d) pCasPP Clu2

Figure 3.1.7: CRISPR-Cas systems for the deletion of *ugdH1*, *ugdH2*, *Clu1Clu2* and *Clu2*. Plasmids were assembled via Gibson Assembly and Golden Gate Cloning.

Figure 3.1.8 shows the successful amplification of fragments 2, 3 and 4 for the assembly of pCasPP ugdH1. Fragments 1 and 5 were correctly amplified in a new PCR as shown in Figure 3.1.10. Fragments 2 and 3 for pCasPP Clu1Clu2 and pCasPP Clu2 were made by PCR as shown in Figure 3.1.9. Fragments 1 and 4 for pCasPP Clu1Clu2 can be seen in Figure 3.1.10. Because fragment 4 is the same for both pCasPP Clu1Clu2 and pCasPP Clu2, it was only necessary to amplify fragment 1 for pCasPP Clu2. Figure 3.1.11 shows the successful amplification of fragment 1 for pCasPP Clu2. All the correct fragments were excised from the gel and purified.

Table 3.1.1: Primers and template DNA used in PCR reactions to amplify the fragments needed for the Gibson Assembly reactions.

Plasmid	Fragment nr.	Primers	Template DNA	Length [bp]
pCasPP ugdH1	1	42+43	pCasPP	4075
	2	44+45	pCasPP	761
	3	46+47	gDNA	994
	4	48+49	gDNA	931
	5	50+51	pCasPP	4501
pCasPP ugdH2	1	42+54	pCasPP	4075
	2	55+56	pCasPP	761
	3	57+58	gDNA	1004
	4	59+60	gDNA	1046
	5	61+51	pCasPP	4501
pCasPP Clu1Clu2	1	42+36	pCasPP	5247
	2	37+38	gDNA	907
	3	39+31	gDNA	1032
	4	32+51	pCasPP	4499
pCasPP Clu2	1	42+27	pCasPP	5246
	2	28+29	gDNA	1034
	3	30+31	gDNA	1034
	4	32+51	pCasPP	4499

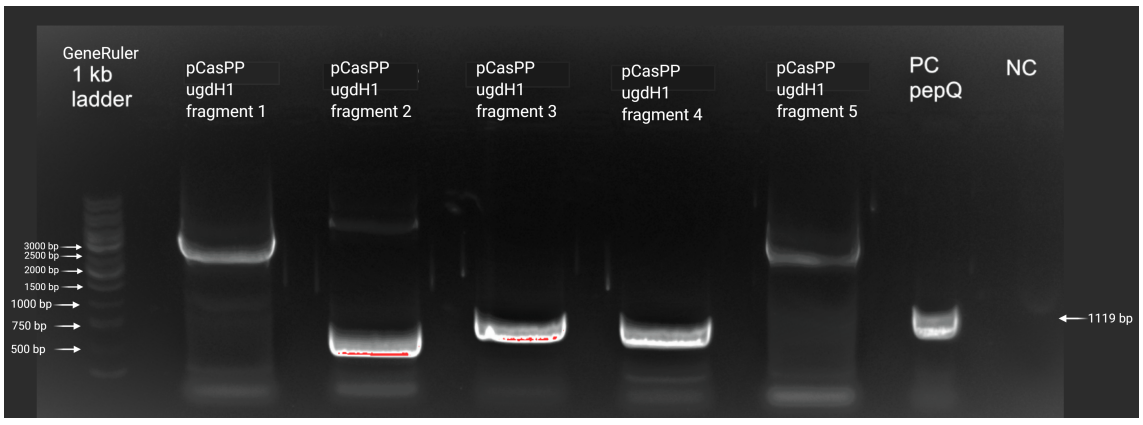


Figure 3.1.8: Successful amplification of fragments 2, 3 and 4 for Gibson Assembly of pCasPP ugdH1. Fragments 1 and 5 had incorrect size. PC was positive control with gDNA and primers 7+8. NC was the negative control.

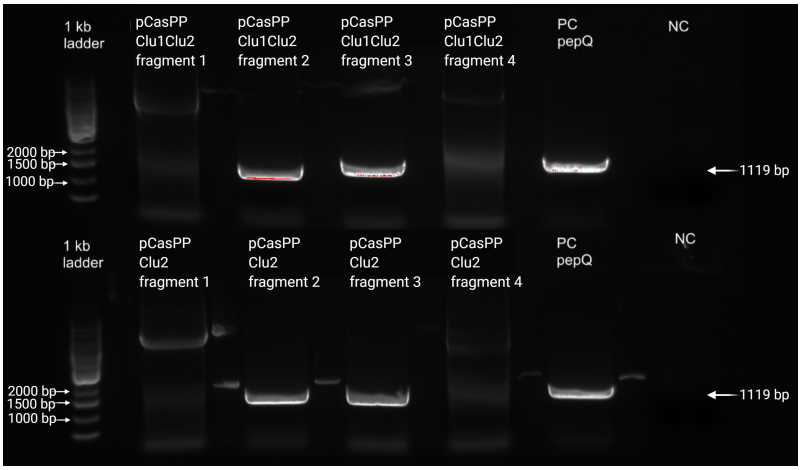


Figure 3.1.9: Correct amplification of fragments 2 and 3 for assembly of pCasPP Clu1Clu2 and pCasPP Clu2. Fragments 1 and 4 were incorrect. PC was positive control with gDNA and primers 7+8. NC was the negative control with gDNA and no primers.

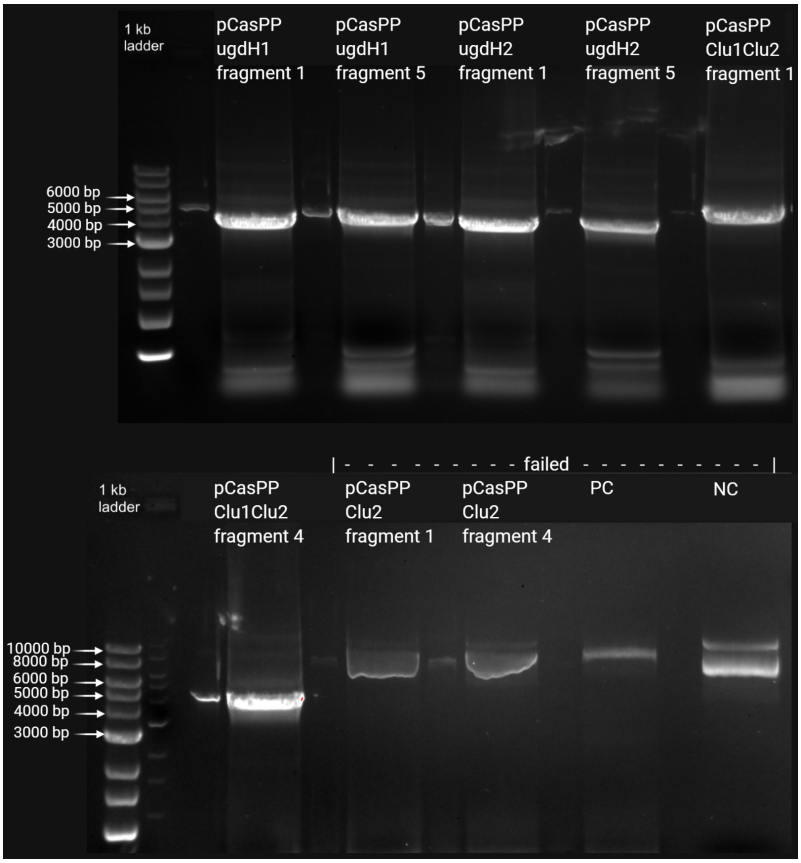


Figure 3.1.10: Fragments 1 and 5 for pCasPP ugdH1 and fragments 1 and 4 for pCasPP Clu1Clu2 were successfully amplified. Fragment 4 fir pCasPP Clu2 is identical to fragment 4 for pCasPP Clu1Clu2. The PCR products visualized in the four last lanes failed due to a faulty dNTP used for these reactions. The positive control (PC) was intended to be a 3127 bp amplicon from pCasPP PL01 and primers 3+5, but shows instead only the pCasPP PL01 plasmid. Negative control (NC) was the pCasPP plasmid.

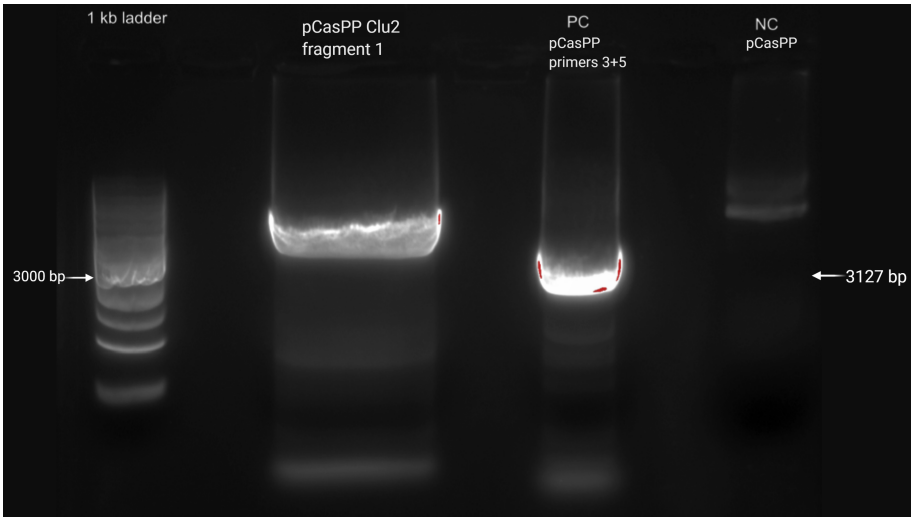


Figure 3.1.11: Amplification of fragment 1 for pCasPP Clu2. Positive control was the amplicon from pCasPP with primers 2+3, on 3127 bp. The negative control (NC) was the pCasPP plasmid without primers.

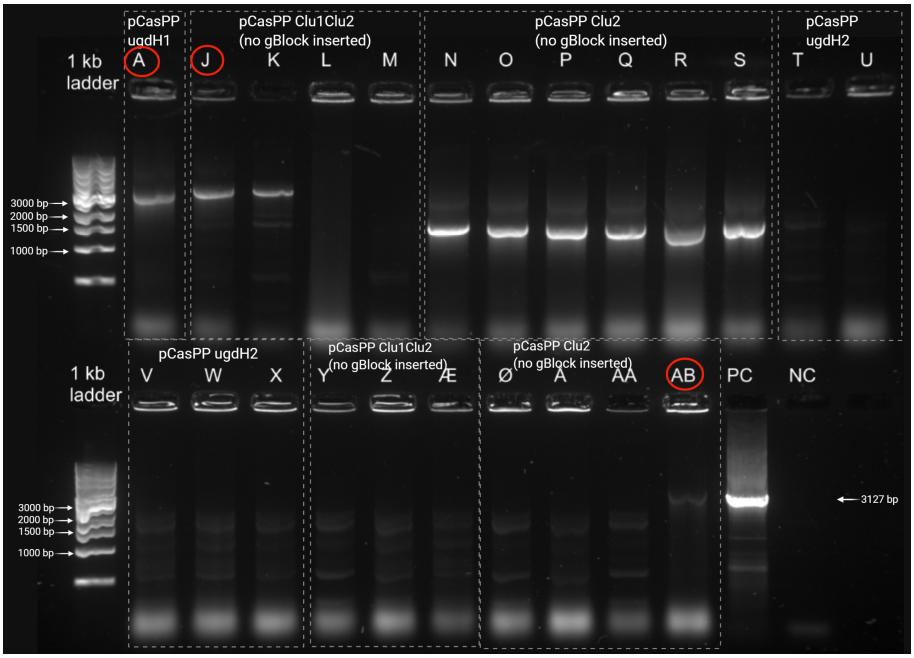


Figure 3.1.12: Screening of correct assembly of pCasPP ugdH1, pCasPP Clu1Clu2_H and pCasPP Clu2_H. Colony A had the correct amplicon for pCasPP ugdH1. Colonies J and K had the correct amplicons for pCasPP Clu1Clu2 without gBlock, where plasmids from colony J were used further. For pCasPP Clu2 without gBlock the correct amplicon was observed for colony AB. PC was the positive control with pCasPP PL01 and primers 3+5. NC was the negative control.

Figure 3.1.12 shows the result of a colony PCR screening with primers 3+5 after Gibson Assembly reactions. Correct assembly of pCasPP ugdH1 would give 3121 bp fragment. Successful Gibson Assembly reaction of fragments 1-4 for pCasPP Clu1Clu2 and pCasPP Clu2 would give amplicons of 3569 bp and 3696 bp respec-

tively. Colonies A, J, K and AB were believed to have the correct product. Plasmids from colony A was purified and sent for sequencing, which confirmed the correct assembly of pCasPP ugdH1. Plasmids from colony J and AB were purified and used for further cloning procedures.

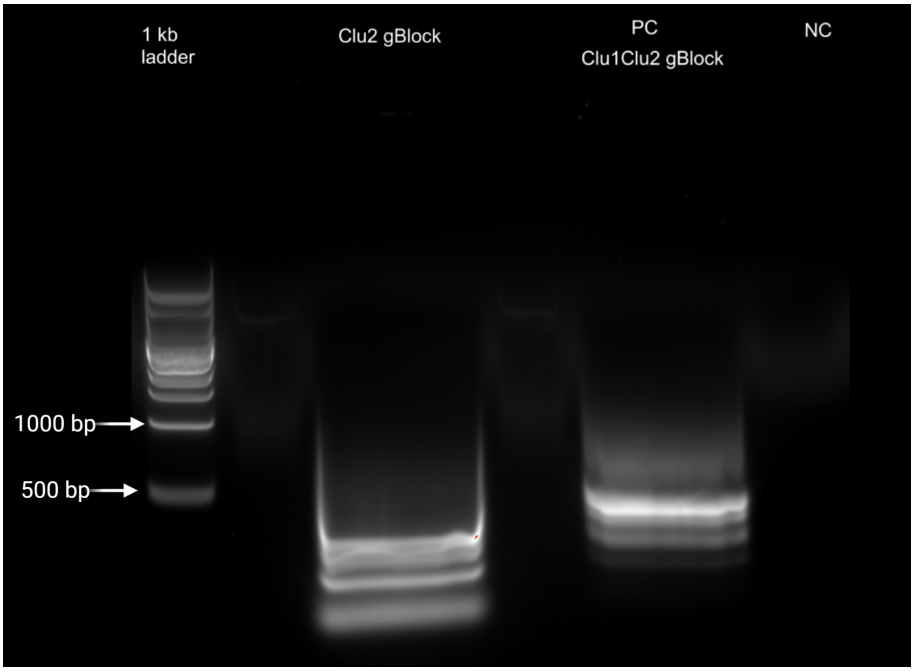


Figure 3.1.13: Visualization of the amplified gBlocks for Clu2. Positive control was the Clu1Clu2 gBlock with primers 25+26. NC was the negative control.

The gBlock for pCasPP Clu2 that was ordered could not be made by the producer because of some problems in the production. There were fortunately no issues with the production of the Clu1Clu2 gBlock. Two new primers, number 63 and 64, were therefore ordered to amplify the new Clu2 gBlock with the correct gRNAs from the Clu1Clu2 gBlock. The Clu1Clu2 gBlock was also amplified with primers 25+26. Figure 3.1.13 shows the amplification of the Clu2 gBlock and the Clu1Clu2 gBlock.

Golden Gate Cloning was performed to insert the Clu1Clu2 gBlock into plasmid J, pCasPP Clu1Clu2 without guide sequences, from the Gibson Assembly reaction. Figure 3.1.14 shows the colony PCR productions after the insertion of the gBlock. Primers 6+41 were used to test if the colonies had plasmids containing the inserted gBlock. The expected fragment size for successful reaction was 726 bp. If the insertion failed, there should not be any bands on the gel. Plasmids from colony A was though to be correct and therefore purified. Sequencing confirmed that this plasmid was the correctly assembled pCasPP Clu1Clu2.

The purified Clu2 gBlock was used for Golden Gate Cloning with plasmids from colony AB from the Gibson Assembly, which was pCasPP Clu2 without the guide sequences. Colonies were screened by a PCR with primers 6+33. Correct insertion of the gBlock would give fragments of 726 bp, while no insertion would not give any

band on the gel. Figure 3.1.15 shows the screening reaction. Plasmids from colony 10 was purified. Sequencing confirmed that the plasmids was in fact the correctly assembled pCasPP Clu2.

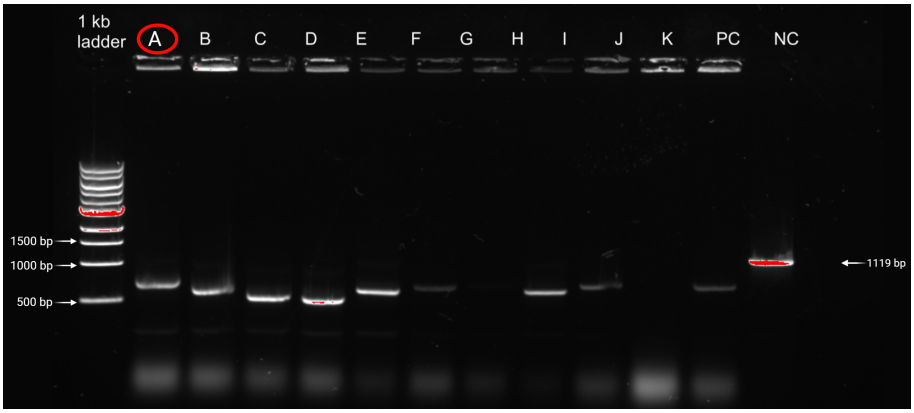


Figure 3.1.14: Screening of colonies for the correct insertion of Clu1Clu2 gBlock. Colony A were believed to have the correct amplicon. PC was the positive control with gDNA and primers 7+8. NC was the negative control.

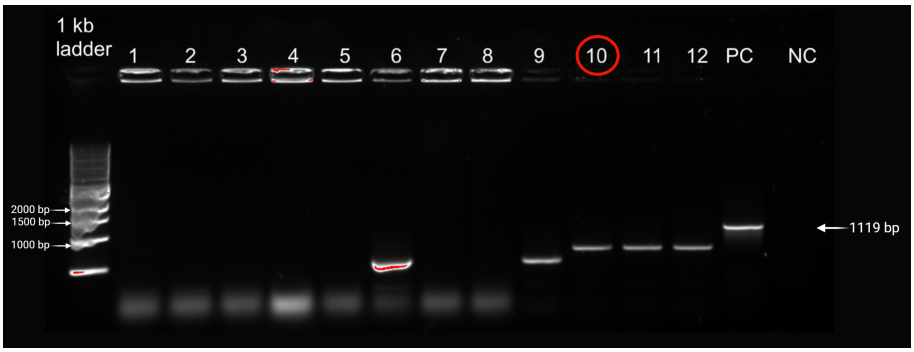


Figure 3.1.15: Screening of colonies for the successful insertion of the Clu2 gBlock to assemble pCasPP Clu2. Colony 10, 11 and 12 were believed to have the correct amplicon. PC was the positive control with gDNA and primers 7+8. NC was the negative control.

The last plasmid to be assembled was pCasPP ugdH2. During the previous PCR reactions it was found that the Q5 High-Fidelity DNA polymerase (Q5) was more successful than Phusion High-Fidelity DNA polymerase, especially for the larger fragments. The fragments needed for Gibson Assembly of pCasPP ugdH2 were therefore amplified with Q5 and the result is visualized in Figure 3.1.16. Transformation of the Gibson Assembly product resulted in only four colonies. Figure 3.1.17 shows the PCR screening of the four colonies using primers 3+5. Colony A and B appeared to have the correct plasmid, and both were purified. Sequencing confirmed that both plasmids were the correctly assembled pCasPP ugdH2.

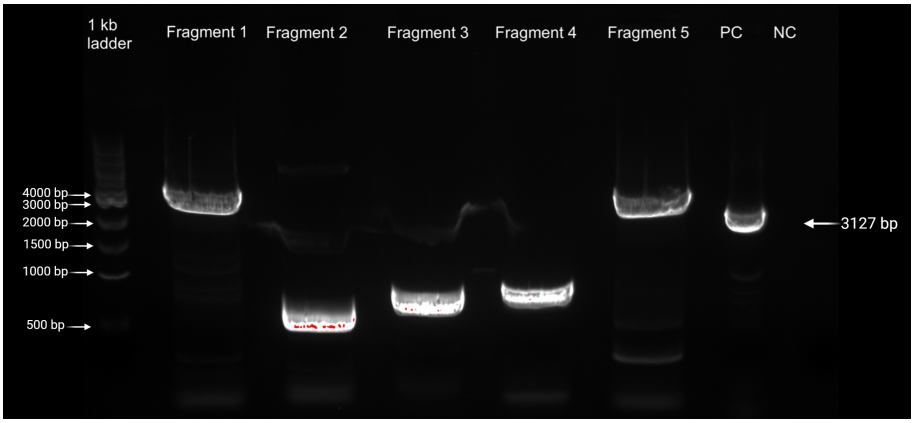


Figure 3.1.16: Successful amplification of all five fragments for the assembly of pCasPP ugdH2. PC is the positive control with pCasPP PL01 and primers 3+5. NC was the negative control.

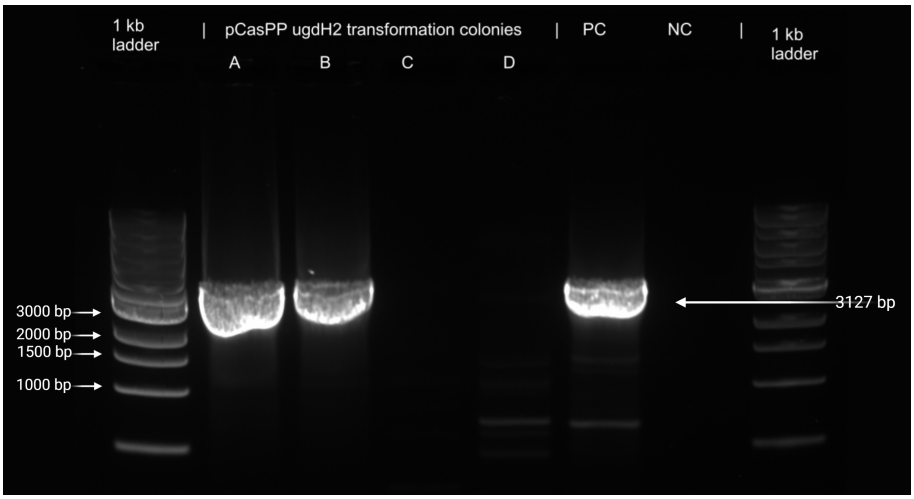


Figure 3.1.17: Screening of colonies for the correctly assembled pCasPP ugdH2. Colony A and B appeared to have the correct amplicons. Positive control, PC, was pCasPP PL01 with primers 3+5. NC was the negative control.

3.2 Gene deletions in *P. polymyxa* strains

CRISPR-Cas systems were used to do targeted gene deletions in strains of *P. polymyxa*. The aim was to both make a deletion strain for each of the deletion targets and to combine all the deletions in one strain. Conjugation was the main mechanism used for transfer of plasmid into *P. polymyxa*. Attempts were also made to establish electroporation as a plasmid transfer technique, but only one of the electrocompetent *P. polymyxa* strains yielded a positive result. Table 3.2.1 shows an overview of all the attempted gene deletions where the transfer of plasmid into *P. polymyxa* was successful. A full list of all attempted deletions can be found in Appendix, Section B.1, Table B.1.

Table 3.2.1: CRISPR-Cas systems were used to make targeted gene deletions in *P. polymyxa* strains. The systems were transferred to this strain by either conjugation or electroporation. After the successful transfer of the CRISPR-Cas system into the *P. polymyxa* strain, deletion was found to be either correct (Deleted=Yes), incorrect (Deleted=No) or not known (Deleted=NK).

Starting strain	**Genes to delete**	**Plasmid transfer mechanism**	**Successful transfer**	**Deleted**
P. polymyxa DSM 365	PL01	Conjugation	Yes	Yes
P. polymyxa ΔSpoIIE	PL01	Conjugation	Yes	Yes
P. polymyxa DSM 365	PLLP19	Conjugation	Yes	Yes
P. polymyxa ΔSpoIIE	PLLP19	Conjugation	Yes	Yes
P. polymyxa ΔSpoIIE ΔPL01	PLLP19	Conjugation	Yes	Yes
P. polymyxa ΔPL01	PLLP19	Conjugation	Yes	Yes
P. polymyxa DSM 365	SacB	Conjuagtion	Yes	No
P. polymyxa ΔSpoIIE ΔPL01 ΔPLLP19	SacB	Conjuagtion	Yes	No
P. polymyxa ΔPL01 ΔPLLP19	SacB	Conjuagtion	Yes	No
P. polymyxa ΔClu1	PLLP19	Electroporation	Yes	Yes
P. polymyxa DSM 365	ugdH1	Conjugation	Yes	NK
P. polymyxa ΔSpoIIE ΔPL01 ΔPLLP19	ugdH1	Conjugation	Yes	NK
P. polymyxa ΔSpoIIE ΔPL01 ΔPLLP19	Clu1Clu2	Conjugation	Yes	NK
P. polymyxa ΔSpoIIE ΔPL01 ΔPLLP19	Clu2	Conjugation	Yes	NK
P. polymyxa DSM 365	ugdH2	Conjugation	Yes	NK

The pCasPP PL01 plasmid was transferred to *P. polymyxa* DSM 365 and *P. polymyxa* ΔSpoIIE by conjugation. Colonies were cured and tested by colony PCR. Figure 3.2.1 shows that three of the five tested colonies seemed to have the correct deletion with amplicons of 715 bp. Two of the colonies did not appear to have a deletion of *PL01*, but amplicons of 985 bp. Sequencing confirmed the deletion of *PL01* for both *P. polymyxa* DSM 365 in colony C, and for *P. polymyxa* ΔSpoIIE in colony E.

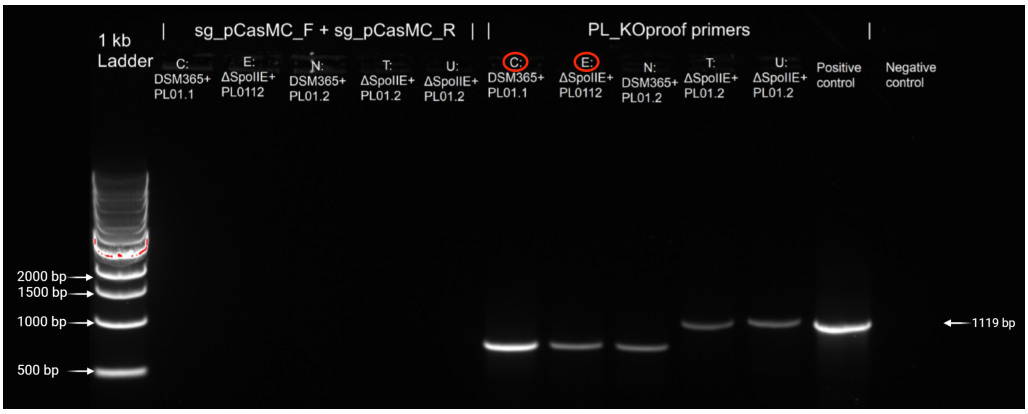


Figure 3.2.1: Deletion of *PL01* in *P. polymyxa* DSM 365 and *P. polymyxa* ΔSpoIIE verified by colony PCR. The colonies were tested with primers 3+5 in the five first lanes to see if the plasmids were cured, which all of them appeared to be. Positive control was the genomic DNA of *P. polymyxa* DSM 365 with primers 11+12.

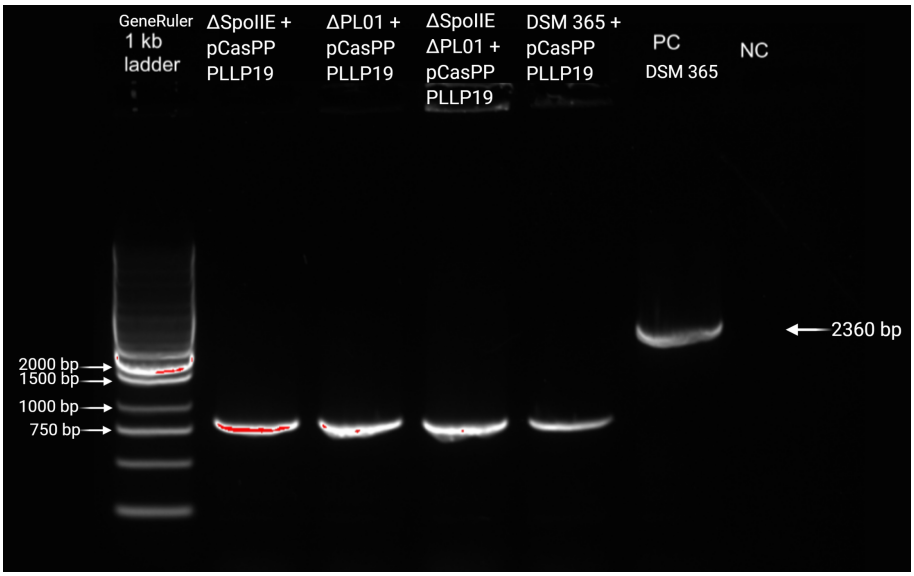


Figure 3.2.2: Verification of the deletion of *PLLP19* in *P. polymyxa* strains. All four strains appeared to have the correct deletion with amplicons between 750 bp and 100 bp. Positive control was the genomic DNA of *P. polymyxa* DSM 365 and primers 21+22. NC was the negative control with the same primers, but without template DNA.

Conjugation was used to transfer pCasPP PLLP19 into *P. polymyxa* DSM 365, *P. polymyxa* ΔPL01, *P. polymyxa* ΔSpoIIEΔPL01, and *P. polymyxa* ΔSpoIIE. After

curing of the plasmid, the deletion was verified by PCR. Figure 3.2.2 shows the deletion of *PLLP19* in the four *P. polymyxa* strains. Primers 21+22 were used to verify the deletion. Correct deletion would give amplicon of 824 bp, while incorrect deletion would give a fragment of 2360 bp. The deletion of *PLLP19* in the four strains were confirmed by sequencing.

To see if electroporation could be used to transfer plasmids into *P. polymyxa* strains, electrocompetent cells were made for *P. polymyxa* DSM 365, *P. polymyxa* ΔClu1 and *P. polymyxa* ΔSpoIIEΔPL01ΔPLLP19. The only strain that were proved to be functional for electroporation was the *P. polymyxa* ΔClu1 strain. pCasPP PLLP19 was transferred to this strain by electroporation. Then five colonies were randomly selected, cured, and tested by PCR. Primers 21+22 were used to test for the deletion in the same way as for the first *PLLP19* deletion. All five colonies had amplicons between 500 bp and 1000 bp, similar to the *P. polymyxa* ΔPLLP19 strain used as positive control. Colony 1 was additionally tested by sequencing, which confirmed the deletion.

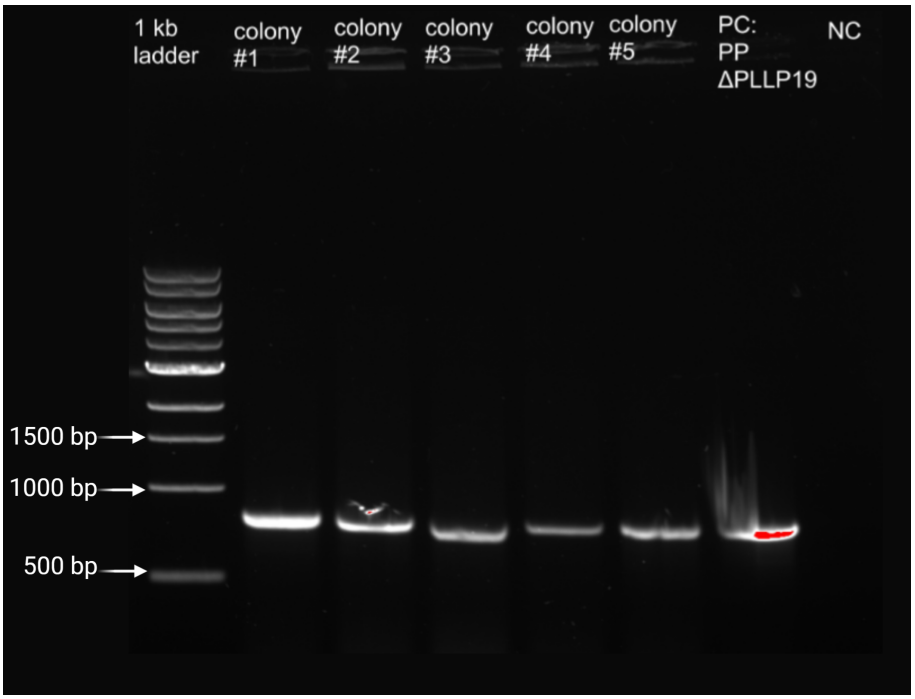


Figure 3.2.3: Deletion of *PLLP19* in *P. polymyxa* ΔClu1 by electroporation. Five colonies from the electroporation were tested by cPCR with primers 21+22. All appeared to have the correct amplicon. PC was the positive control with *P. polymyxa* ΔPLLP19. NC was the negative control with the same primers, but without template DNA.

Conjugation was used to transfer pCasPP sacB to *P. polymyxa* ΔSpoIIEΔPL01ΔPLLP19, *P. polymyxa* ΔPL01ΔPLLP19 and *P. polymyxa* DSM 365. Only selection plates with calcium chloride had any conjugants. Three colonies were cured and tested by PCR with primers 65+66. Correct deletion would give an amplicon of 953 bp, while no deletion of *sacB* would give amplicon of 2453 bp. None of the nine tested colonies appeared to have the correct deletion. The pCasPP sacB plasmid was sent for sequencing to see if it was the correct plasmid. The gRNA sequence was found to

be in the *sacB* gene, so it was concluded that the plasmid most likely was functional.

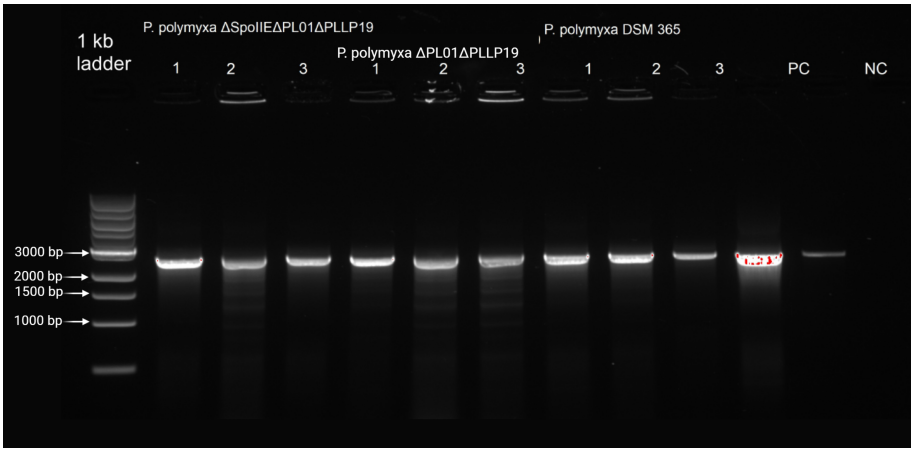


Figure 3.2.4: Deletion of *sacB* appeared to have failed for all the tested colonies. PC was positive control with *P. polymyxa* DSM 365 and primers 65+66. NC was the negative control with the same primers, but without template DNA.

Conjugation and electroporation were used to transfer pCasPP ugdH1, pCasPP ugdH2, pCasPP Clu1Clu2 and pCasPP Clu2 to *P. polymyxa* DSM 365 and *P. polymyxa* ΔSpoIIEΔPL01ΔPLLP19. The electroporation was not functional to transfer these plasmids. For pCasPP ugdH1 conjugation was successful for both strains. Conjugation was only successful for transfer of pCasPP ugdH2 into *P. polymyxa* DSM 365. Both pCasPP Clu1Clu2 and pCasPP Clu2 were only transferred successfully to *P. polymyxa* ΔSpoIIEΔPL01ΔPLLP19 by conjugation. The successful plasmid transfers are summarized in the following list:

- *P. polymyxa* DSM 365 + pCasPP ugdH1
- *P. polymyxa* ΔSpoIIEΔPL01ΔPLLP19 + pCasPP ugdH1
- *P. polymyxa* DSM 365 + pCasPP ugdH2
- *P. polymyxa* ΔSpoIIEΔPL01ΔPLLP19 + pCasPP Clu1Clu2
- *P. polymyxa* ΔSpoIIEΔPL01ΔPLLP19 + pCasPP Clu2

Several attempts were made to verify the deletions, but none of them could neither confirm nor deny the success of the deletions. PCR reactions with Standard Taq DNA polymerase, Phusion High-Fidelity DNA polymerase and Q5 High-Fidelity DNA polymerase failed to give any result at all. Primers 52+53 used for verification of *ugdH1* deletion and primers 62+35 used for verification of deletion of *ugdH2* should give amplicons independent on whether the deletion was successful or not. Primers 40+35 for verification of deletion of *Clu1Clu2* and primers 34+35 for verification of deletion of *Clu2* should not give any fragments if the deletion failed due to their large size. Therefore it was also tested for the negative result. Primers 9+10 were used to test for *pepF*, which is in the *Clu1Clu2* gene cluster and primers 7+8 were used to test for *pepQ*, which is within *Clu2*. If the PCR was successful there should therefore be amplicons both for successful and non-successful recombinations.

To get an indication of whether the deletions were successful or not, colonies from each conjugation were replated on EPS agar plates. The *P. polymyxa* DSM 365 and *P. polymyxa* ΔSpoIIEΔPL01ΔPLLP19 were also plated on EPS for comparison. In Appendix, Section B.0.1 there are pictures of the EPS plates for all the parallels.

The plates with colonies from conjugation with *P. polymyxa* DSM 365 were generally more slimy and appeared to be producing EPS. The colonies with *P. polymyxa* ΔSpoIIEΔPL01ΔPLLP19 and pCasPP ugdH1, pCasPP Clu1Clu2 and pCasPP Clu2 had a flat morphology and did not appear to be producing EPS. The *P. polymyxa* ΔSpoIIEΔPL01ΔPLLP19 alone was slimy and produced EPS similar to *P. polymyxa* DSM 365. This could be an indication that the recombinations might have been successful for these strains. Figure 3.2.5 shows the EPS plate with colonies of *P. polymyxa* ΔSpoIIEΔPL01ΔPLLP19 and pCasPP Clu1Clu2.

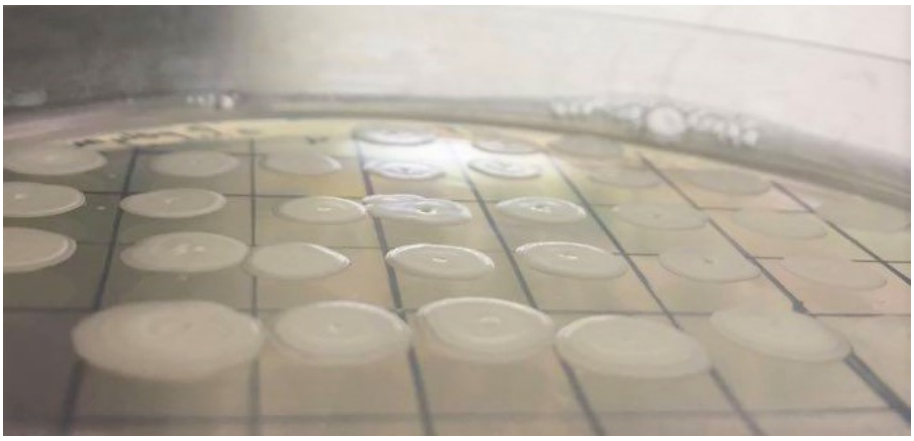


Figure 3.2.5: Colonies from conjugational transfer of pCasPP Clu1Clu2 to *P. polymyxa* ΔSpoIIEΔPL01ΔPLLP19 on EPS plates.

3.3 Pectin assay

Pectin assay was performed on three of the deletion strains in addition to the wild type strain. These three strains had deletion of Pectin lyase, *P. polymyxa* Δ*PL01*, Pectin lyase like protein, *P. polymyxa* Δ*PLLP19*, and both pectin lyase genes, *P. polymyxa* Δ*PL01*Δ*PLLP19*. The assay was performed twice for the four strains. During the first test the cultures were incubated at 30°C and 225 rpm, while for the second test the cultures were incubated at 30°C and 170 rpm.

Figure 3.3.1 shows the absorbance measurements for the four LB cultures. All the strains are growing well during the first 12 hours for both tests. The optical density then decreases gradually to around 0.4 in a varying tempo.

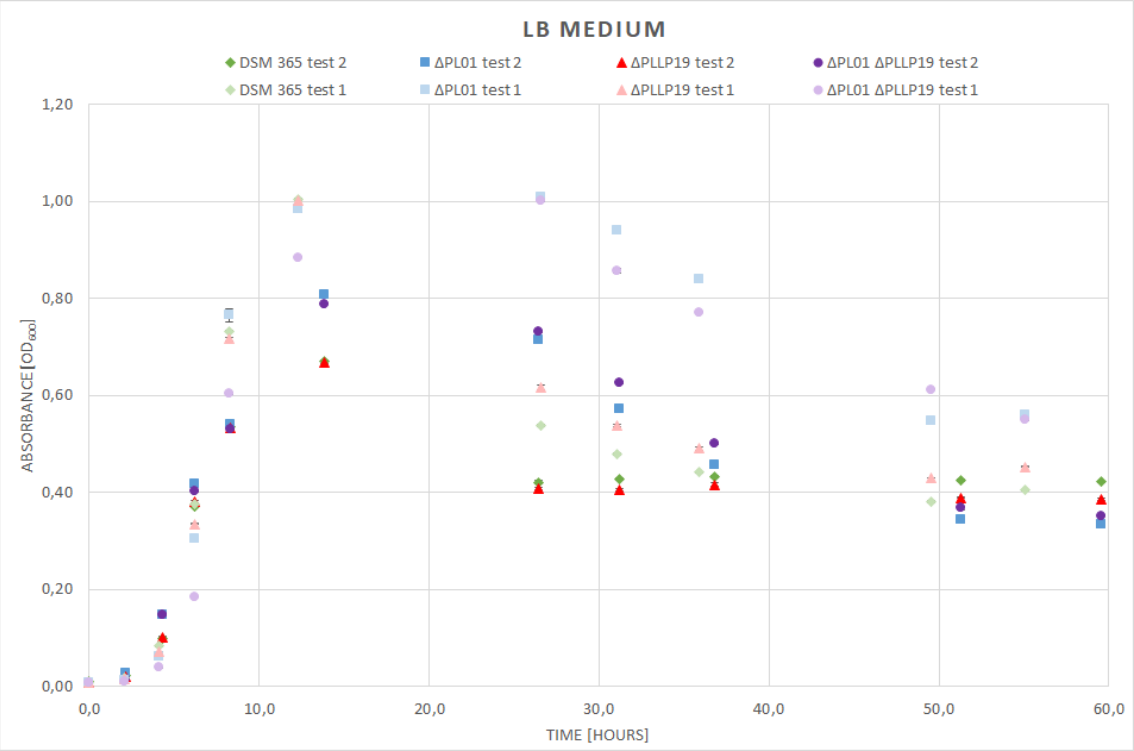


Figure 3.3.1: Absorbance of *P. polymyxa* strains grown in LB medium. The experiment was performed two times and measurements for both of these are included in the diagram.

Figure 3.3.2 shows the absorbance measurements for the four strains grown in pectin broth. The growth appear to follow a similar pattern for both of the tests. All the strains appear to be growing quite well during the first 12 hours. After 25 hours, the growth properties start to vary between the strains. *P. polymyxa* DSM 365 and *P. polymyxa* Δ*PLLP19* appear to have similar growth, slowly increases with time. *P. polymyxa* Δ*PL01* and *P. polymyxa* Δ*PL01*Δ*PLLP19* show a low and stable absorbance after 25 hours.

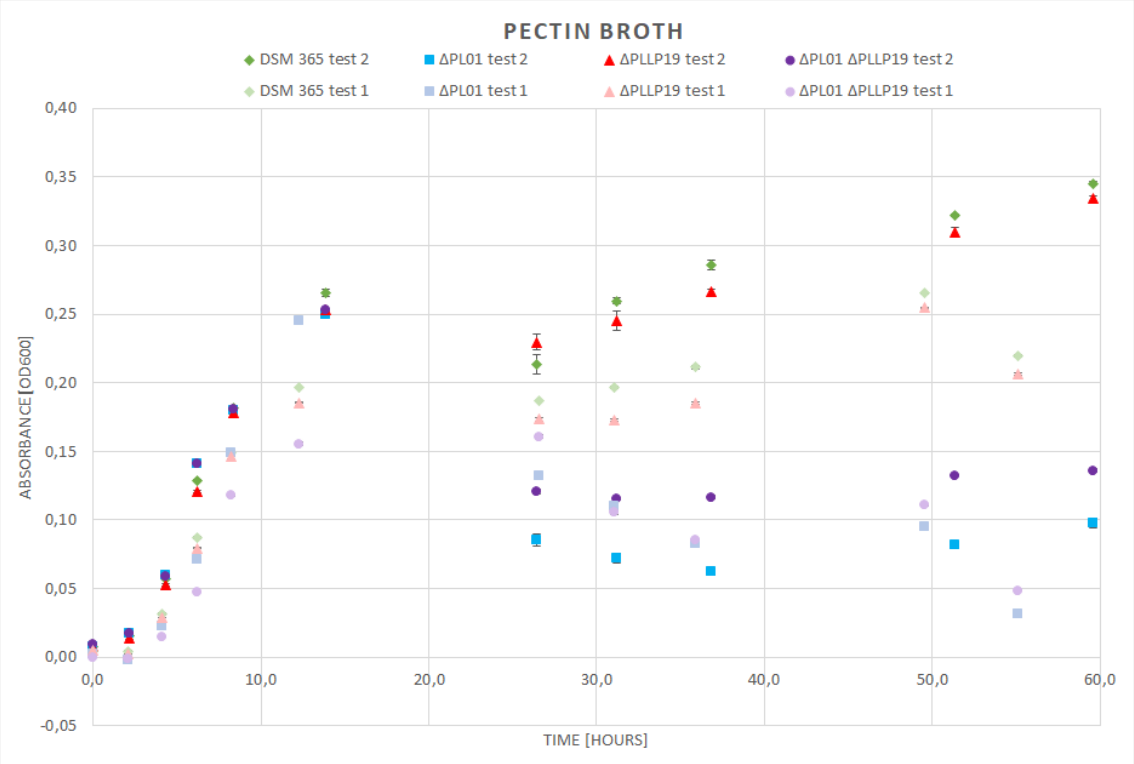


Figure 3.3.2: Growth curves for the *P. polymyxa* strains grown in pectin broth. *P. polymyxa* DSM 365 and *P. polymyxa* ΔPLLP19 appear to grow better, than the strains where *PL01* was deleted.

3.4 Effect of minerals on recombination efficiency

In previous work, it was experienced that when calcium was added to non-selective and selective agar plates during conjugation, the number of colonies obtained were drastically increased. Many of the colonies did however, not give the correct recombination. This could have happened because the calcium chloride prevented damage of the cell membrane by polymyxin, and thus reduced the antibiotic sensitivity [74]. During the deletion of *PLLP19*, selective plates with LB neo/poly/trace elements and LB neo/poly/$CaCl_2$ [60 mM] were used. Non-selective plates were LB with 60 mM $CaCl_2$. Both pCasPP PLLP19A and pCasPP PLLP19B were also used to make the gene deletions. Three colonies were picked from each of the selection plates, except for two plates with pCasPP PLLP19A on LB neo/poly/trace elements were it was only possible to pick two colonies. The strains were tested by PCR after curing of the plasmid. Gel picture can be seen in Appendix B.2 Figure B.2.1. Table 3.4.1 summarizes the finding of this test. 96% of the colonies from LB neo/poly/trace elements plates had the correct deletion, compare to only 33% for the selective plates with calcium chloride. There was not a significant difference between the efficiency of pCasPP PLLP19A and pCasPP PLLP19B.

Table 3.4.1: The observed recombination efficiency for the deletion of *PLLP19*. pCasPP PLLP19A and pCasPP PLLP19B had similar rate of successful recombinations. Selective plates with LB neo/poly/trace elements had a rate of 96 % while calcium chloride plates had only 33%.

Variable	Success rate
pCasPP PLLP19A	68 %
pCasPP PLLP19B	58 %
LB neo/poly/trace elements	96 %
LB neo/poly/$CaCl_2$ [60 mM]	33 %

A conjugation experiment was designed to investigate further the of effect calcium chloride on the recombination efficiency. It was desired to find a balance between obtaining enough colonies and at the same time ensure a recombination efficiency for the colonies.

Deletion of *PL01* was chosen because pCasPP PL01 had previously been shown to be functional. Three types of non-selective plates were tested: LB, LB $CaCl_2$ [30 mM], and LB $CaCl_2$ [60 mM]. Four types of selective plates were tested for each of the non-selective plates: LB neo/poly, LB neo/poly/trace elements, LB neo/poly/$CaCl_2$ [30 mM], and LB $CaCl_2$ [60 mM]. Ten colonies were picked from each parallel and evaluated by PCR and gel electrophoresis. Figure 3.4.1 shows the experimental set up and the results. In Appendix B.2 one can find the gel pictures used to give these results.

The five parallels that had successful recombinations for 100% of the tested colonies were:

- Non-selective plates: LB and selective plates: LB neo/poly
- Non-selective plates: LB and selective plates: LB neo/poly/tace elements
- Non-selective plates: LB $CaCl_2$ [30mM] and selective plates: LB neo/poly
- Non-selective plates: LB $CaCl_2$ [30mM] and selective plates: LB neo/poly/-trace elements
- Non-selective plates: LB $CaCl_2$ [60mM] and selective plates: LB neo/poly

This clearly shows how adding calcium chloride to both non-selective and selective plates drastically reduces the recombination efficiency. Table 3.4.2 shows that calcium chloride added to the selective plates alone could give a recombination efficiency five times lower that of normal LB neo/poly plates. The addition of this mineral to non-selective plates reduced the efficiency by 40%.

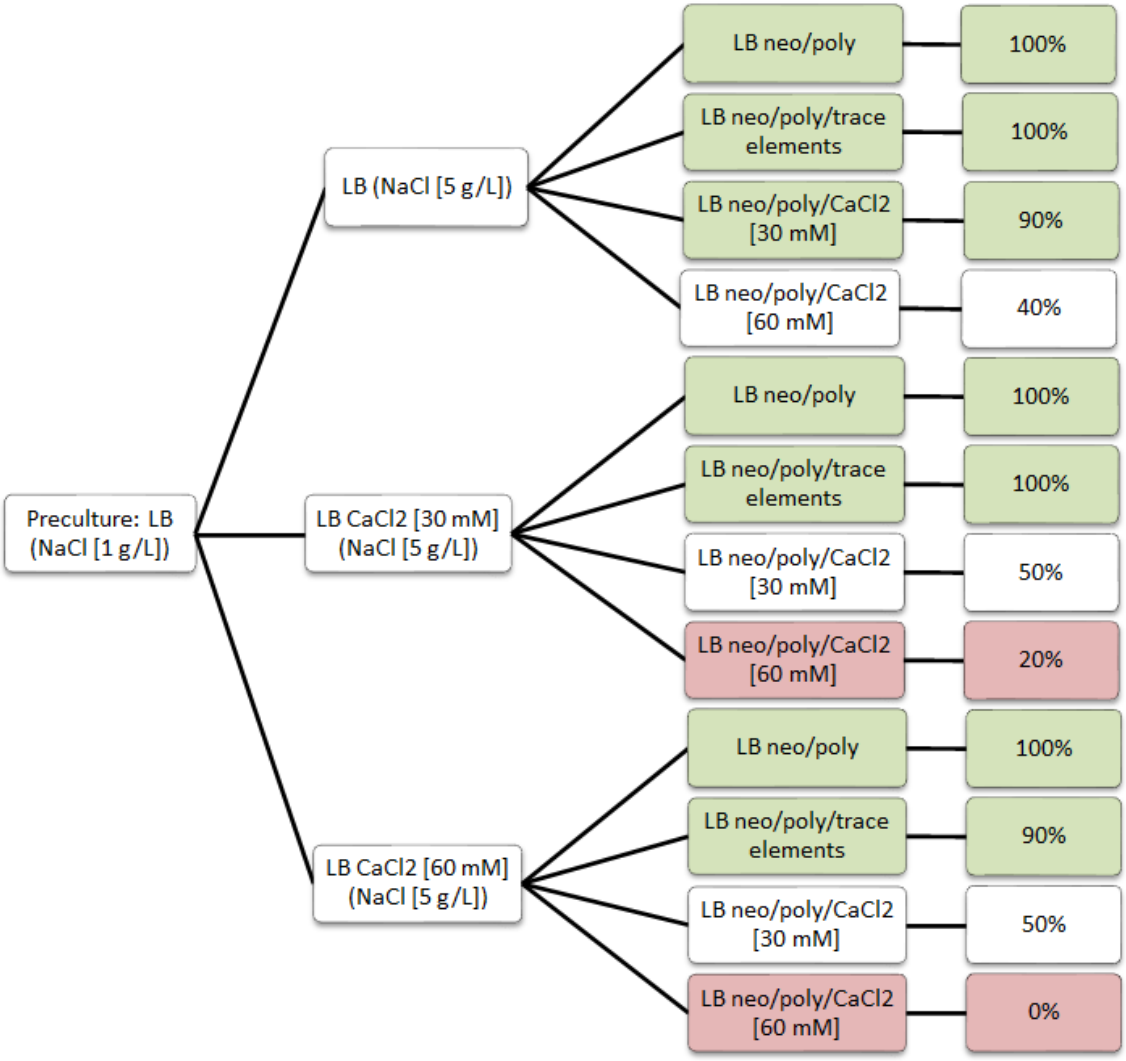


Figure 3.4.1: An experiment was designed to test the effect of calcium chloride on the recombination efficiency. Three types of non-selective plates were tested, and for each of them four types of selective plates were tested. The rate of successful recombinations is indicated to the right for each parallel.

3.5 Antibiotic susceptibility of *P. polymyxa*

Previous attempts at conjugational transfer of plasmids to the *P. polymyxa* ΔClu1 strain had failed to give any result. It was though that this might be because the strain has reduced its resistance to polymyxin. If *P. polymyxa* is going to be used for heterologous EPS production, it is necessary to find a way to transfer the plasmids even when the strain does not appear to be resistant to polymyxin anymore. The antibioitc susceptibility of *P. polymyxa* was therefore tested to see if there are any other antibiotics that could replace polymyxin.

First a antibiogram was made to get an indication of the antibiotic susceptibility of the strain. The antibiotics listed in Table 3.5.1 were tested in the given concentrations.

Table 3.4.2: The average per cent of successful recombinations (Avg. %) for the three types of non-selective plates and for the four selective plates.

Non-selective plates	Avg. %	Selective plates	Avg. %
LB	83	LB neo/poly	100
LB $CaCl_2$ [30 mM]	68	LB neo/poly/trace elements	97
LB $CaCl_2$ [60 mM]	60	LB neo/poly/$CaCl_2$ [30 mM]	63
		LB neo/poly/$CaCl_2$ [60 mM]	20

Table 3.5.1: The antibiotics used in the antibiogram and their concentrations.

	Polymyxin	Neomycin	Kanamycin	Gentamycin	Ampicillin	Chloamphenicol
Conc. [μg/mL]	5, 10, 15, 20	5, 25, 50	5, 25, 50	5, 15, 30	12.5, 25, 50, 100	5, 12.5, 25

Antibiogram was made for *P. polymyxa* DSM 365, *P. polymyxa* ΔClu1 and *P. polymyxa* ΔSpoIIE. Some of the plates with *P. polymyxa* ΔClu1 were contaminated, the results from this strain was therefore not included. Based on the inhibition zones it was determined whether the strain was resistant (R), intermediate (I), or susceptible (S) to the antibiotic. Figure 3.5.1 shows the result from the antibiogram.

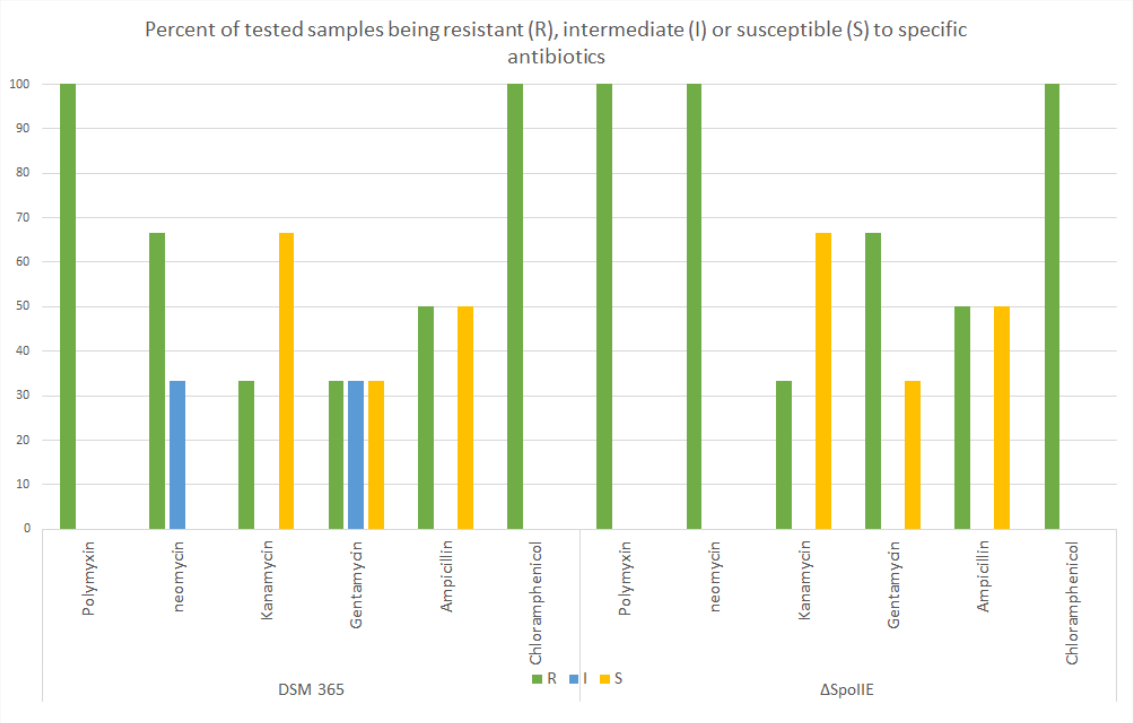


Figure 3.5.1: Antibiogram for *P. polymyxa* DSM 365 (DSM 365) and *P. polymyxa* ΔSpoIIE (ΔSpoIIE). The size of the inhibition zones were used to determine whether the strain was resistant (R), intermediate (I), or susceptible (S) to the tested antibiotic. The two strains showed resistance to polymyxin and chloramphenicol. They were susceptible to kanamycin and ampicillin.

The antibiogram shows that the strains were resistant to polymyxin (poly) and chloramphenicol (clm). It also showed that the strains were quite resistant to neomycin (neo). This was not expected and needed further investigation. *P. polymyxa* appeared to be quite susceptible to ampicillin (amp) and kanamycin (kan) and these antibiotics were considered to not be potential selection agents. To see if gentamycin (genta), clm and poly could be used at specific concentrations, an antibiotic susceptibility test was done for the three *P. polymyxa* strains. Tetracycline (tet) and neo was also included in this test. Table 3.5.2 shows the result of the antibiotic susceptibility test for the *P. polymyxa* strains.

Table 3.5.2: Antibiotic susceptibility test showed that *P. polymyxa* DSM 365 and *P. polymyxa* ΔSpoIIE grew well on all poly concentrations and clm concentrations up to 2.5 μg/mL. *P. polymyxa* ΔClu1 had the same antibiotic susceptibility, except that it was more sensitive to poly and less sensitive to neo.

Type of plates	DSM 365	ΔClu1	ΔSpoIIE
LB poly [1 μg/mL]	Grows well	Grows well	Grows well
LB poly [2 μg/mL]	Grows well	Grows well	Grows well
LB poly [3 μg/mL]	Grows well	Grows poorly	Grows well
LB poly [4 μg/mL]	Grows well	Grows poorly	Grows well
LB poly [5 μg/mL]	Grows well	No growth	Grows well
LB poly [10 μg/mL]	Grows well	No growth	Grows well
LB poly [15 μg/mL]	Grows well	No growth	Grows well
LB poly [20 μg/mL]	Grows well	No growth	Grows well
LB tet [20 μg/mL]	No growth	Not tested	Not tested
LB genta [15 μg/mL]	No growth	No growth	No growth
LB genta [30 μg/mL]	No growth	No growth	No growth
LB clm [12.5 ng/mL]	Grows well	Grows well	Grows well
LB clm [30 ng/mL]	Grows well	Grows well	Grows well
LB clm [0.025 μg/mL]	Grows well	Grows well	Not tested
LB clm [0.25 μg/mL]	Grows well	Grows well	Not tested
LB clm [2.5 μg/mL]	Grows well	Grows well	Not tested
LB clm [5.0 μg/mL]	Grows poorly	Grows poorly	Not tested
LB clm [10.0 μg/mL]	No growth	No growth	Not tested
LB neo [50 μg/mL]	Poor growth	Grows well	Poor growth
LB CaCl2 [60mM]	Grows well	Grows well	Grows well
LB	Grows well	Grows well	Grows well

The antibiotic susceptibility test showed that low concentrations of poly and clm could potentially replace 20 μg/mL poly, used as the current selection agent for *P. polymyxa* strains. To be able to use these antibioitics, it would be necessary that *E. coli* S17-1 were shown to be sensitive to these antibiotics. An antibiotic susceptibility test was then performed for *E. coli* S17-1 holding pCasPP Clu1 as shown in Table 3.5.3.

Table 3.5.3: Antibiotic susceptibility test showed that *E. coli* S17-1 pCasPP Clu1 was able to grow well on poly, up to 3 μg/mL, and clm, up to 2.5 μg/mL. No growth was observed for tet and genta.

Type of plates	*E. coli S17-1* pCasPP Clu1
LB poly [1 μg/mL]	Grows well
LB poly [2 μg/mL]	Grows well
LB poly [3 μg/mL]	Grows well
LB poly [4 μg/mL]	Grows poorly
LB poly [10 μg/mL]	No growth
LB tet [20 μg/mL]	No growth
LB genta [15 μg/mL]	No growth
LB genta [30 μg/mL]	No growth
LB clm [12.5 ng/mL]	Grows well
LB clm [30 ng/mL]	Grows well
LB clm [0.025 μg/mL]	Grows well
LB clm [0.25 μg/mL]	Grows well
LB clm [2.5 μg/mL]	Grows well
LB neo [50 μg/mL]	Grows well
LB CaCl2 [60 mM]	Grows well
LB	Grows well

E. coli S17-1 pCasPP Clu1 and *P. polymyxa* strains had the same resistance to clm. Both were sensitive to tet and genta. *P. polymyxa* ΔClu1 had similar sensitivity to poly as *E. coli* S17-1 pCasPP Clu1. By that, no alternative selection agent to polymyxin was found during the antibiotic susceptibility testing.

3.6 Investigation of polymyxin gene cluster in *P. polymyxa*

3.6.1 BLAST analysis of polymyxin region

The polymyxin synthetase PmxB was located in contig 01 based on RAST annotation of the whole genome of *P. polymyxa* DSM 365. The region ca 25 000 bp upstream and downstream of this gene was analyzed with BLASTx and annotated. Figure 3.6.1 shows the investigated region and most of the resulting features. The small, red feature in the middle of the sequence is the polymyxin synthetase PmxB found by RAST analysis. Two genes encoding ABC transporter substrate-binding protein and iron ABC transporter permease are found upstream of a large region encoding a non-ribosomal peptide synthetase (NRPS). These are displayed as orange features. Table B.3.1 in Appendix B.3 shows a complete list of the features found by BLAST analysis of this region.

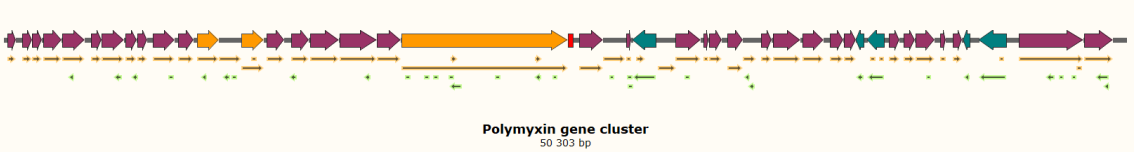


Figure 3.6.1: Analysis of region around the polymyxin synthetase PmxB by the use of BLAST

3.6.2 Comparison of polymyxin gene clusters

AntiSMASH analysis of the genome of *P. polymyxa* DSM 365 showed that the strain has a number of secondary metabolite gene clusters for the production of a number of antimicrobial metabolites. Table 3.6.1 shows the antiSMASH results where the similarity score was 100 %. Most of the antimicrobials are non-ribosomal peptide synthases (NRPSs) and ribosomally synthesized and post-translationally modified peptides (RiPPs).

Table 3.6.1: The biosynthetic gene clusters for antimicrobials in *P. polymyxa* DSM 365 found with 100% similarity score via antiSMASH analysis

Region	Type	From	To	Most similar known cluster
1.4	lanthipeptide	572 038	599 044	paenilan NRP
4.1	lanthipeptide	366 915	393 356	paenicidin B RiPP:Lanthipeptide
6.1	lanthipeptide	122 181	146 546	paenibacillin RiPP:Lanthipeptide
16.1	NRPS	44 787	96 486	fusaricidin B. Polyketide + NRP: Lipopeptide
40.1	NRPS	1	11450	tridecaptin. NRP
41.1	NRPS	1	10 091	polymyxin. NRP
47.1	NRPS	1	1277	anabaenopeptin NZ857/nostamide A. NRP
51.1	NRPS	1	1277	anabaenopeptin NZ857/nostamide A. NRP
60.1	NRPS	1	1043	icosalide A/ icosalide B. NRP: Lipopeptide
63.1	NRPS	1	1018	anabaenopeptin NZ857/nostamide A. NRP
64.1	NRPS	1	1018	xenematide. NRP

The antiSMASH analysis finds polymyxin as the most similar known cluster in region 40.1. This region is identical to contig 42 in the *P. polymyxa* DSM 365 genome. The cluster consist of four domains and these are numbered beginning from the left. Figure 3.6.2 shows the top three results for the ClusterBlast function, where similar clusters to the query sequence are shown. The trend is similar for the nine best results, three out of four domains are identical to the query. These have a NRPS as domain nr 1 (marked in red), an ABC transporter ATP binding protein as domain nr 2 and 3 (marked in blue) and a NRPS as domain nr 4 (white). The white color indicates that the region show no similarity with the query sequence.

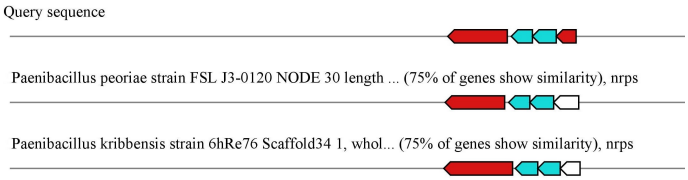


Figure 3.6.2: ClusterBlast result for region 41.1 with 100% similarity to polymyxin gene clusters. The first gene from the left, marked in red, encode NRPS. The second and third genes encode ABC binding protein and are marked in blue. The fourth gene marked in white show no similarity with the query sequence.

A comparison was made between the polymyxin region found by antiSMASH and the nine most similar gene clusters using the BRIG program. Figure 3.6.3 shows the result of the analysis. The innermost circle represent the cluster that is the most similar, while the outermost circle is the least similar of the nine ClusterBlast results. A strong color indicates highly similar or identical region, while weak or gray colored region indicate less similar sequences. *ATP transporter protein* is a shorter version name for the ABC transporter ATP binding protein. The two ATP transporter proteins appear to be highly conserved as these sequences are identical for all the strains. There are some differences in the NRPS sequences, especially at the beginning of the first domain and at the end of the fourth domain.

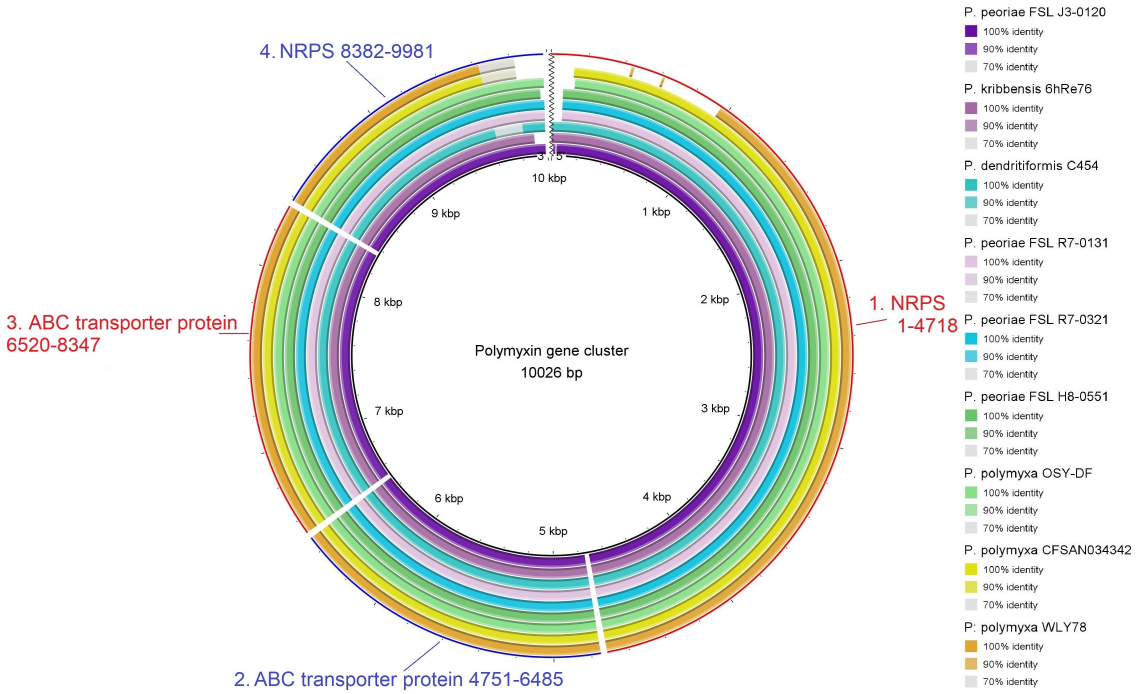


Figure 3.6.3: Visualization of the similarities between the polymyxin gene cluster identified in region 41.1 and the nine most similar gene clusters.

Figure 3.6.4 shows the result from the KnownClusterBlast function in antiSMASH. It shows biosynthetic clusters that are similar to the current region. Polymyxins A and B are the most similar clusters, even though they are larger in size.

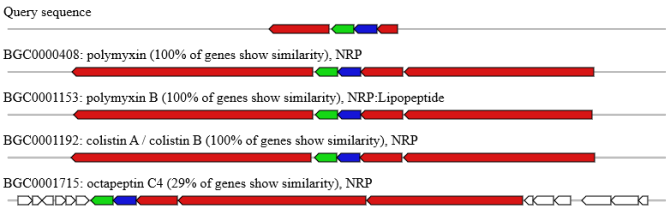


Figure 3.6.4: KnownClusterBlast result for region 41.1. This region show 100 % similarity with regions for polymyxin biosynthesis. The polymyxin gene cluster in *P. polymyxa* DSM 365 appear to be much shorter than for other strains.

The polymyxin cluster in region 41.1 was identical to contig 42. This indicated that the 50 kbp region in contig 01 which was annotated by BLAST analysis, was not likely to be related to the polymyxin production. It was desired to see if the annotated cluster was linked to the biosynthesis of any of the other antimicrobials. BRIG was used to compare the annotated gene cluster to all the 40 biosynthetic gene clusters which were identified by antiSMASH. Figure 3.6.5 shows the BRIG image comparing the gene clusters. One of the gene clusters show high similarity for 45 out of 50 kbp. This cluster has a 53 % similarity to the known clusters for bacillibactin.

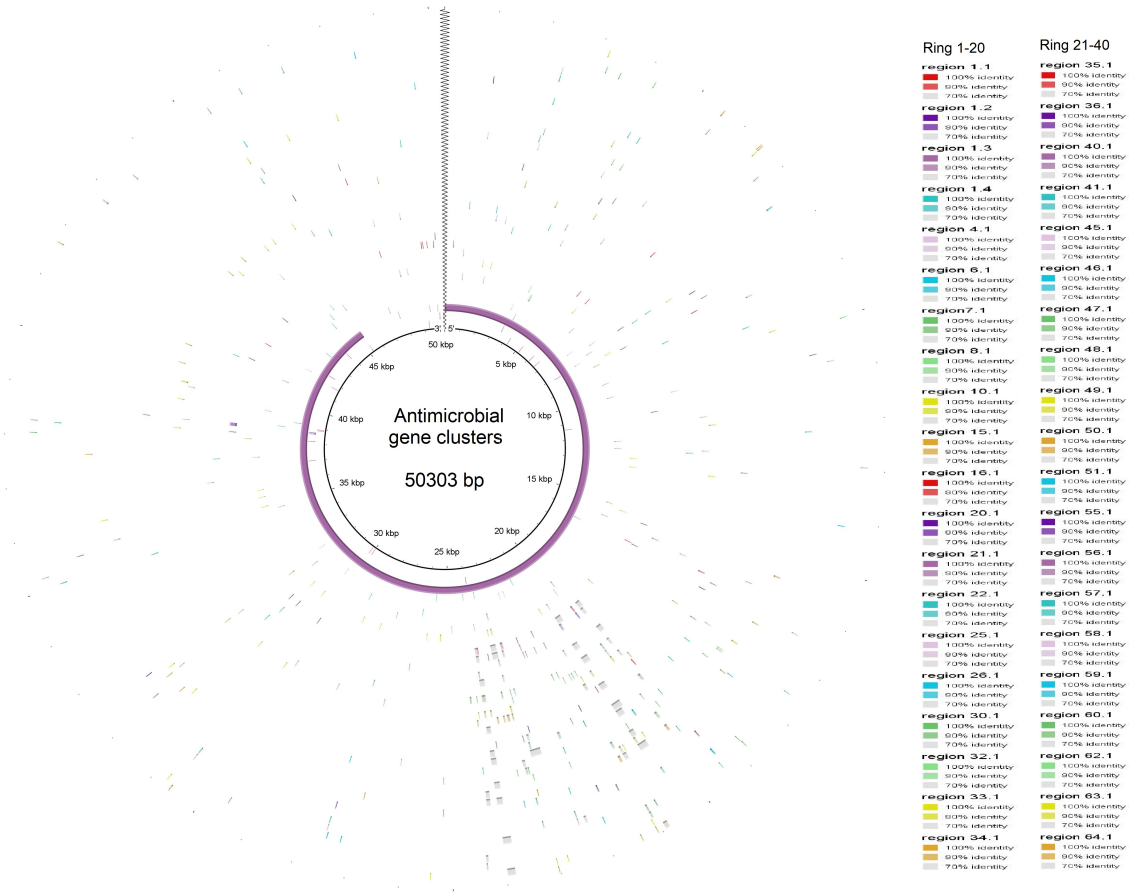


Figure 3.6.5: Comparison of the annotated region in contig 01 and the identified biosynthetic gene clusters found by antiSMASH. Only one of the gene clusters show a high similarity with the reference region.

4 | Discussion

4.1 Targeted gene deletions

CRISPR-Cas systems for the deletion of *PLLP19*, *ugdH1*, *ugdH2*, *Clu1Clu2*, and *Clu2* were successfully constructed and verified by sequencing. *PL01* and *PLLP19* were deleted form the genome of *P. polymyxa* DSM 365 and *P. polymyxa* ΔSpoIIE. The deletions were also combined in both strains. Deletion of the *sacB* gene was attempted, but recombination was not successful in the tested colonies. The plasmid was sequenced and the targeting of *sacB* was verified. This indicated that the plasmid might not have been successfully transferred after all and that the colonies might have survived on the selective plates because of the calcium chloride. Clones were obtained for the transfer of pCasPP ugdH1 and pCasPP ugdH2 to *P. polymyxa* DSM 365 and *P. polymyxa* ΔSpoIIEΔPL01ΔPLLP19. For *Clu1Clu2* and *Clu2* deletion plasmids, the transfer was only successful to the latter strain. The deletion of *ugdH1*, *ugdH2*, *Clu1Clu2*, and *Clu2* from *P. polymyxa* DSM 365 and *P. polymyxa* ΔSpoIIEΔPL01ΔPLLP19 were not verified due to PCR-related problems and limited time in the lab.

Earlier in the project it was experienced some similar issues with the PCRs. After trouble shooting it was discovered that several of the dNTP vials used in the PCRs were non-functional. It was then necessary to order new dNTPs, which solved the problem. If the current problem with the PCR reactions had a similar cause, then it would take too much time to solve the problem. It was therefore determined to postpone the verification of these deletions to future work.

Non-verified clones were transferred to EPS plates and the morphology was investigated. It appeared that the EPS production was close to normal for *P. polymyxa* DSM 365 with plasmids for deletion of *ugdH1* and *ugdH2*. This could either mean that the deletions were not successful, or that the deletion of these genes did not affect the EPS-production. Rütering et al. documented that the deletion of *ugdH1* reduced the EPS production for *P. polymyxa* DSM 365 [34]. By this, it was believed to be more likely that the deletion was unsuccessful for these clones. *P. polymyxa* ΔSpoIIEΔPL01ΔPLLP19 with plasmids for the deletion of *ugdH1*, *Clu1Clu2* and *Clu2* had clones with a distinct morphology. These clones were flat and appeared not to produce any EPS.

A common denominator for these three sets of clones is that all of them delete *ugdH1*, *ugdH2*, or both genes. Therefore it is probable that both *ugdH1* and *ugdH2* are important for the EPS production. The differences between *P. polymyxa* DSM 365 + pCasPP ugdH1 and *P. polymyxa* ΔSpoIIEΔPL01ΔPLLP19 + pCasPP ugdH1

were quite significant. This indicated that the first deletion was unsuccessful. Still, there is a chance that the change in morphology is not solely caused by the deletion of *ugdH* genes, but that a combination of the deletions of *SpoIIE*, *PL01*, *PLLP19* and *ugdH1* or *ugdH2* could lead to a disruption of the EPS production. The deletions of *ugdH1*, *ugdH2*, *Clu1Clu2*, and *Clu2* in all strains should be properly verified in future work. Investigation of growth- and EPS production rates should also be performed to understand the full effect of the genomic modifications.

4.2 Gibson Assembly - an efficient replacement for overlap-extension PCR

Two methods for assembly of CRISPR-Cas plasmids were tested. First, the OE-PCR and Golden Gate Cloning were used to assemble pCasPP PLLP19. Then Gibson Assembly was used to assemble pCasPP ugdH1 and pCasPP ugdH2. pCasPP Clu1Clu2 and Clu2 were assembled by using Golden Gate Cloning after a Gibson Assembly reaction. The assembly of pCasPP PLLP19 took nine days from the first PCR until the plasmid was purified and finished. The assembly of pCasPP ugdH2 took only four days from the start to the end. Even with the insertion of the gBlocks via Golden Gate Cloning after the Gibson Assembly it only took six to seven days in total. This shows how much more efficient it is to use Gibson Assembly to make the CRISPR-Cas constructs contrary to the OE-PCR reaction.

One thing to be cautious about is that all the fragments need to be completely correct to have a successful Gibson Assembly. During this thesis several attempts were made to assemble plasmids with fragments that looked like they had the correct length, but yielded no products from the Gibson Assembly reaction. This was likely to be caused by some error in one or more of the fragments. The solution was simply to start from the beginning once again. Errors, e.g. in overlapping regions between two fragments, could also happen during the OE-PCR. Therefore it is not seen as a significant disadvantage for the method. For future construction of CRISPR-Cas systems based on pCasPP, it is recommended to use the Gibson Assembly procedure.

4.3 Characterization

The pectin lyase activity was investigated in the strains with deletions of *PL01* and *PLLP19*. For the LB growth curve, shown in Figure 3.3.1, one would normally expect a higher absorbance at the plateau phase. It was discovered that the spectrophotometer that was used showed values of up to 0.4 lower than one of the other spectrophotometers. This could indicate that the growth was actually within normal values. The same spectrophotometer was used for all the measurements. Therefore, the measurements still give valuable information about the growth properties of the four strains in both LB and pectin broth.

The only carbon-source in the pectin broth is pectin. Therefore it was necessary for the bacteria to be able to break this down to be able to survive. It looks like the Δ*PL01* and Δ*PL01*Δ*PLLP19* strains were only able to use pectin as a nutrient for a shorter period of time. It appeared as if the DSM 365 and Δ*PLLP19* strain were

able to degrade the pectin better and in a more stable manner. This indicated that the *PL01* gene might be more important for pectin degradation than the *PLLP19* gene. As a consequence it was believed that *PL01* also would be more important for the degradation of pectin-like products made by the strain.

Because all the strains appear to grow quite well in the beginning, this could imply that there are other ways for the bacteria to degrade pectin than by Pectin lyase or Pectin lyase like protein. If there is another way for the strains to break down pectin, then it only seems to be functional for shorter periods of time. Saharan et al. documented that Pectin lyase (E.C.4.2.2.10) activity by *Bacillus subtilis* reached its plateau after 18 to 21 hours. It was also shown that enzyme activity curve correlated with the growth rate[75]. The pectin medium which was used in this assay is not the same as the one used by Saharan et al. Still, it gives an indication that the Pectin lyase (E.C. 4.2.2.10) encoded by the *PL01* gene might have a higher activity after a certain amount of time. From Figure 3.3.2 it can be seen that the growth of *P. polymyxa* DSM 365 and *P. polymyxa* ΔPLLP19 reached a plateau after 12 hours. To investigate this further one could do analytical measurements of the amount of pectin lyases released to the media during growth.

It was planned to do analysis of the polymyxin production in the deletion strains by high performance liquid chromatography (HPLC). This was not possible due to the lock down of campus caused by the corona virus pandemic.

4.4 Optimization of procedures

4.4.1 Improved conjugation protocol

To investigate the effect of calcium chloride in agar plates on the recombination efficiency, a specific conjugation experiment was designed. The rate of successful recombinations were found for all the twelve tested parallels. It was found that increasing concentration of calcium chloride in selective plates significantly reduced the recombination efficiency. Based on the results of this experiment it is recommended to always use either LB neo/poly or LB neo/poly/trace elements as selective plates. Normal LB plates should be sufficient as non-selective plates. If there is experienced any issues with obtaining clones from conjugation it is recommended to only add 30 mM $CaCl_2$ to the non-selective plates.

One weakness with this experiment was that a large number of colonies were obtained from all the parallels. There was in other word not a problem with getting enough clones for this CRISPR-Cas system. This could be because the deletion was of only 270 bp. The deletions of *PLLP19* were not confirmed by sequencing yet at this point. Therefore it was decided to use pCasPP PL01 rather than pCasPP PLLP19, even though pCasPP PLLP19 might have given more relevant results with regards to larger deletions in the future.

The reduced recombination efficiency in calcium chloride containing selection plates could be caused by the a deactivation of the polymyxin by the salt. It is also possible that the presence of calcium chloride reduces the attractive forces between the polymyxin and the LPS layer, and this way shielding the cell membrane from the antibiotic. The resulting effect is an apparent reduced sensitivity to polymyxin.

This would make both *P. polymyxa*, and *E. coli* S17-1 able to survive on the selective plates, and make it less likely to pick the correct clone.

Another explanation could be that the effect of neomycin was reduced by the calcium chloride. Neomycin works by binding irreversibly to proteins in the 30S ribosomal subunit and thereby disrupting protein synthesis[76]. Calcium chloride could possibly interact with either neomycin or the proteins transporting the neomycin into the cell. Then the conjugation would result in clones of *P. polymyxa* both with and without the plasmids. To investigate this further one could analyze the bacterial composition on selective plates at varying concentrations of calcium chloride. If the neomycin is the cause for the reduced recombination efficiency, one could easily replace this antibiotic with another one.

4.4.2 Antibiotic susceptibility test

Antibiogram and antibiotic susceptibility test of *P. polymyxa* showed that none of the tested antibiotics were suitable to replace polymyxin as a selection agent.

Potential candidates to replace neomycin were tetracycline and gentamycine, as both *P. polymyxa* and *E. coli* are sensitive to these antibiotics. The replacement of neomycin would also require a modification of the pCasPP plasmid to introduce the new resistance gene.

To ensure that it would be possible to let non-EPS producing strains be subject to conjugational transfer of CRISPR-Cas systems it would be possible to investigate further if other antimicrobials are released by *P. polymyxa* and if these are effective against *E. coli* S17-1. Another objective could be to focus on using electroporation as a replacement for conjugation. During this thesis it has been shown that electroporation was successful for *P. polymyxa* ΔClu1, but not for *P. polymyxa* DSM 365 and *P. polymyxa* ΔSpoIIEΔPL01ΔPLLP19. This was likely caused by not successfully making the two latter strains electrocompetent. Future work could investigate this further and optimize the procedure of making electrocompetent *P. polymyxa* cells.

4.5 Annotation of polymyxin gene cluster

The biosynthetic gene cluster for polymyxin was first attempted identified by annotating the region around the Polymyxin synthetase PmxB, which was identified during RAST annotation of the whole genome of *P. polymyxa* DSM 365. A more efficient analysis of antimicrobial gene clusters was performed using antiSMASH. This tool identified the polymyxin gene cluster in region 41.1, which is identical to contig 42. This region is only 10 091 bp, which is relatively short compare to the 41 040 bp polymyxin gene cluster identified in *P. polymyxa* E681. The region identified by blast analysis in contig 01 is likely to be responsible for production of bacillibactin, but further research is necessary to confirm this. Future work should also include further analysis and deletion of the polymyxin gene cluster.

5 | Conclusion

Pectin lyase (*PL01*) and Pectin lyase like protein (*PLLP19*) have been deleted from *P. polymyxa* DSM 365. These deletions have been combined into seven novel mutant strains. These included non-sporulating strains and strains where the first part of the paenan biosynthetic gene cluster (*Clu1*) were removed. Four of the seven strains were subject to a pectin assay to investigate the strains ability to degrade pectin. This assay showed that the *PL01* gene was more important than *PLLP19* for the utilization of pectin as a nutrient. The result indicated that it was more important to delete the *PL01* gene to prevent the degradation of pectin-like products.

CRISPR-Cas constructs was successfully assembled for the deletion of two UDP-glucose dehydrogenase genes, *ugdH1* and *ugdH2*. These genes were believed to play a role in the sensitivity to polymyxin. The deletion of these genes from *P. polymyxa* strains were not verified, and thus the polymyxin sensitivity could not be tested. For the deletion of the full paenan cluster (*Clu1Clu2*) and the second half of the same cluster (*Clu2*), CRISPR-Cas systems were assembled. The constructs were transferred to *P. polymyxa* by conjugation, but the deletions were not verified. Levansucrase (*sacB*) was also attempted to be removed from the genome, but the genomic modification was unsuccessful.

Addition of calcium chloride to agar plates increased the number of clones obtained by conjugation. The effect on the recombination efficiency was investigated through a tailored experimental series. It was found that the presence of calcium chloride, especially in the selective plates, drastically reduced the recombination efficiency.

The susceptibility to various antibiotics by *P. polymyxa* were assessed through antibiogram and antibiotic susceptibility tests. No alternative was found to replace polymyxin as selection marker during conjugation. Transfer of plasmids to *P. polymyxa* by electroporation was successful for one of the strains, and can therefore be a valid alternative to conjugation.

The biosynthetic gene cluster for polymyxin (*pmx)* was identified by antiSMASH analysis of the genome of *P. polymyxa* DSM 365. Future work includes the deletion of this gene cluster, and the subsequent analysis of polymyxin production. The polymyxin production and resistance should also be assessed for the *ugdH1* and *ugdH2* deletion strains. For the target chassis organism, the deletions of *Clu1Clu2*, *sacB*, and *pmx* should be combined in the present *P. polymyxa* ΔSpoIIEΔPL01ΔPLLP19 strain. Further characterization of this strain, as well as the genome, would be important for determination of future deletion targets.

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A | Primer data

Table A.0.1: Complete list of primers used in this thesis. Primer number(#), primer name, DNA sequence (in 5'-3' direction), and short descriptions of their purpose are listed. Restriction enzyme cut sites are underlined.

Beginning of Table A.0.1

#	Primer name	Sequence (5'-3')	Purpose
1	27fn	AGAGTTTGATCMTGGCTCAG	16s-rRNA primers
2	1525r	AAGGAGGTGWTCCARCC	16s-rRNA primers
3	seq_sg_pCasMC.REV	CCAGGGGGAAACGCCTGG	Binds to pCasPP. [34]
4	seq_PSG5_F	GGAAAGTCTACACGAACCCTTTGGC	Binds to pCasPP. [34]
5	seq_sg_pCasMC.FOR	CCGAATATATCGGTTATGCGTGG	Binds to pCasPP. [34]
6	seq_Harms_R	GCGATTGAGGAAAAGGCGGC	Binds to pCasPP. [34]
7	pepQ_KOproof_F	TGTATATTCGGTCGCTCATGGGCTT	Verify correct deletion of the *pepQ* gene.
8	pepQ_KOproof_R	ATCCCGGCAATTTTAGCAATCGCAT	Verify correct deletion of the *pepQ* gene.
9	pepF_KOproof_F	GGCTCTGCTGGTGCCGTGCATGC	Verify deletion of the *pepF* gene. [77]
10	pepF_KOproof_R	GCATCGTATGCAGTAAATCCAAACGC	Verify deletion of the *pepF* gene. [77]
11	PL01_KOproof_F	CCTGATTCAGCTGGTTGACGATAGG	Verify correct deletion of the pectin lyase (*PL01*) gene in contig 01.
12	PL01_KOproof_R	CTCATACCGATCATCCTTTCCCAGC	Verify correct deletion of the pectin lyase (*PL01*) gene in contig 01..
13	PLLP19_sg1_F	ACGC GTAGGCACCGATGTTCACAG	Anneal guide RNA for assembly of pCasPP PLLP19A (sg1)
14	PLLP19_sg1_R	AAAC CTGTGAACATCGGTGCCTAC	Anneal guide RNA for assembly of pCasPP PLLP19A (sg1).
15	PLLP19_sg2_F	ACGC GGTTTTCGGGCTTGGTCTGG	Anneal guide RNA for assembly of pCasPP PLLP19B (sg2).
16	PLLP19_sg2_R	AAAC CCAGACCAAGCCCGAAAACC	Anneal guide RNA for assembly of pCasPP PLLP19B (sg2).
17	PLLP19_US_F	GTATCTAG AAAGCTATGAGTGTGTTCAGTAAAGATGG	Amplification of US fragment of PLLP19 HDR-template.

Continuation of Table A.0.1

#	Primer name	Sequence (5'-3')	Purpose
18	PLLP19_US_R	ATATACGGCGTTCCTCATCCCCTTTGTTTTTCTTGT	Amlification of US fragment of PLLP19 HDR-template.
19	PLLP19_DS_F	TGAGGAACGCCGTATATTTTATGACAAACAGAGCATGG	Amplification of DS fragment of PLLP19 HDR-template.
20	PLLP19_DS_R	AATCTAG AAGAGTGGCGCCGACT	Amplification of DS fragment of PLLP HDR-template.
21	PLLP19_KOproof_F	CCTGAACAGACTCAAACCCTGCCTA	Verify correct deletion of the *PLLP19*.
22	PLLP19_KOproof_R	AGGCCGGATTTACTCCCATAGAAGC	Verify correct deletion of *PLLP19*.
23	PL01_KOproof_new_F	TATTCCGGTGGCTGAAGAAAGTGGT	Verify correct deletion of *PL01* without curing deletion construct
24	PL01_KOproof_new_R	AATAGCCACTTCGACTTTGGATGCG	Verify correct deletion of *PL01* without curing deletion construct
25	Clu2_gBlock_F	GAGACATCTTTGAAGACAA ACGC	Amplification of gBlock containing sg1 and sg2 for deletion of Clu2. Can also be used to amplify the Clu1Clu2_gBlock.
26	Clu2_gBlock_R	GCCACGTGAAAGAAGACTT AAAC	Amplification of gBlock containing sg1 and sg2 for deletion of Clu2. Can also be used to amplify the Clu1Clu2_gBlock.
27	Clu2_Frag1_R	GGAGTGTATGTTCCAGCTGGGGCCTTTTTACGGTTCCTGGC	Assembly of pCasPP Clu2.
28	Clu2_Frag2_F	GCCAGGAACCGTAAAAAGGCCCCAGCTGGAACATACACTCC	Assembly of pCasPP Clu2.
29	Clu2_Frag2_R	CGTTTCATATGCAGCCTCCCCTTTATGAGATAAATTGGTGACGGG	Assembly of pCasPP Clu2.
30	Clu2_Frag3_F	CCCGTCACCAATTTATCTCATAAAGGGGAGGCTGCATATGAAACG	Assembly of pCasPP Clu2.
31	Clu2_Frag3_R	CTATGGAAAAACGCCAGCAACGCGGCGATATGGCAGTGGTAAGC	Assembly of pCasPP Clu2.
32	Clu2_Frag4_F	GCTTACCACTGCCATATCGCCGCGTTGCTGGCGTTTTTCCATAG	Assembly of pCasPP Clu2.
33	Clu2_gBlock_insert_F	GACGTTAGCTTCACCTGGTTTTAGAG	Use together with seq_Harms_R to verify insertion of Clu2_gBlock.
34	Clu2_KOproof_F	CTCGAACATTTTACAGGCTGACCGG	Verify deletion of *Clu2* without curing plasmid.
35	Clu2_KOproof_R	CATTGCCCCTCCTTCTGTTGGACTA	Verify deletion of *Clu2* without curing plasmid.
36	Clu1Clu2_Frag1_R	CGGATCTGTGTTCCCTCTCCGGGCCTTTTTACGGTTCCTGGC	Assembly of pCasPP Clu1Clu2.
37	Clu1Clu2_Frag2_F	GCCAGGAACCGTAAAAAGGCCCGGAGAGGGAACACAGATCCG	Assembly of pCasPP Clu1Clu2.
38	Clu1Clu2_Frag2_R	CGTTTCATATGCAGCCTCCCCTGAGGCTTTTCATTCCACTCGC	Assembly of pCasPP Clu1Clu2.
39	Clu1Clu2_Frag3_F	GCGAGTGGAATGAAAAGCCTCAGGGGAGGCTGCATATGAAACG	Assembly of pCasPP Clu1Clu2.

Continuation of Table A.0.1

#	**Primer name**	**Sequence (5'-3')**	**Purpose**
40	Clu1Clu2_KOproof_F	CGAGAATGGTAGCAGGAGGTGAGG	Verify deletion of *Clu1Clu2* without curing plasmid. Use together with Clu2_KOproof_R.
41	Clu1Clu2_gBlock_insert_F	GAGGCAGACGCAGACATGTTTTAG	Use together with seq_Harms_R to verify insertion of Clu1Clu2_gBlock.
42	ugdH1_frag1_F	CGTCGACAAGCTCTTCATCCAGCTGGTGCAGACCTACAACC	Assembly of pCasPP ugdH1.
43	ugdH1_Frag1_R	AACCACAATATTCGGGTGATCCAGCGTATCCCCTTTCAGATACTCG	Assembly of pCasPP ugdH1.
44	ugdH1_Frag2_F	TGGATCACCCGAATATTGTGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGC	Assembly of pCasPP ugdH1.
45	ugdH1_Frag2_R	GCCAAGTGGCTCTAATATCCGCATGGCCTTTTTACGGTTCCTGGC	Assembly of pCasPP ugdH1.
46	ugdH1_Frag3_F	GCCAGGAACCGTAAAAAGGCCATGCGGATATTAGAGCCACTTGGC	Assembly of pCasPP ugdH1.
47	ugdH1_Frag3_R	CCCCGCCACAGTCACTTTCCTCCTTTTAGAAAGTTCACATAGGC	Assembly of pCasPP ugdH1.
48	ugdH1_Frag4_F	GCCTATGTGAACTTTCTAAAAGGAGGAAAGTGACTGTGGCGGGG	Assembly of pCasPP ugdH1.
49	ugdH1_Frag4_R	GGAAAAACGCCAGCAACGCGCTCAACAATGATGCGCGTATTC	Assembly of pCasPP ugdH1.
50	ugdH1_Frag5_F	GAATACGCGCATCATTGTTGAGCGCGTTGCTGGCGTTTTTCC	Assembly of pCasPP ugdH1.
51	ugdH1_Frag5_R	GGTTGTAGGTCTGCACCAGCTGGATGAAGAGCTTGTCGACG	Assembly of pCasPP ugdH1.
52	ugdH1_KOproof_F	ACCAGAACCATTCGGTCAGGTCATT	Verify correct deletion of *ugdH1* without curing plasmid.
53	ugdH1_KOproof_R	TAGAGCATTGCGTTCAATCAGACGC	Verify correct deletion of *ugdH1* without curing plasmid.
54	ugdH2_Frag1_R	CCGAACGGATATCCGTGGTTGCGTATCCCCTTTCAGATACTCG	Assembly of pCasPP ugdH2.
55	ugdH2_Frag2_F	AACCACGGATATCCGTTCGGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGC	Assembly of pCasPP ugdH2.
56	ugdH2_Frag2_R	TGCATAGTTTTCATGGGCTACTGCGGCCTTTTTACGGTTCCTGGC	Assembly of pCasPP ugdH2.
57	ugdH2_Frag3_F	GCCAGGAACCGTAAAAAGGCCGCAGTAGCCCATGAAAACTATGCA	Assembly of pCasPP ugdH2.
58	ugdH2_Frag3_R	CGTTTCATATGCAGCCTCCCCTTGCATCCTGCTTTTACCGACTTC	Assembly of pCasPP ugdH2.
59	ugdH2_Frag4_F	GAAGTCGGTAAAAGCAGGATGCAAGGGGAGGCTGCATATGAAACG	Assembly of pCasPP ugdH2.

Continuation of Table A.0.1

#	**Primer name**	**Sequence (5'-3')**	**Purpose**
60	ugdH2_Frag4_R	CTATGGAAAAACGCCAGCAACGCAGGCTTACCTAAGGCGATATGGC	Assembly of pCasPP ugdH2.
61	ugdH2_Frag5_F	GCCATATCGCCTTAGGTAAGCCTGCGTTGCTGGCGTTTTTCCATAG	Assembly of pCasPP ugdH2.
62	ugdH2_KOproof_F	ATGGAGACACGGTACTCGATTGCAT	Verify correct deletion of the *ugdH2* gene. Use together with Clu2_KOproof_R.
63	Clu2_gBlock_amp_F	TTTGAAGACAAACGCGGAGACGTTAGCTTCACCTGGTTTTAGAGCTAG	Amplify Clu2_gBlock from Clu1Clu2_gBlock.
64	Clu2_gBlock_amp_R	AAAGAAGACTTAAACTTTGTTTTGCCGATAAGATTGCGTATCCCCTTTC	Amplify Clu2_gBlock from Clu1Clu2_gBlock.
65	sacB_KOproof_F	AATTATCGCATTGCTGCCCAGACAG	Verify deletion of *sacB*
66	sacB_KOproof_R	AGATCGGGTTGCTACCAATCTACCG	Verify deletion of *sacB*

End of Table A.0.1

B | Supplementary tables and figures

B.0.1 EPS plates of conjugation products

Clones of *P. polymyxa* DSM 365 + pCasPP ugdH1, *P. polymyxa* ΔSpoIIEΔPL01ΔPLLP19 + pCasPP ugdH1, *P. polymyxa* DSM 365 + pCasPP ugdH2, *P. polymyxa* ΔSpoIIEΔPL01ΔPLLP19 + pCasPP Clu1Clu2, and *P. polymyxa* ΔSpoIIEΔPL01ΔPLLP19 + pCasPP Clu2 were added to EPS plates to study EPS production. The *P. polymyxa* DSM 365 and *P. polymyxa* ΔSpoIIEΔPL01ΔPLLP19 were also plated on EPS for comparison.

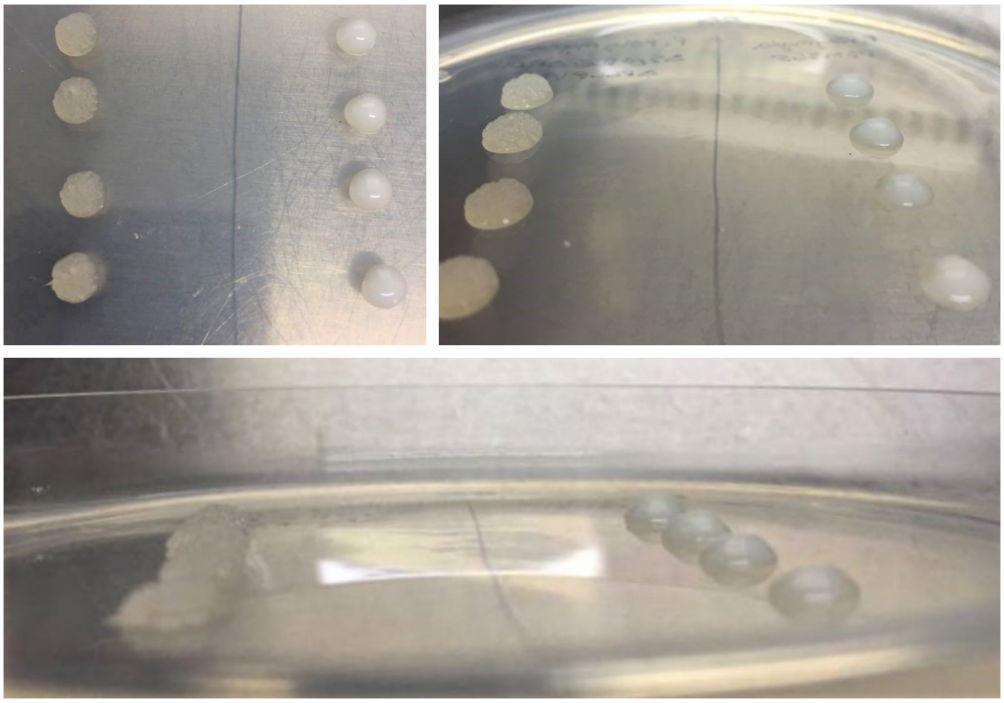


Figure B.0.1: EPS plates with *P. polymyxa* ΔSpoIIEΔPL01ΔPLLP19 on the left side and *P. polymyxa* DSM 365 on the right side

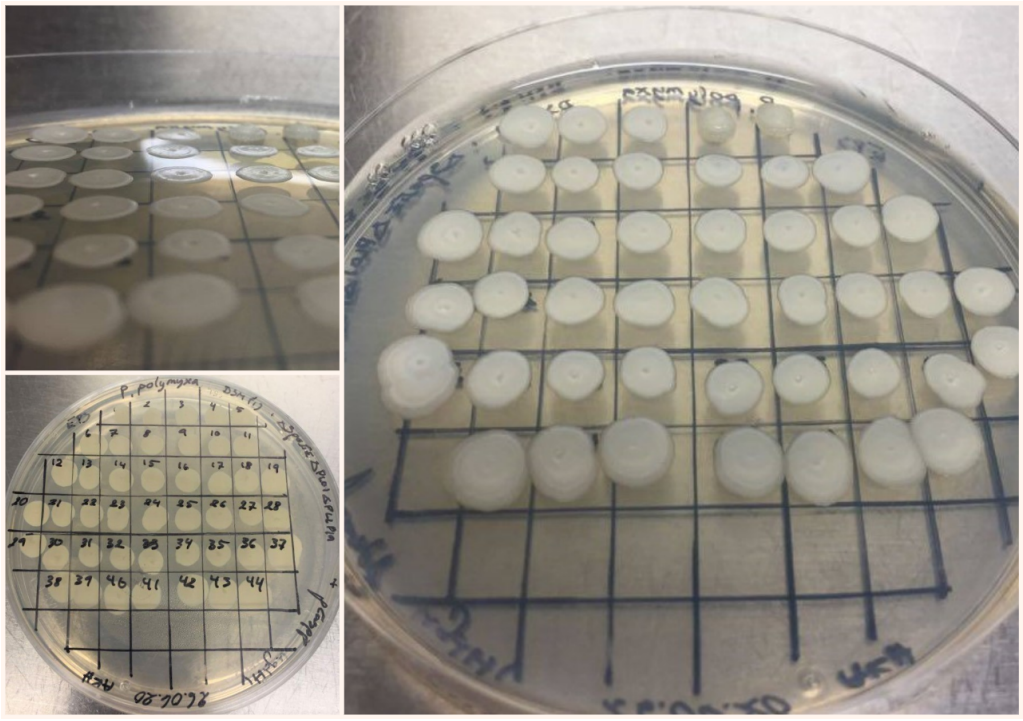


Figure B.0.2: Colonies from conjugational transfer of pCasPP ugdH1 to *P. polymyxa* DSM365 (nr 1 and 2) and to *P. polymyxa* ΔSpoIIE ΔPL01 ΔPLLP19 (nr 3-44) grown on EPS agar. Colonies 1 and 2 are slimy, while colonies 3-44 are flat and non-slimy.

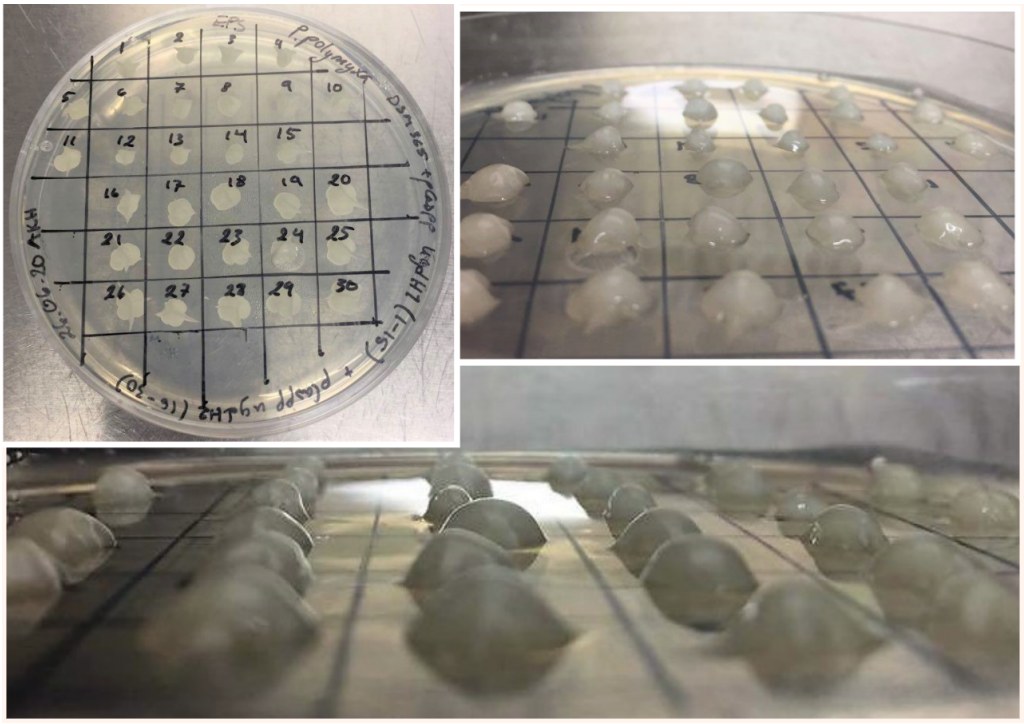


Figure B.0.3: Colonies from conjugational transfer of pCasPP ugdH1 (1-15) and pCasPP ugdH2 (16-30) to *P. polymyxa* DSM 365 on EPS plates.

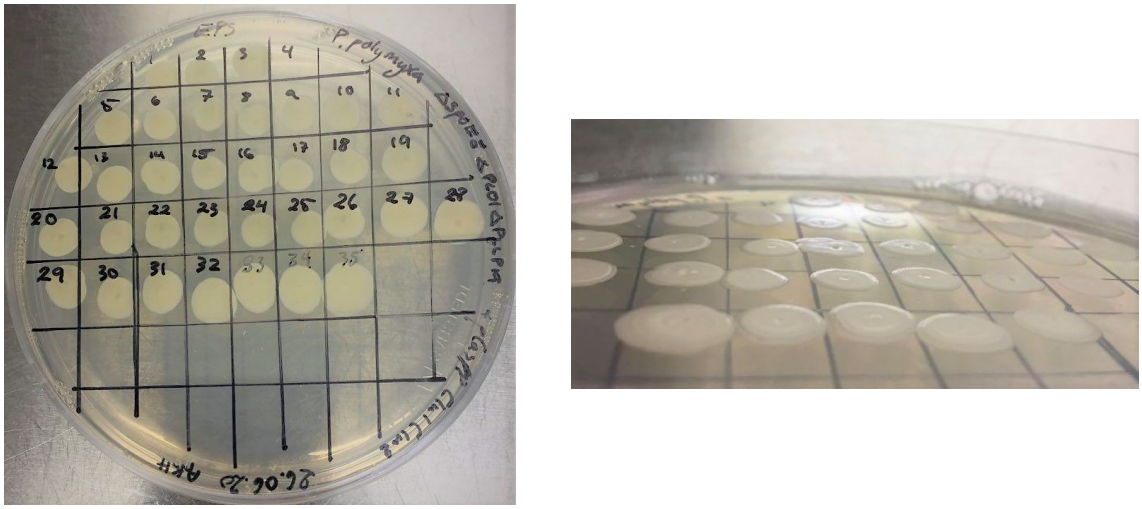


Figure B.0.4: Colonies from conjugational transfer of pCasPP Clu1Clu2 to *P. polymyxa* ΔSpoIIEΔPL01ΔPLLP19 on EPS plates.

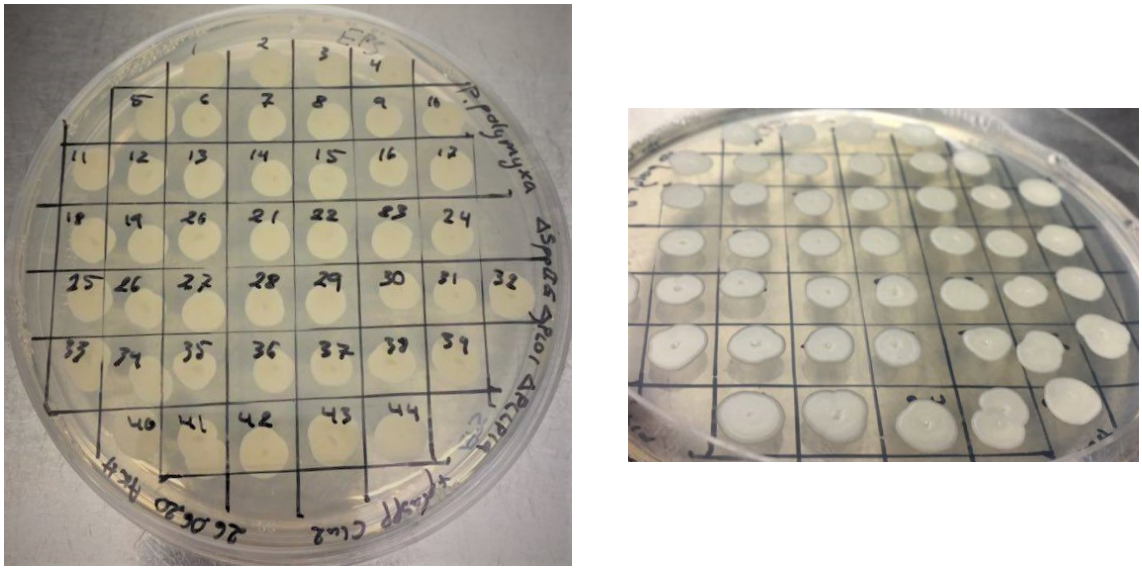


Figure B.0.5: Colonies from conjugational transfer of pCasPP Clu2 to *P. polymyxa* ΔSpoIIEΔPL01ΔPLLP19 on EPS plates. Colonies are flat and does not appear to produce EPS.

B.1 Gene deletions

Table B.1.1: Overview of the attempted CRISPR-Cas based genome modifications. Plasmids containing the deletion systems were transferred by either conjugation or electroporation into the target *P. polymyxa* strain.

Starting strain	Genes to delete	Plasmid transfer mechanism	Successful transfer	Verified deletion	New strain name
P. polymyxa DSM 365	PL01	Conjugation	Yes	Yes	*P. polymyxa* ΔPL01
P. polymyxa ΔSpoIIE	PL01	Conjugation	Yes	Yes	*P. polymyxa* ΔSpoIIEΔPL01
P. polymyxa ΔClu1	PL01	Conjugation	No	-	-
P. polymyxa DSM 365	PLLP19	Conjugation	Yes	Yes	*P. polymyxa* ΔPLLP19
P. polymyxa ΔSpoIIE	PLLP19	Conjugation	Yes	Yes	*P. polymyxa* ΔSpoIIE ΔPLLP19
P. polymyxa ΔSpoIIEΔPL01	PLLP19	Conjugation	Yes	Yes	*P. polymyxa* ΔSpoIIEΔPL01 ΔPLLP19
P. polymyxa ΔPL01	PLLP19	Conjugation	Yes	Yes	*P. polymyxa* ΔPL01 ΔPLLP19
P. polymyxa DSM 365	SacB	Conjuagtion	Yes	No, failed deletion	-
P. polymyxa ΔSpoIIEΔPL01 ΔPLLP19	SacB	Conjuagtion	Yes	No, failed deletion	-
P. polymyxa ΔPL01 ΔPLLP19	SacB	Conjuagtion	Yes	No, failed deletion	-
P. polymyxa DSM 365	PLLP19	Electroporation	No	-	-
P. polymyxa DSM 365	Clu1	Electroporation	No	-	-
P. polymyxa ΔSpoIIEΔPL01 ΔPLLP19	Clu1	Electroporation	No	-	-
P. polymyxa ΔClu1	PLLP19	Electroporation	Yes	Yes	*P. polymyxa* ΔClu1 ΔPLLP19
P. polymyxa DSM 365	ugdH1	Conjugation	Yes	No, verification failed	-
P. polymyxa ΔSpoIIEΔPL01 ΔPLLP19	ugdH1	Conjugation	Yes	No, verification failed	-
P. polymyxa DSM 365	ugdH1	Electroporation	No	-	-
P. polymyxa ΔSpoIIEΔPL01 ΔPLLP19	ugdH1	Electroporation	No	-	-
P. polymyxa DSM 365	Clu1Clu2	Conjugation	No	-	-
P. polymyxa ΔSpoIIEΔPL01 ΔPLLP19	Clu1Clu2	Conjugation	Yes	No, verification failed	-
P. polymyxa DSM 365	Clu1Clu2	Electroporation	No	-	-
P. polymyxa ΔSpoIIEΔPL01 ΔPLLP19	Clu1Clu2	Electroporation	No	-	-
P. polymyxa DSM 365	Clu2	Conjugation	No	-	-
P. polymyxa ΔSpoIIEΔPL01 ΔPLLP19	Clu2	Conjugation	Yes	No, verification failed	-
P. polymyxa DSM 365	Clu2	Electroporation	No	-	-
P. polymyxa ΔSpoIIEΔPL01 ΔPLLP19	Clu2	Electroporation	No	-	-
P. polymyxa DSM 365	ugdH2	Conjugation	Yes	No, verification failed	-
P. polymyxa ΔSpoIIEΔPL01 ΔPLLP19	ugdH2	Conjugation	No	-	-
P. polymyxa DSM 365	ugdH2	Electroporation	No	-	-

B.2 Recombination efficiency test

Evaluation of recombination efficiency for the CRISPR-Cas mediated deletion of *PLLP19* deletion.
Two or three colonies were picked from each conjugation plate and cured. These were then tested by colony PCR with primers 21+22. Correct recombination would give an amplicon of 824 bp, while incorrect deletion would give 2360 bp amplicon.

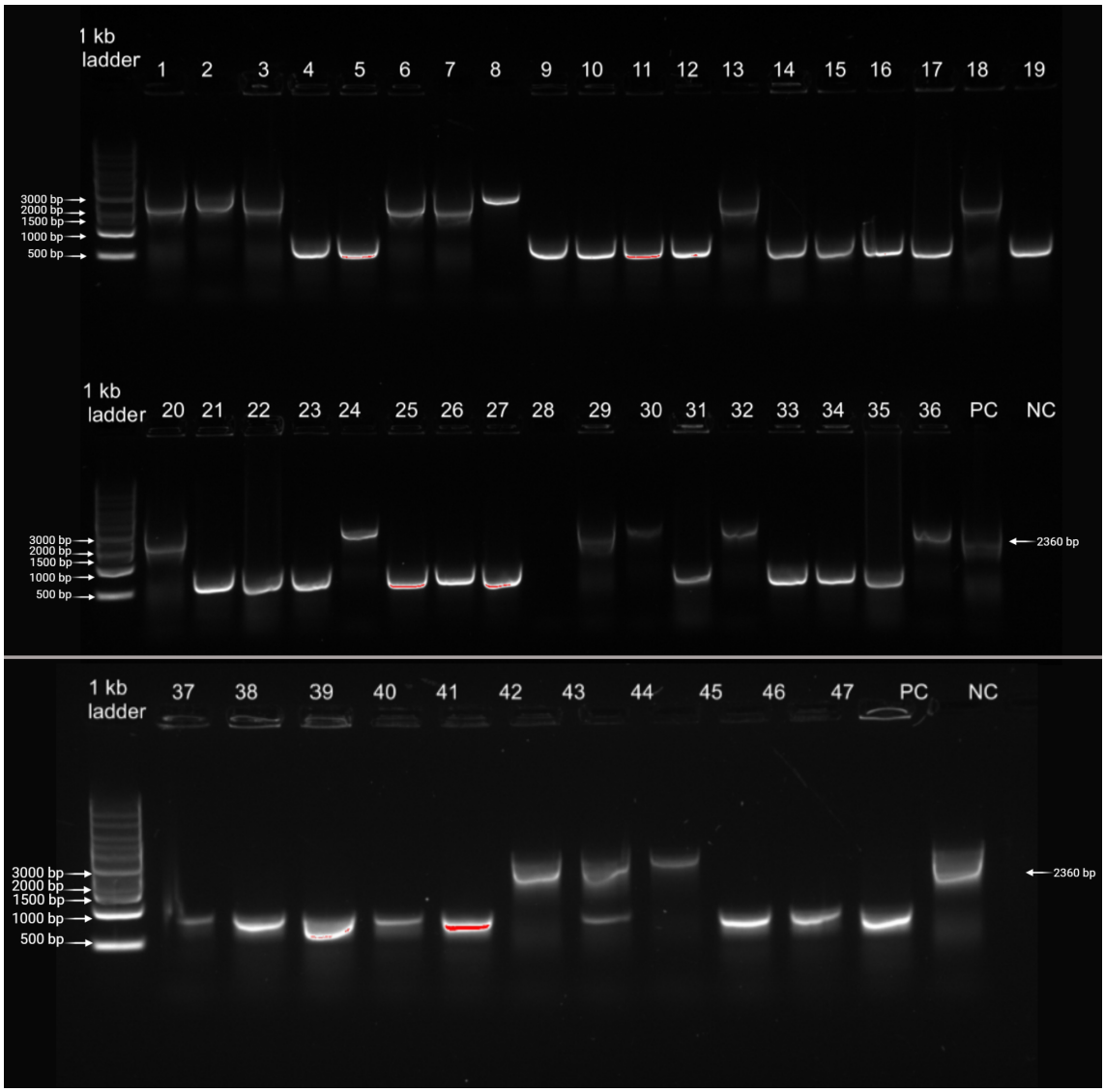


Figure B.2.1: 47 colonies were tested by colony PCR for the correct deletion of *PLLP19*. Of the tested colonies, 29 of them had the correct deletion. Positive control (PC) was gDNA and primers 21+22, which would give amplicon of 2360 bp.

An experiment was designed to evaluate the effect calcium chloride in agar medium had on the recombination efficiency during a conjugation. Figure B.2.2 shows the experimental set up, and the number of colonies obtained from each parallel. The red columns, numbered 1 to 4, were the different selective plates where normal LB plate was used as non-selective plate. The blue columns, numbered 5 to 8, shows

the selective plates where LB $CaCl_2$ [30 mM] was first used as non-selective plate. The yellow columns, numbered 9 to 12, shows the selective plates where LB $CaCl_2$ [60 mM] was first used as non-selective plate. The parallels are referred to by these numbers on the following gel pictures. Ten colonies were picked for each parallel, and these were referred to by decimal numbers to the parallel name. So as an example, the fifth colony picked from the 4th parallel, which had LB as non-selective plate and LB neo/poly/$CaCl_2$ [60 mM] as selective plate, were then referred to as C4.5.

C	*E. coli S17* pCasPP PL01.1											
	LB				LB CaCl2 [30 mM]				LB CaCl2 [60 mM]			
	LB neo/poly	LB neo/poly/ trace elements	LB neo/poly /CaCl2 [30 mM]	LB neo/poly /CaCl2 [60 mM]	LB neo/poly	LB neo/poly /trace elements	LB neo/poly /CaCl2 [30 mM]	LB neo/poly /CaCl2 [60 mM]	LB neo/poly	LB neo/poly /trace elements	LB neo/poly /CaCl2 [30 mM]	LB neo/poly /CaCl2 [60 mM]
	197*	458*	>1000**	>1000**	>1000*	>1000*	>1000***	>1000***	736*	>1000*	>1000***	>1000***
	1	2	3	4	5	6	7	8	9	10	11	12
	*	Single colonies										
	**	Very small, but single colonies										
	***	Small colonies, confluent growth										

Figure B.2.2: Experimental set up for the experiment used to evaluate the effect calcium chloride had on the recombination efficiency.

Both primers 11+12 and 23+24 were used to verify the deletion of *PL01*. The first pair makes it easier to see the deletion, but depend on a complete curing of the plasmid. They give amplicons of 715 bp for correct deletion and 985 bp for incorrect deletion. Primers 23+24 does not depend on the curing of the plasmid, but give bands that are not always as easy to distinguish. Correct deletion would give amplicon of 2278 bp, while incorrect deletion would give 2548 bp. The positive control (PC) for these reactions were always the *P. polymyxa* DSM 365 genome and the same primers as the rest of the samples.

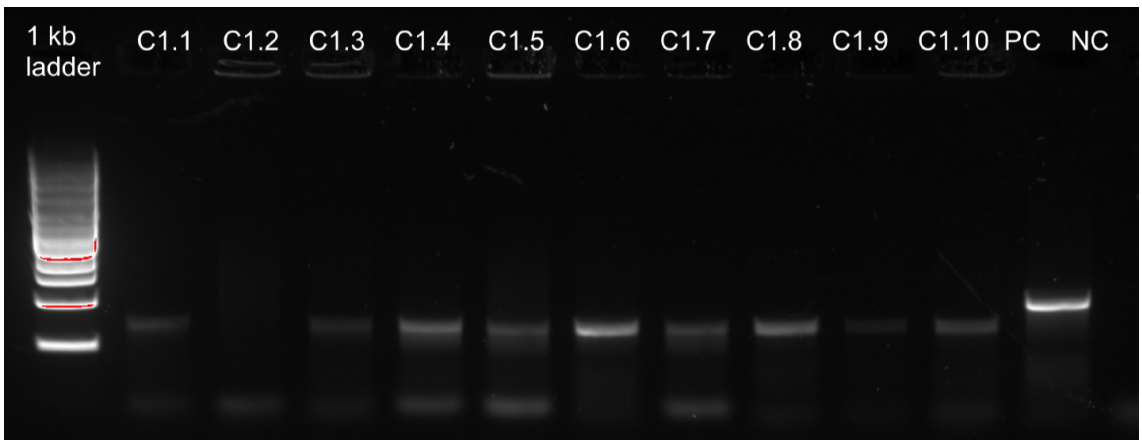


Figure B.2.3: The ten colonies selected from the C1 parallel were tested by PCR for the correct deletion. Primers 11+12 were used in the PCR reaction.

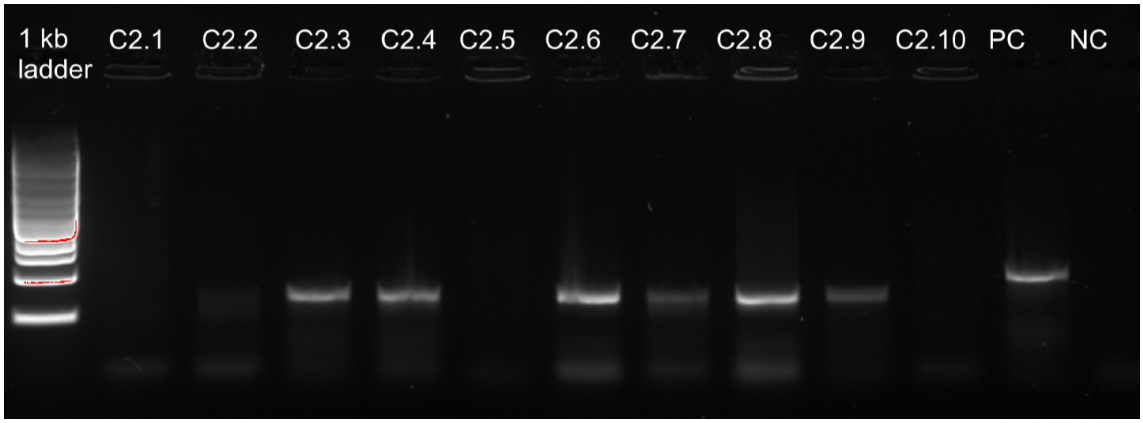


Figure B.2.4: The ten colonies selected from the C2 parallel were tested by PCR for the correct deletion. Primers 11+12 were used in the PCR reaction.

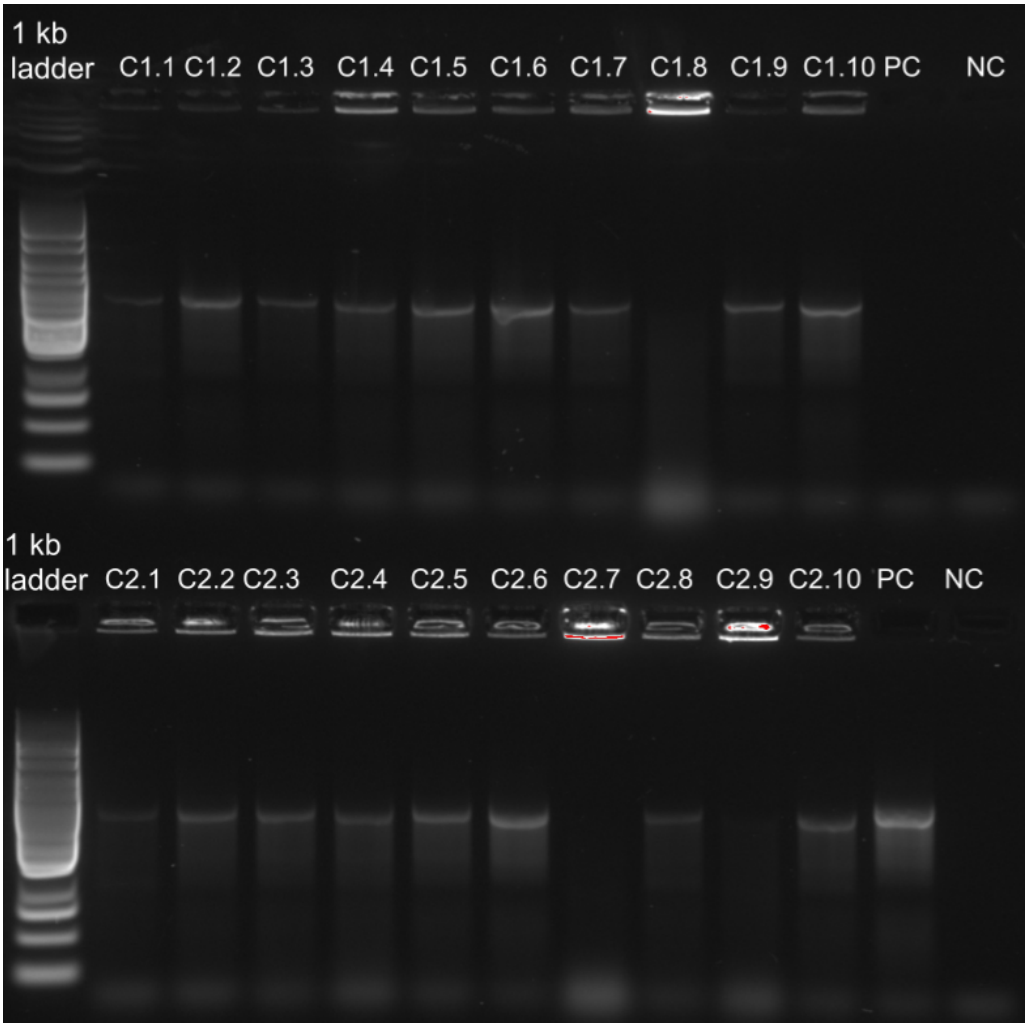
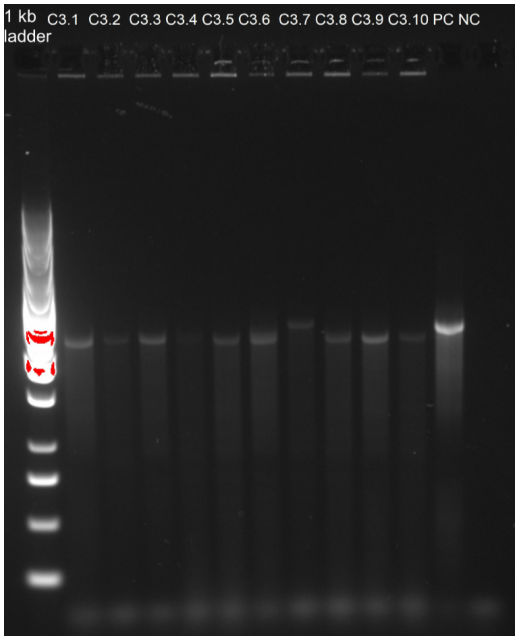
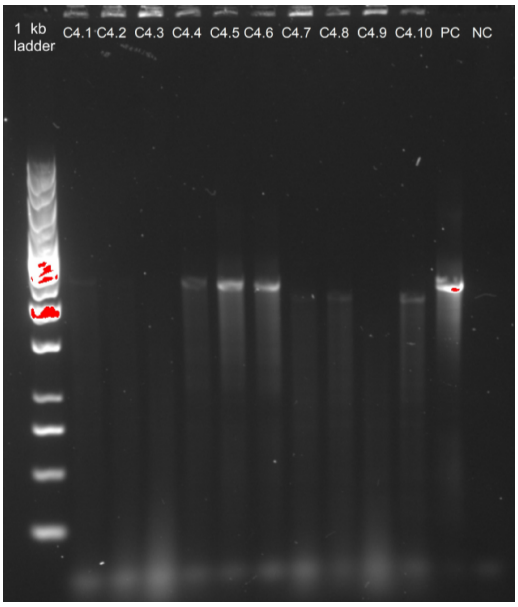


Figure B.2.5: The ten colonies selected from the C1 and C2 parallel were tested by PCR for the correct deletion. Primers 23+24 were used in the PCR reaction.

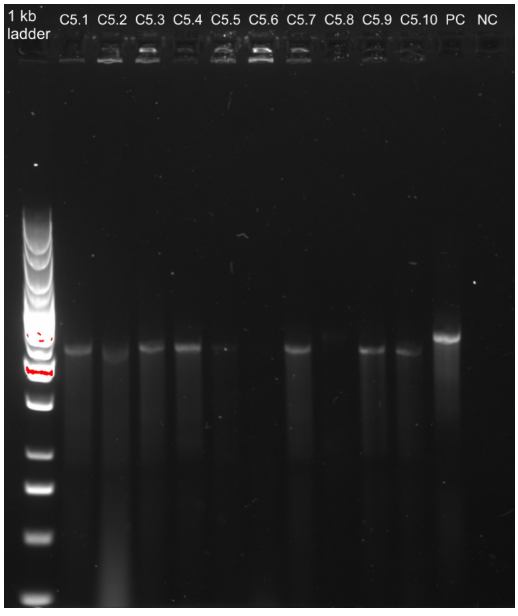


(a) C3

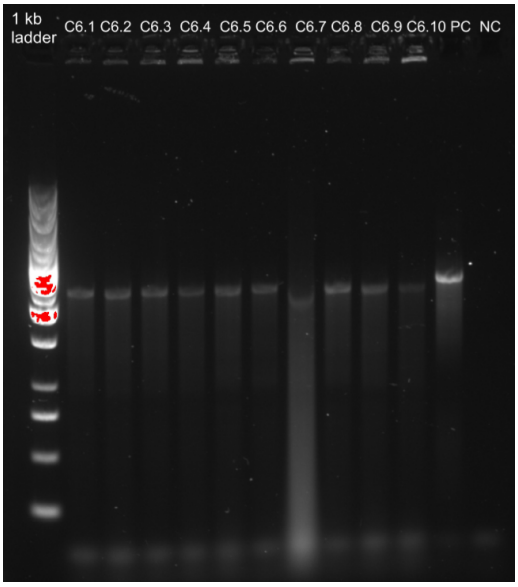


(b) C4

Figure B.2.6: The ten colonies selected from the C3 and C4 parallel were tested by PCR for the correct deletion. Primers 23+24 were used in the PCR reaction. The 1 kb GeneRuler ladder from Thermo Fisher Scientific was used.

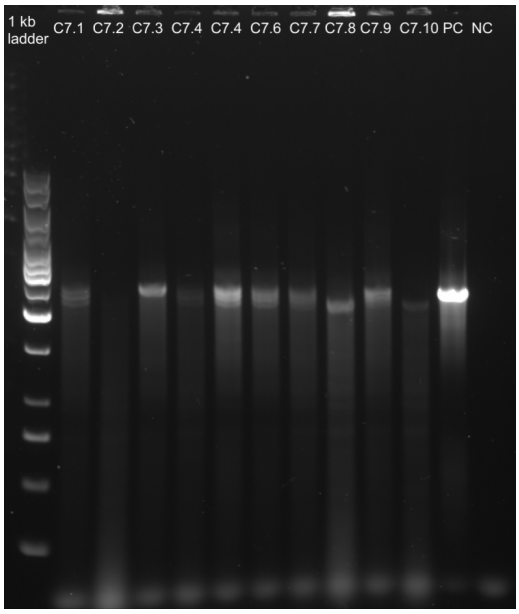


(a) C5

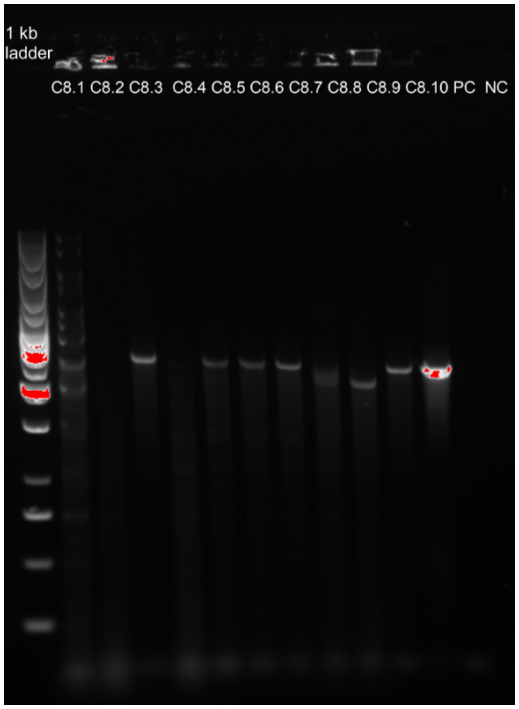


(b) C6

Figure B.2.7: The ten colonies selected from the C5 and C6 parallel were tested by PCR for the correct deletion. Primers 23+24 were used in the PCR reaction. The 1 kb GeneRuler ladder from Thermo Fisher Scientific was used.

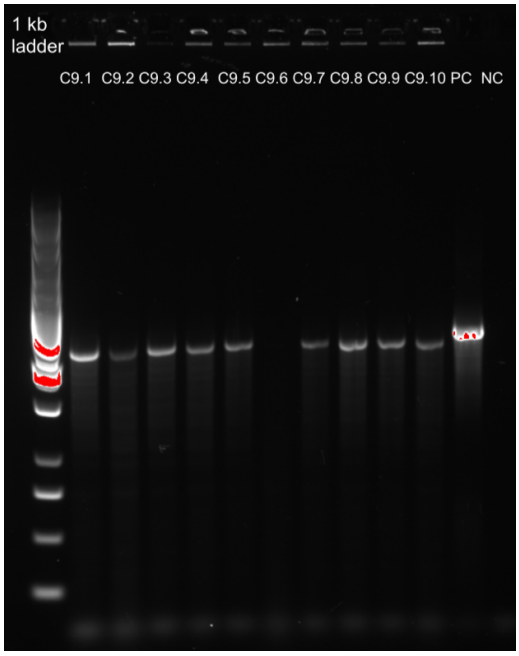


(a) C7

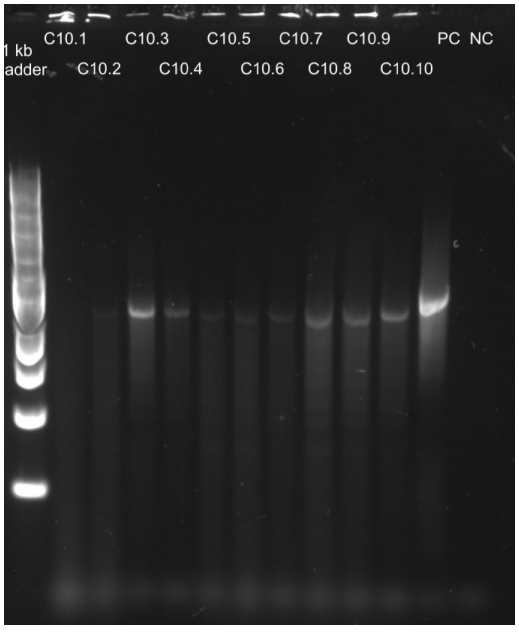


(b) C8

Figure B.2.8: The ten colonies selected from the C7 and C8 parallel were tested by PCR for the correct deletion. Primers 23+24 were used in the PCR reaction. The 1 kb GeneRuler ladder from Thermo Fisher Scientific was used.

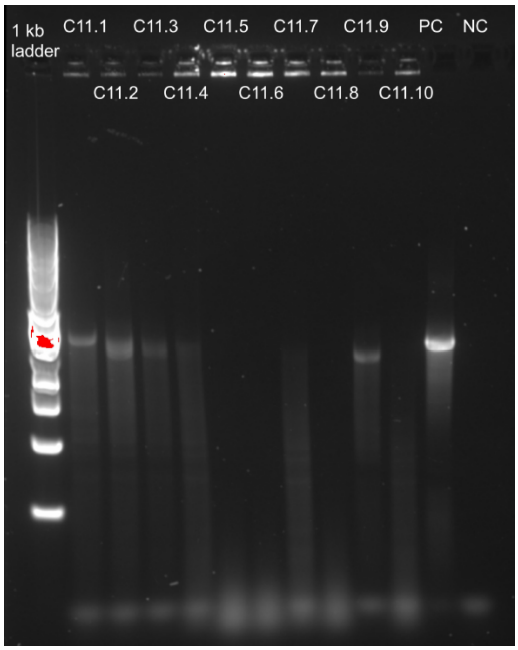


(a) C9

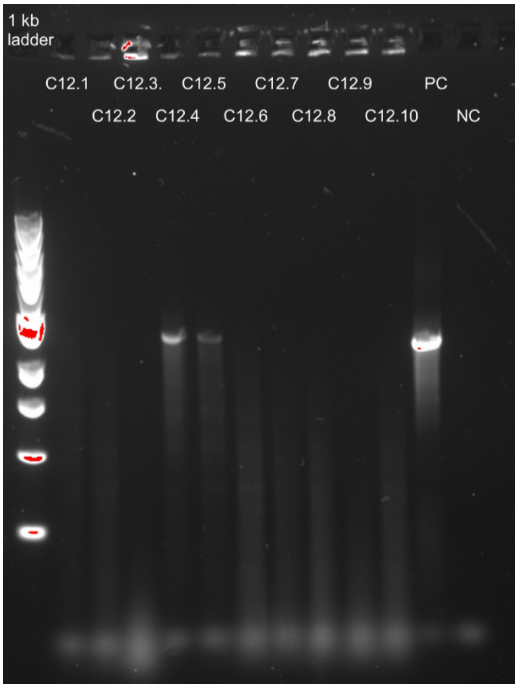


(b) C10

Figure B.2.9: The ten colonies selected from the C9 and C10 parallel were tested by PCR for the correct deletion. Primers 23+24 were used in the PCR reaction. The 1 kb GeneRuler ladder from Thermo Fisher Scientific was used.



(a) C11



(b) C12

Figure B.2.10: The ten colonies selected from the C11 and C12 parallel were tested by PCR for the correct deletion. Primers 23+24 were used in the PCR reaction. The 1 kb GeneRuler ladder from Thermo Fisher Scientific was used.

Figures B.2.12-B.2.15 shows the new PCR test of the samples that had inconclusive results in the previous tests.

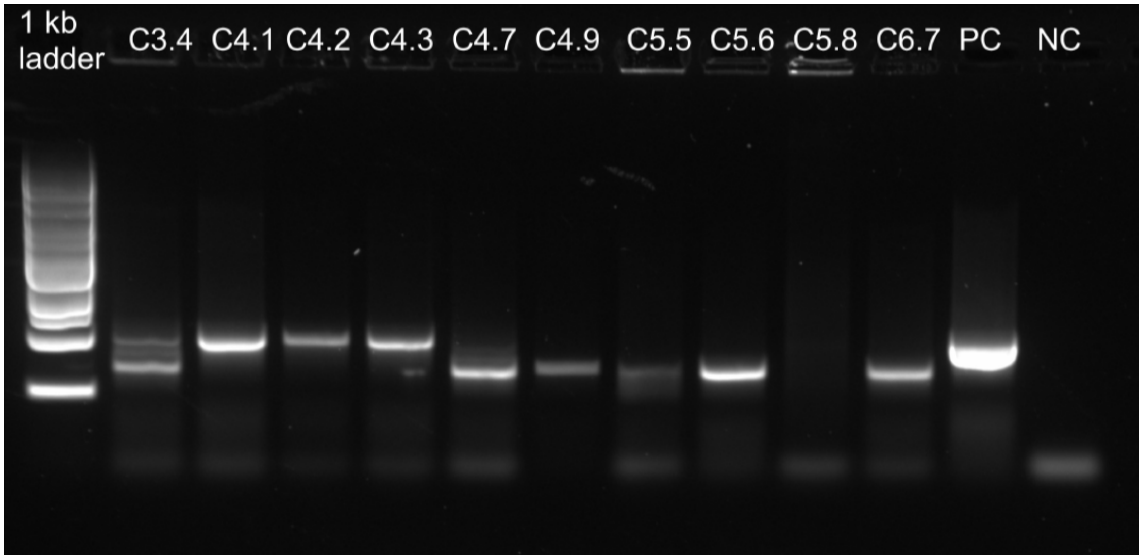


Figure B.2.11: PCR reactions with primers 11+12 and samples with previously inconclusive results.

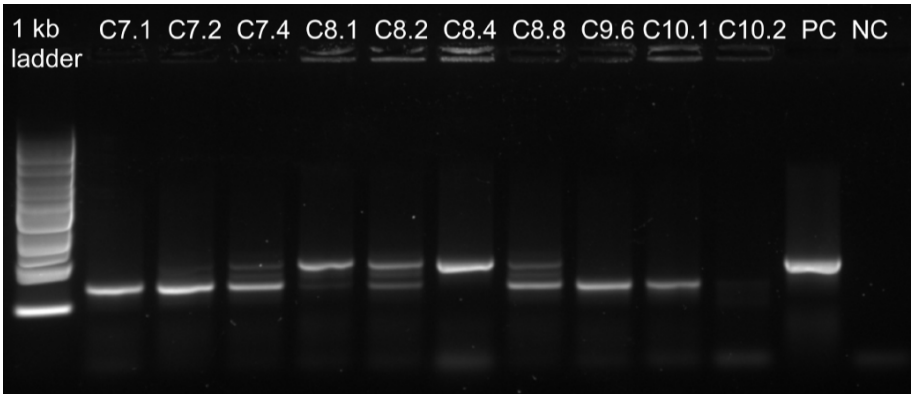


Figure B.2.12: PCR reactions with primers 11+12 and samples with previously inconclusive results.

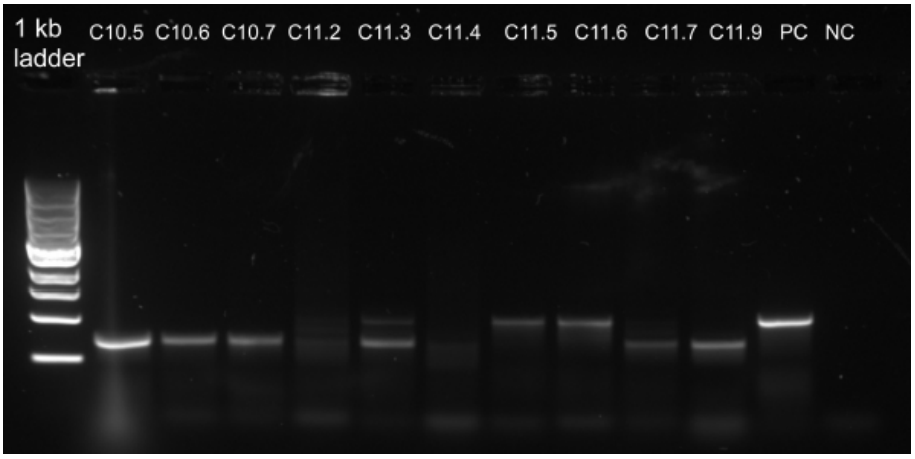


Figure B.2.13: PCR reactions with primers 11+12 and samples with previously inconclusive results.

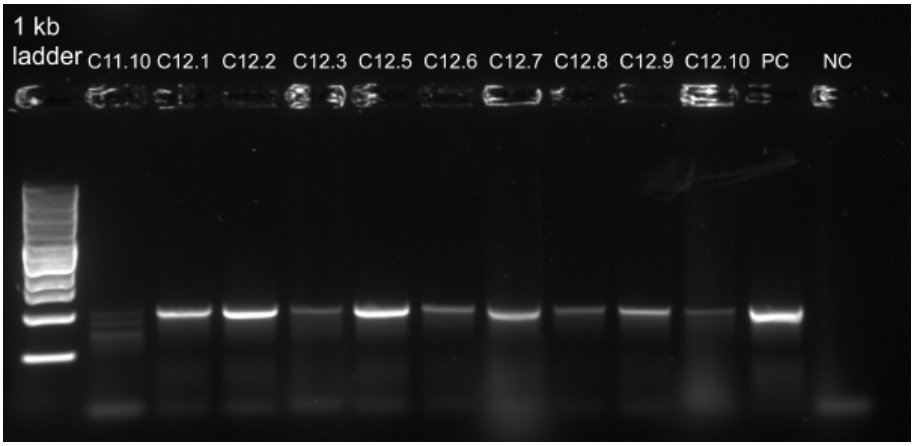


Figure B.2.14: PCR reactions with primers 11+12 and samples with previously inconclusive results.

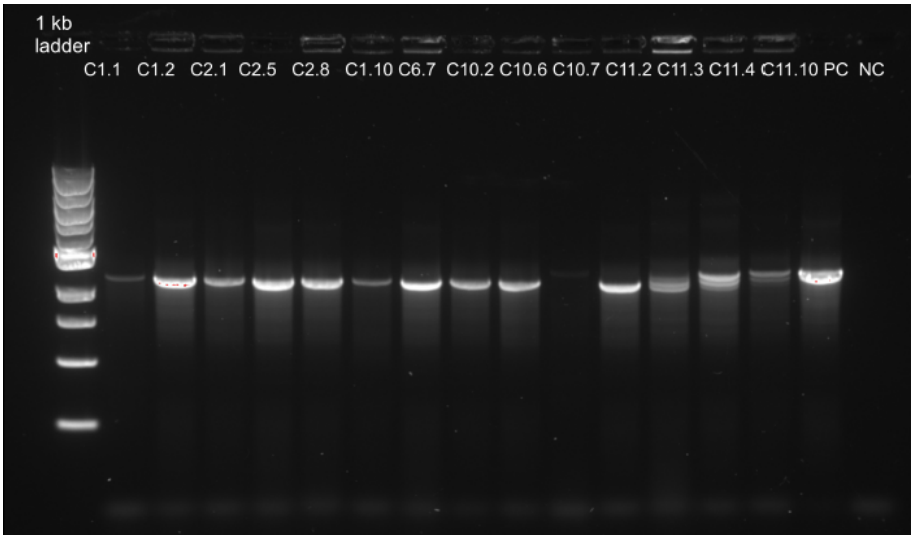


Figure B.2.15: PCR reactions with primers 23+24 and samples with previously inconclusive results.

B.3 Annotation of polymyxin gene cluster by BLAST analysis

The polymyxin synthethase PmxB was identified by RAST annotation of the genome of *P. polymyxa* DSM 365. A 50 kbp region centered around this gene was annotated by BLASTx analysis of the open reading frames. Table B.3.1 Shows the annotated regions with location, size, directionality and type.

Table B.3.1: Features from BLAST search on polymyxin region

Beginning of Table B.3.1				
Feature	**Location**	**Size**	**Dir**	**Type**
Hypothetical protein	183 - 536	354	→	CDS
GNAT family N-acetyltransferase	833 - 1270	438	→	CDS
Hypothetical protein	1271 - 1732	462	→	CDS
Helix-turn-helix domain-containing protein	1769 - 2584	816	→	CDS
Aldo/keto reductase	2645 - 3646	1002	→	CDS
8-Oxo-dGTP diphosphatase	3913 - 4404	492	→	CDS
Phosphotransferase	4407 - 5405	999	→	CDS
GNAT family N-acetyltransferase	5453 - 5974	522	→	CDS
GNAT family N-acetyltransferase	5726 - 6037	312	←	CDS
Hypothetical protein	6003 - 6452	450	→	CDS
NAD(P)/FAD-dependent oxidoreductase	6699 - 7676	978	→	CDS
HD domain-containing protein	7823 - 8506	684	→	CDS
ABC transporter substrate-binding protein	8671 - 9651	981	→	CDS
Iron ABC transporter permease	9662 - 10669	1008	→	CDS
Iron ABC transporter permease	10662 - 11684	1023	→	CDS
Alpha/beta hydrolase	11803 - 12573	771	→	CDS
2,3-dihydro-2 3-dihydroxybenzoate dehydrogenase	12885 - 13676	792	→	CDS
Isochorismate synthase DhbC	13754 - 15040	1287	→	CDS
(2,3-dihydroxybenzoyl)adenylate synthase	15071 - 16726	1656	→	CDS
Isochorismatase family protein	16753 - 17820	1068	→	CDS

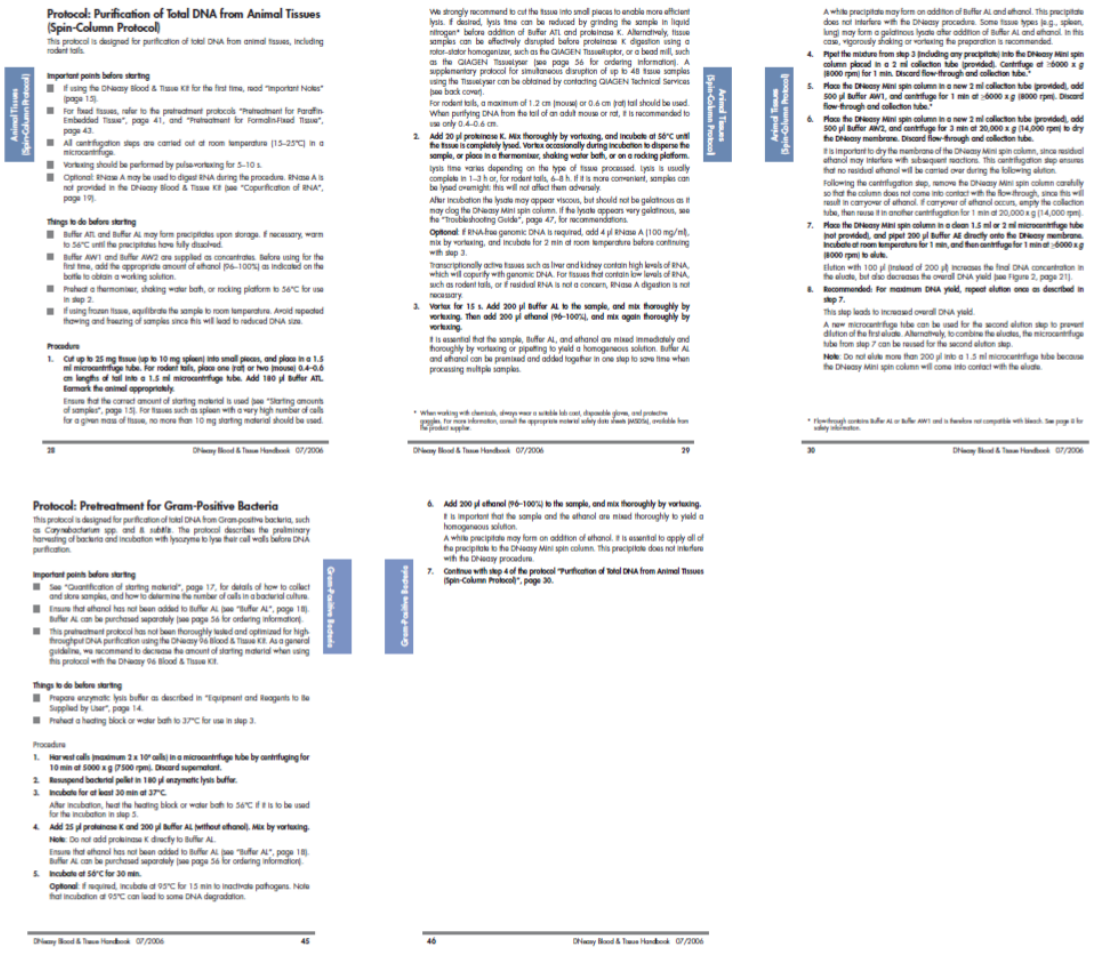
Continuation of Table B.3.1

Feature	**Location**	**Size**	**Dir**	**Type**
Non-ribosomal peptide synthetase	17835 - 25307	7473	→	CDS
Polymyxin synthetase PmxB	25304 - 25522	219	-	misc feature (RAST)
Membrane protease subunit SPFH domain	25824 - 26885	1062	→	CDS
Sugar kinase	26882 - 27883	1002	→	CDS
nadF	27913 - 28140	228	→	CDS
GAF domain-containing protein	28217 - 29233	1017	←	CDS
GAF domain-containing protein	28365 - 28787	423	→	CDS
Class I SAM dependent methyltransferase	29355 - 30170	816	→	CDS
Saccharopine dehydrogenase	30121 - 31248	1128	→	CDS
Hypothetical protein	31394 - 31624	231	→	CDS
SMI1/KNR9 family protein	31639 - 32196	558	→	CDS
HAD family hydrolase	32447 - 33160	714	→	CDS
GNAT- family N-acetyltransferase	33157 - 33744	588	→	CDS
DUF2809 domain-containing protein	33968 - 34450	483	→	CDS
Lysophospholipase	34511 - 35722	1212	→	CDS
Class I SAM-dependent methyltransferase	35838 - 36782	945	→	CDS
Hypothetical protein	37053 - 37646	594	→	CDS
GNAT family N-acetyltransferase	37677 - 38237	561	→	CDS
GIY-YIG nuclease family protein	38239 - 38580	342	←	CDS
DUF2087 domain-containing protein	38736 - 39494	759	←	CDS
DUF2087 domain-containing protein	38865 - 39092	228	→	CDS
DUF2087 domain-containing protein	39249 - 39479	231	→	CDS
HEAT repeat domain-containing protein	39704 - 40192	489	→	CDS
Hypothetical protein	40355 - 40870	516	→	CDS
Barnase inhibitor	40890 - 41747	858	→	CDS
Helix-turn-helix transcriptional regulator	42010 - 42255	246	→	CDS
Hypothetical protein	42564 - 42983	420	→	CDS
Hypothetical protein	43032 - 43337	306	←	CDS
MFS transporter	43751 - 44950	1200	←	CDS
MFS transporter	44643 - 44882	240	→	CDS
2-Oxoglutarate dehydrogenase E1 component	45542 - 48433	2892	→	CDS
2-Oxoglutarate dehydrogenase E1 component	48105 - 48365	261	→	CDS
2-Oxoglutarate dehydrogenase complex dihydrolipoyllysine-residue succinyltransferase	48463 - 49737	1275	→	CDS

End of Table B.3.1

C | Standard protocols

C.1 DNeasy Blood and Tissue Kit



Protocol: Purification of Total DNA from Animal Tissues (Spin-Column Protocol)

This protocol is designed for purification of total DNA from animal tissues, including rodent tails.

Animal Tissues (Spin-Column Protocol)

Important points before starting

- If using the DNeasy Blood & Tissue Kit for the first time, read "Important Notes" (page 15).
- For fixed tissues, refer to the pretreatment protocols "Pretreatment for Paraffin-Embedded Tissue", page 41, and "Pretreatment for Formalin-Fixed Tissue", page 43.
- All centrifugation steps are carried out at room temperature (15–25°C) in a microcentrifuge.
- Vortexing should be performed by pulse-vortexing for 5–10 s.
- Optional: RNase A may be used to digest RNA during the procedure. RNase A is not provided in the DNeasy Blood & Tissue Kit (see "Copurification of RNA", page 19).

Things to do before starting

- Buffer ATL and Buffer AL may form precipitates upon storage. If necessary, warm to 56°C until the precipitates have fully dissolved.
- Buffer AW1 and Buffer AW2 are supplied as concentrates. Before using for the first time, add the appropriate amount of ethanol (96–100%) as indicated on the bottle to obtain a working solution.
- Preheat a thermomixer, shaking water bath, or rocking platform to 56°C for use in step 2.
- If using frozen tissue, equilibrate the sample to room temperature. Avoid repeated thawing and freezing of samples since this will lead to reduced DNA size.

Procedure

1. **Cut up to 25 mg tissue (up to 10 mg spleen) into small pieces, and place in a 1.5 ml microcentrifuge tube. For rodent tails, place one (rat) or two (mouse) 0.4–0.6 cm lengths of tail into a 1.5 ml microcentrifuge tube. Add 180 µl Buffer ATL. Earmark the animal appropriately.**

   Ensure that the correct amount of starting material is used (see "Starting amounts of samples", page 15). For tissues such as spleen with a very high number of cells for a given mass of tissue, no more than 10 mg starting material should be used.

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We strongly recommend to cut the tissue into small pieces to enable more efficient lysis. If desired, lysis time can be reduced by grinding the sample in liquid nitrogen* before addition of Buffer ATL and proteinase K. Alternatively, tissue samples can be effectively disrupted before proteinase K digestion using a rotor–stator homogenizer, such as the QIAGEN TissueRuptor, or a bead mill, such as the QIAGEN TissueLyser (see page 56 for ordering information). A supplementary protocol for simultaneous disruption of up to 48 tissue samples using the TissueLyser can be obtained by contacting QIAGEN Technical Services (see back cover).

For rodent tails, a maximum of 1.2 cm (mouse) or 0.6 cm (rat) tail should be used. When purifying DNA from the tail of an adult mouse or rat, it is recommended to use only 0.4–0.6 cm.

2. **Add 20 µl proteinase K. Mix thoroughly by vortexing, and incubate at 56°C until the tissue is completely lysed. Vortex occasionally during incubation to disperse the sample, or place in a thermomixer, shaking water bath, or on a rocking platform.**

   Lysis time varies depending on the type of tissue processed. Lysis is usually complete in 1–3 h or, for rodent tails, 6–8 h. If it is more convenient, samples can be lysed overnight; this will not affect them adversely.

   After incubation the lysate may appear viscous, but should not be gelatinous as it may clog the DNeasy Mini spin column. If the lysate appears very gelatinous, see the "Troubleshooting Guide", page 47, for recommendations.

   Optional: If RNA-free genomic DNA is required, add 4 µl RNase A (100 mg/ml), mix by vortexing, and incubate for 2 min at room temperature before continuing with step 3.

   Transcriptionally active tissues such as liver and kidney contain high levels of RNA, which will copurify with genomic DNA. For tissues that contain low levels of RNA, such as rodent tails, or if residual RNA is not a concern, RNase A digestion is not necessary.

3. **Vortex for 15 s. Add 200 µl Buffer AL to the sample, and mix thoroughly by vortexing. Then add 200 µl ethanol (96–100%), and mix again thoroughly by vortexing.**

   It is essential that the sample, Buffer AL, and ethanol are mixed immediately and thoroughly by vortexing or pipetting to yield a homogeneous solution. Buffer AL and ethanol can be premixed and added together in one step to save time when processing multiple samples.

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

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Animal Tissues (Spin-Column Protocol)

A white precipitate may form on addition of Buffer AL and ethanol. This precipitate does not interfere with the DNeasy procedure. Some tissue types (e.g., spleen, lung) may form a gelatinous lysate after addition of Buffer AL and ethanol. In this case, vigorously shaking or vortexing the preparation is recommended.

4. **Pipet the mixture from step 3 (including any precipitate) into the DNeasy Mini spin column placed in a 2 ml collection tube (provided). Centrifuge at ≥6000 x g (8000 rpm) for 1 min. Discard flow-through and collection tube.***
5. **Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 µl Buffer AW1, and centrifuge for 1 min at ≥6000 x g (8000 rpm). Discard flow-through and collection tube.***
6. **Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 µl Buffer AW2, and centrifuge for 3 min at 20,000 x g (14,000 rpm) to dry the DNeasy membrane. Discard flow-through and collection tube.**

   It is important to dry the membrane of the DNeasy Mini spin column, since residual ethanol may interfere with subsequent reactions. This centrifugation step ensures that no residual ethanol will be carried over during the following elution.

   Following the centrifugation step, remove the DNeasy Mini spin column carefully so that the column does not come into contact with the flow-through, since this will result in carryover of ethanol. If carryover of ethanol occurs, empty the collection tube, then reuse it for another centrifugation for 1 min at 20,000 x g (14,000 rpm).

7. **Place the DNeasy Mini spin column in a clean 1.5 ml or 2 ml microcentrifuge tube (not provided), and pipet 200 µl Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1 min, and then centrifuge for 1 min at ≥6000 x g (8000 rpm) to elute.***

   Elution with 100 µl (instead of 200 µl) increases the final DNA concentration in the eluate, but also decreases the overall DNA yield (see Figure 2, page 21).

8. **Recommended: For maximum DNA yield, repeat elution once as described in step 7.**

   This step leads to increased overall DNA yield.

   A new microcentrifuge tube can be used for the second elution step to prevent dilution of the first eluate. Alternatively, to combine the eluates, the microcentrifuge tube from step 7 can be reused for the second elution step.

   Note: Do not elute more than 200 µl into a 1.5 ml microcentrifuge tube because the DNeasy Mini spin column will come into contact with the eluate.

* Flow-through contains Buffer AL or Buffer AW1 and is therefore not compatible with bleach. See page 8 for safety information.

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Protocol: Pretreatment for Gram-Positive Bacteria

This protocol is designed for purification of total DNA from Gram-positive bacteria, such as *Corynebacterium* spp. and *B. subtilis*. The protocol describes the preliminary harvesting of bacteria and incubation with lysozyme to lyse their cell walls before DNA purification.

Gram-Positive Bacteria

Important points before starting

- See "Quantification of starting material", page 17, for details of how to collect and store samples, and how to determine the number of cells in a bacterial culture.
- Ensure that ethanol has not been added to Buffer AL (see "Buffer AL", page 18). Buffer AL can be purchased separately (see page 56 for ordering information).
- This pretreatment protocol has not been thoroughly tested and optimized for high-throughput DNA purification using the DNeasy 96 Blood & Tissue Kit. As a general guideline, we recommend to decrease the amount of starting material when using this protocol with the DNeasy 96 Blood & Tissue Kit.

Things to do before starting

- Prepare enzymatic lysis buffer as described in "Equipment and Reagents to Be Supplied by User", page 14.
- Preheat a heating block or water bath to 37°C for use in step 3.

Procedure

1. **Harvest cells (maximum 2 x 10¹⁰ cells) in a microcentrifuge tube by centrifuging for 10 min at 5000 x g (7500 rpm). Discard supernatant.**
2. **Resuspend bacterial pellet in 180 µl enzymatic lysis buffer.**
3. **Incubate for at least 30 min at 37°C.**

   After incubation, heat the heating block or water bath to 56°C if it is to be used for the incubation in step 5.

4. **Add 25 µl proteinase K and 200 µl Buffer AL (without ethanol). Mix by vortexing.**

   Note: Do not add proteinase K directly to Buffer AL.

   Ensure that ethanol has not been added to Buffer AL (see "Buffer AL", page 18). Buffer AL can be purchased separately (see page 56 for ordering information).

5. **Incubate at 56°C for 30 min.**

   Optional: If required, incubate at 95°C for 15 min to inactivate pathogens. Note that incubation at 95°C can lead to some DNA degradation.

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Gram-Positive Bacteria

6. **Add 200 µl ethanol (96–100%) to the sample, and mix thoroughly by vortexing.**

   It is important that the sample and the ethanol are mixed thoroughly to yield a homogeneous solution.

   A white precipitate may form on addition of ethanol. It is essential to apply all of the precipitate to the DNeasy Mini spin column. This precipitate does not interfere with the DNeasy procedure.

7. **Continue with step 4 of the protocol "Purification of Total DNA from Animal Tissues (Spin-Column Protocol)", page 30.**

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Figure C.1.1: Protocol for genome extraction using the *DNeasy Blood and Tissue Kit* from Qiagen.

C.2 GeneJET Plasmid Miniprep Kit #K0502, #K0503

PURIFICATION PROTOCOLS

Note

- Read IMPORTANT NOTES on p.3 before starting.
- All purification steps should be carried out at **room temperature**.
- All centrifugations should be carried out in a table-top microcentrifuge at **>12000 × g** (10 000-14 000 rpm, depending on the rotor type).

Use 1-5 mL of *E. coli* culture in LB media for purification of **high-copy** plasmids.

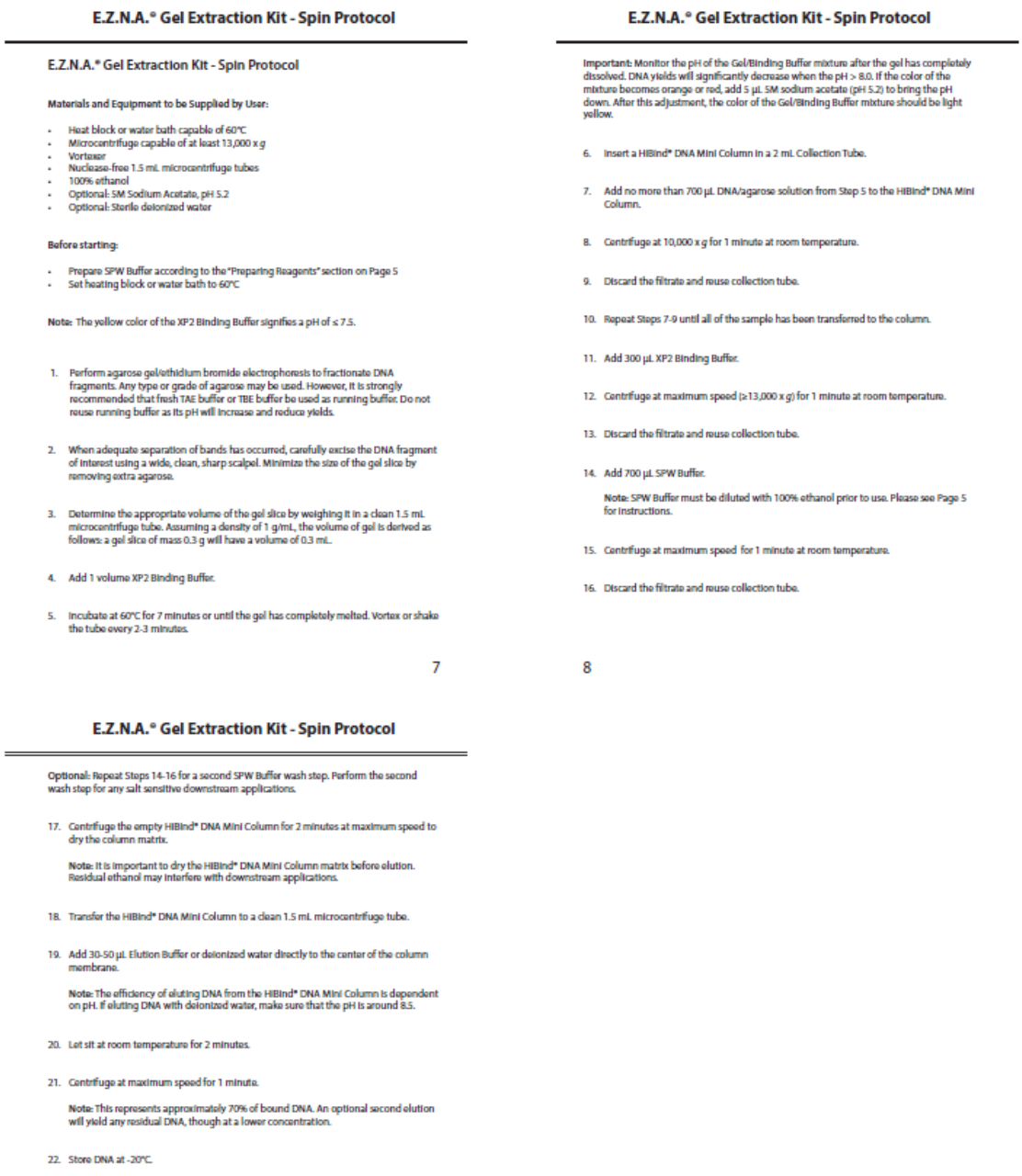
For **low-copy** plasmids use up to 10 mL of culture.

Protocol A. Plasmid DNA purification using centrifuges

Step	Procedure
1	Resuspend the pelleted cells in **250 µL of the Resuspension Solution**. Transfer the cell suspension to a microcentrifuge tube. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain. **Note.** Ensure RNase A has been added to the Resuspension Solution (as described on p.3)
2	Add **250 µL of the Lysis Solution** and mix thoroughly by inverting the tube 4-6 times until the solution becomes viscous and slightly clear. **Note.** Do not vortex to avoid shearing of chromosomal DNA. Do not incubate for more than 5 min to avoid denaturation of supercoiled plasmid DNA.
3	Add **350 µL of the Neutralization Solution** and mix immediately and thoroughly by inverting the tube 4-6 times. **Note.** It is important to mix thoroughly and gently after the addition of the Neutralization Solution to avoid localized precipitation of bacterial cell debris. The neutralized bacterial lysate should become cloudy.
4	Centrifuge for 5 min to pellet cell debris and chromosomal DNA.
5	Transfer the supernatant to the supplied GeneJET spin column by decanting or pipetting. Avoid disturbing or transferring the white precipitate. **Note. Close the bag with GeneJET Spin Columns tightly after each use!**
6	Centrifuge for 1 min. Discard the flow-through and place the column back into the same collection tube. **Note.** Do not add bleach to the flow-through, see p.8 for Safety Information.
7 for EndA+ strains only	*Optional:* use this preliminary washing step only if EndA+ strains which have high level of nuclease activity are used. Wash the GeneJET spin column by adding 500 µL of Wash Solution I (#R1611, diluted with isopropanol) and centrifuge for 30-60 sec. Discard the flow-through. **Note.** This step is essential to remove trace nuclease activity.
8	Add **500 µL of the Wash Solution** (diluted with ethanol prior to first use as described on p.3) to the GeneJET spin column. Centrifuge for 30-60 seconds and discard the flow-through. Place the column back into the same collection tube.
9	Repeat the wash procedure (step 8) using **500 µL of the Wash Solution**.
10	Discard the flow-through and centrifuge for an additional 1 min to remove residual Wash Solution. This step is essential to avoid residual ethanol in plasmid preps.
11	Transfer the GeneJET spin column into a fresh 1.5 mL microcentrifuge tube (not included). Add **50 µL of the Elution Buffer** to the center of GeneJET spin column membrane to elute the plasmid DNA. Take care not to contact the membrane with the pipette tip. Incubate for 2 min at room temperature and centrifuge for 2 min. **Note.** An additional elution step (optional) with Elution Buffer or water will recover residual DNA from the membrane and increase the overall yield by 10-20%. For elution of plasmids or cosmids >20 kb, prewarm Elution Buffer to 70°C before applying to silica membrane.
12	Discard the column and store the purified plasmid DNA at -20°C.

Figure C.2.1: Protocol for plasmid isolation using Thermo Scientific's *GeneJET Plasmid Miniprep Kit.*

C.3 E.Z.N.A. Gel extraction Kit



E.Z.N.A.® Gel Extraction Kit - Spin Protocol

E.Z.N.A.® Gel Extraction Kit - Spin Protocol

Materials and Equipment to be Supplied by User:

- Heat block or water bath capable of 60°C
- Microcentrifuge capable of at least 13,000 x g
- Vortexer
- Nuclease-free 1.5 mL microcentrifuge tubes
- 100% ethanol
- Optional: 5M Sodium Acetate, pH 5.2
- Optional: Sterile deionized water

Before starting:

- Prepare SPW Buffer according to the "Preparing Reagents" section on Page 5
- Set heating block or water bath to 60°C

Note: The yellow color of the XP2 Binding Buffer signifies a pH of ≤ 7.5.

1. Perform agarose gel/ethidium bromide electrophoresis to fractionate DNA fragments. Any type or grade of agarose may be used. However, it is strongly recommended that fresh TAE buffer or TBE buffer be used as running buffer. Do not reuse running buffer as its pH will increase and reduce yields.
2. When adequate separation of bands has occurred, carefully excise the DNA fragment of interest using a wide, clean, sharp scalpel. Minimize the size of the gel slice by removing extra agarose.
3. Determine the appropriate volume of the gel slice by weighing it in a clean 1.5 mL microcentrifuge tube. Assuming a density of 1 g/mL, the volume of gel is derived as follows: a gel slice of mass 0.3 g will have a volume of 0.3 mL.
4. Add 1 volume XP2 Binding Buffer.
5. Incubate at 60°C for 7 minutes or until the gel has completely melted. Vortex or shake the tube every 2-3 minutes.

7

E.Z.N.A.® Gel Extraction Kit - Spin Protocol

Important: Monitor the pH of the Gel/Binding Buffer mixture after the gel has completely dissolved. DNA yields will significantly decrease when the pH > 8.0. If the color of the mixture becomes orange or red, add 5 µL 5M sodium acetate (pH 5.2) to bring the pH down. After this adjustment, the color of the Gel/Binding Buffer mixture should be light yellow.

6. Insert a HiBind® DNA Mini Column in a 2 mL Collection Tube.
7. Add no more than 700 µL DNA/agarose solution from Step 5 to the HiBind® DNA Mini Column.
8. Centrifuge at 10,000 x g for 1 minute at room temperature.
9. Discard the filtrate and reuse collection tube.
10. Repeat Steps 7-9 until all of the sample has been transferred to the column.
11. Add 300 µL XP2 Binding Buffer.
12. Centrifuge at maximum speed (≥13,000 x g) for 1 minute at room temperature.
13. Discard the filtrate and reuse collection tube.
14. Add 700 µL SPW Buffer.

    Note: SPW Buffer must be diluted with 100% ethanol prior to use. Please see Page 5 for instructions.
15. Centrifuge at maximum speed for 1 minute at room temperature.
16. Discard the filtrate and reuse collection tube.

8

E.Z.N.A.® Gel Extraction Kit - Spin Protocol

Optional: Repeat Steps 14-16 for a second SPW Buffer wash step. Perform the second wash step for any salt sensitive downstream applications.

17. Centrifuge the empty HiBind® DNA Mini Column for 2 minutes at maximum speed to dry the column matrix.

    Note: It is important to dry the HiBind® DNA Mini Column matrix before elution. Residual ethanol may interfere with downstream applications.
18. Transfer the HiBind® DNA Mini Column to a clean 1.5 mL microcentrifuge tube.
19. Add 30-50 µL Elution Buffer or deionized water directly to the center of the column membrane.

    Note: The efficiency of eluting DNA from the HiBind® DNA Mini Column is dependent on pH. If eluting DNA with deionized water, make sure that the pH is around 8.5.
20. Let sit at room temperature for 2 minutes.
21. Centrifuge at maximum speed for 1 minute.

    Note: This represents approximately 70% of bound DNA. An optional second elution will yield any residual DNA, though at a lower concentration.
22. Store DNA at -20°C.

Figure C.3.1: Protocol for gel purification with *E.Z.N.A. Gel Extraction Kit* from Omega Bio-tek.

C.4 PCR Protocol for Phusion High-Fidelity DNA Polymerase (M0530)

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PCR Protocol for Phusion® High-Fidelity DNA Polymerase (M0530)

Protocols.io also provides an interactive version of this protocol where you can discover and share optimizations with the research community.

Overview

The following guidelines are provided to ensure successful PCR using Phusion® DNA Polymerase. These guidelines cover routine PCR. Amplification of templates with high GC content, high secondary structure, low template concentrations or long amplicons may require further optimization.

Protocol

1. **Reaction Setup:** We recommend assembling all reaction components on ice and quickly transferring the reactions to a thermocycler preheated to the denaturation temperature (98°C). All components should be mixed and centrifuged prior to use. It is important to add Phusion DNA Polymerase last in order to prevent any primer degradation caused by the 3′→ 5′ exonuclease activity. Phusion DNA Polymerase may be diluted in 1X HF or GC Buffer just prior to use in order to reduce pipetting errors. Please note that protocols with Phusion DNA Polymerase may differ from protocols with other standard polymerases. As such, conditions recommended below should be used for optimal performance.

Component	20 µl Reaction	50 µl Reaction	Final Concentration
Nuclease-free water	to 20 µl	to 50 µl	
5X Phusion HF or GC Buffer	4 µl	10 µl	1X
10 mM dNTPs	0.4 µl	1 µl	200 µM
10 µM Forward Primer	1 µl	2.5 µl	0.5 µM
10 µM Reverse Primer	1 µl	2.5 µl	0.5 µM
Template DNA	variable	variable	< 250 ng
DMSO (optional)	(0.6 µl)	(1.5 µl)	3%
Phusion DNA Polymerase	0.2 µl	0.5 µl	1.0 units/50 µl PCR

Notes: Gently mix the reaction. Collect all liquid to the bottom of the tube by a quick spin if necessary. Overlay the sample with mineral oil if using a PCR machine without a heated lid.

Transfer PCR tubes from ice to a PCR machine with the block preheated to 98°C and begin thermocycling:

Thermocycling conditions for a routine PCR:

STEP	TEMP	TIME
Initial Denaturation	98°C	30 seconds
25-35 Cycles	98°C 45-72°C 72°C	5-10 seconds 10-30 seconds 15-30 seconds per kb
Final Extension	72°C	5-10 minutes
Hold	4-10°C	

General Guidelines:

Template:
Use of high quality, purified DNA templates greatly enhances the success of PCR. Recommended amounts of DNA template for a 50 µl reaction are as follows:

DNA	Amount
genomic	50 ng–250 ng
plasmid or viral	1 pg–10 ng

If the template DNA is obtained from a cDNA synthesis reaction, the volume added should be less than 10% of the total reaction volume.

2. Primers:
Oligonucleotide primers are generally 20–40 nucleotides in length and ideally have a GC content of 40–60%. Computer programs such as Primer3 can be used to design or analyze primers. The final concentration of each primer in a reaction using Phusion DNA Polymerase may be 0.2–1 µM, while 0.5 µM is recommended.

3. Mg^{++} and additives:
Mg^{++} is critical to achieve optimal activity with Phusion DNA Polymerase. The final Mg^{++} concentration in 1X Phusion HF and GC Buffer is 1.5 mM. Excessive Mg^{++} can prevent full denaturation of DNA as well as cause non-specific binding of primers. The optimal Mg^{++} concentration is affected by dNTP concentration, the template being used and supplements that are added to the reaction. This can also be affected by the presence of chelators (e.g. EDTA). Mg^{++} can be optimized in 0.5 mM increments using the $MgCl_2$ provided.

Amplification of difficult targets, such as those with GC-rich sequences or secondary structure, may be improved by the presence of additives such as DMSO (included). A final concentration of 3% DMSO is recommended, although concentration can be optimized in 2% increments. It is important to note that if a high concentration of DMSO is used, the annealing temperature must be lowered as it decreases the primer T_m (2). Phusion DNA polymerase is also compatible with other additives such as formamide or glycerol.

4. Deoxynucleotides:
The final concentration of dNTPs is typically 200 µM of each deoxynucleotide. Phusion cannot incorporate dUTP.

Figure C.4.1: Protocol for amplification of DNA using the Phusion High-Fidelity DNA polymerase, provided by New England BioLabs.

C.5 PCR Protocol for Q5 High-Fidelity DNA Polymerase (M0491)

Home > Protocols > PCR Using Q5® High-Fidelity DNA Polymerase (M0491)

PCR Using Q5® High-Fidelity DNA Polymerase (M0491)

Protocols.io also provides an interactive version of this protocol where you can discover and share optimizations with the research community.

1. Please note that protocols with Q5 High-Fidelity DNA Polymerase may differ from protocols with other polymerases. Conditions recommended below should be used for optimal performance.

   Reaction Setup:
   We recommend assembling all reaction components on ice and quickly transferring the reactions to a thermocycler preheated to the denaturation temperature (98°C). All components should be mixed prior to use. Q5 High-Fidelity DNA Polymerase may be diluted in 1X Q5 Reaction Buffer just prior to use in order to reduce pipetting errors.

COMPONENT	25 µl REACTION	50 µl REACTION	FINAL CONCENTRATION
5X Q5 Reaction Buffer	5 µl	10 µl	1X
10 mM dNTPs	0.5 µl	1 µl	200 µM
10 µM Forward Primer	1.25 µl	2.5 µl	0.5 µM
10 µM Reverse Primer	1.25 µl	2.5 µl	0.5 µM
Template DNA	variable	variable	< 1,000 ng
Q5 High-Fidelity DNA Polymerase	0.25 µl	0.5 µl	0.02 U/µl
5X Q5 High GC Enhancer (optional)	(5 µl)	(10 µl)	(1X)
Nuclease-Free Water	to 25 µl	to 50 µl	

   Notes: Gently mix the reaction. Collect all liquid to the bottom of the tube by a quick spin if necessary. Overlay the sample with mineral oil if using a PCR machine without a heated lid.

   Transfer PCR tubes to a PCR machine and begin thermocycling.

   Thermocycling Conditions for a Routine PCR:

STEP	TEMP	TIME
Initial Denaturation	98°C	30 seconds
25–35 Cycles	98°C *50–72°C 72°C	5–10 seconds 10–30 seconds 20–30 seconds/kb
Final Extension	72°C	2 minutes
Hold	4–10°C	

Figure C.5.1: Protocol for amplification of DNA using the Q5 High-Fidelity DNA polymerase, provided by New England BioLabs.

C.6 PCR Protocol for Taq DNA Polymerase with Standard Taq Buffer (M0273)

Home > Protocols > PCR Protocol for *Taq* DNA Polymerase with Standard *Taq* Buffer (M0273)

PCR Protocol for *Taq* DNA Polymerase with Standard *Taq* Buffer (M0273)

Protocols.io also provides an interactive version of this protocol where you can discover and share optimizations with the research community.

Overview

PCR

The Polymerase Chain Reaction (PCR) is a powerful and sensitive technique for DNA amplification (1). *Taq* DNA Polymerase is an enzyme widely used in PCR (2). The following guidelines are provided to ensure successful PCR using NEB's *Taq* DNA Polymerase. These guidelines cover routine PCR. Amplification of templates with high GC content, high secondary structure, low template concentrations, or amplicons greater than 5 kb may require further optimization.

Protocol

Reaction setup:

We recommend assembling all reaction components on ice and quickly transferring the reactions to a thermocycler preheated to the denaturation temperature (95°C).

Component	25 µl reaction	50 µl reaction	Final Concentration
10X Standard *Taq* Reaction Buffer	2.5 µl	5 µl	1X
10 mM dNTPs	0.5 µl	1 µl	200 µM
10 µM Forward Primer	0.5 µl	1 µl	0.2 µM (0.05–1 µM)
10 µM Reverse Primer	0.5 µl	1 µl	0.2 µM (0.05–1 µM)
Template DNA	variable	variable	<1,000 ng
Taq DNA Polymerase	0.125 µl	0.25 µl	1.25 units/50 µl PCR
Nuclease-free water	to 25 µl	to 50 µl	

Notes: Gently mix the reaction. Collect all liquid to the bottom of the tube by a quick spin if necessary. Overlay the sample with mineral oil if using a PCR machine without a heated lid.

Transfer PCR tubes from ice to a PCR machine with the block preheated to 95°C and begin thermocycling.

Thermocycling conditions for a routine PCR:

STEP	TEMP	TIME
Initial Denaturation	95°C	30 seconds
30 Cycles	95°C 45-68°C 68°C	15-30 seconds 15-60 seconds 1 minute/kb
Final Extension	68°C	5 minutes
Hold	4-10°C	

General Guidelines:

1. Template:

   Use of high quality, purified DNA templates greatly enhances the success of PCR. Recommended amounts of DNA template for a 50 µl reaction are as follows:

DNA	Amount
genomic	1 ng–1 µg
plasmid or viral	1 pg–1 ng

2. Primers:

   Oligonucleotide primers are generally 20–40 nucleotides in length and ideally have a GC content of 40–60%. Computer programs such as Primer3 (http://frodo.wi.mit.edu/primer3) can be used to design or analyze primers. The final concentration of each primer in a reaction may be 0.05–1 µM, typically 0.1–0.5 µM.

3. Mg^{++} and additives:

   Mg^{++} concentration of 1.5–2.0 mM is optimal for most PCR products generated with *Taq* DNA Polymerase. The final Mg^{++} concentration in 1X Standard *Taq* Reaction Buffer is 1.5 mM. This supports satisfactory amplification of most amplicons. However, Mg^{++} can be further optimized in 0.5 or 1.0 mM increments using $MgCl_2$.

   Amplification of some difficult targets, like GC-rich sequences, may be improved with additives, such as DMSO (3) or formamide (4).

4. Deoxynucleotides:

Figure C.6.1: Protocol for amplification of DNA using the Standard Taq DNA polymerase, from New England BioLabs

C.7 GeneJET PCR Purification Kit #K0702

PURIFICATION PROTOCOLS

Note

- Read IMPORTANT NOTES on p. 3 before starting.
- All purification steps should be carried out at **room temperature**.
- All centrifugations should be carried out in a table-top microcentrifuge at **>12000 × g** (10 000-14 000 rpm, depending on the rotor type).

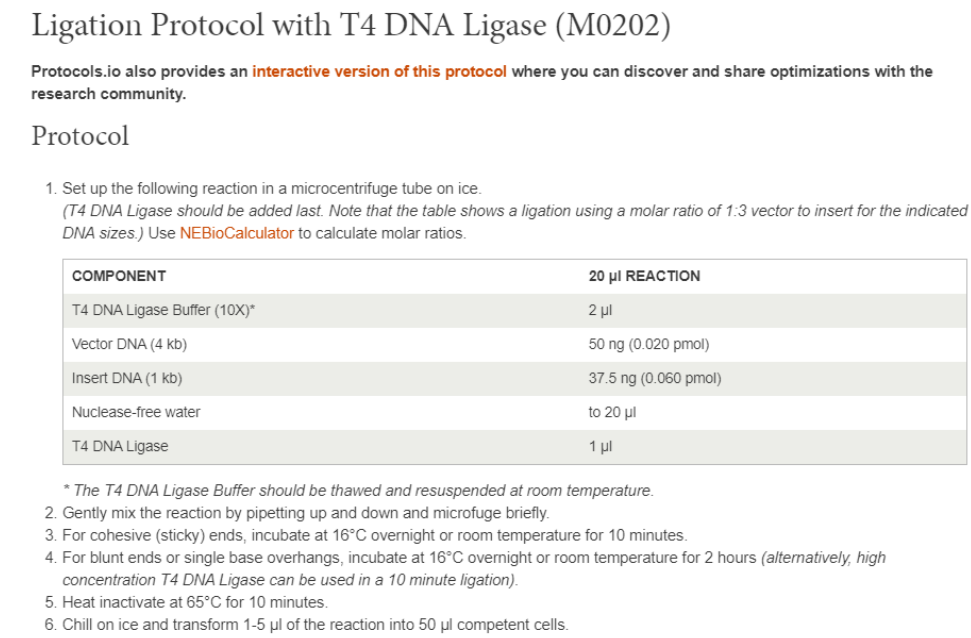
Protocol A. DNA purification using centrifuge

Step	Procedure
1	Add a **1:1 volume** of **Binding Buffer** to completed PCR mixture (e.g. for every 100 µL of reaction mixture, add 100 µL of Binding Buffer). Mix thoroughly. Check the color of the solution. A yellow color indicates an optimal pH for DNA binding. If the color of the solution is orange or violet, add 10 µL of 3 M sodium acetate, pH 5.2 solution and mix. The color of the mix will become yellow.
2 for DNA ≤500 bp	*Optional:* if the DNA fragment is ≤500 bp, add a 1:2 volume of 100% isopropanol (e.g., 100 µL of isopropanol should be added to 100 µL of PCR mixture combined with 100 µL of Binding Buffer). Mix thoroughly. **Note.** If PCR mixture contains primer-dimers, purification without isopropanol is recommended. However, the yield of the target DNA fragment will be lower.
3	Transfer up to 800 µL of the solution from step 1 (or optional step 2) to the GeneJET purification column. Centrifuge for 30-60 s. Discard the flow-through. **Notes.** If the total volume exceeds 800 µL, the solution can be added to the column in stages. After the addition of 800 µL of solution, centrifuge the column for 30-60 s and discard flow-through. Repeat until the entire solution has been added to the column membrane. **Close the bag with GeneJET Purification Columns tightly after each use!**
4	Add **700 µL** of **Wash Buffer** (diluted with the ethanol as described on p. 3) to the GeneJET purification column. Centrifuge for 30-60 s. Discard the flow-through and place the purification column back into the collection tube.
5	Centrifuge the empty GeneJET purification column for an additional 1 min to completely remove any residual wash buffer. **Note.** This step is essential as the presence of residual ethanol in the DNA sample may inhibit subsequent reactions.
6	Transfer the GeneJET purification column to a clean 1.5 mL microcentrifuge tube (not included). Add **50 µL** of **Elution Buffer** to the center of the GeneJET purification column membrane and centrifuge for 1 min. **Note** • For low DNA amounts the elution volumes can be reduced to increase DNA concentration. An elution volume between 20-50 µL does not significantly reduce the DNA yield. However, elution volumes less than 10 µL are not recommended. • If DNA fragment is >10 kb, prewarm Elution Buffer to 65 °C before applying to column. • If the elution volume is 10 µL and DNA amount is ≥5 µg, incubate column for 1 min at room temperature before centrifugation.
7	Discard the GeneJET purification column and store the purified DNA at -20 °C.

4

Figure C.7.1: Protocol for purification of PCR products using the *GeneJET PCR Purification Kit* from Thermo Scientific.

C.8 Ligation Protocol with T4 DNA Ligase (M0202)



Ligation Protocol with T4 DNA Ligase (M0202)

Protocols.io also provides an interactive version of this protocol where you can discover and share optimizations with the research community.

Protocol

1. Set up the following reaction in a microcentrifuge tube on ice.
   (T4 DNA Ligase should be added last. Note that the table shows a ligation using a molar ratio of 1:3 vector to insert for the indicated DNA sizes.) Use NEBioCalculator to calculate molar ratios.

COMPONENT	20 µl REACTION
T4 DNA Ligase Buffer (10X)*	2 µl
Vector DNA (4 kb)	50 ng (0.020 pmol)
Insert DNA (1 kb)	37.5 ng (0.060 pmol)
Nuclease-free water	to 20 µl
T4 DNA Ligase	1 µl

   ** The T4 DNA Ligase Buffer should be thawed and resuspended at room temperature.*
2. Gently mix the reaction by pipetting up and down and microfuge briefly.
3. For cohesive (sticky) ends, incubate at 16°C overnight or room temperature for 10 minutes.
4. For blunt ends or single base overhangs, incubate at 16°C overnight or room temperature for 2 hours *(alternatively, high concentration T4 DNA Ligase can be used in a 10 minute ligation).*
5. Heat inactivate at 65°C for 10 minutes.
6. Chill on ice and transform 1-5 µl of the reaction into 50 µl competent cells.

Figure C.8.1: Protocol for ligation of fragments cut with type II restriction enzymes, provided by New England BioLabs.

Anne Kristin Haraldsvik

Development of a chassis organism based on CRISPR-Cas engineering in *Paenibacillus polymyxa*

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Norwegian University of
Science and Technology