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Deletion of a ribosomal dimerization factor of the 30S ribosomal subunit in the hyperthermophile *Pyrococcus furiosus*

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Abstrakt (deutsch)

Ribosomale Dimerisierungsfaktoren spielen eine zentrale Rolle in der Regulation der Translation in Organismen. Während die Dimerisierungsvorgänge der Ribosomen in Bakterien und Eukaryoten gut erforscht sind, ist deren Funktion in Archaeen noch nicht genau bekannt. Ziel dieser Studie war es, einen Deletionsstamm von *Pyrococcus furiosus* zu erzeugen, bei dem der ribosomale Dimerisierungsfaktor PF0560 der Archaeen fehlt, um seine Funktion in diesem Organismus zu untersuchen. Dieses Protein ist für Bildung eines 30S-30S-Dimers unter Nahrungsmangelbedingungen verantwortlich und spielt hierbei eine wichtige Rolle bei der Regulation der Ribosomen im Zuge der Translation in Archaeen. Zudem könnte PF0560 in *Pyrococcus furiosus*, einem Organismus, der unter extrem hohen Temperaturen gedeiht, eine zentrale Rolle bei der Regulierung der energieintensiven Translation übernehmen. Dies könnte den Organismen eine Strategie zur Überlebenssicherung unter extremen Bedingungen bieten. Da die genaue Rolle noch nicht bekannt ist, wie dieser Faktor Ribosomen in ihrer Funktion beeinflusst, ist die Forschung der ribosomalen Faktoren in Archaea essentiell. Um daher mehr Einblicke in die funktionelle Rolle des ribosomalen Dimerisierungsfaktors (aRDF) der Archaeen zu gewinnen, wurde das Protein im Zuge dieser Arbeit im Organismus deletiert und das Wachstum dieser resultierenden Mutante analysiert. Hierbei wurde eine Deletionsstrategie verwendet, die ein genetisches Manipulationssystem ermöglichte. Grundlage dieser Strategie war die homologe Rekombination, bei der das Gen *pf0506* mittels eines Doppel-Crossover ausgetauscht wurde. Hierzu wurden die Regionen, die den aRDF ,codiert durch *pf0560*, flankieren, amplifiziert und mit einem für *Pyrococcus furiosus* essentiellen Gen verbunden. Das zusammengesetzte Konstrukt wurde in eine auxotrophe Mutante eingeführt, dem dieses essentielle Gen fehlt und für das Wachstum der hyperthermophilen Archaeen entscheidend ist. Auf diese Weise wurden homologe Regionen flankierend um den aRDF bereitgestellt, die mit dem essentiellen Gen an der Stelle des aRDF verknüpft waren. Nach der Transformation konnte ein Doppel-Crossover an den homologen Regionen stattfinden, der zum Austausch von *pf0560* durch das essentielle Gen führte. Mit dieser Strategie konnten positive Transformanten direkt anhand ihres Wachstums identifiziert werden. In der weiteren Arbeit wurde die Deletionsmutante im Wachstum überwacht und es konnte beobachtet werden, dass der ribosomale Dimerisierungsfaktor eine wichtige Rolle bei der Stabilität der Zellen spielt, insbesondere in der stationäre Phase. Darüber hinaus wurde dieses Deletionssystem in dieser Arbeit verwendet, um ein kleineres ribosomales Protein in *Pyrococcus furiosus* zu deletieren. Hierbei sollte das ribosomale Protein L41e, das als ribosomales Protein in mehreren Lebensbereichen verbreitet ist, in *Pyrococcus furiosus* entfernt werden. Das Protein L41e ist dafür bekannt, mit der 50S-Untereinheit der Ribosomen zu interagieren und spielt eine wichtige Rolle bei der Stabilisierung der Kontakte zwischen verschiedenen ribosomalen Proteinen und rRNA-Regionen. Insbesondere beeinflusst das Protein die Effizienz der Proteinsynthese. Da die Funktion von L41e in anderen Lebensdomänen besser bekannt ist, bleibt allerdings die essentielle Rolle von L41e in Archaeen bislang unklar. Daher wurde im Rahmen dieser Arbeit das Protein L41e in *Pyrococcus furiosus* deletiert, um seinen Einfluss in Archaeen zu untersuchen. Auch hierbei wurde das genetische Deletionssystem verwendet, basierend auf der Erzeugung von homologen Regionen stromauf- und stromabwärts von L41e in der auxotrophen Mutante. Durch Verbinden der upstream- und downstream-Regionen von L41e mit dem essentiellen Gen sollte L41e mittels homologer Rekombination deletiert werden. Allerdings konnte L41e mit diesem Ansatz nicht entfernt werden. Dies deutet darauf hin, dass L41e in Archaeen möglicherweise eine wesentlichere Rolle spielt als bisher angenommen. Auf Basis dieser Studie wird daher deutlich, wie notwendig die Forschung an Archaeen und deren ribosomalen Proteinen ist, um die Mechanismen zu verstehen, die diesen Organismen das Leben unter extrem unterschiedlichen Bedingungen ermöglichen.

Abstract (english)

Ribosomal dimerization factors play important roles in the regulation of protein synthesis in organisms. Since the processes underlying ribosome dimerization are well studied in bacteria and eukaryotes, its functions in archaea remain poorly understood. In this study, the goal was to create a deletion strain in *Pyrococcus furiosus*, in which the archaeal ribosomal dimerization factor PF0560 was missing, in order to investigate its function in this organism. As PF0560 mediates the formation of a 30S-30S dimer during starving conditions, the factor might play an important role in regulating the translation in archaea. Moreover, as *Pyrococcus furiosus* exists as an organism thriving under extremely high temperatures, PF0560 might serve as a key player in down regulating the energy consuming translation event. This might provide a strategy for the organisms to survive under those extreme conditions. But, how PF0560 influences the cell in its functions remains still elusive. In order to gain more insight into the functional role of the archaeal ribosomal dimerization factor, namely aRDF, during this work the protein was deleted in the organism and further, the growth of the mutant strain was monitored. Hereby, a deletion strategy was utilized, that enabled a genetic manipulation system. In this strategy, homologous recombination was used as the basis for deleting the gene via a double cross over. To do so, regions flanking the aRDF, encoded by *pf0560*, were amplified and connected to an essential gene in *Pyrococcus furiosus*. The assembled construct was introduced into an auxotroph mutant strain, missing this essential gene, which is crucial for the growth in hyperthermophile archaea. In this way, homologous regions flanking aRDF, connected to the essential gene at the position of the aRDF, were provided in the organism. Thereby, after the transformation into the auxotroph mutant a double cross over on the homologous regions could take place, that led to exchange of *pf0560* with the essential gene. Providing this strategy, positive transformants could directly be screened based on their growth. In the further work, the deletion mutant missing *pf0560* was monitored in its growth. Moreover, it could be observed during this work, that *pf0560* might play an important role in the stability of cells, especially in cells entering the stationary phase.

In addition, during this work the deletion system was used to delete a smaller ribosomal protein in *Pyrococcus furiosus*. Moreover, the ribosomal protein L41e, that is common as a ribosomal protein spread among several domains of life, should be deleted in *Pyrococcus furiosus*. Further, the ribosomal protein L41e is known to interact with the 50S subunit of ribosomes and seems to play an important role in the stabilization of the contact among various ribosomal proteins and rRNA regions. Especially, the ribosomal protein influences the efficiency of the protein synthesis. But, due to the fact that the function of L41e is better understood in the other domains of life, the essential role of L41e in archaea remains still unknown. Thereby, during this work the ribosomal protein L41e was deleted in *Pyrococcus furiosus* in order to investigate, the influence of L41e in archaea. To do so, likewise, in this experiment, the genetic deletion system was utilized. In this way, homologous regions flanking L41e upstream and downstream, were provided in the auxotroph mutant version of *Pyrococcus furiosus* after transformation. Via assembling the upstream and downstream regions of L41e to the essential gene, L41e should be deleted via homologous recombination in *Pyrococcus furiosus*. Based on this work, L41e could not be deleted via using this approach. Moreover, this elucidated the finding that L41e might play a more crucial role in archaea than further expected. Still, more research has to be done in order to get more insight into the fundamental role of L41e in the third domain of life. Reliant on this study, this sheds light on the necessity of research in archaea in important ribosomal protein factors, in order to understand its processes enabling archaea to make their living under different living conditions.

1. Introduction

Archaea- the third domain of life

Due to understand the classification of members belonging to different domains of life, a crucial premise is the comprehension of the definition of life. Novel studies term that "Life is an organized matter that provides genetic information metabolism". (Tetz&Tetz, 2020) Thereby, a crucial feature determining organic matter to life is the property of reproduction, growth, adaption, or genetic information inheritance. (Tetz&Tetz, 2020) Based on these features, organisms are classified into different domains of life, each containing two or more kingdoms. During the first phylogenetic analysis, living organisms were classified in two domains, consisting of the prokaryotes and eukaryotes, which is nowadays outdated. Regarding this outdated two domain of life theory, the first domain belong to the prokaryotes, determined as a single-celled organism without harboring a nucleus, consisted of the kingdoms bacteria and archaebacteria. (Pace, 2006)

Further, the second domain belong to the eukaryotes, which are determined harboring a nucleus with the genetic information (DNA) within a membrane-bound organelle, which separates the nucleus from the cytoplasm. Thereby, its cell organization differs profoundly from the prokaryotes. (Vellai & Vida, 1999) Moreover, eukaryotes belong to complex organisms, consisting of highly complex cell machineries. Further, transcription and translation is located in different compartments, whereby transcription takes place in the nucleus and translation takes part in the cytoplasm. (Kachaev et al., 2021) This requires the presence of other machineries, e.g. that can transport the transcribed mRNA from the nucleus into the cytoplasm to the ribosomes. This is mediated via additional factors, proteins and especially compartments in eukaryotes. (Li et al., 2021) Thereby, eukaryotes are highly complexed organisms, which distinguishes them tremendously from prokaryotes. Eukaryotes are further subclassified into the different kingdoms. (Simpson& Roger, 2004)

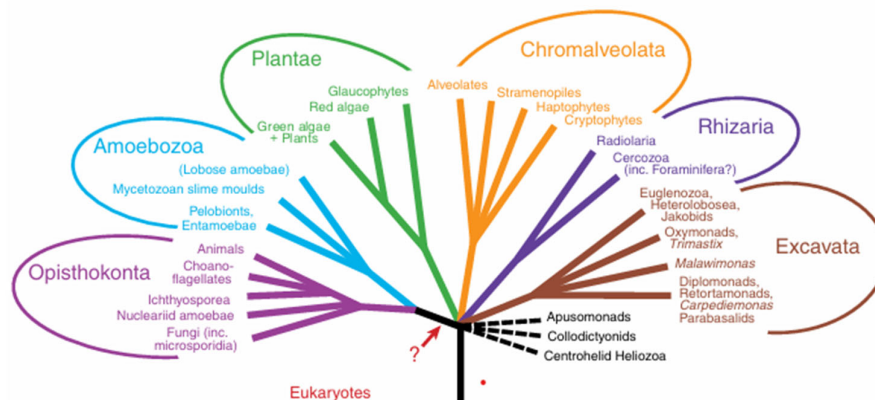


Figure 1: Second domain of life, the eukaryotes. Classification of the eukaryotes into its kingdoms: Opisthokonta, Amoebozoa, Plantae, Chromalveolata, Rhizaria and Excavata. Each kingdoms are further subdivided into the respective members. (Simpson & Roger, 2004)

Moreover, the eukaryotic domain is classified into the kingdoms Opisthokonta, Amoebozoa, Plantae, Chromalveolata, Rhizaria and Excavata, each kingdom consisting of its respective members. (Simpson & Roger, 2004)

But, after several years of studies the previous characterization of the prokaryotes as the first domain of life and the solely two domain of life theory, turned outdated. Moreover, through decades of research and several investigations over the past years, living organisms were

reidentified into three domains of life. (Woese et al., 1990) Especially, with increasing research and advances in microbial techniques, e.g. in genome sequencing via Next generating sequencing, the domain of prokaryotes was reclassified. Moreover, the kingdoms bacteria and archaeobacteria were classified into two distinct domains. (Pace, 2006) Moreover comparisons of ribosomal RNAs elucidates that bacteria and archaeobacteria are more dissimilar to each other than previously assumed. This led to a new classification of the domains of life. Further, bacteria and archaea were classified into different domains leading to a domain of bacteria and one domain of archaea. (Simpson& Roger, 2004) In addition, further advanced research revealed that archaea consist of a more complex cell machinery, that distinguishes from the bacteria and even resembles more the eukaryotic cell machineries. (Koonin, 2015) Also, archaeal membranes consist of ether lipids, which seem to be a unique feature, distinguishing them from bacteria and eukaryotes. (Rezanka et al., 2023) Thereby, archaea were newly termed as a ‘third’ domain of life, sharing both features of the domain of bacteria and eukaryotes, but also harboring structures, which are unique for this domain.

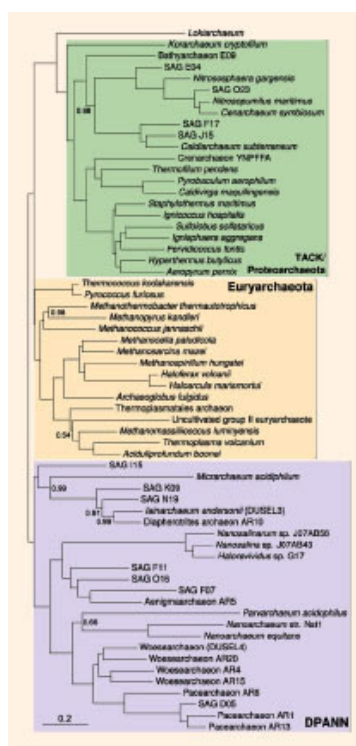


Figure 2: Classification of the third domain of life, the archaea into the related kingdoms and respective members. Depending on the kingdom, archaea share different evolutionary features with the other two domains of life. (Williams et al., 2017)

Based on Figure 2 , the domain of the archaea is divided into different subgroups, consisting of the Phyla Euryarchaeota, Crenarchaeota, Thaumarchaeota, Korarchaeota, Nanoarchaeota and Lokiarchaeota. The last representative belongs to the eukaryote resembling group. Regarding the similarities of archaea with eukaryotes, this third domain of life, especially the Lokiarchaeota, might play a more crucial role in evolution besides of the bacteria, as previously assumed. (Spang et al., 2015)

Importance of Archaea in evolution

As archaea nowadays belong to a unique domain, they harbor specific features classifying them. Moreover, archaea are single celled organisms with complexed cell structures, sharing features of both the eukaryotes and bacteria. Features related with prokaryotes, belong to their small size and single-celled shape. Furthermore, they share similarities with its gene arrangement and morphology, e.g. its cell wall. (Zillig, 1991) But, having a closer look into their function, archaea provide more similarities with eukaryotes. Furthermore, their transcriptional and translational machinery, the presence of complexed cell structures (e.g. Histones, complex RNA polymerases), its promoter structure and translation proteins are similar to each other. (Wenck & Santangelo, 2020) This classifies archaea being more related to eukaryotes. Based on this, archaea might play an important role in the evolution of eukaryotes, moreover in the evolution of complex life. (Baker et al., 2020) Hereby, eukaryotes might evolve of both the prokaryotes and the archaea. Recent studies assume, that eukaryotes emerged from an archaea-related host cell and a bacteria related endosymbiont. (Vosseberg et al., 2024) In this context, archaea tend to serve as a key player in the evolution of the complex life on earth.

Archaeal diversity through extreme habitats

Further, archaea belong to organisms, that are prone to live under extreme conditions. Especially, they can live under conditions, where a tremendous part of the organisms on earth would not survive. (Angelakis et al., 2024) Moreover, archaea are able to live in hot springs, salt lakes and in the deep sea. (Medina-Chávez & Travisano, 2022) Depending on the living space, archaea can be categorized into different groups (Table 1).

Table 1: Characterization of extreme habitats occupied by archaea. Depending on the living habitat, archaea are categorized into different groups. Each group represents an extreme environmental living condition.

Archaeal Groups	Habitat	Archaeal agents
Halophiles	High saline environment, e.g.: salty lakes	<i>Halobacterium salinarum</i>
Methanogens	Anaerobic, humid environment, e.g.: soil, gastrointestinal tract	<i>Methanobrevibacter smithii</i> , <i>Methanosarcina barkeri</i>
Psychrophiles	Cold environment (T-optimum between 10 °C-20 °C), e.g.: arctic sediments	<i>Polaromonas vacuolate</i> , <i>Methanogenium frigidum</i>
Acidophiles	Environment with low pH, e.g.: volcanic sediment	<i>Sulfolobus acidocaldarius</i>
Alkaliphiles	Environment with high pH (pH>9), e.g.: alkaline salt seas	<i>Natronococcus occultus</i> , <i>Natronomonas pharaonis</i>
Thermophiles	High temperatures (T-optimum between 40 °C-70 °C), e.g.: hot springs	<i>Methanothermobacter thermoautotrophicus</i>
Hyperthermophiles	Extreme high temperatures (T-Optimum between 90 °C – 115 °C), e.g.: hydrothermal vents	<i>Pyrolobus fumarii</i> , <i>Pyrococcus furiosus</i>

Due to this huge diversity in existing through various biotopes, the domain archaea increases interest in molecular biology. Further, to get to know the molecular biological and ecological features for thriving under extreme conditions, archaea arrives more and more into the focus.

Features of Hyperthermophile Archaea

Archaea thriving under extremely high temperatures belong to the category Hyperthermophiles. Hereby, archaea harbor specific features, that enable to resist heat. Moreover, the DNA of archaea contains a high G+C content, which enhances the stability of DNA. (Trivedi et al., 2006) In addition, the RNA obtains strong modifications, that protects against degradation. One crucial feature, that is only occurring in archaea belongs to its stable membrane. Further, their membranes consist of Ether lipids. Those Ether lipids are composed of a Phytanyl side chain, that spans the membrane and provides a high stabilized membrane. (Madigan et al., 2006, Fig. 4-19b) In addition, also its proteins are stabilized due to building up ionic interactions, less exposed sensitive residues and high compactness. (Atomi, 2005)

Pyrococcus furiosus-an archaeal Extremophile

One important representative of the hyperthermophile archaea, that also was used as basis during this work, is *Pyrococcus furiosus*. This organism belongs to the phyla Euryarcheota in the order Thermococcales. This archaea thrives as a heterotrophic organism strict anaerobically under high temperature at 100 °C. (Fiala & Stetter, 1986) Moreover, they are found at marine sediments with a pH optimum of 7. The first isolation of this organism took place in sediments of the Porto Levante, on the island Vulcano in Italy by Karl Stetter and Gerhard Fiala, 1986. *Pyrococcus furiosus* grows optimally on nutrients as starch, maltose, peptone and complex organic substrates. (Fiala & Stetter, 1986) But, interestingly, this organism is able to reduce elemental Sulfur into hydrogen sulfide. (Kengen, 2017) Furthermore, *Pyrococcus furiosus* is highly motile (Herzog & Wirth, 2012), composed of a spherical shape with a diameter of 0,8- 2,5 µm and monopolar polytrichous flagelles. (Fiala & Stetter, 1986) Under optimal conditions its doubling time consists of solely 37 minutes. Besides, this organism belongs to the group of hyperthermophiles with the optimal growth at 95-100 °C. Due to the fact, that *Pyrococcus furiosus* thrives under such extreme conditions, it possesses proteins that are highly thermostable and resistant to several chemical and organic solvents (Lee et al., 2009), making this organism of considerable scientific and biotechnological interest.

Pyrococcus furiosus as a model organism for genetic systems

Especially, *Pyrococcus furiosus* serves as a model organism for analysis in molecular biology and biochemistry of archaea. Due to unravelling the complete genome sequence of three species in *Pyrococcus*, the creation of genetic systems became more accessible via using the hyperthermophile organism as basis. (Robb et al., 2004) For instance, initial gene disruption systems were introduced in the Euryarchaeota *Thermococcus kodakarensis*. Hereby, an Uracil-auxotrophic mutant served as the basis for transforming a construct with homologous regions with complementation of Uracil. In this way, genetic systems are produced, where the mutants serve as kind of a selection marker in the further analysis. (Sato et al., 2003) In addition, earliest genetic systems were established in *Pyrococcus furiosus*. Hereby, one genetic tool was developed based on a modified shuttle vector system derived from pYS2 from *Pyrococcus abyssi*. (Waage et al., 2010) In this shuttle vector the *pyrE* gene, which previously was used as an auxotrophic selection marker, was substituted by the gene encoding 3-hydroxy-3-methylglutaryl coenzyme A reductase gene (HMG-CoA). Further, this gene was placed under the control of a strong *gdh* promoter from *Pyrococcus furiosus*, enabling overexpression, as an overexpression of

HMG-CoA lead to the resistance to simvastatin. By transforming the modified shuttle vector into *Pyrococcus furiosus*, the plasmid replicates extrachromosomally. The transformants harbor an additional copy of the HMG-CoA gene, resulting in elevated levels of HMG-CoA reductase leading to the compensation of the inhibitory effect of the antibiotic simvastatin. This selection system provides a basis for future manipulation strategies without the usage of auxotrophic mutant strains. (Waage et al., 2010)

Transformation system used for the creation of deletion mutants

During this work, a genetic modification strategy was used based on homologous recombination. Hereby, the manipulation system is reliant on the usage of an auxotroph mutant strain, lacking an essential gene. (Schick, 2012) Moreover, upstream and downstream regions flanking the target gene, that should be deleted, are being produced via PCR. In addition, the essential gene, which was knocked out in the parental strain, is amplified via PCR. (Grünberger et al., 2021) All those three fragments are then assembled together with a vector, in a way that the upstream and downstream region of the target gene are flanking the amplified essential gene. In this way, the essential gene functions as a selection marker. In order to assemble the fragments together, a Gibson Assembly can be used. Next, the assembled gene construct is then introduced into the auxotrophic mutant. Via introducing the construct providing homologous regions in the auxotroph mutant, homologous recombination is expected to happen, at the upstream region and the downstream region via a double cross-over. In this way, the gene assembly should be inserted into the organism and the target gene should be deleted and exchanged for the essential gene. (Knüppel et al., 2021) Further, the transformants are selected onto media without the addition of intermediates only being produced by the essential gene. In this way, a selection system can be provided, whereby transformants, that grow in the absence of this supplement, can be validated as positive knock out candidates.

Further, in a previous study, the auxotrophic mutant strain lacking this essential namely *pf1623*, encoding an enzyme with a key role in the polyamine biosynthesis, was generated by using the same strategy (Schick, 2012) and used as a basis during this work. Moreover, this auxotrophic mutant was constructed by assembling the upstream and downstream regions of *pf1623* around the previously established simvastatin resistance selection marker. (Waage et al., 2010) Introduction of this assembled construct into *Pyrococcus furiosus*, resulted in the deletion of the essential gene *pf1623* by homologous recombination. (Schick, 2012) This resulting mutant strain, was used as the parental strain for the experiments during this study. In addition, the same strategy based on the use of homologous regions was applied to generate the deletion mutants in this study.

The essential gene *pf1623*, encoding the Pyruvoyl-Dependent Arginine Decarboxylase

Reliant on the previous produced auxotroph mutant, the gene *pf1623* was knocked out. In particular, this gene encodes for the Pyruvoyl dependent Arginine Decarboxylase mediating as an essential role in *Pyrococcus furiosus*. Since *Pyrococcus furiosus* thrives under high temperatures at 100 °C, the living under such extreme conditions demands considerable effort for the organisms to cope with this environment. There is shown, that Polyamines may play an important role to protect organisms during high temperatures from degradation and are essential for its normal cell growth and viability. (Terui et al., 2018) Thereby, the organism needs features that enables to withstand those extreme conditions. Hereby, especially long branched Polyamine chains appear to have a significant influence on the stability of the DNA and on the survival of extreme thermophiles. (Hidese et al., 2018) Further, long Polyamines, such as Caldoptamine and Caldohexamine, seem to protect DNA against depurination. (Oshima et al., 2011)

Depurination represents the process, where purine bases are released from the DNA by the hydrolysis of N-glycosidic bonds. (An et al., 2014) This is mediated by high temperatures, radical oxygen species or hydrogen peroxide. Further, as Polyamines consist of hydrocarbons and amino groups, they serve as polycations, which enable due to its high amount of cations the protection of the DNA from depurination. (Terui et al., 2018)

Therefore, the presence of long branched Polyamines is crucial to hyperthermophiles for making a living under its harsh environment. Building up those long complexed structures, the precursor and major compounds are Polyamines like Putrescine, Spermidine or Spermine, whereby the synthesis of those molecules is essential for its growth and survival. (Hidese et al., 2018) Hereby, the biosynthetic pathway of Polyamines plays an important role and is depicted in Figure 3 below for the archaea *Thermococcus kodakaraensis*. As the hyperthermophile *Thermococcus kodakaraensis* is distinct related to *Pyrococcus furiosus* (Fukui et al., 2005), the organisms resembles the same pathway.

Based on this metabolism, the Polyamine biosynthesis originates from the amino acid Arginine (Fig. 3A) Further, Arginine gets decarboxylated into Agmatine catalyzed via the Pyruvoyl dependent Arginine Decarboxylase. In the next step, Agmatine gets further converted via intermediates into the Polyamines, which are then further synthesized into the essential long branched Polyamine chains. (Morimoto et al., 2010)

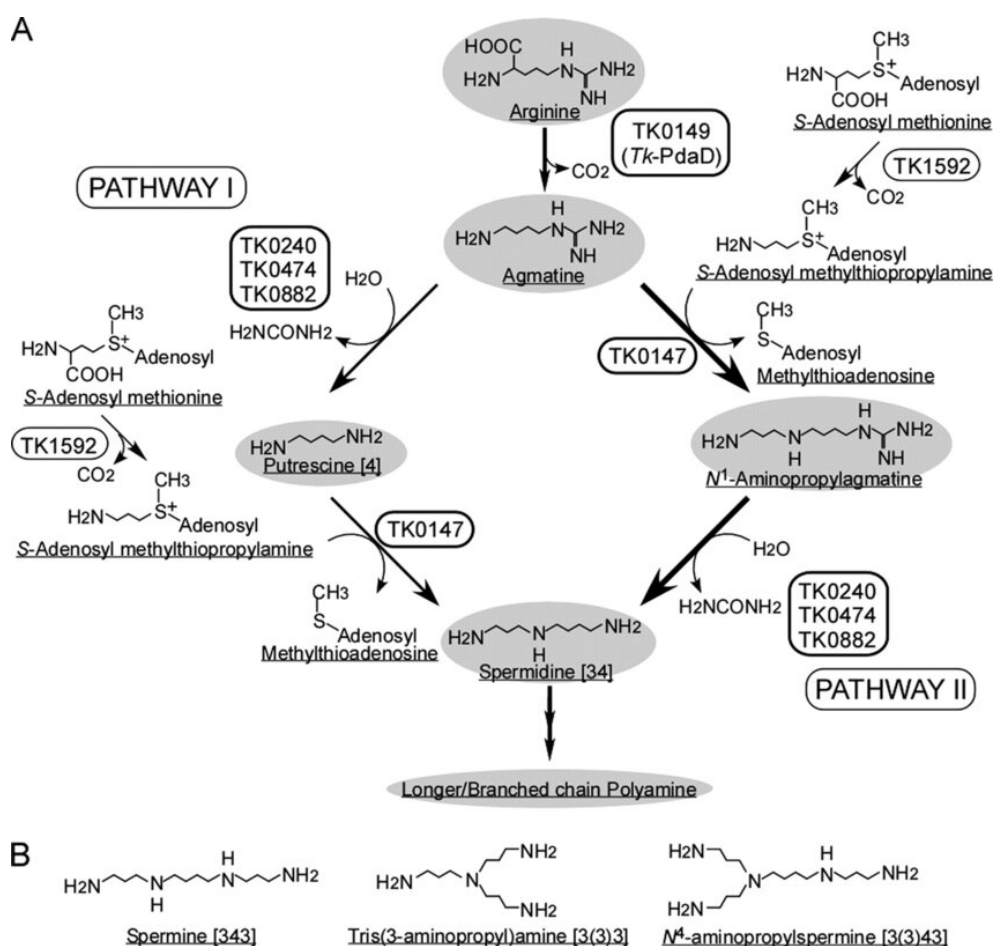


Figure 3: Metabolism of Polyamine synthesis in hyperthermophile archaea *Thermococcus kodakaraensis*. A. Arginine as the primary source for the synthesis of the precursor molecule Agmatine. Pyruvoyl dependent Arginine decarboxylase PdaD serves as the key enzyme via mediating the decarboxylation of Arginine into Agmatine. (Morimoto et al., 2010)

Hereby, Agmatine serves as the precursor compound for Polyamines (Fig.3A) Therefore, the synthesis of Agmatine, which is mediated by the enzyme Pyruvoyl dependent Arginine Decarboxylase, plays the central role in the biosynthesis. Moreover, the Pyruvoyl dependent Arginine Decarboxylase, PdaD, is essential for the synthesis of Agmatine in archaea and thereby, crucial for the production of Polyamines and the survival of archaea. (Sakamoto et al., 2022)

Pyruvoyl dependent Arginine Decarboxylase auxotroph mutant as a basis for creating the genetic system

Further, as the Pyruvoyl dependent Arginine Decarboxylase, PdaD, represents an essential enzyme for the synthesis of Agmatine and thereby, for the further growth of hyperthermophile archaea, several studies investigated the effect of the enzyme PdaD via deleting it. As a result, archaeal strains lacking PdaD were not able to grow anymore. Moreover, the mutated strains only grew in supplementation with Agmatine. (Fukuda et al., 2008)

In this context, the auxotroph mutant strain lacking PdaD served as the parental strain for the transformation of a genome assemblage providing homologous regions in the target organism during this work. To do so, genes flanking upstream and downstream the target gene were designed and assembled around the gene, encoding for the PdaD, at the position, where the target gene is localized usually. This gene construction should be integrated into the auxotroph mutant via transformation. Thereby, after the transformation on the one hand, the PdaD should be complemented. On the other hand, the target gene should be deleted via exchange of the PdaD. Further, it was able to proof, if the PdaD was complemented, via observing a growth of transformants without the addition of Agmatine. Using this strategy, PdaD could be utilized as a selection marker. Thereby, only transformants, which were able to grow, could be confirmed in its incorporation of the genetic system.

Archaeal Ribosomal dimerization factor PF0560

Further, ribosomes serve as key players mediating the synthesis of proteins, which is an essential mechanism for the processes of life. (Green & Noller.,1997). Given that protein synthesis is a highly energy-intensive process, cells may possess mechanisms in order to downregulate the function of ribosomes. Especially, one such strategy involves ribosome hibernation, whereby ribosomes emerge into a dormant state observed during stress conditions. This mechanism is well established in bacteria and eukaryotes. In particular, bacteria entering stress conditions react in inactivating 70 S ribosomes or forming 100 S ribosomal dimers, that are functionally inactive, in order to save energy during starving conditions. (Hassan et al., 2005) However, the conceptual processes of ribosome hibernation in archaea remain poorly understood. A recent study investigated that one dimerization factor might play an important role in regulating the cost intensive translation by dimerizing ribosomes. (Hassan et al., 2005) In particular, the ribosomal dimerization factor namely PF0560 elucidated to function as a down regulator of the archaeal cell machinery. (Hassan et al., 2005) Moreover, this protein mediates the formation of 30S-30S dimers, whereby the formation of the 70S complex is blocked. The protein PF0560, nowadays called archaeal ribosomal dimerization factor aRDF, forms two homodimers when interacting with two 30 S subunits. This leads to the stabilization of a 30S dimer, which in the further step prevents the 50S subunit from associating with the 30S dimer. (Hassan et al., 2025) In this way, the ribosome subunit joining, which is essential for the translation start, is suppressed. In the further way, the translation is blocked. This process seems to function as an adaptation to extreme environmental conditions. Moreover since *Pyrococcus furiosus* thrives under high temperatures and thereby, handles with extreme conditions, there used to be processes in the cell, that downregulate cell machinery processes, in order to maintain extreme conditions.

(Prossliner et al., 2018) Hereby, this ribosomal dimerization factors PF0560 appears to play an essential role in archaea. But, how PF0560 functions and which influence it profoundly has on the cell, remains elusive.

Ribosomal protein L41e

Further, as ribosomes are highly organized cell structures, they are mediating with several additional factors. In particular, the factors that are involved during cell machinery processes differ depending on the domain of life. (Woese et al., 1990) Still, there are multiple factors present, that are conserved through the whole domains of life. Moreover, the ribosomal protein L41e illustrates one factor that is present from mammalian and yeast through archaea. (Yu & Warner, 2001) This factor is located on the surface of the large ribosomal subunit and seems to stabilize the contact among various ribosomal proteins and rRNA regions. Furthermore, it influences the efficiency of the protein synthesis. L41e is known as a small protein, encoded only by 118 bp. Thereby, it is composed of a small amount of amino acids, whereby the most proportion of amino acids consists of Arginine or Lysine. (Yu & Warner, 2001) Despite of several investigations of this protein in yeast and eukaryotes, the full function of L41e in archaea is largely unknown and still has to be studied.

Purpose of the study

Since the function of the ribosomal dimerization factor and of the ribosomal protein still remain unknown in archaea, the influence of these proteins should be investigated in this work in *Pyrococcus furiosus*. Thereby, in this study, the aim was to generate deletion mutants lacking the target proteins.

Therefore, firstly a genetic system was performed to delete the archaeal ribosomal dimerization factor (aRDF) PF0560. The resulting knock out strain enabled to assess how PF0560 influences the cells and especially its impact via monitoring its growth under different conditions.

Further, since L41e is conserved across different domains of life, but, its function in archaea remains poorly understood the function of the ribosomal protein L41e was investigated in this study. Therefore, an additional deletion approach was generated, whereby L41e should be deleted in several approaches, in order to assess the impact of L41e in archaea.

Strategy to delete *pf0560* in *Pyrococcus furiosus*:

In order to delete the ribosomal dimerization factor PF0560, that is encoded by the gene *pf0560* with a length of 903 base pairs, the genomic regions upstream and downstream of *pf0560* were generated.

Based on the genomic organization, the upstream region flanking the target gene *pf0560* represents *pf0559*. This gene consists a length of 2322 base pairs and encodes for a Carbamoyl transferase. The gene localized downstream of *pf0560*, represents the gene *pf0561*, that consists of 504 base pairs encoding for the KAP NTPase domain-containing protein.

The organization of the respective genes are depicted in the Figure 4 below, in which the orientation of the genes with its promoter are shown.



Figure 4: Genome loci of the respective genes. Upstream and downstream regions with its promoter and the orientation in the genome are depicted in blue. The regions are flanking *pf0560* in the genome. The target gene *pf0560* with its promoter and orientation in the genome is depicted in green color. (Biorender)

The respective upstream and downstream genes were utilized to generate the genetic manipulation system in order to delete *pf0560*. Hereby, the genes were fused to the selection gene *pf1623*, which encodes a Pyruvoyl-dependent arginine Decarboxylase. Using the genetic construct, *pf0560* will be replaced by the selection marker PdaD, thereby enabling the targeted deletion of the gene.

In the further investigation, the mutant strain should be analyzed in its growth behavior, in order to investigate how PF0560 influences the cell according to its absence.

Strategy to delete the ribosomal protein L41e in *Pyrococcus furiosus*

In order to delete the ribosomal protein L41e, two experimental approaches were designed. In the first experiment, L41e was deleted together with its adjacent gene. In the second experiment, the genetic construct was performed to delete only the small ribosomal protein L41e.

First experimental set up

The ribosomal protein L41e is encoded by the gene *pf1018*, representing a small gene with the size of 114 base pairs. In order to delete this gene, genomic regions upstream and downstream of *pf1018* were generated.

Based on the genomic organization, the upstream region represents *pf1017*. This gene consists a length of 789 base pairs and encodes for a CN hydrolase domain-containing protein. The organization of the respective genes are depicted in the Figure 5 below.

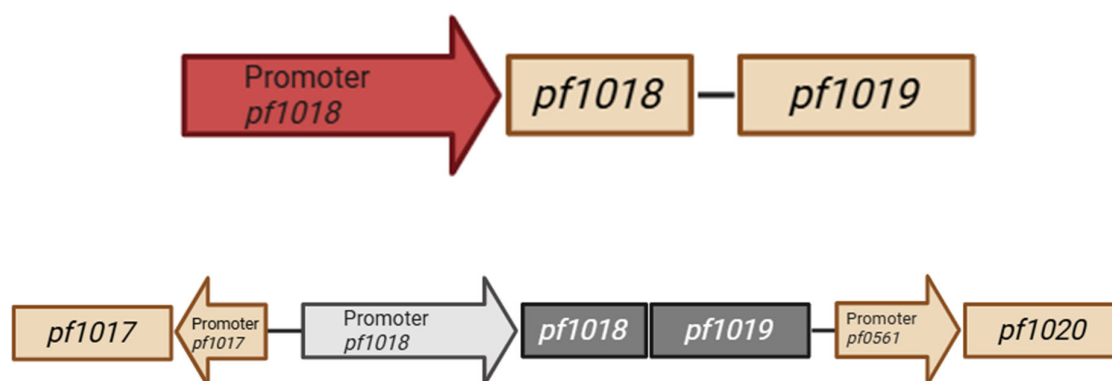


Figure 5: Genome loci of the respective genes. The red arrow depicts the Promoter of *pf1018*, co-transcribing the genes *pf1018* and *pf1019*. (above) B. Upstream and downstream regions flanking *pf1018* and *pf1019* are depicted in yellow. The genome localization of the target region, that was deleted in this experiment, is depicted in grey color (below). (Biorender)

With regard to the arrangement in the genomic DNA, *pf1018* appears to be co-transcribed with its adjacent gene *pf1019*. Thereby, in the first experimental approach, the small gene *pf1018* was deleted together with the downstream gene *pf1019* to increase the likelihood of a successful deletion. Thereby, the gene localized downstream of the target region represents the gene *pf1020*, that consists of 1239 base pairs encoding for a Phosphatase permease.

The depicted genomic regions should be deleted in *Pyrococcus furiosus* based on a genetic manipulation system. Further, the growth of the deletion mutant should be monitored, in order to analyze the function of L41e on the organism.

Second experimental setup

In a further approach, a second deletion experiment of L41e was designed. Hereby, solely the small ribosomal protein L41e should be deleted. Thereby, the adjacent gene *pf1019*, that appears to be co-transcribed with *pf1018*, was generated and provided as the downstream region for the genetic manipulation system. Based on Figure 6A the fragments were generated and connected to each other in a way that only L41e should be deleted and exchanged for the selection gene *pf1623*. (Fig. 6A,B)

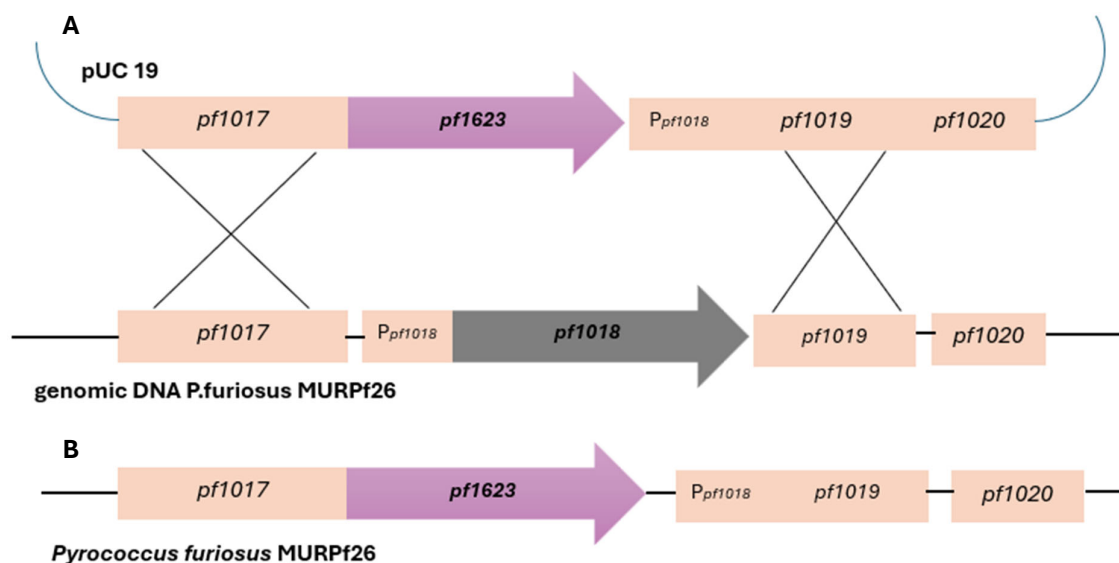


Figure 6: Genetic construct for the deletion of the ribosomal protein L41e (*pf1018*) via homologous recombination. A. The promoter of *pf1018* and the downstream gene *pf1019* were generated and assembled to the vector-*pf1017*-*pf1623* construction. After further introduction of the assemblage into the auxotroph mutant of *P.furiosus* ($\Delta PdaD$), the small gene *pf1018* should be deleted via double cross over. B. The new mutant strain complemented the missing PdaD at the position of *pf1018*. Thereby, the gene *pf1018* could be deleted via homologous recombination.

Via providing homologous regions, after introducing the assembled construct into the auxotroph mutant of *Pyrococcus furiosus*, in this way, only the gene *pf1018*, should be deleted (Fig. 6A), in order to investigate the function of L41e in archaea.

2. Material and Methods:

2.1. Materials:

2.1.1. Bacterial and archaeal strains:

<i>Organism</i>	<i>Strain</i>
<i>Escherichia coli</i>	<i>DH5α</i>
<i>Pyrococcus furiosus</i>	Δpf1623 (MURPf26)
<i>Pyrococcus furiosus</i>	DSM 3638

2.1.2. Plasmids:

<i>Plasmid</i>	<i>Usage</i>
pUC19 (pMUR6)	Basal plasmid for the construction of the genetic constructs
pMUR435	Basal plasmid for the amplification of <i>pf1623</i>
pMUR685	Genetic construct for the transformation into MURPf26, for the deletion of <i>pf0560</i>
pMUR686	Genetic construct for the transformation into MURPf26, for the deletion of <i>pf1777</i>
pMUR687	Genetic construct for the transformation into MURPf26, for the deletion of <i>pf1018</i>
pMUR691	Genetic construct for the transformation into MURPf26, for the deletion of <i>pf1018</i>

2.1.3. Enzymes:

<i>Enzyme</i>	<i>Manufacturer</i>
Phusion High Fidelity DNA-Polymerase	New England Biolabs, Frankfurt
Proteinase K	Fermentas, Burlington (USA)
RNase A	New England Biolabs, Frankfurt
Gibson Assembly Mix	New England Biolabs, Frankfurt
DpnI	New England Biolabs, Frankfurt

2.1.4. Extraction Kits:

<i>Kit</i>	<i>Manufacturer</i>
Wizard R SV Gel and PCR Clean Up	Promega, Mannheim
Nucleo Spin* Gel and PCR Clean Up	Macherey-Nagel, Düren
DNA, RNA and protein purification	Macherey-Nagel, Düren

2.1.5. Chemicals:

Chemical	Manufacturer
Chloroform/Isoamyl alcohol	Roth, Karlsruhe
Phenol/Chloroform/Isoamyl alcohol	Roth, Karlsruhe
Agarose	Roth, Karlsruhe
Agmatin-Sulfat	Sigma, St. Louis
Ampicillin	Merck, Darmstadt
Calcium Chloride	Merck, Darmstadt
BacLigth	Thermo Fisher Scientific, Dreieich
EDTA	Merck, Darmstadt
Ethanol, 99,9%	Roth, Karlsruhe
Gelrite	Serva, Heidelberg
GeneRuler™ 1 kb DNA Ladder	Thermo Fisher Scientific, Dreieich
GeneRuler™ 100 bp DNA Ladder	Thermo Fisher Scientific, Dreieich
Glycerin	Merck, Darmstadt
Yeast extract	Difco, Lawrence (USA)
Magnesium Chloride	Merck, Darmstadt
Natrium Chloride	VWR, Leuven (BE)
Natrium Sulfide	VWR, Leuven (BE)
Oligonucleotides	Microsynth, Balgach (Suisse)
Phosphate	Merck, Darmstadt
Phusion™ HF- Buffer (5x)	New England Biolabs, Frankfurt
Bacto™ Peptone	Difco, Lawrence (USA)
Resazurine	Serva, Heidelberg
Tris	USB Corporation, Cleveland USA
Tryptone	Difco, Lawrence (USA)
SYBR™ Safe DNA Gel Stain	Thermo Fisher Scientific, Dreieich

2.1.6. Buffer and Solutions:

1x TAE

Tris, pH 8,0	40 mM
Acetic acid	20 mM
EDTA	1 mM

TE 10:1

Tris, pH 8,0	10 mM
EDTA	1 mM

TE 10:0,1

Tris, pH 8,0	10 mM
EDTA	0,1 mM

Pyrococcus furiosus Transformation buffer

CaCl ₂ x H ₂ O	80 mM
Natrium Pyruvate	40 mM

NH ₄ Cl	4,67 mM
½ SME (without KH ₂ PO ₄), pH 7,0	

Glycerol solution for E.coli Stocks

Glycerol	65 %
MgSO ₄ x 7 H ₂ O	0,1 M
Tris, pH 8,0	0,025 mM

Loading Dye for Agarose Gels (6x)

Saccharose	50 %
Urea	4,0 M
EDTA	50 Mm
Bromphenolblue	0,1 %

2.1.7. Media and Supplements:

½ SME-Media

<i>Solution</i>	<i>Amount</i>
SME Stock Solution (1x)	500,0 ml
KH ₂ PO ₄	0,5 g
NH ₄ Cl	0,5 g
Yeast extract (0,1%)	1,0 g
(Bacto-) Pepton (0,1%)	1,0 g
Natriumpyruvate (40mmol)	4,4 g
Wolfe's Mineral/10x/pH 1,0	1,0 ml
Resazurin, 0,2%	0,5 ml
Na ₂ S x 7-9 H ₂ O	0,3 g
H ₂ O bidest, ad	1000,0 ml

Gelrite Media Plates

<i>Solution</i>	<i>Amount</i>
SME Stock Solution (1x)	375 ml
KH ₂ PO ₄	0,375g
NH ₄ Cl	0,188 g
Yeast extract (0,1%)	0,75 g
(Bacto-) Pepton (0,1%)	0,75 g
Starch	0,75 g
Wolfe's Mineral/10x/pH 1,0	0,75 ml
Na ₂ S x 7-9 H ₂ O	0,2 g
H ₂ O bidest, ad	600,0 ml

LB-Media

Tryptone	1 %
Yeast extract	0,5 %
NaCl	0,5 %
pH 7,5	

LB-Agar Media

Agar	1,8 %
Tryptone	1 %
Yeast extract	0,5 %
NaCl	0,5 %
pH 7,5	

2.2. Methods:

2.2.1. Molecular biological Methods

2.2.1.1. PCR-Polymerase Chain Reaction:

The Polymerase Chain Reaction (PCR) represents a widely used molecular biologic technique, that enables the amplification of a specific DNA sequence. The method is partitioned into several steps, including the denaturation step, primer annealing and DNA synthesis. In the first step, the double stranded DNA template gets denatured by heat and separated into two single strands. Further, primers, which consist of short synthetic oligonucleotides, anneal specifically to the complementary sequence flanking the target region during the annealing step. In the further extension step, a DNA Polymerase synthesizes a new DNA strand via incorporating nucleotides that are complementary to the template. Those steps are repeated in respective amount of cycles, whereby the target DNA is amplified and can be used for the further experiments.

2.2.1.1.1. Phusion PCR:

The Phusion PCR is based on using the Phusion High Fidelity DNA-Polymerase, which consists of the Pfu Polymerase and DNA binding protein. The Phusion High Fidelity DNA-Polymerase enables high yield and lower error rates. Furthermore, this Polymerase provides higher speed and high processivity by harboring a DNA binding domain.

The samples were prepared via manufacturer instruction. Further, the PCRs were prepared as instructed in Table 2 using different Annealing Temperatures depending on the GC-content. The extension time was adjusted depending on the length of the amplified fragment.

Table 2: Utilized PCR Program. Time of Extension varied depending on the length the amplifying fragment. Annealing Temperature varied depending on the G+C content in the fragment.

Step	Temperature	Time	Cycles
Initial Denaturation	98 °C	30 sec	1
Denaturation	98 °C	10 sec	30
Primer Annealing	°C	45 sec	
Extension	72 °C	15-30 sec/kb	
Final Extension	72 °C	10 min	1
Hold	12 °C	∞	1

2.2.1.2. PCR Purification:

The PCR Products were purified using the Gel and PCR Clean Up Kit of Macherey-Nagel following the manufacturer instructions.

2.2.1.3. DpnI restriction:

In order to cleave of methylated DNA, the template DNA derived from *E.coli* and therefore methylated, was treated with the restriction enzyme *DpnI* after Purification. This enabled only having DNA present, that arose from the PCR amplification, which is not methylated. Moreover, the enzyme cleaves only when its recognition site GATC is methylated. In this way, non-methylated DNA gets not cleaved and remains. Using *DpnI* (New England Biolabs), 20 U of the enzyme was added to the PCR approach and incubated at 37 °C overnight.

2.2.1.4. Nanodrop:

To measure the concentration of the purified DNAs, isolated Plasmids or genomic DNAs, a Nanodrop instrument was utilized. Beside of analyzing the concentration of DNA, the device gave

information about the quality of the DNA by measuring the A260/280 and A260/230 ratios. As DNA/RNA has its absorption at 260 nm, A260 in the ratio gives a hint about the DNA/RNA content in the sample. In contrast, A280 demonstrates the protein content in the sample, as proteins have its absorption at 280 nm. An A260/280 ratio between 1,8-2,0 is expected to have a good quality of DNA/RNA without any contamination of protein or RNA. Further, the A260/230 ratio gives a hint about the contamination of the DNA with organic solvents and should have a ratio between 2,0-2,2 for pure DNA/RNA.

In addition, the DNA was loaded in a specific concentration calculated back from the measured Nanodrop concentration on a gel, in order to adjust the measured concentration of the DNA, if the intensity of the DNA on the gel differed to the Nanodrop value. Hereby, samples of known concentration served as standards.

2.2.1.5. Agarose Gel Electrophoresis:

Agarose Gel electrophoresis provides a molecular biological technique, that enables the separation of nucleic acids according to their size. Hereby, the DNA/RNA moves due to its negatively charged backbone from the negative pole to the positive pole. The fragments are migrating distinct depending on their size. Furthermore, larger fragments are migrating slower than smaller fragments.

Using Agarose Gel electrophoresis, dependent on the size of the fragments an agarose gel with different percentage of agarose was prepared. Moreover, using smaller sized fragments an agarose gel with higher percentage was used, as smaller fragments are able to penetrate easier through higher concentrated agarose gel. In contrast, larger fragments were analyzed with less percentage of agarose, which facilitated the migrating of the fragments through the gel.

In this way, 1-3% of Agarose Gels were prepared depending on the size of the DNA Fragments. As a running buffer a 1x TAE Solution was utilized. The gel solutions were stained with SYBR Safe (2,5 µL), which enabled the intercalation of the DNA and the visualization of DNA under UV Light. For loading the samples on the gel, the probes were mixed with a loading dye (6x). As a marker, usually the 1 kb Gene Page Ruler served for analyzing fragments of higher size and the 100bp Gene Page Ruler for analyzing smaller fragments. The samples ran on the gel with a voltage of 100 at a time of 30-35 min.

2.2.1.6. Gibson Assembly:

Gibson Assembly is a technique, which enables the assembly of multiple fragments in only one reaction mainly performed by three enzymes, the Exonuclease, DNA Polymerase and DNA Ligase. This technique provides the advantage that multiple fragments can be combined together without the usage of restriction enzymes.

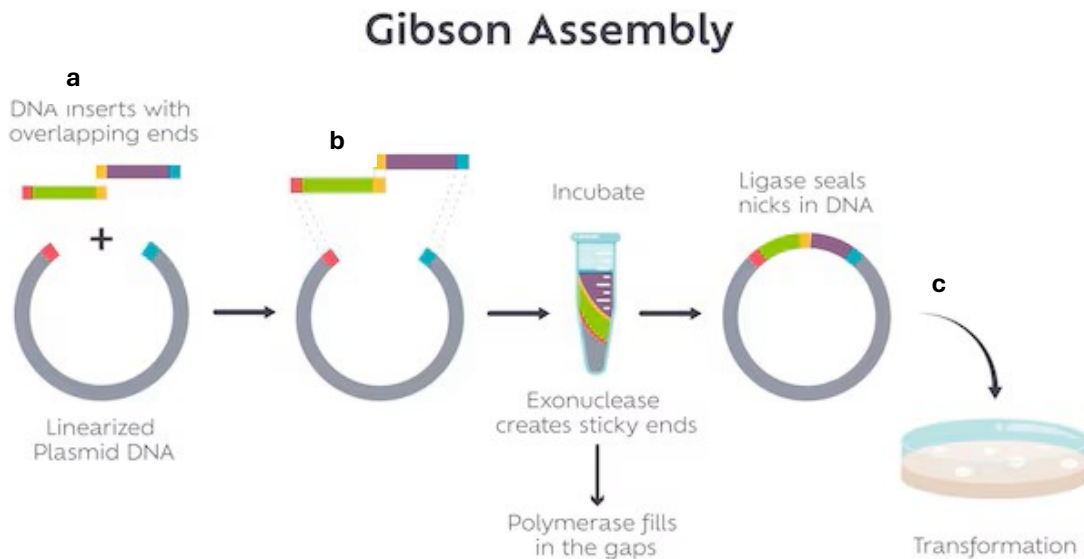


Figure 7: Procedure of the Gibson Assembly. a. Fragments designed with overhangs to the respective DNA fragment and vector are introduced together into the vector and anneal with each other. b. Via adding Gibson Assembly Mix and incubating, the fragments are generated to each other. c. Transforming into a recipient strain, provides the screening for the assembled clone. (Biorender)

The method is based on providing overlapping overhangs of the DNA Fragments, which can anneal to each other (Fig.7a). In the first step of the reaction, the Exonuclease chews away the DNA fragment at the 5'end via creating sticky ends (Fig.7b). Thereby, the DNA fragments build 3' single stranded regions that are complementary to each other and hybridize. In a second step, the DNA Polymerase refills the gaps in the new synthesized DNA strands with nucleotides (Fig.7b). In a final step, the DNA Ligase connects the DNA fragments covalently via providing phosphodiester bonds between the end of the 3' Hydroxyl group and the 5' Phosphate (Fig 7b). These steps happen all in one isothermal reaction by providing all 3 enzymes at the same time. Further, the new created plasmid is transformed into recipient strains and can be further used. (Fig. 7c)

Using the method in this work, the fragments flanking the target gene for deletion, were assembled to the selection marker PdaD and to the vector.



Figure 8: Principle of genome assembly in this work. Gene flanking upstream of the target gene is designed harboring overlapping overhangs to the vector at its 5' end and overlapping overhangs at its 3' end to the Pyruvoyl dependent Decarboxylase (PdaD). Likewise, the region flanking the downstream part of the region, is designed with overhangs to the selection marker at its 5' end and overlapping overhangs at its 3' end to the vector. The designed regions are amplified and in this way, assembled to each other via the Gibson Assembly.

As described in Figure 8, the constructs were assembled with respective overhangs in a way, that the upstream and downstream region of the target gene were connected to the Pyruvoyl dependent Arginine decarboxylase (PdaD) at the position where the target gene is located in the target genome. In addition, the downstream and upstream fragments were assembled with respective overhangs to a vector pUC19. All fragments were assembled simultaneously to each other via the Gibson Assembly in a one reaction approach.

2.2.1.6.1. Calculations for equimolar amounts of the fragments in the Gibson Assembly reaction:

To calculate the inserted volume for the Gibson Assembly Reaction to have equimolar amounts of DNA, following equation was used:

$$X = \frac{\text{length of the fragment [bp]}}{\text{length of the vector [bp]}} (1)$$

The quotient was calculated into the required concentration of DNA in ng, when using 50ng of DNA.

$$\text{DNA amount [ng]} = X \times 50\text{ng} (2)$$

Further, the required amount of DNA were adjusted to the intensities of each fragments. Hereby 50ng of each fragment was loaded on to a gel. Differences in the intensity of the fragments observed on the gel were adjusted to ensure equimolar amounts of all DNA fragments.

To get to know the Volume for equimolar amounts of the fragments, following equation was used:

$$\text{Volume [\mu L]} = \frac{\text{DNA amount [ng]}}{\text{Nanodrop concentration of purified PCR fragment}} (3)$$

Table 3: General scheme used for the Gibson Assembly approach. The Volume of the fragments was calculated using the equations.

Volume	Purified PCR-Fragment	Excess
1 μL	pUC19 Vector [50ng]	1x
x μL	Pyruvoyl Carboxylase	3x
x μL	Upstream flanking geneX	3x
x μL	Downstream flanking geneX	3x
4 μL	Gibson-Assembly Mix (2,5x)	
x μL	H ₂ O	
10 μL per reaction		

To assemble the fragments with the Gibson Assembly Mix together the respective solutions, as described in Table 3 were pipetted into one PCR Tube. As a control, one approach was prepared where only the vector and the Pyruvoyl dependent Arginine Decarboxylase were assembled together. To increase the probability for the annealing of the fragments with the vector, the respective upstream and downstream fragment and PdaD amplified fragment were added in a 3x molar excess towards the vector, if not mentioned differently. The reaction mix was incubated for 100 minutes at 50°C.

2.2.2. Cultivation and Transformation of *E.coli*

2.2.2.1. Plasmid Isolation

The *E.coli* transformants were inoculated in 10 ml solid LB Media with 10 µL Ampicillin [100 mg/ml] and incubated at 37 °C with shaking overnight. The Plasmids were isolated using the DNA, RNA and protein purification Kit and Protocol of Macherey-Nagel.

2.2.2.2. Transformation in *E.coli*:

For transforming the plasmids created via Gibson Assembly into *E.coli*, competent cells of *E.coli* DH5α were used. 1-4 µL of the Gibson Assembly Reaction were added to 100 µL *E.coli* DH5α and incubated on ice 30 minutes. As a negative control a Gibson Assembly reaction without addition of the fragment and as a positive control 1 ng pUC19 Vector was used. Further, the heat shock was performed incubating 1 minute at 42 °C. 800 µL LB Media was added and samples were incubated at 37°C for 1 hour. 100 µL of each reaction were plated on LB-Media plates, supplemented with Ampicillin, and incubated at 37°C overnight.

2.2.3. Cultivation and Transformation of *Pyrococcus furiosus*

2.2.3.1. Cultivation of *Pyrococcus furiosus*

Cultivation of *Pyrococcus furiosus* in Liquid culture:

To cultivate *P. furiosus*, the clones were incubated in 40 ml ½ SME Media flask flushed with 0,8 bar N₂. The culture was incubated 20-25 hours at 85 °C.

Cultivation of *Pyrococcus furiosus* onto Gelrite Media Plates:

Pyrococcus furiosus was cultivated on Gelrite Media Plates in an anaerobe jar flushed with 1 bar N₂ at 85 °C.

2.2.3.2. Transformation in *Pyrococcus furiosus*:

The respective DNA constructs were transformed into *Pyrococcus furiosus*, providing an Agmatine auxotroph deletion strain as the recipient strain (MURPf 26). The recipient strains utilized for the transformation, were selected based on their growth rate. Only recipient strains obtaining the exponential phase ($1-2 \times 10^8$ cell count/ml) were selected for the transformation.

The transformation steps were prepared in an anaerobic chamber providing an anaerobic condition. Moreover, 8 ml of fresh *Pyrococcus furiosus* (MURPf26) culture were centrifugated 10 minutes at 9000 rpm. Supernatant was removed additional 8 ml of fresh culture were added and centrifugated 10 minutes at 900 rpm. Supernatant was removed. 600 µL Transformation buffer was added to the Pellets and resuspended. Further, per transformation approach 100 µL were added per PCR Reaction Tube. The transformation approach was set into a Thermocycler for 1 ½ hours at 4 °C, after ½ hour linear DNA [0,5 pmol] was added to the reaction. Heat shock was performed 3 minutes at 85 °C. Samples were incubated 10 minutes at 4°C. The transformation approach was plated undiluted onto Gelrite Media Plates. The plates were incubated in an anaerobe jar flushed with 1bar N₂.

2.2.3.2.1. Calculation to have 0,5 pmol DNA of the fragment

To have 0,5 pmol of the linear DNA fragment in excess to the vector, following equation was utilized:

$$0,33 \mu\text{g dsDNA of } 1\text{kb length} = 0,5 \text{ pmol (4)}$$

2.2.3.3. Isolation of gDNA via Phenol/Chloroform:

For the Isolation of genomic DNA of *Pyrococcus furiosus*, a DNA Extraction using Phenol and Chloroform was conducted. Herefore, 10-20 ml of an exponential grown culture [$1,8 \times 10^8$ cell counts/ml] were centrifugated at 20000 rpm over 2 minutes. Supernatant was removed and Pellet was resuspended with 567 μ L TE-Buffer (10:1). 25,38 μ L SDS [10%], 5,95 μ L RNase A [20mg/ml] and 6 μ Proteinase K [20 mg/ml] were added, the solution was mixed and incubated 15 minutes at 60 °C. 36 μ L Natrium chloride [0,5M] were added. 700 μ L Phenol/Chloroform/Isoamyl alcohol (25:24:1) was added, vortexed and centrifugated 5 minutes at 20000 rpm. The upper, aqueous phase was placed into a new Eppendorf Tube and the Phenol Extraction was repeated. In the next step, 700 μ L Chloroform/Isoamyl alcohol was added, vortexed and centrifugated 5 minutes at 20000 rpm. The upper, aqueous phase was placed into a new Eppendorf Tube. 0,6 Volume Isopropanol was added and centrifugated 20 seconds, 20000 rpm. Supernatant was removed and Pellet was resuspended in 1ml Ethanol (70%). The sample was centrifugated 2 minutes at 20000 rpm and Supernatant was removed. Pellet was dried for 30 minutes at room temperature and resuspended in 100-200 μ L TE-Buffer (10:0,1).

2.2.4. Molecular biological Techniques

2.2.4.1. Measuring archaeal growth with an OD measuring device:

To investigate the growth of archaeal cells a OD-measurement was utilized. In this way, a measuring device came into play (Grünberger et al., 2021), that could detect the exposed light of the archaeal cell abstracted from the media in the probe during the incubation time.

2.2.4.2. Microcopy with a live dead staining:

To visualize dead and living cells, probes were prepared under the microscope with the dye BacLight, which consists of two stains, SYTO 9 and Propidium iodide. In particular, the dye SYTO 9 binds DNA and can enter dead and living cells. Through entering into the cell, all cells get fluorescently green labelled. The second stain, Propidium iodide can only enter cells with an impaired membrane. Those attached cells get fluorescently red labelled. Furthermore, only cells that are referred to death get red labelled. Living cells keep the staining of SYTO 9, as the PI stain is not able to enter the intact membrane of the living cell. In order to visualize the staining, a GFP filter and a Tx Red filter were used under the light microscope.

For the preparation of the samples 5 μ L of each probe was treated with 1 μ L BacLight and 1 μ L Phosphate. The sample was heated up over 3 minutes at 80 °C and immediately visualized under the microscope. Due to the heating and the addition of Phosphate, the cell should be more active.

2.2.5. Construction and Transformation of the DNA Construct for the deletion of the ribosomal dimerization factor PF0560

In order to delete pf0560 in *Pyrococcus furiosus*, a genetic construct was created. Based on this construct, the upstream fragment (pf0559) and downstream fragment (pf0561) flanking pf0560 were amplified. In addition, the selection marker gene pf1623, which encodes for the Pyruvoyl dependent Arginine decarboxylase, and the vector pUC19 were amplified. In the next step the fragments were connected to each other via Gibson Assembly in a way, that pf0559 and pf0561 were flanking the selection gene pf1623 at the position, where pf0560 is located in *Pyrococcus furiosus*. Likewise, in the one step reaction the pUC19 vector has been assembled to the fragments. This enabled the construct to be carried on a plasmid, to further transform it into *E.coli*. Thereby, clones could be picked and screened for the correct sequence. The clone with the desired sequence of the genome assembly was further amplified using primers targeting the

upstream and downstream region of *pf0560*. This fragment was further introduced into a *Pyrococcus furiosus* strain (MURPf26), where the selection gene *pf1623* was not present.

Further, via transforming the genetic construct into MURPf26, homologous regions were provided upstream and downstream of *pf0560*. Thereby, it was expected that homologous recombination took place at the position of *pf0559* and *pf0561*. Furthermore, as the gene *pf1623* was not present in the target gene, *pf1623* was expected to be complemented and exchanged at that position for *pf0560*. The incorporation of the genetic construct into MURPf26 was further screened on the growth in absence of Agmatine.

2.2.5.1. Plasmid pUC19-*pf0559*-*PdaD*-*pf0561*

The fragments flanking the upstream region of the gene *pf0560*, namely *pf0559*, and downstream region of the gene *pf0560*, namely *pf0561*, were assembled together to *pf1623* and the vector pUC19 via Gibson Assembly.

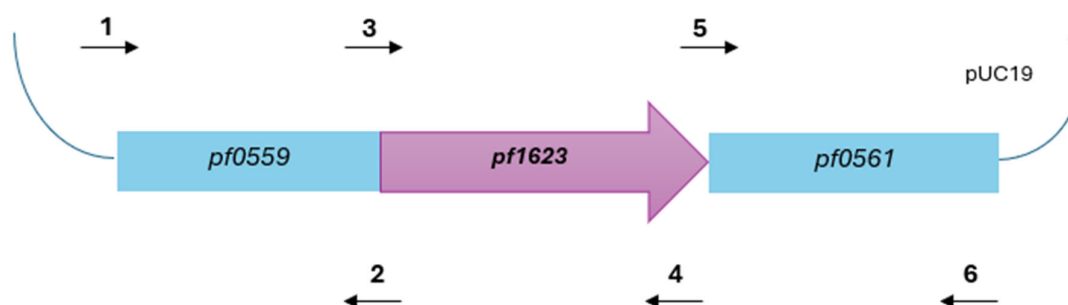


Figure 9: Fragments with the respective overhangs assembled to each other. The fragment *pf0559*, encoding the upstream region of *pf0560*, with the overhangs to the vector and *pf1623*. The selection gene *pf1623* harboring overhangs to *pf0559* and *pf0561*. The fragment *pf0561*, encoding the downstream region of *pf050*, harboring overhangs to *pf1623* and to the vector.

The respective fragments were designed with 16-25 nucleotide overhangs complementary to the respective adjacent fragment in the final construct. (Fig.9) The following primers used for the amplification of the fragment are listed in Table 4 below.

Table 4: Primers and Sequence to amplify the regions for the genetic construct.

Nr.	Primer	Sequence
1	pUC_M13F_UpPf0560_F	5'-GTAAACGACGGCCAGTCCCAGGAGGGGATTTGGCC-3'
2	Agma_Ups_Pf0560_R	5'-CTGCGGTGTTTTGTAGGATAAGAAAAAAGGAAGGCCTAGGTAG-3'
3	Agma_RK-F	5'-TCCTACAAAACACCGCAGATTAAAC-3'
4	Agma_Rk_R	5'-TTATGAAGTGAGCAAAATAGCAAGAAAG-3'
5	Agma_DowPf0560_F	5'-GCTATTTTGCTCACTTCATAACCATGTCTAGGAATTTAACTCTATCGG-3'
6	pUC_M13R_DowPf0560_R	5'-CAGGAAACAGCTATGACCCAGCTTATCCTAACAGTATCGCTTCC-3'

For amplifying the upstream region of *pf0560*, which obtains *pf0559*, Primer 1 and 2 were utilized. Amplifying the downstream region of *pf0560*, namely *pf0561*, Primer 5 and 6 were used. To produce the selection marker, encoded by *pf1623*, Primers 3 and 4 were used. (Table 4)

The Annealing temperatures for generating the fragments are listed in Table 5 below.

Table 5: Annealing Temperatures for the PCRs to amplify the respective fragments.

Nr.	Fragment	Annealing temperature
1 2	<i>pf0559</i>	63,5 °C
3 4	<i>pf1623</i>	63,0 °C
5 6	<i>pf0561</i>	63,0 °C

To generate the Vector pUC19 the Primer Ampl_pUC_F with the Sequence 5'-GGTCATAGCTGTTTCCTGTGTG-3' and Ampl_pUC_R with the Sequence 5'-ACTGGCCGTCGTTTACAACG-3' were utilized. As a template for the amplification, the plasmid pMUR6 was used.

Plasmid pUC19-*pf1776*-PdaD-*pf1778* as control

Likewise, a mutant version should be created, which functioned as a control for the transformation process. In this case a transposon, encoded by *pf1777*, should be deleted. To do so, likewise genomic regions flanking this gene upstream and downstream should be amplified and connected to the selection marker and to the vector pUC19.

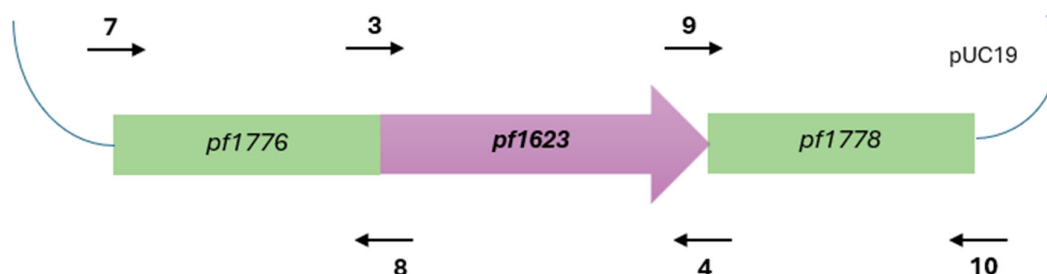


Figure 10: Fragments with the respective overhangs assembled to each other. The fragment *pf1776*, encoding the upstream region of *pf1777*, with the overhangs to the vector and *pf1623*. The selection gene *pf1623* harboring overhangs to *pf1776* and *pf1778*, encoding the downstream region of *pf1777*. The fragment *pf1778* harboring overhangs to *pf1623* and to the vector.

Following primers were used to amplify the upstream and downstream fragments of a transposon (Table 6). Amplifying the upstream region of *pf1777*, encoded by *pf1776*, Primer 7 and 8 were utilized. Amplifying the downstream region *pf1778*, Primer 9 and 10 were used.

Table 6: Primers and Sequence to amplify the regions for the genetic construct.

Nr.	Primer	Sequence
7	pUC_M13F_UpTransp_F	5'-GTAAAACGACGGCCAGTCCGCTTCAAGTTGATTCATTCTCC-3'
8	Agma_Upst_Transp_R	5'-CTGCGGTGTTTTGTAGGAGGGTTCTGCATATGAGGTAATTAATC-3'
3	Agma_RK-F	5'-TCCTACAAAACACCGCAGATTAAAC-3'
4	Agma_Rk_R	5'-TTATGAAGTGAGCAAAATAGCAAGAAAG-3'
9	Agma_DowTransp_F	5'-GCTATTTTGCTCACTTCATAACAGTATCCAGGAGGATTAGGTAACG-3'
10	pUC_M13R_DowTransp_R	5'-CACACAGGAAACAGCTATGACCCGGGTTTCATCTCTATGGGGG-3'

The Annealing temperatures for the amplification of the fragments are listed in Table 7 below.

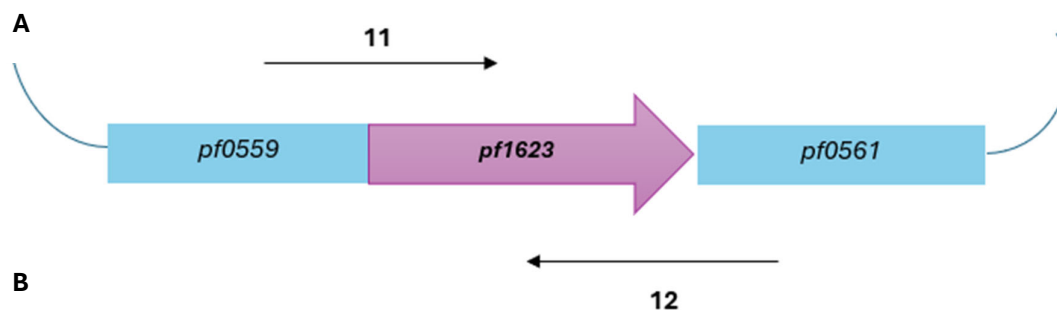
Table 7: Annealing Temperatures for the PCRs to amplify the respective fragments.

Nr.	Fragment	Annealing temperature
7 8	<i>pf1776</i>	63,5 °C
3 4	<i>pf1623</i>	63,0 °C
9 10	<i>pf1778</i>	63,0 °C

2.2.5.2. DNA Fragments for the Transformation in *Pyrococcus furiosus*

DNA Fragment *pf0559*-PdaD-*pf0561* for deleting *pf0560*

In order to provide homologous regions flanking the target gene *pf0560* when introducing into *Pyrococcus furiosus*, a part of the region *pf0559*, flanking upstream of the target gene, and of the region *pf0561*, flanking downstream of the target gene, were amplified with harboring the selection gene, *pf1623*, between instead of the target gene *pf0560*. In this way, homologous regions could be provided for the transformation in *Pyrococcus furiosus*, where *pf0560* is exchanged for the *pf1623* encoding for the selection marker, the Pyruvoyl dependent Arginine Decarboxylase.



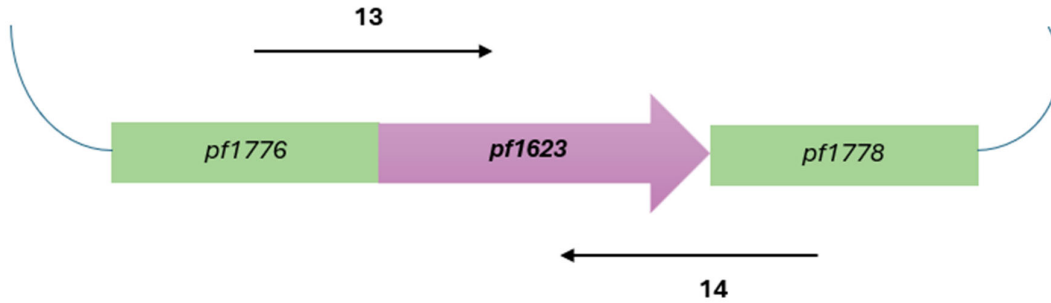
Nr.	Primer Name	Sequence
11	Ampl_Trafo_PF0560_F	5'-GGTGCTTGATGCCTTCTCGG-3'
12	Ampl_Trafo_PF0560_R	5'-CCACTTATCGTTGCCTTAGTTTGTCTCCTC-3'

Figure 11: Target region for the introduction into *Pyrococcus furiosus*. A. Respective primers were used to amplify a region of the genetic construct, that was further transformed into the recipient strain of *Pyrococcus furiosus*. B. Number of the Primer and its Sequence.

The primers used for the amplification of the fragment that was introduced into *Pyrococcus furiosus*, are listed in Figure 11B.

DNA Fragment *pf1766*-PdaD-*pf1778* for the control transformation

In the same way a part of genes flanking upstream and downstream the gene *pf1777*, encoding for a transposon, were amplified in the construct with harboring the selection gene, *pf1623*, between instead of the target gene *pf1777*, to provide homologous regions for the transformation in *Pyrococcus furiosus*.

A**B**

Nr.	Primer Name	Sequence
13	Ampl_Trafo_Transp_F	5'-GGATGAGGACATCAGCTATCGCTG-3'
14	Ampl_Trafo_Transp_R	5'-GCTCTGGCCTCTTACCTTTGGATC-3'

Figure 12: Target region for the introduction into *Pyrococcus furiosus*. A. Respective primers were used to amplify a region of the genetic construct, that was further transformed into the recipient strain of *Pyrococcus furiosus*. B. Number of the Primer and its Sequence.

The primers used for the amplification of the fragment that was introduced into *Pyrococcus furiosus*, are listed in Figure 12B.

2.2.5.3. Screening of PF0560 in the deletion mutant

To investigate, if *pf0560* was still present in the created deletion strain, a PCR Screening was done. Hereby, Primers were used that target the gene *pf0560*. Thereby, this analysis could give an information, whether *pf0560* is still in the genome. The primers and sequence of the primers are listed in Table 8 below.

Table 8: Respective Primer Name and its sequence used for the Screening of *pf0560*.

Nr.	Primer Name	Sequence
15	PF0560_F	5'-AAAGTAGTAGGAATTAAGGGAAGTGTC-3'
16	PF0560_R	5'-CTTGGGATATACTAATAGCGTTCTCC-3'

2.2.6. Construction and Transformation of the DNA Constructs for the deletion of the ribosomal protein L41e

2.2.6.1. Construction of pUC19-pf1017-PdaD-pf1020

Based on this construct, the upstream fragment flanking *pf1018*, namely *pf1017* was amplified. In addition the fragment, that targets the downstream region of *pf1019*, which is located downstream of *pf1018*, was amplified. In this approach, *pf1018* should be deleted together with its adjacent gene *pf1019*, in order to facilitate the deletion process. The amplified fragments were connected via Gibson Assembly to the selection gene and the vector pUC19. The further steps were performed in the same way as described in 2.2.5.

2.2.6.1.1. Plasmid pUC19-pf1017-PdaD-pf1020

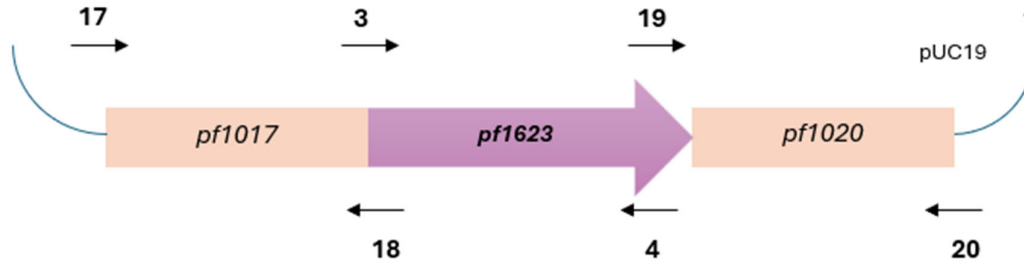


Figure 13: Fragments with the respective overhangs assembled to each other. The fragment *pf1017*, encoding the upstream region of *pf1018*, with the overhangs to the vector and *pf1623*. The selection gene *pf1623* harboring overhangs to *pf1017* and *pf1020*, encoding downstream of *pf1018*. The fragment *pf1020* harboring overhangs to *pf1623* and to the vector.

Hereby, the upstream fragment of *pf1018* (*pf1017*) was generated with an 5' overhang to the vector and a 3' overhang to the gene *pf1623*. To generate *pf1623*, primers were used with an 5' overhang to the upstream fragment and a 3' overhang to the gene *pf1020*. Further, the gene *pf1020* was generated with an 5' overhang to the selection gene and a 3' overhang to the vector. (Fig. 13)

Table 9: Primers and Sequence to amplify the regions for the genetic construct.

Nr.	Primer	Sequence
17	pUC_M13F_UpL41e_F	5'-GTAAACGACGGCCAGTGCCCTCTTCTCTCCTACTCTATC-3'
18	Agma_Ups_L41e_R	5'-CTGCGGTGTTTTGTAGGATACATTTCCCAAGTCTTAGGAC-3'
3	Agma_RK-F	5'-TCCTACAAAACACCGCAGATTAAAC-3'
4	Agma_Rk_R	5'-TTATGAAGTGAGCAAAATAGCAAGAAAG-3'
19	Agma_DowL41e_F	5'-CTATTTTGCCCTTCATAACTAATCAACGATCTATATAGAATACTGAGC-3'
20	pUC_M13R_DowL41e_R	5'-CAGGAAACAGCTATGACCCAAGATCAGTGGCTGG-3'

As depicted in Table 9 following primers were used to amplify the fragments. For amplifying the upstream region of *pf1018*, which obtains *pf1017*, Primer 17 and 18 were utilized.

Amplifying the downstream region of *pf1019*, namely *pf1020*, Primer 19 and 20 were used. The Annealing temperatures for the primers are listed in Table 10 below.

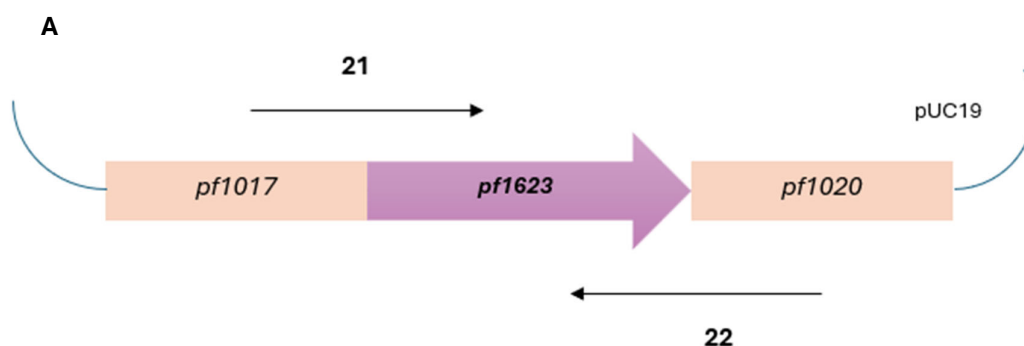
Table 10: Annealing Temperatures for the PCRs to amplify the respective fragments.

Nr.	Fragment	Annealing Temperature
17	<i>pf1017</i>	63,0 °C
18		
3	<i>pf1623</i>	63,0 °C
4		
19	<i>pf1020</i>	63,0 °C
20		

2.2.6.1.2. DNA Fragment for the transformation in *Pyrococcus furiosus*

In order to provide homologous regions flanking the target gene *pf1018* when introducing into *Pyrococcus furiosus*, a part of the region *pf1017*, flanking upstream of the target gene, and of the region *pf1020*, flanking downstream of the target gene, were amplified with harboring the selection gene, *pf1623*, between instead of the target gene *pf0560*.

In this way, homologous regions could be provided for the transformation in *Pyrococcus furiosus*, where *pf0560* is exchanged for the *pf1623*. Further, a part of the genetic construct was amplified for the transformation into *Pyrococcus furiosus*. This fragment should contain a part of flanking the upstream region of *pf1018*, connected to *pf1623*, the selection marker and a part of flanking the downstream region of *pf1020*. (Fig. 14A)



B

Nr.	Primer Name	Sequence
21	Ampl_Trafo_L41e_F	5'-GGTGCTTGATGCCTTCTCGG-3'
22	Ampl_Trafo_L41e_R	5'-CCACTTATCGTTGCCTTAGTTTGTCTCCTC -3'

Figure 14: Target region for the introduction into *Pyrococcus furiosus*. A. Respective primers were used to amplify a region of the genetic construct, that was further transformed into the recipient strain of *Pyrococcus furiosus*. B. Number of the Primer and its Sequence.

To amplify this construct, where the upstream and downstream regions are connected to the selection marker (Fig. 14A), the primers listed in Figure 14B were utilized. This fragment was then inserted into *Pyrococcus furiosus*.

2.2.6.2. Construction of pUC19-*pf1017*-*PdaD*-*P_{pf1018}*-*pf1019*-*pf1020*

In order to provide a deletion mutant, where only the small protein L41e was deleted, a second deletion approach was performed.

Hereby, additional fragments were amplified encoding for the Promotor of L41e and encoding for *pf1019* with the respective overhangs, all fragment were connected via Gibson Assembly to each other (Fig. 6) and introduced into *Pyrococcus furiosus* using the steps as described in 2.2.5.

2.2.6.2.1. Plasmid pUC19-*pf1017*-*PdaD*-*P_{pf1018}*-*pf1019*-*pf1020*

The fragments flanking the upstream region of the gene *pf1018* and downstream region of the gene *pf1018* were assembled together to *pf1623* and the vector pUC19 via Gibson Assembly. (Fig. 15).



Figure 15: Fragments with the respective overhangs assembled to each other. The fragment *pf1017*, encoding the upstream region of *pf1018*, with the overhangs to the vector and *pf1623*. The selection marker *pf1623* harboring overhangs to *pf1017* and *Ppf1018*. The fragment, encoding the Promotor of *pf1018* with overhangs to *pf1623* and *pf1019*, connected to the downstream fragment of *pf1018*, namely *pf1019*.

Hereby, to generate the Promoter of *pf1018* primers were designed with an 5' overhang to *pf1623* and primer with an 3' overhang of *pf1019*. The downstream fragment *pf1019* was generated with a 5' overhang to the Promoter of *pf1018* and a 3' overhang to the gene *pf1020*. The selection gene *pf1623* was generated with an 5' overhang to *pf1017* and a 3' overhang to the Promoter of *pf1018*. The vector pUC19 and *pf1017* were generated in the same way as described in the first deletion approach. (2.2.6.1) In this way the genes could be connected via Gibson Assembly to each other as illustrated in Figure 15.

Table 11: Primers and Sequence to amplify the regions for the genetic construct.

Nr.	Primer	Sequence
17	pUC_M13F_UpL41e_F	5'-GTAACGACGCGCCAGTGCCTCTTTCTCTCCTACTCTATC-3'
18	Agma_Ups_L41e_R	5'-CTGCGGTGTTTGTAGGATACATTTCCCAAGTCTTAGGAC-3'
3	Agma_RK-F	5'-TCCTACAAAACACCGCAGATTAAAC-3'
4	Agma_Rk_R	5'-TTATGAAGTGAGCAAAATAGCAAGAAAG-3'
23	AgmaRK_PromL41ePhospho_F	5'-GCTATTTTGCTCACTTCATAAAAAACCAATAAACTTAAATGACTTAGAAGG-3'
24	PromL41e R	5-ATCATCACCTCCTTATTTTCCC-3'
25	PromL41e_Phosphoesterase_F	5'-GGAAAATAAGGAGGTGATGATCATGGCTTTTGAAGATTTTCCC-3'
20	pUC_M13R_DowL41e_R	5'-CAGGAAACAGCTATGACCCAAGATCAGTGGCTGG-3'

As depicted in Table 11, following primers were used to amplify the fragments. For amplifying the upstream region of *pf1018*, which obtains *pf1017*, the vector pUC19 and the selection marker, the Primer 17 and 4 were utilized. Amplifying the Promotor of L41e, the Primer 23 and 24 were used. For the amplification of the downstream region of *pf1018*, moreover the amplification of *pf1019* and *pf1020*, Primer 25 and 20 served.

The Annealing temperatures for the primers are listed in Table 12 below.

Table 12: Annealing Temperatures for the PCRs to amplify the respective fragments.

Nr.	Fragment	Annealing Temperature
23	<i>P_{pf1018}</i>	52,0 °C
24		
25	<i>pf11019-pf1020</i>	68,0 °C
20		
17	pUC19- <i>pf1017</i> - <i>pf1623</i>	68,0 °C
4		

2.2.6.2.2. DNA Fragment for the Transformation in *Pyrococcus furiosus*

In order to introduce the genetic construct into *Pyrococcus furiosus* the respective fragment was firstly amplified using the Primers 21 and 22 (Fig. 16).



Figure 16: Target region for the introduction into *Pyrococcus furiosus*. A. Respective primers were used to amplify a region of the genetic construct, that was further transformed into the recipient strain of *Pyrococcus furiosus*.

2.2.6.2.3. Primer for Screening for L41e

In order to investigate, if *pf1018*, encoding for the ribosomal protein L4e, was present in the genome after the transformation, a PCR Screening was performed, targeting specifically the region of L41e. Hereby, Primers were used that target the gene *pf1018*, encoding for L41e. The respective primers are listed in Table 13.

Table 13: Respective Primer Name and its sequence used for the Screening of *pf1018*.

Nr.	Primer Name	Sequence
26	L41e_F	5'-GGAGAGGAGACCAAGGAAATGG-3'
27	L41e_R	5'-CAGATTAGACCTCTCTTTCTTCC-3'

3. Results

3.1. Deletion of the ribosomal dimerization factor PF0560

To create a deletion mutant, where the archaeal dimerization factor PF0560 is knocked out, the fragments flanking the gene *pf0560* upstream and downstream were amplified and connected via Gibson Assembly to the selection marker and the vector. As a control for the genetic system, a transposon encoded by *pf1777* was simultaneously knocked out via amplifying in the first step the regions flanking this gene upstream and downstream and connecting via Gibson Assembly to *pf1623* and to the vector.

3.1.1. Assembly of the genetic construct

Basis for the deletion of the archaeal ribosomal dimerization factor PF0560, which is encoded by *pf0560*, was the homologous recombination. Hereby, the upstream and downstream region of *pf0560* were connected to a selection marker, the Pyruvyl dependent Arginine Decarboxylase PdaD, encoded by *pf1623*, and to a vector pUC19 (Fig. 17A). This genome assembly was further introduced into an auxotrophic *Pyrococcus furiosus* strain, with a deletion of the PdaD, namely the MURPf26 strain.

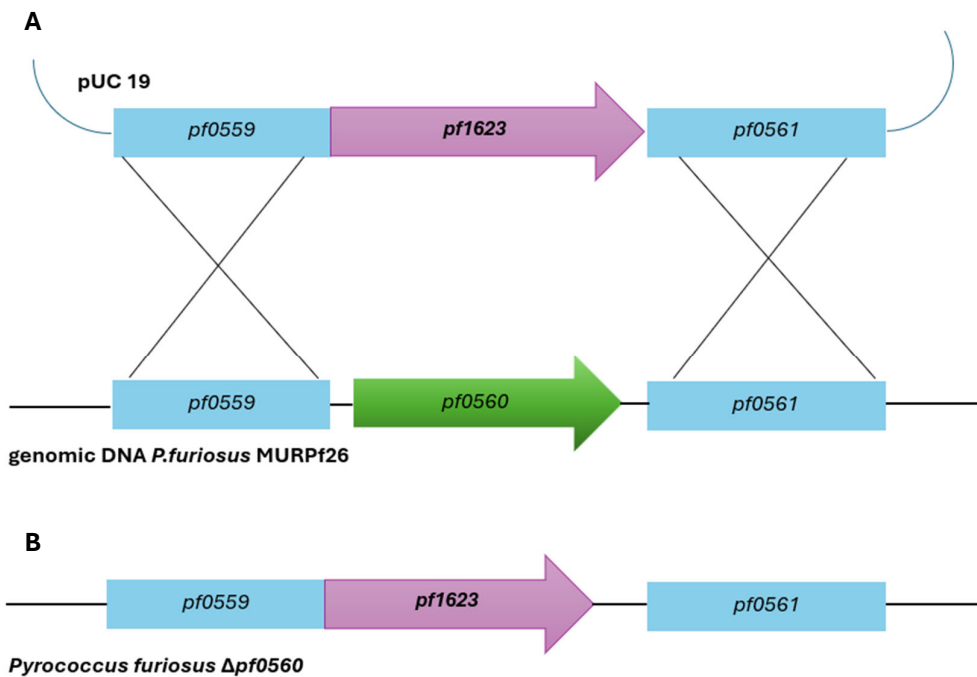


Figure 17: Genetic construct for deleting *pf0560*. A. *Pf0559* and *pf0561* are connected to *pf1623*, the PdaD and the vector pUC19. Via introducing the construct into MURPf26, homologous regions upstream and downstream of *pf0560* lead to a double cross over at those positions. B. As a result, *pf0560* gets deleted in MURPf26.

Accordingly, via transforming the construct into the recipient strain MURPf26, homologous regions were provided (Fig. 17A), whereby the upstream and downstream fragments flanking *pf0560* lead due to a double cross over to the exchange of *pf0560* with *pf1623*, encoding for the PdaD. In this way, the new mutant strain was performed, whereby *pf0560* was deleted in *Pyrococcus furiosus*. (Fig. 17B)

3.1.2. Amplification and Purification of the fragments flanking *pf0560*

In the first step, the fragments flanking the gene *pf0560* upstream and downstream were amplified using *Pyrococcus furiosus* (DSM3638) as template. In addition, the selection marker PdaD and vector pUC19 were amplified using Phusion PCR. (2.2.1.1.1)

Hereby, pMUR435 was used as a template to amplify the selection marker. To amplify the vector, pMUR6 was used as template. The respective PCR-products were further purified and the concentration was measured via Nanodrop (Table 14).

Table 14: Nano Drop concentration of the purified PCR fragments *pf0559*, *pf0561*, *pf1623* and *pUC19* vector.

Fragment	Concentration [ng/μL]
<i>pf0559</i>	92,1
<i>pf0561</i>	71,2
<i>pf1623</i>	93,9
pUC19	112,4

In order to estimate the intensity of each fragment, the purified fragments were loaded onto a gel with an amount of 50ng (Fig. 18).

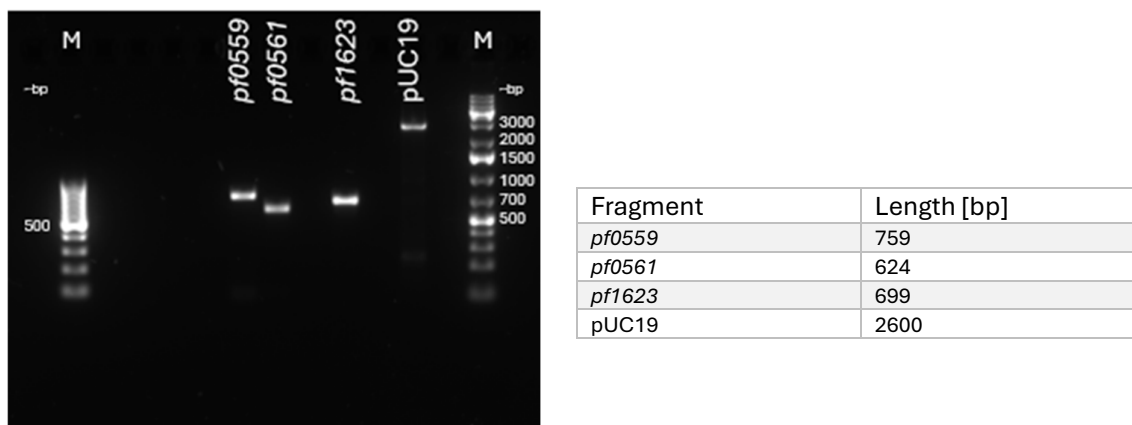


Figure 18: Amplified and purified PCR Fragments. The regions flanking *pf0560* upstream (*pf0559*) and downstream (*pf0561*), *pf1623* and the vector pUC19 were amplified using Phusion PCR. The fragment *pf0559* was amplified with the Primers 1 and 2. The fragment *pf0561* was amplified with the Primers 5 and 6. The selection gene *pf1623* was amplified with Primer 3 and 4, the vector pUC19 with the Primer *AmpI_pUC_F* and *AmpI_pUC_R*. The respective size of the fragments are listed in the right table. Each Fragment was loaded onto gel with 50 ng.

Regarding the intensity of the fragment on the gel (Fig. 18), *pf0561* had $\frac{1}{4}$ less intensity compared to the other fragments, and pUC19 showed half of the intensity on the gel. This was considered in the calculation for equimolar amounts of DNA for the Gibson Assembly.

Further, as shown in Figure 18, all fragments could be amplified and showed the expected size.

The pUC19 template was treated with the restriction enzyme *DpnI*, which hydrolyzes methylated DNA. Thereby, only DNA, which is new synthesized and not methylated, should be present after cleavage. The restriction digestion took place overnight.

Amplification and Purification of the fragments flanking the transposon as the control

In the same principle, the fragments flanking the transposon upstream and downstream were amplified. All fragments could be identified at their respective sizes (Figure 19). The fragments were purified and measured via Nanodrop (Table 15).

Table 15: Nano Drop concentration of the purified PCR fragments *pf1776* and *pf1778*.

Fragment	Concentration [ng/μL]
<i>pf1776</i>	76,9
<i>pf1778</i>	118,7

The intensity of the fragments was analyzed loading the probes in an amount of 50 ng onto the gel (Fig.19) based on the measured Nanodrop concentration as a basis.

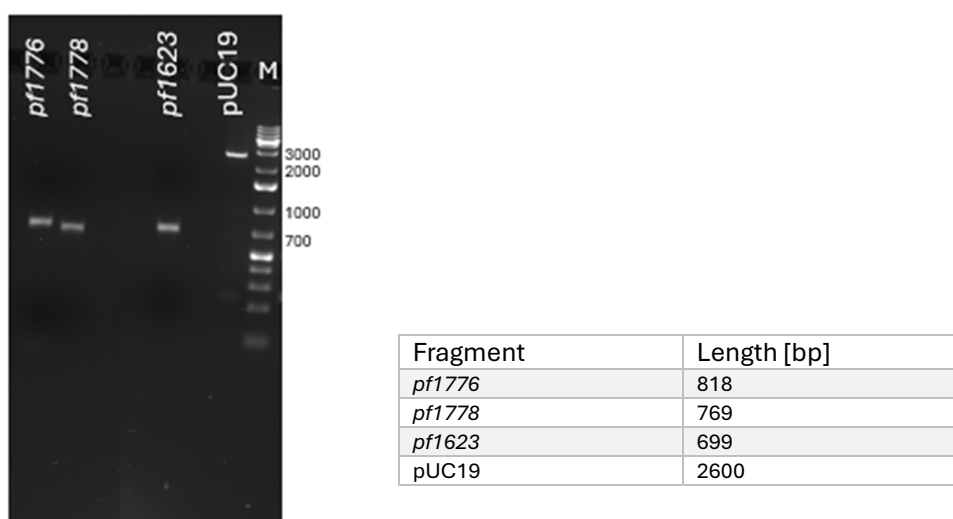


Figure 19 Amplified and purified PCR Fragments. The regions flanking *pf1776* and *pf1778*, the selection gene (*pf1623*) and the vector pUC19 amplified using Phusion PCR. The fragment *pf1776* amplified with the Primers 7 and 8. The fragment *pf1778* amplified with the Primers 9 and 10. The respective size of the fragments are listed in the right table. Each Fragment was loaded onto gel with 50 ng.

As shown in Figure 19, the fragments could be identified at their respective size.

Regarding the intensity of the fragment on the gel, *pf1776* and *pf1778* had 1/3 less intensity compared to the other fragments (Fig.19), which was considered in the further Gibson Assembly Reaction.

3.1.3. Gibson Assembly of pUC19-pf0559-pf1623-pf0561

In the further step, the resulting fragments were connected to each other via Gibson Assembly. For calculating the Volume needed (Table 16) to have equimolar amounts of all fragments (with an 3 fold excess of the upstream and downstream fragments and of the selection gene), the equation (1), (2) and (3) were used.

Table 16: Amount of DNA used for equimolar amounts of the fragments to delete *pf0560*.

Fragment	Equimolar amount [ng]	Amount of DNA with molar excess [ng]	Volume for equimolar amount [μ L]
pf0559	14,6 ng	43,8 ng (~3x)	0,49
pf0561	12 ng	40 ng * (~3x)	0,56
pf1623	13,4 ng	40,2 ng (~3x)	0,44
pUC19	50 ng	50 ng	1**

*Regarding the intensity of the fragment on the gel, *pf0561* had $\frac{1}{4}$ less intensity compared to the other fragments (Fig. 18), which was summed up in this calculation

** Regarding the intensity of the fragment on the gel, pUC19 showed half of intensity on the gel compared to the other fragments (Fig.18)

The Volume used for the Gibson Assembly are listed in Table 17.

Table 17: Gibson Assembly Reaction Mix.

Volume	Purified PCR-Fragment	Excess
1 μ L	pUC19 Vector	1x
1,32 μ L (1:3 diluted)	Pyruvoyl Carboxylase	3x
1 μ L (1:2 diluted)	Upstream flanking <i>pf0560</i>	3x
1,12 μ L(1:2 diluted)	Downstream flanking <i>pf0560</i>	3x
4 μ L	Gibson-Assembly Mix (2,5x)	
1,56 μ L	H ₂ O	
10 μL total		

Gibson Assembly of the control pUC19-pf1776-pf1623-pf1778

Likewise, the fragments to generate the construct for the control were connected via Gibson Assembly using the same strategy. The amount in order to have equimolar amounts of DNA in ng and μ L are listed in the Table 18 below.

Table 18: Amount of DNA used for equimolar amounts of the fragments to delete *pf1777*.

Fragment	Equimolar amount [ng]	Amount of DNA with molar excess [ng]	Volume for equimolar amount [μ L]
pf1776	15,8 ng	33 ng*** (~3x)	0,43
pf1778	14,8 ng	30 ng *** (~3x)	0,25

*** Regarding the intensity of the fragment on the gel, *pf1776* and *pf1778* had $\frac{1}{3}$ less intensity compared to the other fragments (Figure 19), which was summed up in this calculation.

The Volume used for the Gibson Assembly are listed in Table 19.

Table 19: Gibson Assembly Reaction Mix.

Volume	Purified PCR-Fragment	Excess
1 μ L	pUC19 Vector	1x
1 μ L (1:4 diluted)	Pyruvoyl Carboxylase	3x
1,7 μ L (1:4 diluted)	Upstream flanking <i>pf1776</i>	3x
1 μ L (1:4 diluted)	Downstream flanking <i>pf1778</i>	3x
4 μ L	Gibson-Assembly Mix (2,5x)	
1,3 μ L	H ₂ O	
10 μ L total		

The approaches were further transformed into *E.coli* DH5 α as described in 2.2.2.2 and incubated for 20h at 37 °C.

3.1.4. Screening of the genomic assembly in *E.coli*

9 transformants were inoculated into solid LB_{Amp} Media and incubated for 17h, 37°C. Further, the Plasmids of all 9 clones were isolated and further PCR Screening was done for picking a clone which had the correct gene construction assembled (Fig. 20).

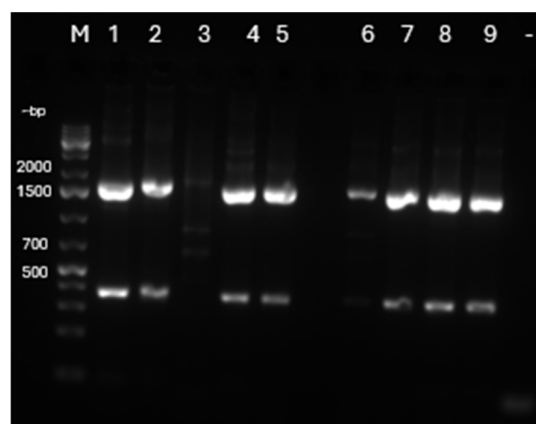


Figure 20: Respective clones from the Gibson Assembly. 9 clones amplified with Primer 11 and 12. The respective size of the fragment is expected to be 1682 bp. As Marker (left) Gene Ruler™ 1 kb Plus DNA Ladder was utilized.

Clone 2 was sent for sequencing, in order to validate the insertion of the genetic construct.

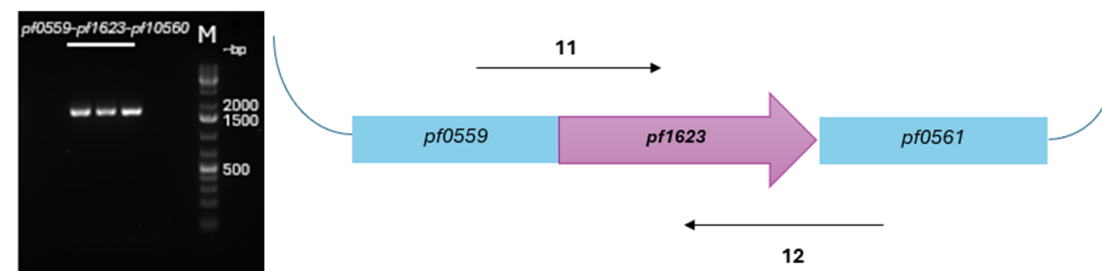


Figure 21: Transformant harboring the right genomic sequence. Transformant amplified in three approaches with an Annealing Temperature at 74 °C using Primer 11 and 12. On the righten side the resulted construct after amplification is shown.

The clone 2 harbored the right sequence and all PCR approaches (Fig. 21) were purified and used in the further experiment.

Screening of the genomic assembly of the control pUC19-*pf1776-pf1623-pf1778*

Likewise, 3 approaches of a Gibson Assembly clone of the control construct, where regions flanking *pf1777* upstream and downstream were connected to *pf1623*, were prepared and screened for the correct gene assembly via PCR (Fig. 22) and via Sequencing.

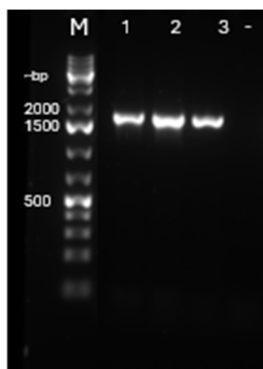


Figure 22: Clone from the Gibson Assembly. 3 approaches amplified with Primer 13 and 14 in varying Annealing Temperatures. Lane 1 was amplified at 65,2 °C, Lane 2 at 66,5 °C and Lane 3 at 65,2 °C. The respective size of the fragment is expected to be 1742 bp. As Marker (left) Gene Ruler™ 1 kb Plus DNA Ladder was used.

The clone had the correct construction assembled and was amplified with the corresponding Primers. Further, the amplified PCR Products were purified using the Gel and PCR Purification Kit from Macherey-Nagel.

3.1.5. Transformation of the genetic constructs into MURPf26

Further, the genetic constructs were transformed into the recipient strain MURPf26 (*Pyrococcus furiosus* ΔPdaD) as described in 2.2.3.2. The transformation approaches were plated undiluted onto Gelrite Media Plates (2.2.3.1) and incubated for 66 hours at 85°C in an anaerobe jar flushed with 1 bar N₂. Simultaneously, the control was transformed into MURPf26 on the same principle.

The amount of DNA used for the transformation into *Pyrococcus furiosus* is shown in Table 20 below. In order to transform 0,5 pmol DNA the equation (4) was utilized calculating back the needed amount of the DNA insert.

Table 20: Amount of linear DNA of the construct for 0,5 pmol DNA-insert.

Genetic construct	Length of the fragment	Amount of linear DNA
<i>pf0559-pf1623-pf0561</i>	1,682 kb	555 ng
<i>pf1776-pf1623-pf1778</i>	1,742 kb	575 ng

3.1.6. Incubation of transformants and Isolation of genomic DNA

As a result, 5 Δ*pf0560* knock out candidates could be observed on Gelrite Plates in comparison to 7 knock out candidates of the control (Table 21). As the amount of transformants was similarly high, the transformation efficiency appeared to be similarly in the experiments.

Table 21: Total number of transformants.

Mutant strain	Total number of transformants
<i>Pyrococcus furiosus</i> Δ <i>pf0560</i>	5
<i>Pyrococcus furiosus</i> Δ <i>pf1777</i>	7

Further, to investigate, if the gene *pf0560* could be deleted in *Pyrococcus furiosus*, transformants were incubated in ½ SME Media for 25 hours (2.2.3.1.) and gDNA was isolated via Phenol/Chloroform using the Protocol described in 2.2.3.3. Likewise, transformants of the control were incubated and gDNA was isolated.

3.1.7. PCR Screening for the gene construct in MURPf26

The potential knock out candidates were tested on the presence of the genetic construct and the absence of PF0560 via amplifying the specific region via PCR.

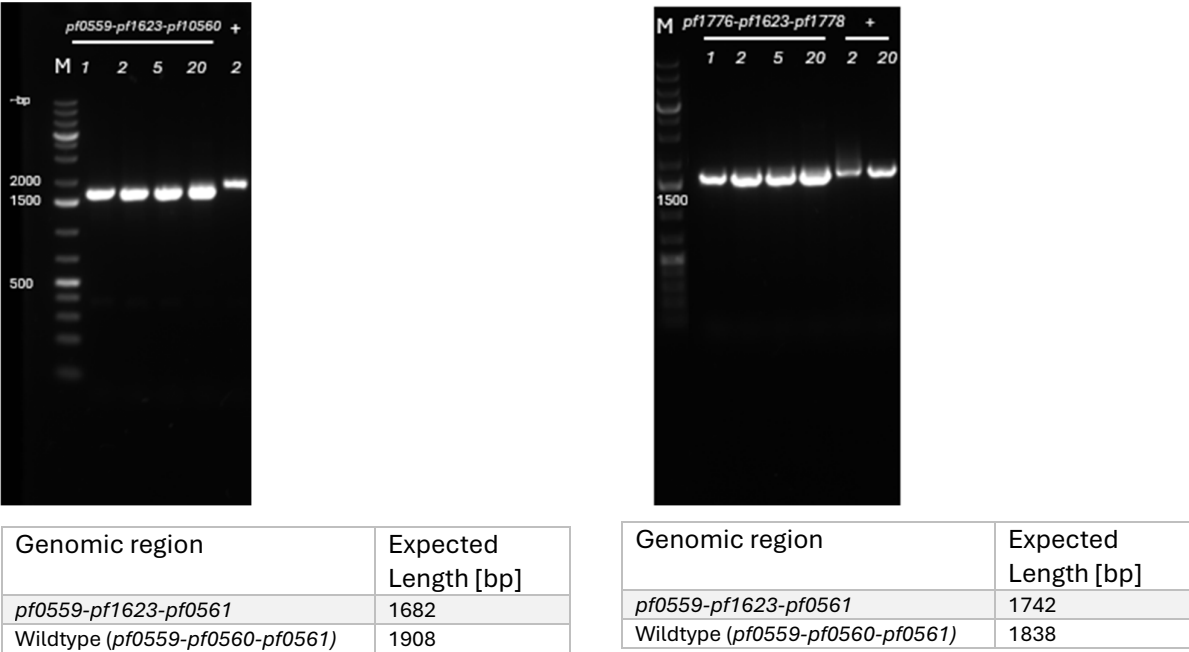


Figure 23: Transformants amplified with the respective primers. MURPf26 (Δ pf0560) clone amplified in varying concentrations. (1ng, 2ng, 5 ng, 20ng). (left) MURPf26 (Δ pf1777) clone amplified in varying concentrations (1ng, 2ng, 5 ng and 20 ng). (right) As a positive control, the Wildtype of *Pyrococcus furiosus* serves. The expected sizes of the fragments in comparison to the size of the genomic region in the wildtype are listed in the Table.

As shown in Figure 23, the genomic region, which was incorporated into MURPf26, could successfully be amplified via PCR. As a result, the expected length of 1682 base pairs in the Δ pf0560 knockout was obtained (Figure 23, left). In comparison, the PCR Product of amplifying the Wildtype revealed a band migrating slightly higher compared to the knock out variant. Further, the band migrates nearly below 2000 base pairs, corresponding to the expected size of 1908 base pairs of the Wildtype probe.

Further, in the Δ pf1777 knock out the expected sizes were obtained after amplification of the expected genomic assembly, appearing slightly smaller than the amplified wildtype fragment (Figure 23, right).

3.1.8. PF0560 Screening

To further investigate, if the gene *pf0560* was missing in the transformant, a PF0560 Screening was done by PCR using the primers targeting *pf0560*.

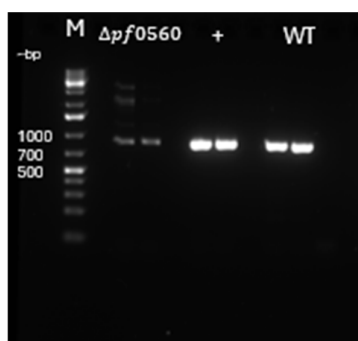


Figure 24: *pf0560* Screening. The knock out candidate tested on the presence of *pf0560* using Primer 15 and 16. As a positive control the produced MURPf26 ($\Delta pf1777$) knock out serves. WT refers to the Wildtype of *Pyrococcus furiosus*.

As shown in Figure 24, the clones still showed signals when Screening for PF0560, which indicates that the gene *pf0560* was still present in the clone. To investigate, if there were clones with a genotype not harboring PF0560, the culture was plated to isolate single colonies. Using this strategy, the culture was isolated into single cells and should lead to the removal of multiple heterogenous genome copies. Thereby, 1 ml of the culture was plated onto Gelrite Media plates and incubated in an anaerobe jar flushed with 1 bar N_2 at 85°C, 68h.

Clones on the dilution plate were inoculated into SME Media as described in 2.2.3.1 genomic DNA was isolated

PF560 Screening after single cell resolution

Further, the PF0560 Screening by PCR using the primers targeting the genomic region of PF0560 was repeated.

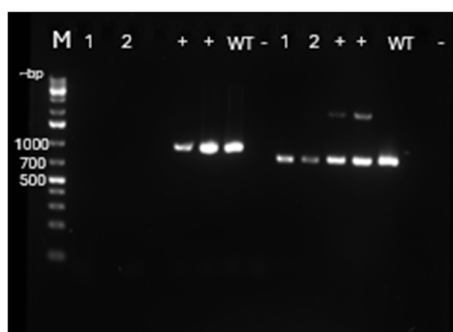


Figure 25: *pf0560* Screening after dilution plating. Lane 1 and 2 refer to the knock out candidate. The positive control refers to the produced knock out for the control. On the left side of the gel, the *pf0560* Screening using Primer 15 and 16 is shown. On the right side, the amplification of the selection gene (using Primer 3 and 4) as a quality control is shown. The size of the selection gene used to be 699 bp.

As a result, the clones after dilution plating could not reveal a signal in the PCR. Further, this implicated that *pf0560* could be deleted in *Pyrococcus furiosus*.

3.1.9. Growth curve

To investigate the growth of the *Δpf0560 mutant* strain compared to the control mutant, the strains were incubated 95 hours in an OD- measurement chamber. Both strains harbored the same cell count at the beginning of incubation.

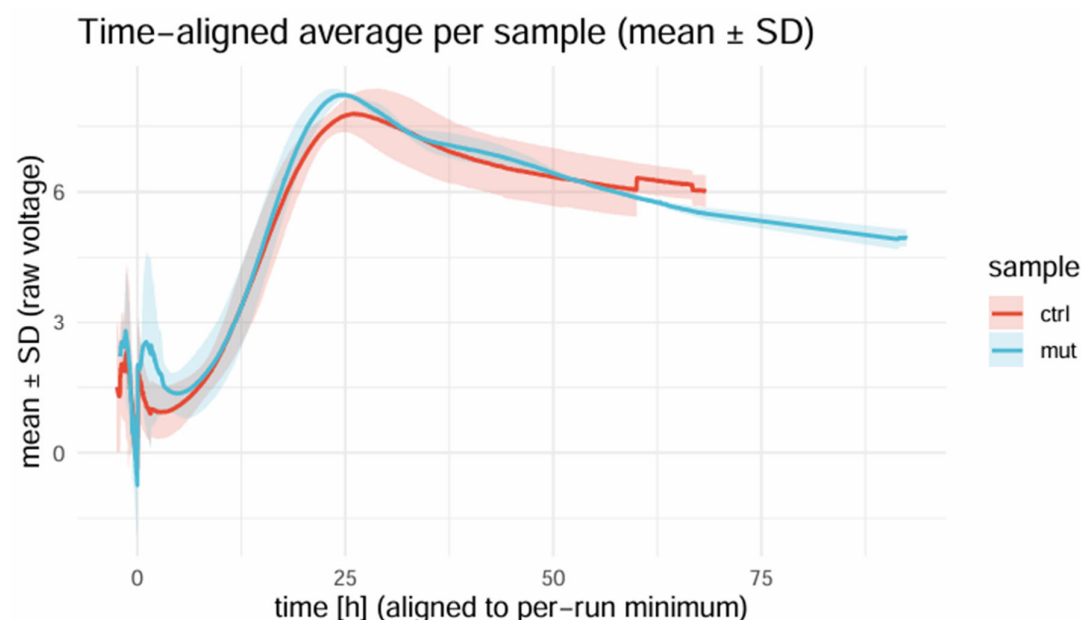


Figure 26: Growth curve of the *Δpf0560 mutant* cells in comparison to the control mutant. The blue line depicts the mutant strain. The red line depicts the control.

Four cultures were incubated 95 hours and the growth was monitored. The growth curve in Figure 26 depicts the average of all four growth curve analysis.

Simultaneously, the cell counts of all cultures incubated after 95 hours were calculated using a Thoma counting chamber under the microscope and the vitality of the cells was assessed in a 40x magnification. The average of the total cell counts and the assessed amount of viable cells in the culture are listed in Table 22 below.

Table 22: Average of the total cell numbers calculated under the microscope with the corresponding amount of vital cells.

Mutant strain	Cell count	Number of vital cells
<i>Pyrococcus furiosus Δpf0560</i>	$3,0 \times 10^8$	11
<i>Pyrococcus furiosus Δpf1777 (control)</i>	$2,7 \times 10^8$	20

With regard to the microscopic visualization (Table 22), the average of the calculated cell counts across all incubated cultures appeared to be similarly high. However, the *Δpf0560* mutant cells exhibited a slightly higher total cell count compared to the control (Table 22). In contrast, the proportion of viable cells was reduced. Consistently, the *Δpf0560* strains showed fewer viable cells overall, indicating a higher proportion of dead cells within the total count compared to the control strain.

Regarding the growth curve (Fig. 26), a lower growth could be observed in the *Δpf0560* strains after 95h in the beginning of the stationary phase. Moreover, after approximately 50 hours, the mutant

strains tend to reach a lower growth level, compared to the control. In contrast, the $\Delta pf0560$ strains appear to reach a higher plateau phase in the exponential phase, since after 25 hours the growth increase of the mutant cells appears higher compared to the control. Moreover, $\Delta pf0560$ cells enter the exponential growth phase more rapidly compared to the control, indicating a higher growth rate. But, however, after approximately 65 hours of incubation, the growth curve of the control cells exhibits a distinct break, where the curve no longer continues. This finding has to be considered in the future experimental setups.

3.1.10. Live dead staining of the mutant strain vs. control

To investigate, how many cells of the mutant actually were alive after growing the culture into the stationary phase in comparison to the control, the cells were analyzed under the microscope with a live-dead staining.

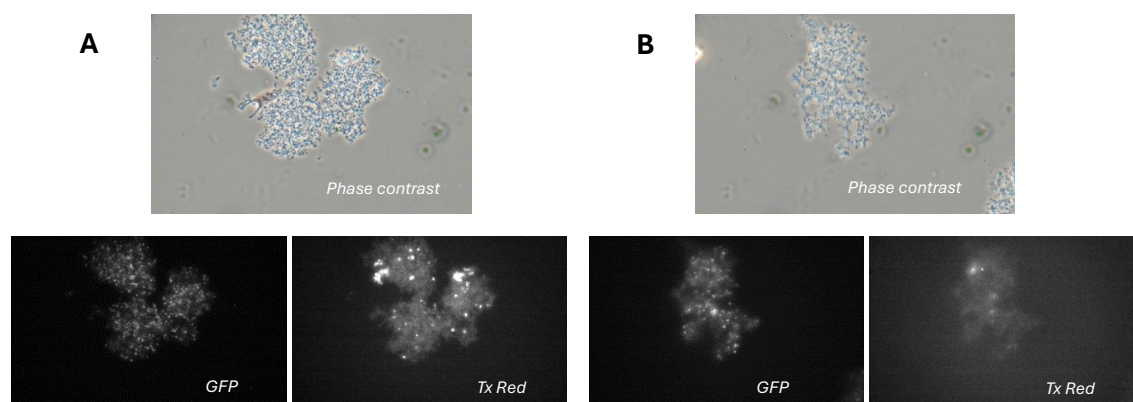


Figure 27: Live dead staining of the $\Delta pf0560$ mutant cells and the control mutant after incubation of 95 hours. A. The $\Delta pf0560$ mutant cells with a GFP Staining (left) and TxRed Staining (right) compared to the total cell amount in the phase contrast. B. Mutant control cells with a GFP Staining (left) and TxRed Staining (right) compared to the total cell amount in the phase contrast.

Analyzing the staining, the GFP filter visualizes the green staining, that gives an insight into the amount of living cells. Every dot in the picture hereby represents a cell. The amount of living cells refers to the amount of the total cells present in the spot. (Fig.27A) Contrarily, the Tx Red staining visualizes the cells, which are referred to death.

As a result, the tendency shows a higher abundance of dead cells in relation to the total amount of cells in the mutant strain. (Fig. 27A) Moreover, more cells appear to be dead than alive, which indicates that $pf0560$ may observe a negative growth influence on *Pyrococcus furiosus* during stationary phase. In contrast, the control appears to consist of a low proportion of cells in total. (Fig. 27B) But, the proportion of dead cells tends to be lower than that of the active cells relative to the total cell population, suggesting that cells still harboring $pf0560$, may experience a higher viability of the cells during the stationary phase.

3.2. Deletion of the ribosomal protein L41e

3.2.1. First experimental set up

3.2.1.1. Assembly of the genetic construct

In this approach, the goal was to create a deletion mutant, where the ribosomal protein L41e is knocked out. Thereby, the fragments flanking the gene *pf1018* upstream and flanking its adjacent gene *pf1019* downstream, namely *pf020*, were amplified, since *pf1019* is being co-transcribed with *pf1018* in the genome. The fragments were likewise connected via Gibson Assembly to the selection marker and to the vector. (Fig. 28) In this way, the gene *pf1020*, that is flanking *pf1019* downstream, was amplified and assembled for providing homologous regions in *Pyrococcus furiosus*.

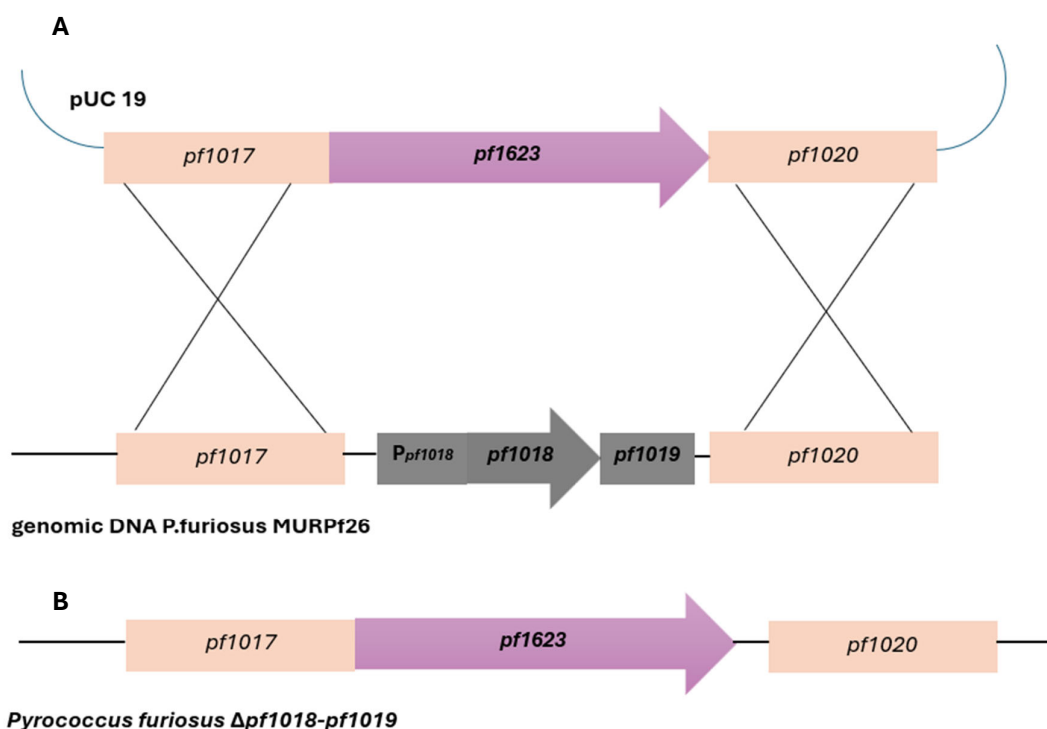


Figure 28: Genetic construct for deleting *pf1018*. A. *pf1017* and *pf1020* are connected to *pf1623*, the PdaD. Via introducing the construct into MURPf26, homologous regions upstream and downstream of *pf1018* lead to a double cross over at those positions. B. As a result, *pf1018* and *pf1019* are deleted in MURPf26.

Further, via transformation of the construct into the recipient strain MURPf26, homologous regions were provided (Fig. 28), whereby the upstream and downstream fragments flanking *pf1018* lead due to a double cross over to the exchange of *pf1018* with *pf1623*, encoding for the PdaD. In this way, the new mutant strain was performed, in which *pf1018* was deleted in *Pyrococcus furiosus*. (Fig. 28)

3.2.1.2. Amplification and Purification of the fragments flanking *pf1018*

As described in the first experiment, the fragment flanking the gene encoding for the ribosomal protein L41e upstream was amplified and downstream were amplified using Phusion PCR. (2.2.1.1.1) The respective PCR-products were further purified and the concentration was measured via Nanodrop (Table 23).

Table 23: Nano Drop concentration of the purified PCR fragments *pf1017* and *pf1020*.

Fragment	Concentration [ng/μL]
<i>pf1017</i>	62,8
<i>pf1020</i>	57,0

In order to estimate the intensity of each fragment, the purified fragments were loaded onto a gel with an amount of 50ng, likewise as in the first experiment.

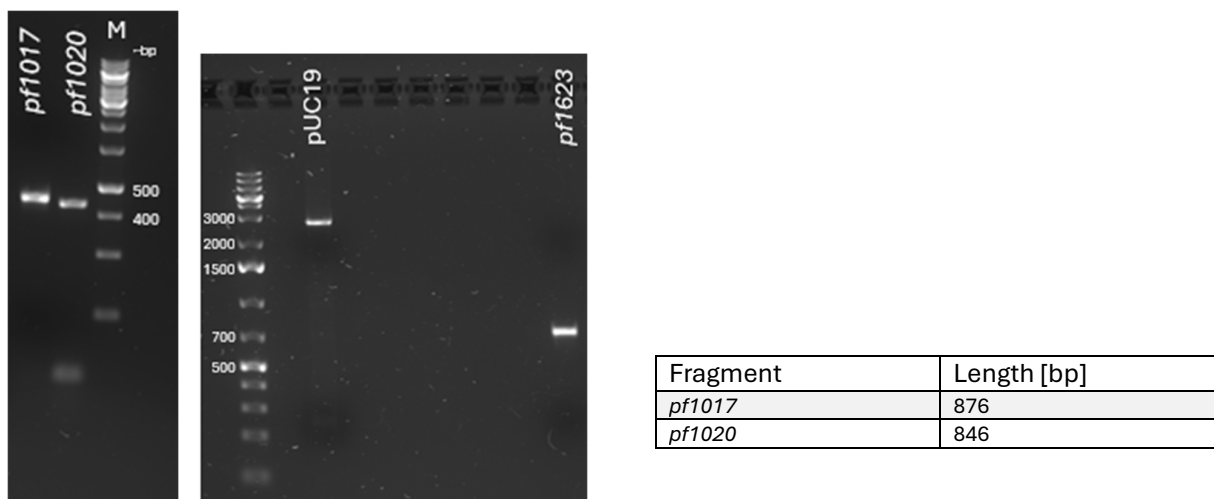


Figure 29: Amplified PCR Fragments. The regions flanking *pf1018* upstream (*pf1017*) and downstream (*pf1020*) amplified using Phusion PCR using Primer 17 and 18 for the upstream fragment and Primer 19 and 20 for the downstream part. (left) The fragments pUC19 and *pf1623* loaded on the gel with an amount of 50ng. (right) The respective size of the fragments are listed in the right table.

As shown in Figure 29, the fragments could be amplified at their respective size.

Regarding the intensity of the fragment on the gel (Fig.29), pUC19 had 3x less intensity compared to the other fragments. This was considered in the calculation for equimolar amounts of DNA for the Gibson Assembly.

3.2.1.3. Gibson Assembly of pUC19-pf1017-pf1623-pf1020

In the next step, the resulting fragments were connected to each other via Gibson Assembly.

For calculating the Volume needed (Table 24) to have equimolar amounts of all fragments (with an 3 fold excess of the upstream and downstream fragments and of the selection marker), the equation (1), (2) and (3) were used.

Table 24: Amount of DNA used for equimolar amounts of the fragments to delete pf1018.

Fragment	Equimolar amount [ng]	Amount of DNA with molar excess [ng]	Volume for equimolar amount [μ L]
pf1017	16,9 ng	35 ng (~3x)	0,56
pf1020	16,3 ng	35 ng (~3x)	0,61

The Volume used for the Gibson Assembly are listed in Table 25.

Table 25: Gibson Assembly Reaction Mix.

Volume	Purified PCR-Fragment	Excess
1 μ L	pUC19 Vector	1x
1 μ L (1:3 diluted)	Pyrovoyl Carboxylase	3x
1,7 μ L (1:3 diluted)	Upstream flanking pf1017	3x
1,8 μ L (1:3 diluted)	Downstream flanking pf1020	3x
4 μ L	Gibson-Assembly Mix (2,5x)	
0,5 μ L	H ₂ O	
10 μL total		

3.2.1.4. Screening of the genomic assembly in E.coli

7 transformants of the Gibson Assembly reaction were inoculated into solid LB_{Amp} Media and incubated for 24 h, 37°C. Further, the Plasmids of all 7 clones were isolated and further PCR Screening was done for picking a clone which had the correct gene construction assembled (Fig. 30).

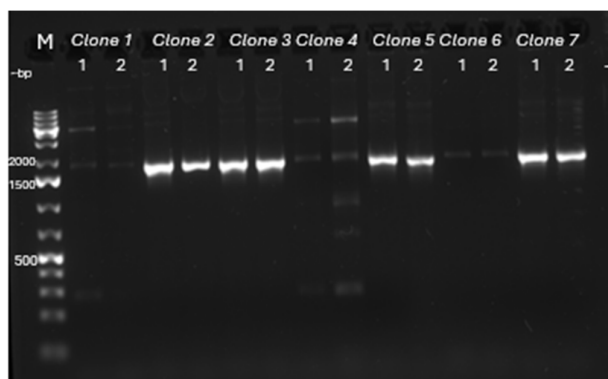


Figure 30: : Respective clones from the Gibson Assembly. 7 clones amplified with Primer 21 and 22. Number 1 refers to an Annealing Temperature of 67,4 °C and Number 2 to an Annealing Temperature of 65,2 °C. The respective size of the fragment is expected to be 1735 bp. As Marker (left) Gene Ruler™ 1 kb Plus DNA Ladder was utilized.

Clone 2 of the Gibson Assembly could reveal the correct insertion of the gene construct (Fig. 31) and was further used in the next experiments.

Further, Clone 2 was amplified with Primer 21 and 22 in 3 approaches, purified and used for the further experiment (Fig. 31).

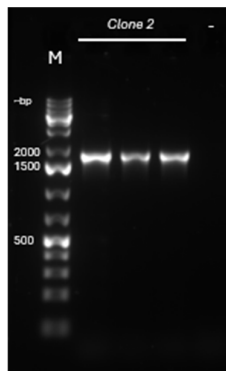


Figure 31: Transformant harboring the right genome sequence. Lane 1-3 were amplified at an Annealing Temperature at 64,7 °C.

3.2.1.5. Transformation of the genetic construct into MURPf26

The genetic construct was transformed into the recipient strain MURPf26 (*Pyrococcus furiosus* ΔPdaD) as described in 2.2.3.2.1. The transformation approach was plated undiluted onto Gelrite Media Plates and incubated for 66h at 85°C in an anaerobe jar flushed with 1 bar N₂.

The amount of DNA used for the transformation into *Pyrococcus furiosus* is shown in Table 26 below using the equation (4).

Table 26: Amount of linear DNA of the construct for 0,5 pmol DNA-insert.

Genetic construct	Length of the fragment	Amount of linear DNA
<i>pf1017-pf1623-pf1020</i>	1,735 kb	573 ng

3.2.1.6. Incubation of transformants and Isolation of genomic DNA

As a result, the number of transformants on the plate, depicted in Table 27, was similarly high, indicating a similar transformation efficiency.

Table 27: Total number of transformants.

Mutant strain	Total number of transformants
<i>Pyrococcus furiosus</i> Δ <i>pf1018-pf1019</i>	20
<i>Pyrococcus furiosus</i> Δ <i>pf1777</i>	25

Further, to investigate, if the gene *pf1018* could be deleted in *Pyrococcus furiosus*, transformants were incubated in ½ SME Media for 25h as described in 2.2.3.1 and gDNA was isolated via Phenol/Chloroform.

3.2.1.7. PCR Screening for the gene construct in MURPf26

The potential knock out candidates were tested on the presence of the genetic construct via amplifying the region of the genetic construct. (Fig. 32) The approach was further sent for sequencing to analyze the exact sequence.

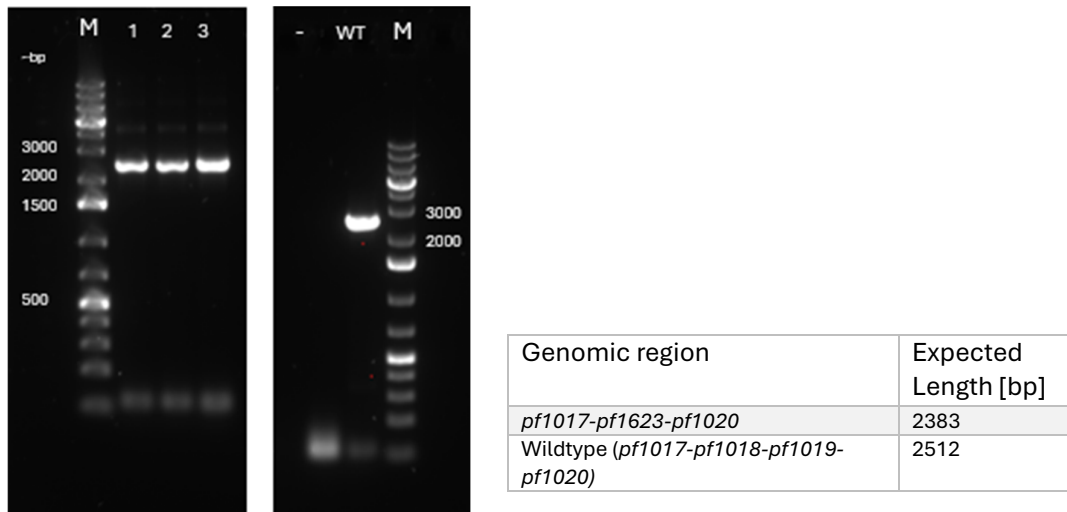
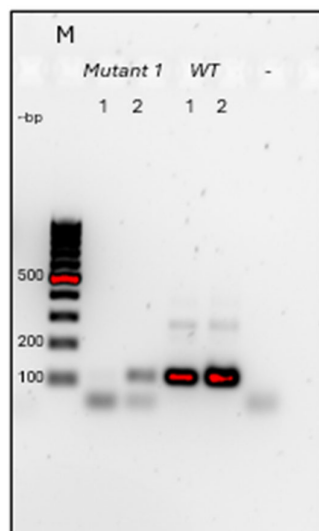


Figure 32: Transformants amplified with the respective primers. MURPf26 ($\Delta pf1018$) clone amplified with Primer 17 and 20 in varying Annealing Temperatures. Lane 1 was amplified with an Annealing Temperature at 69,1 °C, Lane 2 with 70,1 °C and Lane 3 with 71,9 °C. The expected size of the fragment in comparison to the size of the genomic region in the wildtype are listed in the Table.

As a result, in the transformants amplified in varying Annealing Temperatures (Fig. 32), a band corresponding to the expected size of 2383 was obtained (Figure 32, left). Correspondingly, the PCR Product of the wildtype obtained a band at the expected size of 2512 base pairs (Figure 32, right).

3.2.1.8. L41e Screening

To further investigate the absence of the ribosomal protein L41e in the new assembled clone and, a L41e Screening was done via PCR, using Primers targeting the ribosomal protein L41e.



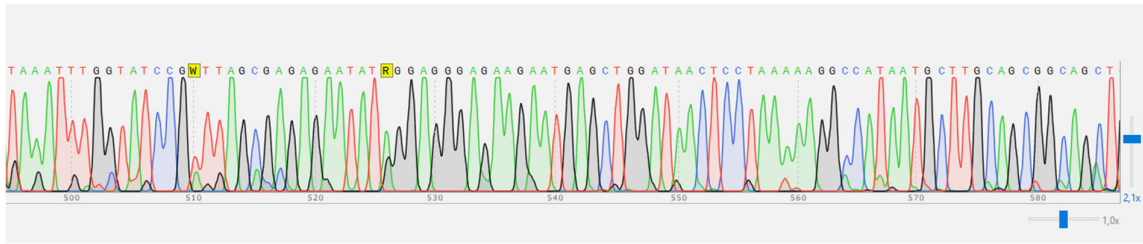


Figure 33: *pf1018* Screening. The knock out candidate tested on the presence of *pf1018* using Primer 26 and 27. WT refers to the Wildtype of *Pyrococcus furiosus*. Lanes with number 1 were amplified with an Annealing Temperature at 61,1 °C. Lanes with number 2 were amplified with an Annealing Temperature at 63,2 °C. Below, the chromatogram of the Sanger sequencing is shown. The clone was prepared with Primer 21 and sent for sequencing.

Regarding Figure 33, traces of L41e still were present in the sample. In particular, there may still have been copies of wildtype sequences present. This is consistent with the corresponding chromatogram, as several peaks are overlapping (Figure 33). To eliminate those remaining wild type genome copies, the culture was plated onto Gelrite Medium, in order to isolate single colonies. Hereby, 100 µL of the culture was plated and incubated 48 hours in an anaerobe jar flushed with 1bar Nitrogen. As a result, clones grew on the plate, whereby one clone was inoculated into fresh Synthetic Seawater and incubated for 25 ½ hours.

L41e Screening after single cell resolution

In the further step, the L41e Screening was repeated by PCR using the primers specifically targeting the genomic region of L41.

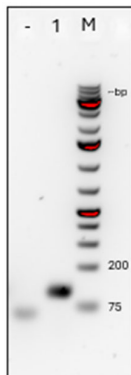


Figure 34: *pf1018* Screening after dilution plating. Lane number 1 refers to the knock out candidate. Annealing Temperature is 63 °C.

According to the PCR Result, after the dilution plating, a band corresponding to the size of the L41e protein could be identified in the probe. Thereby, the clone still tended to harbor sequences of the wild type.

3.2.2. Second experimental set up

In order to create a deletion mutant, where only L41e is knocked out, the fragment flanking downstream of L41e was amplified and used in the genetic construct to knock out L41e via homologous recombination.

3.2.2.1. Assembly of the genetic construct

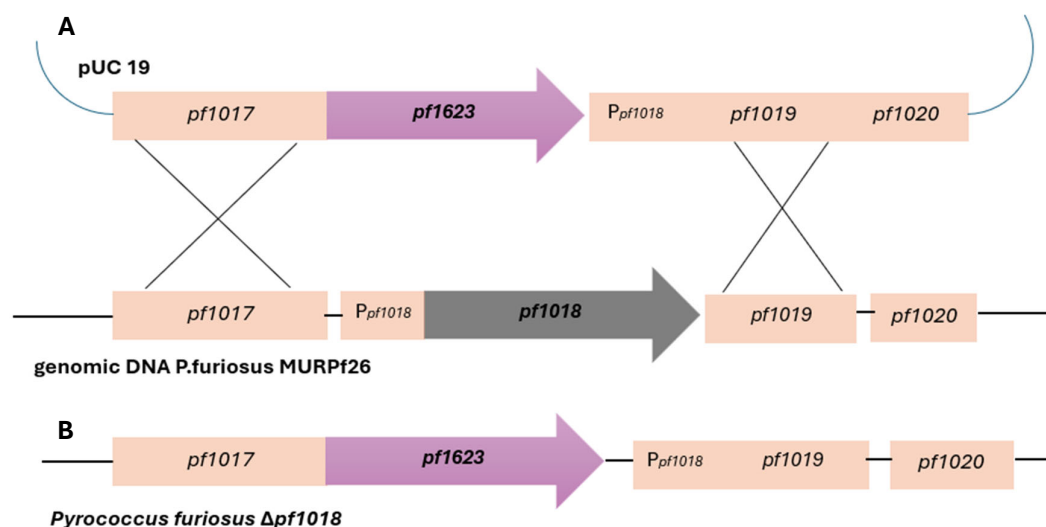


Figure 35: A. Genetic construct for deleting *pf1018*. *pf1017* and *pf1019-1020* are connected to *pf1623*, the PdaD. Via introducing the construct into MURPf26, homologous regions upstream and downstream of *pf1018* lead to a double cross over at those positions. B. As a result, *pf1018* gets deleted in MURPf26.

By introducing this construct into the recipient strain MURPf26, homologous regions were provided harboring fragments flanking upstream of *pf1018* and the region flanking downstream of *pf1018* (Fig. 35A). Thereby, via a double cross over only *pf1018* should be exchanged for *pf1623*. In this way, a mutant strain should be created, whereby only *pf1018* could be deleted as the basis.(Fig. 35B)

3.2.2.2. Amplification and Purification of the fragment flanking *pf1018*

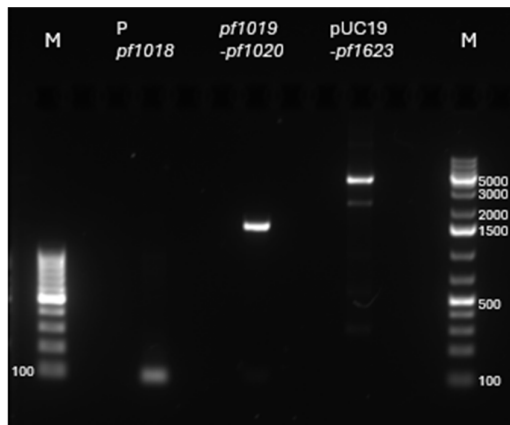
For this experiment, the Promotor L41e, a fragment flanking the Promotor L41e and the gene *pf1019*, which is located downstream of *pf1018* (L41e) were amplified using Phusion PCR via 1.1.1.1. In addition, a fragment was generated via PCR containing the pUC19 vector and the selection gene *pf1623*. Hereby, the isolated plasmid from the first Experiment of deleting L41e (3.2.1.4) was used as template. The fragments were purified with the Gel and PCR Clean Up Kit from Macherey-Nagel and the concentration was measured via Nanodrop (Table 28).

Table 28: Nano Drop concentration of the purified PCR fragments *P_{pf1018}*, *pUC19-pf1623* and *pf1019-pf1020*.

Fragment	Concentration [ng/μL]
<i>P_{pf1018}</i>	59,4
<i>puc19-pf1623</i>	145,1
<i>pf1019-pf1020</i>	218,3

The vector was digested with *DpnI* for 20h at 37 °C. In this way, via providing this system, only the small protein L41e should be deleted.

In order to adjust the intensity of the fragments, the fragments were loaded onto a gel in an amount of 50ng.



Fragment	Length [bp]
P_{pf1018}	85
<i>pf1019-pf1020</i>	1502
pUC19- <i>pf623</i>	4158

Fragment	Length [bp]
P_{pf1018}	85
<i>pf1019-pf1020</i>	1502
pUC19- <i>pf623</i>	4158

Figure 36: Amplified and purified PCR Fragments. The fragment pUC19-*pf1623*, the fragment *pf1019-pf1020*, and the Promotor of L41e (P_{pf1018}) amplified using Phusion PCR. The respective size of the fragments are listed in the right table. The fragments were loaded on the gel in an amount of 50ng.

As shown in Figure 36 all three fragments could be amplified. Regarding the intensity, the Promotor L41e is estimated about 10 times less intense in contrast to the other fragments.

3.2.2.3. Gibson Assembly of pUC19-*pf1017-pf1623-Ppf1018-pf1019-pf1020*

Further, the Gibson Assembly was performed to connect the respective fragments to each other. For calculating the Volume needed (Table 29) to have equimolar amounts of all fragments (with an 3 fold excess of the upstream and downstream fragments and of the selection marker), the equation (1), (2) and (3) were used.

Table 29: Amount of DNA used for equimolar amounts of the fragments to delete *pf1018*.

Fragment	Equimolar amount [ng]	Amount of DNA with molar excess [ng]	Volume for equimolar amount [μ L]
P_{pf1018}	1 ng	40 ng (~4x)***	0,68
pUC19- <i>pf1923</i>	50 ng	50 ng	0,35
<i>pf1019-pf1020</i>	18 ng	36 ng (~2x)	0,16

***the Promotor was in addition adjusted with 10x more concentration regarding its less intensity on the gel

The Volume used for the Gibson Assembly are listed in Table 30.

Table 30: Gibson Assembly Reaction Mix.

Volume	Purified PCR-Fragment	Excess
1,1 μ L (1:3 diluted)	pUC19 Vector- Pyrovoyl Carboxylase	1x
1,4 μ L (1:2 diluted)	Promotor L41e	4x
1,1 μ L (1:7 diluted)	Downstream flanking <i>pf1019-pf1020</i>	2x
4 μ L	Gibson-Assembly Mix (2,5x)	
2,4 μ L	H ₂ O	
10 μL total		

3.2.2.4. Screening of the genomic assembly in *E.coli*

6 transformants of the Gibson Assembly reaction were inoculated into LB_{Amp} Solid Media and incubated for 20 h, 37°C. Further, the Plasmids of all 7 clones were isolated and further PCR Screening was done for picking a clone which had the correct gene construction assembled (Fig. 37).

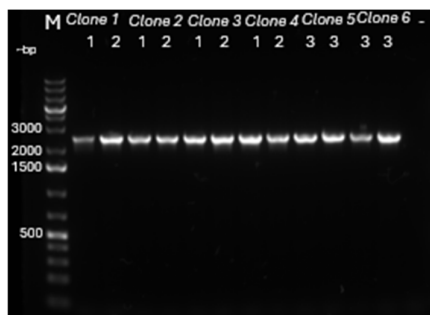


Figure 37: Respective clones from the Gibson Assembly. 6 clones amplified with Primer 21 and 22. The respective size of the fragment is expected to be 2450 bp. As Marker (left) Gene Ruler™ 1 kb Plus DNA Ladder is utilized.

Clone 1 of the Gibson Assembly was screened for its sequence and could reveal the correct insertion of the gene construct (Fig. 37). The clone 1 was amplified with Primer 21 and 22 in two approaches shown in Figure 38 below.

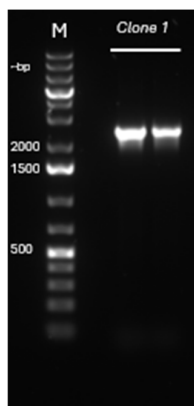


Figure 38: Transformant with the expected genetic construct. 2 approaches of the transformant amplified with Primer 21 and 22.

The respective PCR Products (Fig. 38) were purified and utilized in the further experiments.

3.2.2.5. Transformation of the genetic construct into MURPf26

Further, the genetic construct was transformed into the recipient strain MURPf26 (*Pyrococcus furiosus* ΔPdaD) The transformation approach was plated undiluted onto Gelrite Media Plates and incubated for 68 hours at 85°C in an anaerobe jar flushed with 1 bar N₂.

The amount of DNA used for the transformation into *Pyrococcus furiosus* is shown in Table 31 below using the equation (4).

Table 31: Amount of linear DNA of the construct for 0,5 pmol DNA-insert.

Genetic construct	Length of the fragment	Amount of linear DNA
pUC19-pf1623-pf1017-P _{pf1018} -pf1019-pf1020	2,453 kb	573 ng

3.2.2.5. Incubation of the transformants and Isolation of genomic DNA

As a result, more transformants were observed on $\Delta pf1018$ knock out candidates compared to the control knock out (Table 32), indicating that the transformation efficiency was lower in the control mutant.

Table 32: Total number of transformants

Mutant strain	Total number of transformants
<i>Pyrococcus furiosus</i> $\Delta pf1018$	19
<i>Pyrococcus furiosus</i> $\Delta pf1777$	7

Further, to investigate, if the gene *pf1018* could be deleted in *Pyrococcus furiosus*, transformants were incubated in ½ SME Media for 25h and gDNA was isolated via Phenol/Chloroform.

3.2.2.6. PCR Screening for the gene construct in MURPf26

The potential knock out candidates were tested on the presence of the genetic construct via amplifying the region of the genetic construct (Fig. 39). The approach was further sent for sequencing to analyze the exact sequence.



Figure 39: Knock out candidate amplified with Primer 17 and 20. The clone amplified in different concentrations. The expected size of the fragment in comparison to the size of the genomic region in the wildtype are listed in the Table. Below, the chromatogram of the Sanger sequencing is shown. The clone was prepared with Primer 3 and sent for sequencing.

As depicted in Figure 39, the knock out candidate could reveal after PCR amplification a fragment corresponding to the expected size of 3098 base pairs. But, the analysis revealed a double band, whereby an additional fragment could be amplified, which migrates at a lower position on the same height as the PCR Product of the wildtype. This indicates that there still may be mixed genome copies present, whereby wildtype genome copies still were obtained. A sequencing analysis confirmed the finding, that L41e still was present in the genome in a high amount, which approved the presence of multiple heterogenous copies. (Fig.39) To further, investigate, if the

mixed genome could be split into a homogenous genome, the isolation of single colonies via plating was performed as utilized in Section 3.2.1.8.

In the next step, from the clones of the plating genomic DNA was isolated and a PCR was repeated with Primers targeting the upstream and downstream regions of L41e.

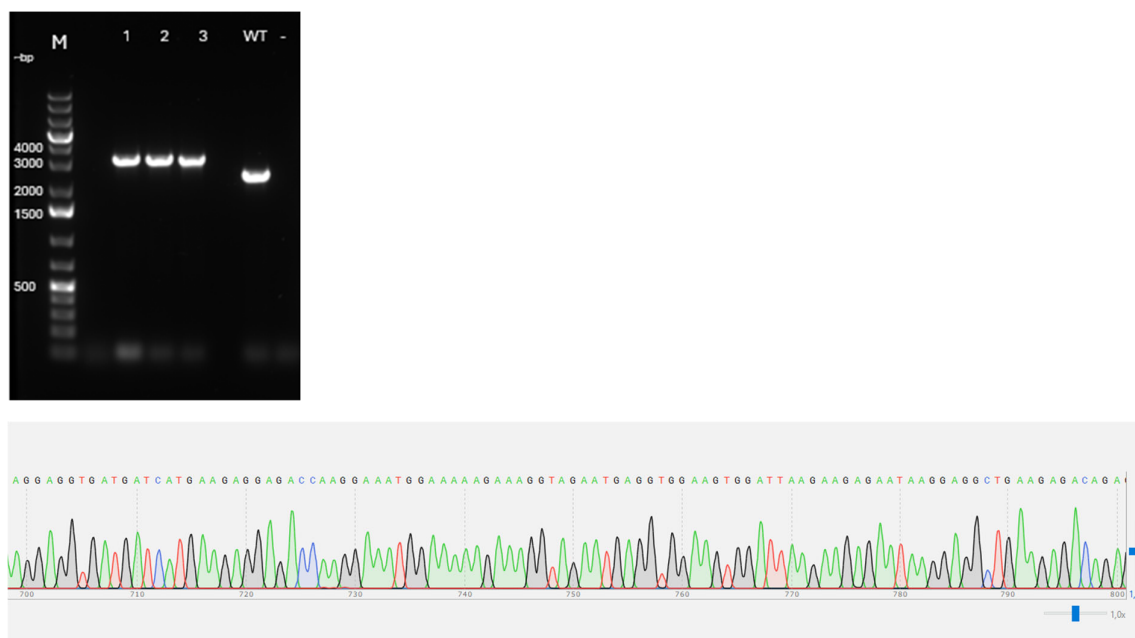


Figure 40: Knock out candidate from the dilution plating amplified with Primer 17 and 20. The clone amplified in different concentrations. Lane 1 with a concentration of 0,7 ng, Lane 2 with 1,4ng and Lane 3 with a concentration of 2,1ng. The respective size of the fragment is 3098 bp. WT refers to the Wildtype. Below, the chromatogram of the Sanger sequencing is shown. The clone was prepared with Primer 3 and sent for sequencing.

Regarding the PCR Product in Figure 40, after the dilution process only signals revealed to the corresponding size of the expected construct. Further, to ensure, if a homogenous copy was present, the sequencing was repeated. Consistently, the chromatogram of the Sanger sequencing analysis revealed less overlapping peaks. (Fig.40) Still, aligning the sequence to the wild type genome revealed a clear match to the genomic region of L41e., indicating the presence of L41e in the approach.

3.2.2.7. L41e Screening

In addition, a Screening was performed via PCR via using Primers that bind specific to the genomic region of L41e.

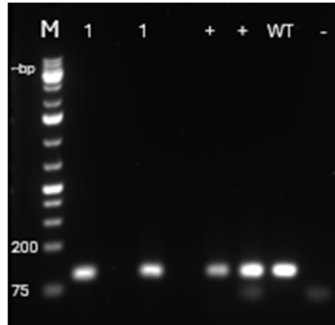


Figure 41: *pf1018* Screening after dilution plating. The mutant tested on the presence of *pf1018* using Primer 26 and 27. WT refers to the Wildtype of *Pyrococcus furiosus*. The positive control belongs to the produced knock out used as a control. Lanes labelled with number 1 refer to the mutant. The respective size of *pf1018* is 85 bp.

According to this finding, L41e could be targeted in all samples, confirming the continuous presence of L41e. These findings led to the result, that L41e could not be deleted in the experiment.

4. Discussion

4.1. Deletion of the ribosomal protein PF0560

Successful complementation of the selection gene *pf1623* and deletion of *pf0560* using the genetic system

In this approach, using the genetic system, the selection gene *pf1623*, encoding the Pyruvoyl dependent Arginine Decarboxylase, could be complemented in the auxotroph mutant strain of *Pyrococcus furiosus*. Moreover, the genetic construct, in which *pf1623* was inserted at the position of *pf0560*, could be successfully introduced into *Pyrococcus furiosus*. This allowed the ribosomal dimerization factor PF0560 to be effectively deleted and replaced with the selection marker as designed in the genetic construct. In this way, the resulting mutant strain was able to complement the PdaD deficiency in the auxotroph mutant strain. As a result, this approach enabled the generation of a deletion mutant, in which the gene *pf0560* was knocked out. Specifically, a clone was obtained as expected, containing upstream and downstream regions of *pf0560*, with homologous recombination occurring at those positions via a double cross over (Section 3.1.4.). Furthermore, by providing homologous regions to both the upstream and downstream sequences of *pf0560* in *Pyrococcus furiosus*, after the transformation of those regions into *Pyrococcus furiosus* the recombination could occur at those two positions in the target organism. Moreover, the double cross over event then resulted in the precise excision of the genomic region (*pf0560*), flanked by these homologous sequences.

Further, the Pyruvoyl dependent Arginine decarboxylase PdaD served as a selection marker, enabling the identification of positive knock out candidates based on growth in agmatine-free media. The analysis of positive knock out candidates was performed using specified media plates, specifically Gelrite medium. Using this plating based selection system, the parental strain, which resembled the auxotroph mutant lacking PdaD, was only able to grow on Gelrite plates when supplemented with agmatine, the precursor for polyamine metabolism via PdaD. After the transformation of the genetic construct into the auxotroph mutant, clones were identified on Gelrite plates after plating in the absence of agmatine. These clones were able to grow without external supplementation of agmatine, indicating successful complementation of PdaD using this genetic system. Subsequently, knock out candidates, that exhibited growth in the absence of agmatine, were further analyzed by PCR. Hereby, PCRs were performed using primers specifically targeting the PdaD to confirm its presence. Consistent with the growth observed on Gelrite plates, PCR analysis revealed bands corresponding to the expected size of PdaD (Section 3.1.8, Fig. 25). Using this approach, the selection marker PdaD was successfully introduced into the auxotroph mutant strain of *Pyrococcus furiosus*. Consequently, this genetic system represents a powerful tool for gene knockout studies in archaea without relying on non-native elements occurring in the target organisms, e.g. restriction enzymes.

Further, the main goal to produce a mutant strain with a deletion of the ribosomal dimerization factor PF0560 was successfully achieved. Moreover, *pf0560* could be deleted by providing homologous regions upstream and downstream of this gene. As a consequence, *pf1623*, encoding for the selection marker PdaD, was located at the position of *pf0560* in the new mutant strain, as expected based on the creation of the genetic construct. Thereby, using this technology, the target gene *pf0560* could be successfully deleted by homologous recombination via double cross over. This genome deletion strategy can offer several advantages in the microbiology. On the one hand, a system can be provided, that more closely resembles the wildtype conditions, as they avoid the introduction of genes, that are not naturally occurring in the organism. On the other

hand, the technique enables a double cross over event occurring in a single experimental step. Moreover, the absence of selectable markers facilitates the clearer interpretation of recombination and deletion events, as no foreign sequence interferes with genomic rearrangements. In this way, during this study, a system could be provided that can be used in further experiments to facilitate the deletion of target genes in microbiological research.

Increased death in the stationary phase

Based on the findings of this study, cells with a $\Delta pf0560$ phenotype exhibited an increased proportion of dead cells during the stationary phase. Furthermore, the microscopic analysis in this study revealed a lower number of viable cells in the $\Delta pf0560$ mutant strains compared with the control (Section 3.1.9., Table 22), indicating a reduced proportion of living cells in the absence of *pf0560*. Still, as this observation is based on only four measurements, additional research is required to fully confirm this trend.

In addition, in the growth curve analysis after 95h (Section 3.1.9., Fig. 26) the control sample could not be followed for the entire 95 hour incubation period. Moreover, the control replicates ended earlier, after approximately 65 hours, due to a stop of data recording. Thereby, to enable a more accurate comparison of stationary phase growth, a new growth curve should be performed with a shorter incubation period. Still, the current data indicate, that $\Delta pf0560$ cells undergo a higher decrease of cells when entering the stationary phase, at approximately 65 hours, in comparison to the control (Section 3.1.9., Fig. 26).

Simultaneously, microscopic observations (Section 3.1.10., Fig. 27) revealed, that $\Delta pf0560$ cells displayed slight defects in the viability during the stationary phase. This is consistent with previous studies suggesting, that cells are more prone to death under early starving conditions. Moreover, ribosomal dimerization factors tend to protect ribosomes during quiescence. Especially, these factors are expressed at higher levels under nutrient-limiting conditions and tend to exert the key function in building ribosomal dimers during these phases. (Basu & Yap, 2016) Moreover, by mediating the dimerization of 30 S ribosomes, this protein inhibits the subunit joining and consequently, the protein synthesis. Due to the fact, that translation is an energy-consuming event for the cell, those factors seem to play important roles in regulating the translation level under starving conditions. (Prossliner et al., 2021) In this way, the cost intensive translation process is hold on a basal level, that might help the cells to survive under those conditions. In addition, as ribosomal dimerization factors show an increased expression during the stationary phase, and mutants with a lack of those factors, as shown in previous studies, show a higher death rate during this phase, it is conclusive that ribosomal dimerization factors are key players in maintaining the viability of cells during starvation via forming storage forms of ribosomes, when conditions become harsh. (Feaga et al., 2020) Moreover, with the help of dimerization factors the cell can maintain a basis level without consuming too much energy. (Hassan et al., 2025) Furthermore, this might prevent the death of the cell over longer time.

Regarding the result in this study, the growth analysis of $\Delta pf0560$ revealed a similar trend. Moreover, the observed decline in cells during the stationary phase may be attributed to the absence of *pf0560*, as this factor is important during this state for maintaining the basis level of stable cells. Thereby, without the ribosomal dimerization factor, cells may lose a substantial part of ribosomes, ultimately leading to cell death over extended periods.

Taking the live dead staining of the cells into account, a higher proportion of $\Delta pf0560$ strains were found to be dead (Section 3.1.10., Fig. 27A.). This may indicate, that upon entering the stationary phase, a high number of $\Delta pf0560$ cells lose its viability, resulting in a lower proportion of living

cells. These findings may suggest, that the absence of *pf0560* exhibits a negative growth effect during the stationary phase. Moreover, this implies, that PF0560 might play an important role in maintaining cell viability under these conditions. Still, in order to affirm these findings, more research is necessary to clarify the actual influence of PF0560 during the stationary phase.

Higher growth during exponential phase in absence of *pf0560*

Contrarily, *Pyrococcus furiosus* $\Delta pf0560$ strains exhibited a sharp increase after approximately 13 hours (Section 3.1.9., Fig. 26), following a higher plateau phase at 25 hours in comparison to the control strains. This observation may indicate that cells lacking the ribosomal dimerization factor PF0560 appeared to have an advantage in growth during the exponential phase. Consistently, previous studies investigated, that the ribosomal dimerization factor mediates the dimerization of 30 S ribosomal subunit (Yaeshima et al., 2022) This dimerization primarily occurs in the stationary phase, with the factor being thereby expressed from the transition from exponential to stationary phase. (Feaga et al., 2020) Contrarily, during exponential phase the dimerization factor appears to be expressed at reduced levels.(Yamagishi, et al., 1993) However, recent studies concluded, that the expression of the ribosomal dimerization factor is influenced by the growth rate. (el-Sharoud & Niven, 2005) Specifically, there appears to be an inverse relation between growth rate and expression of ribosomal dimerization factors (el-Sharoud & Niven, 2005), whereby faster growing organism tend to express lower levels of the dimerization factor. This suggests, that organisms with a higher growth are associated with a reduced expression of ribosomal dimerization factor.

Regardingly, in this study, strains lacking the aRDF exhibited a greater growth during the exponential phase, suggesting, that the factor may have an negative influence during this growth phase. Furthermore, due to the fact that the dimerization factor is knocked out, its expression during this growth phase is shut off. This could provide an advantage for the mutant cells during this phase, as the expression of aRDF, which still occurs at low levels under normal conditions, is entirely eliminated in the mutant strain. Although aRDF is transcriptionally silent under normal conditions, its low-level presence during exponential growth may still require cellular energy. In the absence of the factor, the mutant cells may conserve this energy, thereby facilitating the overserved increase in growth. This could explain, why the archaeal cells lacking the dimerization factor exhibit to display an growth advantage during the exponential phase.

However, to confirm, that the higher growth rate observed in the exponential phase, truly reflects increased cellular activity, it is important to determine how many cells really were active in the exponential phase. Previous studies reported higher levels of ribosome degradation in mutant strains lacking aRDF. (Basu & Yap, 2016), indicating that ribosomal dimerization factors positively influence ribosome stability and protect ribosomes from degradation. Conclusional, although $\Delta pf0560$ mutant strains exhibited a higher growth rate in the exponential phase based on these findings, their ribosomes may be less stable and more prone to degradation. Thereby, further investigations, including cell counting of actively growing cells, are required to clarify the actual activity and viability of the cells during this phase.

Moreover, the underlying mechanism resulting in the higher growth could be based on the influence of the aRDF. As the archaeal ribosomal dimerization factor, encoded by *pf0560*, mediates the formation of two 30S ribosomal dimers and thereby, blocks the formation of the 70S ribosome (Hassan et al., 2025), the absence of *pf0560* might be the cause for the altered growth. Previous studies investigated that the dimerization of ribosomes during starving conditions can be dissociated into the single ribosomes to reinitiate growth, when conditions become more favorable. (Prossliner et al., 2021) Thereby, based on the findings in this study, a plausible

explanation for the faster initiation of the exponential phase can rely on the fact that no dimerization complex of ribosomes is present, that first has to be reassembled to start the protein synthesis. Moreover, the mutant cells can initiate the ribosome subunit joining and immediately enter the translation and subsequently the exponential growth phase.

Further, one plausible mechanism for the higher growth in absence of *pf0560* is that a greater number of cells are able to enter the growth phase due to the absence of the dimerization factor. As the dimerization factor is blocking the cells from translation during stationary phase (Feaga et al., 2020), there might still be cells under favorable conditions blocked as well, and moreover, still adhering in a dormant state. In contrast, in the $\Delta pf0560$ strains those cells would not stick in the dormant state and thereby, more cells were present that could be translated and enter the exponential growth phase.

But, since barely is known about this underlying mechanism, more research has to be done. Moreover, to validate and confirm those findings claimed in this study, also the cell count of strains in the exponential phase has to be taken into account. Thereby, it has to be ensured, if the cells, that were monitored in its growth, were truly active cells, or if cells that already were dead were included in the analysis. In this way, regarding the findings in the exponential phase, a conclusive result could not be observed and it is concerned to investigate more in that subject to fully understand the role of *pf0560* as the dimerization factor in archaea.

PF0560 as a key player in regulating sufficient growth during stationary phase

Based on this study, a greater proportion of $\Delta pf0560$ mutant cells were observed to exhibit reduces viability during the stationary phase. These findings suggest, that cells without the dimerization factor *pf0560*, exhibit to be at a profound disadvantage during the stationary phase. Moreover, this highlights, that PF560 may play a more crucial role during this growth phase, than previously expected. Supporting this, previous studies have shown, that the loss of ribosomal dimerization factors leads to strong defects in the cell viability and even to the loss of rRNA. (Feaga et al., 2020) Moreover, it has been shown, that over long periods of time the absence of ribosomal dimerization factors can cause a dramatic loss of intact ribosomes (Prossliner et al., 2021), which in further steps can profoundly impact the cell. Additionally, recent studies suggest, that the lack of ribosomal dimerization factors even can succeed into ribosome occupancy, which in further steps may lead to the perturbation of the polysome organization. (Basu & Yap, 2016) In this context, to fully understand the role of dimerization factors in archaea and the ongoing effects of this loss for the cell, it is important to investigate the key principles and functions of ribosomal dimerization factors.

As previous studies reported, the aRDF mediates the formation of two 30S-30S dimers via interacting with two 30 S ribosomal subunits. (Hassan et al., 2025) Moreover, aRDF homodimers interact with the ribosomal protein eS32, which is positioned in the 30 S ribosomal subunit and essential for the ribosome subunit joining. Via its interaction with eS32, the ribosomal 50S subunit is disturbed in associating with the 30 S ribosomal subunit. Thereby, the formation of the 70S ribosome complex, which is crucial for the translation process, is blocked. (Hassan et al., 2025) In this way, the aRDF functions as an anti-association factor and can mediate the ribosomal dimer formation and thereby, suppresses the protein synthesis via blocking the formation of the 70S ribosomes. Furthermore, aRDF binds the 16 S rRNA and forms a homodimer, whereby the two 30 S ribosomal subunits get stabilized. This stabilization in the further way hinders the joining of the 50S ribosomal subunit. (Hassan et al., 2025)

This process is observed to play a role in regulating the translation during environmental stressors. Since *Pyrococcus furiosus* thrives at extremely high temperatures, the ribosomal dimerization may serve as an essential mechanism for the organism to conserve resources under these harsh conditions. On the other hand, the function of aRDF may share functional similarities to the IF3/eIF3 factors found in bacteria/ eukaryotes. Despite obtaining different binding modes, as IF3 acts in a multi-subunit complex, whereas aRDF appears to function solely as a homodimer, the role of these factors may be similar. Specifically, aRDF may act as the archaeal agent, that prevents premature ribosome assembly by stabilizing 30S ribosomal dimers and moreover, contributes to the regulation of the protein synthesis. (Hassan et al., 2025)

Moreover, the non-occurrence of the stabilization of the two 30S ribosomal subunits shows to have a profound disadvantage in cells entering the stationary phase. This could be justified that cells in this phase, under normal requirements tend to enter a sort of dormant state. In addition with the extreme temperatures that *Pyrococcus furiosus* deals with, the organism may need a state of quiescence under conditions, where nutrients become limited, in order to survive over longer periods of time in its extreme environment. Furthermore, the lack of factors, namely aRDF, mediating a translational suppression tend to observe negative effects on the cell. In other words, those cells may suffer from too much energy cost intensive events mediated by increased translational processes, that in longer periods of time may lead to the loss of ribosomes and in the further way to the increased dead of archaeal cells.

Regrowth of *Pyrococcus furiosus* Δ pf0560 strains as a further profound investigation

Based on this study, it was able to proof that the ribosomal dimerization factor PF0560 reveals significant impacts on *Pyrococcus furiosus* during the stationary phase. Specifically, the absence of this protein was obtained to affect growth dynamics and in particular, the cell mortality. Furthermore, cells lacking *pf0560* exhibited a reduced growth rate during the stationary phase and an increased proportion of dead cells, consistent with prior studies.

In the further research, an important aim to learn more about the function and impact of PF0560 on *Pyrococcus furiosus*, relies on analyzing *Pyrococcus furiosus* Δ pf0560 strains in their regrowth after starving conditions. Since in this study, it could be observed, that cells in the stationary phase experienced a high rate of mortal cells, it would be a profound investigation to analyze how cells are behaving by their regrowth into fresh media. Moreover, the growth of those cells with taking the proportion of dead and living cells into account would lead to further insights, how *pf0560* and furthermore ribosomal dimerization is influencing cells.

Based on further studies, it is expected that cells derived from starving conditions tend to exhibit reduced growth, when regrowing into new fresh medium. (Akanumu et al., 2016) The underlying mechanisms may be linked to severe defects in the cell viability of those cells and in particular, to a substantial loss of ribosomes. These observations suggest, that ribosome dimers are playing important key roles in the regrowth of archaeal cells. Investigating, how the ribosome dimerization is involved during regrowth and how cells lacking this factor respond in translational regulation, is therefore crucial. Hereby, it is important understanding the process how the ribosome subunit joining during protein synthesis functions and whether the cells observe a disadvantage in translation processes due to a possible less proportion of ribosomes present.

In summary, to investigate the complete role and fundamental functions of *pf0560* in archaea, further studies are necessary. Since PF0560 appears to exert its greatest influence on cells on ribosome dimerization during the stationary phase, a profound study will provide to analyze the regrowth of Δ pf0560 mutant strains from the stationary phase. Further, by additionally assessing

the proportion of dead cells during regrowth, this could provide crucial insights into the impact of PF0560 on cell viability and recovery.

Summary:

Overall, during this work, a deletion mutant of *Pyrococcus furiosus* lacking the ribosomal dimerization factor PF0560, could be successfully generated using a modified genetic system combined with a dilution plating method. Using this approach, cells with a $\Delta pf0560$ phenotype could be generated and were generally viable. Cells lacking *pf0560* exhibited moderate growth, suggesting that *pf0560* is not essential for the general cell proliferation. Moreover, as the mutant strains remained viable and did not require *pf0560* for its general growth, it can be suggested, that the ribosomal dimerization factor PF0560 is likely a non-essential gene in *Pyrococcus furiosus*, not necessarily needed for its ability to grow. However, *pf0560* may play important regulatory functions, particularly in modulating growth and translation under nutrient-limited conditions. Further, this ribosomal dimerization factor appears to exert more profound effects on the cell over longer periods of time. Furthermore, the dimerization factor may play a critical role in regulating cellular energy level under starvation conditions. In particular, since *Pyrococcus furiosus* thrives at extremely high temperatures, the ribosomal dimerization factor likely contribute to the maintenance of the cells under such extreme conditions (Hassan et al., 2025), thereby enabling the organism to survive over long periods of time.

In addition, the resulting $\Delta pf0560$ mutant exhibited a higher growth rate during the exponential phase. However, further research is required to elucidate the underlying mechanisms of PF0560 during this growth phase. In comparison, the mutant cells lacking *pf0560* displayed pronounced defects in growth and viability during the stationary phase, with a tendency for increased mortality upon entry into this phase. To further investigate the fundamental role of the dimerization factor PF0560 on the organism, it is essential to analyze its functions across different growth phases. Moreover, an important lead would be to examine how mutant cells lacking ribosomal dimerization factor behave when regrown in fresh media. Assessing their viability and the proportion of dead cells after regrowing them, could provide critical insights into the function of the dimerization factor in archaea. Additionally, the involvement of this protein in the regulation mechanisms to maintain the survival of archaea during extreme environmental conditions could be observed based on those findings.

Taken together, the findings indicate, that *pf0560* plays a crucial role in the regulation of archaeal cells. However, in order to understand its actual importance in the regulation and how *pf0560* influences cells in its absence, further research is needed considering its conceptional function and moreover its effect on archaea over longer time periods.

4.2. Deletion of the ribosomal protein L41e

Successful complementation of the Pyruvoyl dependent Arginine Decarboxylase as selection marker into the auxotroph mutant strain

Regarding the second experiment, the Pyruvoyl dependent Arginine Decarboxylase could be successfully complemented into the auxotroph mutant version of *Pyrococcus furiosus*. Moreover, it was able to introduce *pf1623* via homologous regions provided by the system. As a result, clones could be identified on Gelrite Media plates in absence of Agmatine. Consistent with the findings of the first experiment, the genetic system could hereby provide a mechanism, which enabled a deletion technique without using not naturally occurring elements by providing homologous regions in the genome of *Pyrococcus furiosus*.

Mixed genome populations after the transformation of the construct into *Pyrococcus furiosus*

Clones, that were isolated from the transformation approach, revealed hints, that L41e was still present in the genome after introducing homologous regions into the target organism. Moreover, in the first deletion approach of L41e, a Phosphoesterase was deleted together with L41e as this Phosphoesterase, encoded by *pf1019*, as this gene is co-transcribed with *pf1018* by the promotor of *pf1018*. As shown in section 3.2.1.8., a PCR Screening targeting L41e in this approach indicated, that there were still fragments corresponding to the size of L41e. In addition, Sanger sequencing analysis suggested the possible presence of residual wild-type genome contamination. (Section 3.2.1.8) This findings suggested that more than one DNA sequence variant may have been present in the sample. This interpretation was supported by the corresponding chromatogram (Section 3.2.1.8, Fig. 33), which displayed overlapping peaks at a specific position within the L41e locus, potentially indicating a mixed genome population. These observations may be attributable either to low-level contamination with wild-type DNA or to the presence of multiple insert variants within the sample. Furthermore, some genome copies might have carried the expected recombination, while others may not have undergone the recombination event or only partially. As described in recent studies, the presence of mixed genome copies after transformations can underly several reasons, whereby a population with multiple genome copies is the consequence. (Velappan et al., 2007) As a result, the population exhibits a mixed genotype, with the proportion of genome copies varying depending on the homologous recombination events that occurred (Scanlon et al., 2009). To resolve this heterogenous genotype, different strategies can be employed to isolate genetically homogeneous clones, that carry only the expected recombination event, with all unintended variants eliminated. (Scanlon et al., 2009) To achieve this, isolating single colonies by plating can serve as an effective experimental approach. In this study, this technique was employed to determine, whether the clones could be isolated into genetically pure populations. Moreover, it allowed the investigation, whether the presence of *pf1018* was due to contamination or if the gene was still present in the majority of the genome copies. To this end, the clones were repurified into pure populations using this method. (Scanlon et al., 2009) In this way, each clone was able to grow into a separate, homogeneous colony, with each colony originating from a single cell.

Besides, in the second L41e deletion approach, whereby only the target protein L41e was expected to be knocked out, the presence of L41e appeared to persist after the transformation. In particular, PCR analysis described in Section 3.2.2.6, using primers targeting the region of the assembled genetic construct, revealed two distinct bands. One band corresponded to the expected size of the deletion mutant at 3098 base pairs, whereas the second band migrated slightly above and was consistent with the size of the wild-type genome. Furthermore, Sanger

sequencing analysis suggested, that L41e was still present in the genome at a relatively high abundance. Alignment of the obtained sequence to the wild-type reference genome resulted in a clear match to the L41e region, indicating that the gene was still present. In contrast, alignment of the sequence to the expected deletion construct lacking L41e showed deletions at the intended L41e locus. Correspondingly, the chromatogram displayed overlapping peaks in the L41e locus as shown in Section 3.2.2.6. in Figure 39. All in all, these observations strengthen the assumption, that L41e was not fully removed from the genome. Thereby, to further investigate, whether L41e could be eliminated or to assess the possibility, that a complete knockout of L41e might not be feasible, a single colony isolation approach was applied, analogous to the procedures used in the previous experiments.

L41e persists after single-cell resolution

Furthermore, the clones isolated after the plating approach described in Section 3.2.1.8, still revealed traces of the gene encoding for L41e. Moreover, regarding the first deletion experiment of *pf1018*, there were still contaminations of wild type genomes according to the PCR Screening left (Section 3.2.1.8, Figure 34). Furthermore, the PCR Screening for *pf1018*, encoding L41e, revealed the band at the corresponding size for this gene. In this way, there may still be copies of wild type genome present and the produced clones appeared to be heterozygote. On the one hand, there might be clones present which had the expected deletion pattern. On the other hand, there might be clones that still had wildtype copies left. The underlying mechanisms, why the complete deletion did not function in this experiment rely on multiple reasons. Furthermore, there might have appeared unexpected DNA repair mechanisms or insufficient editing (Bekaert et al., 2024), that led to the persistence of the ribosomal protein L41e. Thereby, in order to clarify the profound reasons, more research is necessarily to be done.

Accordingly, plating clones from the second approach, similarly indicated the continued presence of *pf1018* in the genome. Moreover, PCR Screening as described in Section 3.2.2.7 in Figure 41 still yielded a fragment corresponding to the size of the L41e gene (85 base pairs). In addition, the sequencing analysis revealed the same result as prior to serial diluting the clone. Nevertheless, the PCR analysis targeting the genetic construct in this clone (Section 3.2.2.6, Fig. 40) revealed only a single band of the expected size of the genetic construct. This result was consistent with the Sequencing chromatogram depicted in Section 3.2.2.6 in Fig. 40, that showed reduced evidence of mixed genome populations. These findings suggests that the plating approach still may have lowered the proportion of mixed genomes. However, since L41e could still be targeted in this approach, the method did not yield the expected outcome.

Moreover, these findings strongly indicate that the gene encoding for L41e remained present in the newly generated mutant strain. These findings suggest that the deletion approach was not successful and that L41e persisted after the transformation step. The pronounced detection of L41e in the second experiment may indicate that L41e encodes a protein, that may be essential for *Pyrococcus furiosus*. However, it cannot be excluded that technical challenges contributed to the unsuccessful deletion, particularly given that L41e encodes a relatively small protein.

In this context, the deletion of a single small gene may pose greater technical challenges than the deletion of larger gene regions, as performed in the first deletion approach. To elucidate the underlying mechanisms responsible for the unsuccessful deletion of L41e, further investigations will be required.

L41e could not be deleted in *Pyrococcus furiosus* using the genetic system

As L41e appeared to remain present in the genome in both experimental setups at different apparent levels, the deletion procedure could not be successfully completed. Furthermore, several technical and biological challenges were observed that may have limited the efficiency of the deletion process. In this context, unanticipated DNA repair mechanisms or insufficient genome editing at the target locus may have occurred. These observations may suggest that *pf1018*, encoding the ribosomal protein L41e, cannot be readily knocked out and may represent an essential gene in *Pyrococcus furiosus*.

Moreover, given that ribosomal proteins play critical roles in ribosome assembly and translation, the deletion of such factors may not be viable for the organism. (Zhou et al., 2015) However, further experimental evidence will be required to conclusively determine the essentiality of L41e.

Furthermore, L41e has been reported to stabilize the ribosomal 50 S subunit in archaea and to act as an inter-subunit bridge mediating the correct assembly of the 50S and the 30S ribosomal subunit. (Hassan et al., 2025) In this way, L41e might function as a key player in the ribosome stability, whereby its deletion might not be achievable. Further, the function of L41e increases the translation efficiency (Yu & Warner, 2001), indicating that this ribosomal protein plays an important role in archaeal cells. However, to completely determine, whether L41e can be depleted, further research will be required.

Moreover, the first experimental set up of deleting L41e, led to the result, that there were still amounts of wild type copies present in the genome. Further, the PCR Screening for L41e revealed signals referring to the size of L41e. Thereby, there might only have been minimal contaminations of wild type sequences, that could further be removed with further ongoing approaches. But, as this only relies on assumptions, still more research is required to ensure those findings.

In contrast, regarding the second experimental set up, as a result L41e still persisted in the genome and appeared to be essential for the organism. Moreover, PCR Screening for L41e showed strong signals and the Sequencing analysis confirmed the presence of sequences aligning with L41e. These results led to the finding, that L41e could not be deleted using this approach. However, as previous studies have successfully deleted L41e in yeast cells (Yu & Warner, 2001), additional investigations are needed to clarify why deletion of L41e was not achievable using this approach in archaea. Moreover, to better understand the underlying processes of L41e in archaea, further research based on those findings has to be done. In addition, since successful deletion of L41e has been reported in organisms such as yeast, but no prior studies have addressed its deletion in archaea, the role and potential essentiality of this gene in archaea require further investigation in future research.

Summary

Overall, using the genetic system, both approaches allowed the introduction of the selection gene, the pyruvoyl-dependent arginine cascade, into the basal auxotroph mutant of *Pyrococcus furiosus*. This enabled the new strain to grow on Gelrite media plates and in liquid media in the absence of agmatine. Through this strategy, the archaeal cells were able to regain the enzyme PdaD, allowing them to convert agmatine independently as a precursor for polyamine metabolism. In this way, a genetic deletion system could be established based on using the enzyme PdaD as the selection marker.

However, in both approaches, the ribosomal protein L41e could not be fully deleted. Furthermore, the two genetic constructs introduced led to different outcomes. In the first approach, which aimed to delete *pf1018* together with *pf1019*, multiple genome copies were observed after transformation, and wild-type sequences persisted, suggesting contamination. In the second approach, which targeted deletion of *pf1018* alone, L41e remained fully present. This may indicate incomplete editing, where the selection gene *pf1623* was incorporated without replacing L41e.

To further investigate, why L41e could not be deleted, additional research is necessary. For the first approach, an extra dilution step could help to determine, whether the heterogeneous genotype could be more effectively segregated. Since the second approach did not achieve deletion, further studies are needed to assess the efficiency of the genetic system, particularly when targeting the deletion of smaller proteins.

In addition, to determine whether L41e can be deleted in archaea, further research should focus on its function, particularly in archaeal organisms, to assess whether L41e may be an essential gene, making its deletion unachievable.

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6. Sequence data

In the following, the DNA sequences of the utilized constructs and the respective inserts for the transformation are shown below. The regions utilized for the design of the primers are highlighted in different colors. The color code is defined in the corresponding single-stranded sequences.

Construct for the deletion of *pf0560*

Double stranded sequence of the construct *pUC19-pf0559-PdaD-pf0561*

```

      10              30              50
TCGCGCGTTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCT 70
AGCGCGCAAAGCCACTACTGCCACTTTTGGAGACTGTGTACGTCGAGGGCCTCTGCCAGTGTGCAACAGA

      80              100             120
GTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGGGCTGG 140
CATTGCGCTACGGCCCTCGTCTGTTGCGGCAGTCCCGCGCAGTCGCCCACAACCGCCACAGCCCCGACC

      150             170             190
CTTA ACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCATATGCGGTGTGAAATACCGCACAGAT 210
GAATTGATACGCCGTAGTCTCGTCTAACATGACTCTCACGTGGTATACGCCACACTTTATGGCGTGTCTA

      220             240             260
GCGTAAGGAGAAAATACCGCATCAGGCGCCATTGCCATTAGGCTGCGCAACTGTTGGGAAGGGCGATC 280
CGCATTCTCTTTTATGGCGTAGTCCGCGGTAAGCGGTAAGTCCGACGCGTTGACAACCCTTCCCGCTAG

      290             310             330
GGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAAGTTGGGTA 350
CCACGCCCCGAGAAGCGATAATGCGGTCGACCGCTTCCCCCTACACGACGTTCCGCTAATTCAACCCAT

      360             380             400
ACGCCAGGGTTTTCCAGTCACGACGTTTAAACGACGGCCAGTCCAGGAGGGGATTTGGCCAGCTAC 420
TGCGGTCCCAAAGGGTCAGTGCTGCAACATTTTGCTGCCGGTCAGGGTCTCCCTAAACCGGTCGATG

                                pUC_M13F_UpPF0560 F
                                AmpL_pUC_R
      430             450             470
TATCCCTTGAGGGCCTTAATTGGAATACTCAGCTTAAACACGACTTAGAGGAAGTTGAGAAAATCATAA 490
ATAGGGAACCTCCGGAATTAACCTTATGAGTCGAATTTGGTGCTGAATCTCCTTCAACTCTTTTAGTATT

      500             520             540
GGGAGTTCTGTCCAAATGCAATAAAGAGCTTAAAGTATGGGGAAACAGAGTTTAGGGTAATTATGAGGCA 560
CCCTCAAGACAGGTTTACGTTATTTCTCGAATTCATACCCCTTTGTCTCAAATCCCATTAATACTCCGT

      570             590             610
ACTCAGCAGCGGGATAAACGTTGCCTATGCCTCTTCAACGGGAAGGGTGCTTGATGCGCTTCTCGGTACTT 630
TGAGTCGTCGCCCTATTTGCAACGGATACGGAGAAGTTGCCCTTCCCACGAACACGGAAGAGCCATGAA

                                Ampl_Trafo PF0560 F
      600             620             680
TTGAACGTTTCTACAGGAGGCACTATGAGGGAGAGCCTGCGATGAAGCTGGAGAGCTTTGCATACCAAG 700
AACTTGCAAAGGATGTCTCCGTGATACTCCCTCTCGGACGCTACTTCGACCTCTCGAAACGTATGGTTC

      710             730             750
GAAAGAACGATCTAAAGCTCACGGCTCCAATTGAAGGTGAGGAAATAAAGGTTTCAGAGTTGTTTGAGGA 770
CTTTCTTGCTAGATTTGAGTGCCGAGGTTAACTTCCACTCCTTTATTTCAAAGTCTCAACAAACTCCT

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780	800	820	
AGTTCTTGAGCTGATGGGCAAGGCCAATCCTAAAGACATAGCTTACTCCGTTCACTTAGCCTTAGCTAGG			840
TCAAGAACTCGACTACCCGTTCCGGTTAGGATTTCTGTATCGAATGAGGCAAGTGAATCGGAATCGATCC			
850	870	890	
GCATTTGCTGAAGTTAGCGTGGAGAAAGCTAAGGAGTTTGGAGCTAAAAGTGTGTTTTGGGTGGGGGAG			910
CGTAAACGACTTCAATCGCACCTCTTTCGATTCTCAAACCTCGATTTTGACAGCAAAACCCACCCCTC			
920	940	960	
TAGGGTACAATGAGCTAATAGTTAAGACGATAAGAAAGATAGTAGAGGGGAGAGGGCTAAGGTTCTTAAC			980
ATCCCATGTTACTCGATTATCAATTCTGCTATTCTTCTATCATCTCCCCTCTCCCGATTCCAAGAATTG			
990	1010	1030	
AACTTACGAAGTTCCCAGGGGAGATAATGGAATTAATGTAGGCCAGGCCTTCCTGGGAGGATTGTACTTG			1050
TTGAATGCTTCAAGGGTCCCCTCTATTACCTTAATTACATCCGGTCCGGAAGGACCCTCCTAACATGAAC			
1060	1080	1100	
GAAGGATACTTAAATAGGGAAGATTTGAGCATTTAGTTTGTCTCTACCTAGGCCTTCCTTTTTTTCTTAT			1120
CTTCCTATGAATTTATCCCTTCTAAACTCGTAAATCAACAGAGATGGATCCGGAAGGAAAAAAGAATA			
1130	1150	1170	
CCTACAAACACCGCAGATTAACTTCTCCTACTTACGAAAACCTTTAAAAGATAATAAATTTGGTATCCG			1190
GGATGTTTTGTGGCGTCTAATTTGAAGAGGATGAATGCTTTTGAAATTTCTATTATTTAAACCATAGGC			
Agma_Ups_PF0560 R			
1200	1220	1240	
TTTAGCGAGAGAATATAGGAGGGAGAAGAATGAGCTGGATAACTCCTAAAAAGGCCATAATGCTTGCAGC			1260
AAATCGCTCTCTTATATCCTCCCTCTTCTTACTCGACCTATTGAGGATTTTCCGGTATTACGAACGTGG			
1270	1290	1310	
GGCAGCTGAAGGCGGGACAAAGCTTAATGCTTTCGATAATGCTCTATTAAAGATGGGAATTGGAAATGTG			1330
CCGTCGACTTCCGCCCTGTTTCGAATTACGAAAGCTATTACGAGATAATTTCTACCCTTAACCTTTACAC			
1350	1370	1380	
AACCTAGTAAAGCTCAGCAGTGTTATACCGGCTCATATAGAGTGGCTGGATGAGTTGCCAAAGAACATTC			1400
TTGGATCATTTTCGAGTCGTACAATATGGCCGAGTATATCTCACCGACCTACTCAACGGTTTCTTGTAAG			
1410	1430	1450	
CTATAGGGATGTTGTTACCAACAGTCTACACCCACATAGAGAGCGATGAGCCAGGTTCAACGATAAGCGC			1470
GATATCCCTACAACAATGGTTGTCAGATGTGGGTGTATCTCTCGCTACTCGGTCCAAGTTGCTATTCGCG			
1480	1500	1520	
AGCTTTAGGIAIIGGGCTTAGCGAAGGCAAIIGAAGGTGGGCTAATCTAIGAAIATTCAAGGATACTGCAAG			1540
TCGAAATCCATAACCCGAATCGCTTCCGTTACTTCCACCCGATTAGATACTTATAAGTCCATGACGTTT			
1550	1570	1590	
AAGGAAGAGGCTGAGGAAATGGTTAAGAAGATGGTTGAGGAAGGTTTTAGGGTTAGAGGATGGAAACTCA			1610
TTCCTTCTCCGACTCCTTTACCAATTCTTCTACCAACTCCTTCCAAAATCCCAATCTCTACCTTTGAGT			
1620	1640	1660	
AGGAAATAAAGATAGTCTCCGCTGAAATAACGGTTAAGGACAAGCCAGCGGCTGCAGTTGCTGCAGTAGT			1680
TCCTTTATTTCTATCAGAGGCGACTTTATTGCCAATTCCTGTTTCGGTCGCCGACGTCACGACGTCATCA			
1690	1710	1730	
TATGTTCCCATAGCAAGAGCTTTTCTTACCTCTTCTATTACCTCATTCATTGCAATCCCAACACATAA			1750
ATACAAGGGTATGATCGTTCTCGAAAAGAATGGAGAAGATAATGGAGTAGTAACGTTAGGGTTGTGTATT			
1760	1780	1800	
CCTCAAAGCTGGGAACCTTACACTAATCCTCACTTTTCTCTTTCTTGTATTTTGTCTCACTTCATAACC			1820
GGAGTTTCGACCCTTGAGAATGTGATTAGGAGTGAAAAGAGAAAGAACGATAAAACGAGTGAAGTATTGG			
Agma_RK-R			76

1830 1850 1870 1890
ATGTCTAGGAATTTAACTCTATCGGAAAGTATTTAAGAGCTATTATAACAATAGTTAGTTATGAGGTGAA
TACAGATCCTTAAATTGAGATAGCCTTTTATAAATTCTCGATAATATTGTTATCAATCAATACTCCACTT

1900 1920 1940 1960
TTACCATGACGCCCATGGAGACATCTAATGAGCAAATTTATAATATCTATCTTCCTAAGGTAGCAGAAGA
AATGGTACTGCGGGTACCTCTGTAGATTACTCGTTTAAATATTATAGATAGAAGGATTCCATCGTCTTCT

1970 1990 2010 2030
GTCTCCTATCGATTCTTTAGAAAAAATTGAACCTGTTGGTAAGATTAGAAATTACATCGACATAAGTTTT
CAGAGGATAGCTAAGAAATCTTTTTTAACCTGGACAACCATCTAATCTTTAATGTAGCTGTATTCAAAA

2040 2060 2080 2100
GAAGGTATCAAGCGGGTCGAAAATGGAGTCTCAGCATTTTATCCCATGGCAATCTTTGGATACTATGGGA
CTTCCATAGTTCGCCCAGCTTTTACCTCAGAGTCGTAATAAGGGTACCGTTAGAAACCTATGATACCTT

2110 2130 2150 2170
GCGGAAAAACATTCTTTGTGAGAAAGCTATCGGAGAAGATAGTGAAAGAGTTGGAAAGAACTATACCTTT
CGCCTTTTTGTAAAGAACTCTTTTCGATAGCCTCTTCTATCACTTTCTCAACCTTTCTTGATATGGAAA

2180 2200 2220 2240
GCACTTTTATCTAGGACAGACATCTTATTTGACTTGATTAAATTTGTGGACGAACTGATAGAAAGTATA
CGTGAAAATAGATCCTGTCTGTAGAATAAAGCTGAACCTAATTTAAACACCTGCTTGACTATCTTTCATAT

2250 2270 2290 2310
GAGGTCTTTATTGAAAGAGGAGACAAAATAAGGCAACGATAAGTGGATATAATCCAGAAAAGTGGATGA
CTCCAGAAATAACTTTCTCCTCTGTTTGTATTCCGTTGCTATTACCTATATTAGGTCTTTTTCACCTACT

Ampl_Trafo PF0560 R

2320 2340 2360 2380
AGTATCTAGAGGTTTTAAAAGCAGCTAGAGAGAAAATTTCTAATGAGGAGGCAGAAAAAGCAATGTGGA
TCATAGATCTCCAAAATTTTCGTGATCTCTCTTTTAAAGATTACTCCTCCGTCTTTTTTCGTTACACCT

2390 2410 2430 2450
AGCGATACTGTTAGGATAAGCTGGGTCTATAGCTGTTTCTGTGTGAAATTGTTATCCGCTCACAATTCCA
TCGCTATGACAATCCTATTTCGACCCAGTATCGACAAAGGACACACTTTAACAATAGGCGAGTGTTAAGGT

pUC_M13R_DowPF0560_R

2460 2480 2500 2520
CACAAACATACGAGCCGGAAGCATAAAGTGTAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAA
GTGTTGTATGCTCGGCCCTTCGTATTTACATTTTCGGACCCACGGATTACTCACTCGATTGAGTGTAATT

2530 2550 2570 2590
TTGCGTTGCGCTCACTGCCCCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCA
AACGCAACGCGAGTGACGGGCGAAAGGTCAGCCCTTTGGACAGCACGGTCGACGTAATTACTTAGCCGGT

2600 2620 2640 2660
ACGCGCGGGGAGAGGCGGTTTTCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCG
TGCGCGCCCCCTCTCCGCCAAACGCATAACCCGCGAGAAGGCGAAGGAGCGAGTGACTGAGCGACGCGAGC

2670 2690 2710 2730
GTCGTTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAAATACGGTTATCCACAGAATCAGGGG
CAGCAAGCCGACGCCGCTCGCCATAGTCGAGTGAGTTTCGCCATTATGCCAATAGGTGTCTTAGTCCCC

2740 2760 2780 2800
ATAACGCAGGAAAGAATCATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCT
TATTGCGTCCTTTCTGTACACTCGTTTTCCGGTCGTTTTCCGGTCCTTGGCATTTTTCCGGCGCAACGA

2810 2830 2850 2870
GGCGTTTTTTCATAGGCTCCGCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGA
CCGCAAAAGGTATCCGAGGCGGGGGGACTGCTCGTAGTGTTTTAGCTGCGAGTTCAGTCTCCACCGCT

2880 2900 2920 2940
AACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCTGTTCCGA
TTGGGCTGTCCTGATATTTCTATGGTCCGCAAAGGGGGACCTTCGAGGGAGCACGCGAGAGGACAAGGCT

2950	2970	2990	
CCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACG			3010
GGGACGGCGAATGGCCTATGGACAGGCGGAAAGAGGGAAGCCCTTCGCACCGCGAAAGAGTATCGAGTGC			
3020	3040	3060	
CTGTAGGTATCTCAGTTCGGTGTAGGTCGTTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTTCTCAG			3080
GACATCCATAGAGTCAAGCCACATCCAGCAAGCGAGGTTTCGACCCGACACACGTGCTTGGGGGGCAAGTC			
3090	3110	3130	
CCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCAC			3150
GGGCTGGCGACGCGGAATAGGCCATTGATAGCAGAACTCAGGTTGGGCCATTCTGTGCTGAATAGCGGTG			
3160	3180	3200	
TGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTG			3220
ACCGTCGTCGGTGACCATTGTCCTAATCGTCTCGCTCCATACATCCGCCACGATGTCTCAAGAACTTCAC			
3230	3250	3270	
GTGGCCTAACTACGGCTACACTAGAAGAACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTC			3290
CACCGGATTGATGCCGATGTGATCTTCTTGTCTATAAACCATAGACGCGAGACGACTTCGGTCAATGGAAG			
3300	3320	3340	
GGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTGTTGCA			3360
CCTTTTTCTCAACCATCGAGAACTAGGCCGTTTGTGGTGGCGACCATCGCCACCAAAAAAACAACGCT			
3370	3390	3410	
AGCAGCAGATTACGCGCAGAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGC			3430
TCGTCTCTAATGCGCGTCTTTTTTCTTAGAGTTCTTCTAGGAACTAGAAAAGATGCCCCAGACTGCG			
3440	3460	3480	
TCAGTGGAACGAAAACCTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATC			3500
AGTCACCTTGCTTTTGAGTGCAATTCCTAAAACAGTACTCTAATAGTTTTTCTAGAAGTGGATCTAG			
3510	3530	3550	
CTTTTAAATTAATAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAACTTGGTCTGACAGTTACC			3570
GAAAATTTAATTTTTACTTCAAAATTTAGTTAGATTTTCAATATACTCATTTGAACCAGACTGTCAATGG			
3580	3600	3620	
AATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTTCGTTTCATCCATAGTTGCCTGACTCCC			3640
TTACGAATTAGTCACTCCGTGGATAGAGTCGCTAGACAGATAAAGCAAGTAGGTATCAACGGACTGAGGG			
3650	3670	3690	
CGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGAC			3710
GCAGCACATCTATTGATGCTATGCCCTCCCGAATGGTAGACCGGGGTACGACGTTACTATGGCGCTCTG			
3720	3740	3760	
CCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTC			3780
GGTGCGAGTGGCCGAGGTCTAAATAGTCGTTATTTGGTTCGGTCGGCCTTCCCGGCTCGCGTCTTCACCAG			
3790	3810	3830	
CTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGAAGCTAGAGTAAGTAGTTTCGCCAGT			3850
GACGTTGAAATAGGCGGAGGTAGGTGAGATAATTAACAACGGCCCTTCGATCTCATTATCAAGCGGTCA			
3860	3880	3900	
TAATAGTTTTCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTACGCTCGTCGTTTGGTATGGCT			3920
ATTATCAAACGCGTTGCAACAACGGTAACGATGTCCGTAGCACCACAGTGCGAGCAGCAAACCATAACGA			
3930	3950	3970	
TCATTAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCATGTTGTGCAAAAAAGCGGTTA			3990
AGTAAGTCGAGGCCAAGGGTTGCTAGTTCCGCTCAATGTACTAGGGGGTACAACACGTTTTTTCGCCAAT			
4000	4020	4040	
GCTCCTTCGGTCTCCGATCGTTGTCAGAAGTAAGTTGGCCGAGTGTTATCACTCATGGTTATGGCAGC			4060
CGAGGAAGCCAGGAGGCTAGCAACAGTCTTCATTCAACCGGCGTCACAATAGTGAGTACCAATACCGTCG			
4070	4090	4110	
ACTGCATAATTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAG			4130
TGACGTATTAAGAGAATGACAGTACGGTAGGCATTCTACGAAAAGACACTGACCACTCATGAGTTGGTTC			
4140	4160	4180	
TCATTCTGAGAATAGTGATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGC			4200
AGTAAGACTCTTATCACATACGCCGCTGGCTCAACGAGAACGGGCCGAGTTATGCCCTATTATGGCGCG			

4210	4230	4250	
CACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACCTCTCAAGGATCTT			4270
GTGTATCGTCTTGAAATTTTCACGAGTAGTAACCTTTTGCAAGAAGCCCCGCTTTTGAGAGTTCCTAGAA			
4280	4300	4320	
ACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTC			4340
TGGCGACAACTCTAGGTCAAGCTACATTGGGTGAGCACGTGGGTTGACTAGAAGTCGTAGAAAATGAAAG			
4350	4370	4390	
ACCGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAGGGAATAAGGGCGACACGGA			4410
TGGTCGCAAAGACCCACTCGTTTTTGTCTTCCGTTTTACGGCGTTTTTCCCTTATTCCCGCTGTGCCT			
4420	4440	4460	
AATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAG			4480
TTACAACCTTATGAGTATGAGAAGGAAAAAGTTATAATAACTTCGTAAATAGTCCCAATAACAGAGTACTC			
4490	4510	4530	
CGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTG			4550
GCCTATGTATAAACTTACATAAATCTTTTTATTTGTTTATCCCCAAGGCGCGTGTAAGGGGGCTTTTCAC			
4560	4580	4600	
CCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAAATAGGCGTATCACGAGGCC			4620
GGTGGACTGCAGATTCTTTGGTAATAATAGTACTGTAATTGGATATTTTTATCCGCATAGTGCTCCGGG			
TTTCGTC			4690
AAAGCAG			

Single stranded sequence of the construct pUC19-pf0559-PdaD-pf0561

pUC19 vector	1-395
<i>pf0559</i> (upstream of <i>pf0560</i>)	396-1119
<i>pf1623</i> (Pyruvoyl-dependent Arginine decarboxylase)	1120-1818
<i>pf0561</i> (downstream of <i>pf0560</i>)	1819-2403
pUC19 vector	2404-4626

TCGCGCGTTTCGGTGATGACGGTGAAAACTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAA
GCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTGGCGGGTGTGGGGCTGGCTTA
ACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCATATGCGGTGTGAAATACCGCACAGATGCGTAAGGA
GAAATACCGCATCAGGCGCCATTGCCATTAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCT
CTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAAGTTGGGTAACGCCAGGGTTTTCC
CAGTCACGACGTTGTAAAACGACGGCCAGTCCAGGAGGGGATTGGCCAGCTACTATCCCTTGAGGGCCTTA
ATTGGAATACTCAGCTTAAACACGACTTAGAGGAAGTTGAGAAAATCATAAGGGAGTTCTGTCCAAATGCAATAAA
GAGCTTAAAGTATGGGGAAACAGAGTTTAGGGTAATTATGAGGCAACTCAGCAGCGGGATAAACGTTGCCTATGC
CTCTCAACGGGAAGGGTGCTTGATGCCCTTCTCGGTACTTTGAACGTTTCTACAGGAGGCACTATGAGGGAGA
GCCTGCGATGAAGCTGGAGAGCTTTGCATACCAAGGAAAGAACGATCTAAAGCTCACGGCTCCAATTGAAGGTG
AGGAAATAAAGGTTTCAGAGTTGTTGAGGAAGTTCTTGAGCTGATGGGCAAGGCCAATCCTAAAGACATAGCTTA
CTCCGTTCACTTAGCCTTAGCTAGGGCATTGCTGAAGTTAGCGTGAGAGAAAGCTAAGGAGTTTGAGCTAAAAC
GTCGTTTTGGGTGGGGGAGTAGGGTACAATGAGCTAATAGTTAAGACGATAAGAAAGATAGTAGAGGGGAGAGGG
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AGAATATAGGAGGGAGAAGAATGAGCTGGATAACTCCTAAAAGGCCATAATGCTTGACGCGGCAGCTGAAGGC
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GCCAGCGGCTGCAGTTGCTGCAGTAGTTATGTTCCCATACTAGCAAGAGCTTTTCTTACCTCTTCTATTACCTCATC
ATTGCAATCCCAACACATAACCTCAAAGCTGGGAACTCTTACACTAATCCTCACTTTTCTTCTTCTGCTATTTTGCT
CACTTCATAACCATGTCTAGGAATTTAACTCTATCGGAAAGTATTTAAGAGCTATTATAACAATAGTTAGTTATGAGGTG
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CTATCGATTCTTTAGAAAAAATTGAACCTGTTGGTAAGATTAGAAATTACATCGACATAAGTTTGAAGGTATCAAGCG
GGTCGAAAATGGAGTCTCAGCATTTTATCCCATGGCAATCTTTGGATACTATGGGAGCGGAAAAACATTCTTTGTGA
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GAGGAGGCAGAAAAAAGCAATGTGGAAGCGATACTGTTAGGATAAGCTGGGTCATAGCTGTTTCTGTGTGAAAT
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GCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAA
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TATCTCAGTTCGGTGTAGGTCGTTCCGCTCAAGCTGGGCTGTGTGCACGAACCCCCCGTTACGCCCCGACCGCT
GCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGTAAGACACGACTTATCGCCACTGGCAGCAGCCACT
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GAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTTCGTTTCATC
CATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAA
TGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACAGCCAGCCGGAAGGGCCGAGC
GCAGAAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTC
GCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTACGCTCGTTCGTTTGGTATGGCT
TCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCATGTTGTGCAAAAAAGCGGTTAGCTCC
TTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCGAGTGTATCACTCATGGTTATGGCAGCACTGCATAATT
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TGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTAAAAGTG
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AGCATTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCG
CGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACTATAAAAAATAGGC
GTATCACGAGGCCCTTTCGTC

Double stranded sequence of the insert pf0559-PdaD-pf0561

10	Ampl_Trafo PF0560 F	30	50	
GGTGCTTGATGCCTTCTCGGTACTTTTGAACGTTTCCTACAGGAGGCACTATGAGGGAGAGCCTGCGATG				70
CCACGAACTACGGAAGAGCCATGAAAACCTTGCAAAGGATGTCCTCCGTGATACTCCCTCTCGGACGCTAC				
80		100	120	
AAGCTGGAGAGCTTTGCATACCAAGGAAAGAACGATCTAAAGCTCACGGCTCCAATTGAAGGTGAGGAAA				140
TTCGACCTCTCGAAACGTATGGTTCCTTTCTTGCTAGATTTGAGTGCCGAGGTAACTTCCACTCCTTT				
150		170	190	
TAAAGGTTTCAGAGTTGTTTGAGGAAGTTCTTGAGCTGATGGGCAAGGCCAATCCTAAAGACATAGCTTA				210
ATTTCCAAAGTCTCAACAACTCCTTCAAGAACTCGACTACCCGTTCCGGTTAGGATTTCTGTATCGAAT				
220		240	260	
CTCCGTTCACTTAGCCTTAGCTAGGGCATTGCTGAAGTTAGCGTGGAGAAAGCTAAGGAGTTTGGAGCT				280
GAGGCAAGTGAATCGGAATCGATCCCGTAAACGACTTCAATCGACCTCTTCGATTCTCAAACCTCGA				
290		310	330	
AAAAGTGTGTTTTGGGTGGGGGAGTAGGGTACAATGAGCTAATAGTTAAGACGATAAGAAAGATAGTAG				350
TTTTGACAGCAAAACCCACCCCTCATCCCATGTTACTCGATTATCAATTCTGCTATTCTTTCTATCATC				
360		380	400	
AGGGGGAGGGCTAAGTTCTTAACAACTTACGAAGTCCCAGGGGAGATAATGGAATTAATGTAGGCCAG				420
TCCCCCTCCCGATTCCAAGAATTGTTGAATGCTTCAAGGGTCCCCTCTATTACCTTAATTACATCCGGTC				
430		450	470	
GCCTTCCTGGGAGGATTGTACTTGAAGGATACTTAAATAGGGAAGATTTGAGCATTTAGTTTGTCTCTA				490
CGGAAGGACCCTCCTAACATGAACCTTCCTATGAATTTATCCCTTCTAAACTCGTAAATCAAACAGAGAT				
500		520	540	
CCTAGGCCTTCCTTTTTTCTTATCTACAAAACACCGCAGATTAAACTTCTCCTACTTACGAAAACCTT				560
GGATCCGGAAGGAAAAAAGAATAGGATGTTTTGTGGCGTCAATTTGAAGAGGATGAATGCTTTTGAAA				
570		590	610	
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TTTTCTATTATTTAAACCATAGGCAAATCGCTCTCTTATATCCTCCCTCTTCTTACTCGACCTATTGAGG				
600		620	680	
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ATTTTTCCGGTATTACGAACGTCGCCGTCGACTTCCGCCCTGTTTCGAATTACGAAAGCTATTACGAGAT				
710		730	750	
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AATTTCTACCCTTAACCTTTACACTTGGATCATTTGAGTCGTCACAATATGGCCGAGTATATCTCACCG				
780		800	820	
TGGATGAGTTGCCAAAGAACATTCTATAGGGATGTTGTTACCAACAGTCTACACCCACATAGAGAGCGA				840
ACCTACTCAACGGTTTCTTGTAAGGATATCCCTACAACAATGGTTGTCAGATGTGGGTGTATCTCTCGCT				
850		870	890	
TGAGCCAGGTTCAACGATAAGCGCAGCTTTAGGTATTGGGCTTAGCGAAGGCAATGAAGGTGGGCTAATC				910
ACTCGGTCCAAGTTGCTATTCGCGTCGAAATCCATAACCCGAATCGCTTCCGTTACTTCCACCCGATTAG				
920		940	960	
TATGAATATTACAGGATACTGCAAGAAGGAAGAGGCTGAGGAAATGGTTAAGAAGATGGTTGAGGAAGGTT				980
ATACTTATAAGTCCTATGACGTTCTTCTTCTCCGACTCCTTTACCAATTCTTACCAACTCCTTCCAA				
990		1010	1030	
TTAGGGTTAGAGGATGGAACTCAAGGAAATAAAGATAGTCTCCGCTGAAATAACGGTTAAGGACAAGCC				1050
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1060	1080	1100	
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TCGCCGACGTCAACGACGTCATCAATACAAGGGTATGATCGTTCTCGAAAAGAATGGAGAAGATAATGGA			
1130	1150	1170	
CATCATTGCAATCCCAACACATAACCTCAAAGCTGGGAACTCTTACACTAATCCTCACTTTTCTCTTTCT			1190
GTAGTAACGTTAGGGTTGTGATTGGAGTTTCGACCCTTGAGAATGTGATTAGGAGTGAAAAGAGAAAGA			
1200	1220	1240	
TGCTATTTTGTCACTTCATAACCATGTCTAGGAATTTAACTCTATCGGAAAGTATTTAAGAGCTATTAT			1260
ACGATAAAACGAGTGAAGTATTGGTACAGATCCTTAAATTGAGATAGCCTTTCATAAATTCTCGATAATA			
Agma_RK-R			
1270	1290	1310	
AACAAATAGTTAGTTATGAGGTGAATTACCATGACGCCCATGGAGACATCTAATGAGCAAATTTATAATAT			1330
TTGTTATCAATCAATACTCCACTTAATGGTACTGCGGGTACCTCTGTAGATTACTCGTTTAAATATTATA			
1350	1370	1380	
CTATCTTCCTAAGGTAGCAGAAGAGTCTCCTATCGATTCTTTAGAAAAAATTGAACCTGTTGGTAAGATT			1400
GATAGAAGGATTCCATCGTCTTCTCAGAGGATAGCTAAGAAATCTTTTTTAACTTGGACAACCATTCTAA			
1410	1430	1450	
AGAAATTACATCGACATAAGTTTTGAAGGTATCAAGCGGGTCGAAAATGGAGTCTCAGCATTTTATCCCA			1470
TCTTTAATGTAGCTGTATTCAAAACTTCCATAGTTCGCCAGCTTTTACCTCAGAGTCGTAAATAGGGT			
1480	1500	1520	
TGGCAATCTTTGGATACTATGGGAGCGGAAAAACATTCTTTGTGAGAAAGCTATCGGAGAAGATAGTGAA			1540
ACCGTTAGAAACCTATGATACCCTCGCCTTTTTGTAAAGAAACACTCTTTTCGATAGCCTCTTCTATCACTT			
1550	1570	1590	
AGAGTTGGAAAGAACTATACCTTTGCACTTTTATCTAGGACAGACATCTTATTTGACTTGATTAAATTT			1610
TCTCAACCTTTCTTGATATGGAAACGTGAAAATAGATCCTGTCTGTAGAATAAAGCTGAACTAATTTAAA			
1620	1640	1660	
GTGGACGAACCTGATAGAAAGTATAGAGGTCTTTATTGAAAGAGGAGACAAAACCTAAGGCAACGATAAGTGG			1681
CACCTGCTTGACTATCTTTCATATCTCCAGAAATAACTTTCTCCTCTGTTTGTATTCCGTTGCTATTCACC			

AmpI_Trafo PF0560 R

Single stranded sequence of the Insert pf0559-PdaD-pf0561

<i>pf0559</i> (upstream of <i>pf0560</i>):	1-514
<i>pf1623</i> (PdaD):	515-1213
<i>pf0561</i> (downstream of <i>pf0560</i>):	1214-1682

GGTGCTTGATGCCTTCTCGGTACTTTTGAACGTTTCCTACAGGAGGCACTATGAGGGAGAGCCTGCGATGAAGCT
GGAGAGCTTTGCATACCAAGGAAAGAACGATCTAAAGCTCACGGCTCCAATTGAAGGTGAGGAAATAAAGGTTTC
AGAGTTGTTTGAGGAAGTTCTTGAGCTGATGGGCAAGGCCAATCCTAAAGACATAGCTTACTCCGTTCACTTAGC
CTTAGCTAGGGCATTGCTGAAGTTAGCGTGGAGAAAGCTAAGGAGTTTGGAGCTAAAAGTGTCTGTTTTGGGTGG
GGGAGTAGGGTACAATGAGCTAATAGTTAAGACGATAAGAAAGATAGTAGAGGGGAGAGGGCTAAGGTTCTTAAC
AACTTACGAAGTTCCCAGGGGAGATAATGGAATTAATGTAGGCCAGGCCTTCCTGGGAGGATTGTACTTGGAAGG
ATACTTAAATAGGGAAGATTTGAGCATTTAGTTTGTCTCTACCTAGGCCTTCCTTTTTTCTTATCCTACAAAAC
ACCGCAGATTAAACTTCTCCTACTTACGAAAACCTTTAAAGATAATAAATTTGGTATCCGTTTAGCGAGAGAATA
TAGGAGGGGAGAAGAATGAGCTGGATAACTCCTAAAAAGGCCATAATGCTTGACGCGGCAGCTGAAGGCGGGACAA
AGCTTAATGCTTTGATAATGCTCTATTAAGATGGGAATTGGAAATGTGAACCTAGTAAAGCTCAGCAGTGTTA
TACCGGCTCATATAGAGTGGCTGGATGAGTTGCCAAAGAACATTCTATAGGGATGTTGTTACCAACAGTCTACA
CCCACATAGAGAGCGATGAGCCAGGTTCAACGATAAGCGCAGCTTTAGGTATTGGGCTTAGCGAAGGCAATGAAG
GTGGGCTAATCTATGAATATTCAGGATACTGCAAGAAGGAAGAGGCTGAGGAAATGGTTAAGAAGATGGTTGAGG
AAGGTTTTAGGGTTAGAGGATGGAACTCAAGGAAATAAGATAGTCTCCGCTGAAATAACGGTTAAGGACAAGC
CAGCGGCTGCAGTTGCTGCAGTAGTTATGTTCCCATCTAGCAAGAGCTTTTCTTACCTCTTCTATTACCTCATC
ATTGCAATCCCAACACATAACCTCAAAGCTGGGAACTCTTACACTAATCCTCACTTTTCTCTTTCTTGCTATTTT
GCTCACTTCATAACCATGTCTAGGAATTTAACTCTATCGGAAAGTATTTAAGAGCTATTATAACAATAGTTAGTT
ATGAGGTGAATTACCATGACGCCCATGGAGACATCTAATGAGCAAATTTATAATATCTATCTTCCTAAGGTAGCA
GAAGAGTCTCCTATCGATTCTTTAGAAAAAATTGAACCTGTTGGTAAGATTAGAAATTACATCGACATAAGTTTT
GAAGGTATCAAGCGGGTCGAAAATGGAGTCTCAGCATTTTATCCCATGGCAATCTTTGGATACTATGGGAGCGGA
AAAACATTCTTTGTGAGAAAGCTATCGGAGAAGATAGTGAAAGAGTTGGAAAGAACTATACCTTTGCACTTTTAT
CTAGGACAGACATCTTATTTGACTTGATTAATTTGTGGACGAACTGATAGAAAGTATAGAGGTCTTTATTGAA
AGAGGAGACAAAACTAAGGCAACGATAAGTGG

Construct for the deletion of *pf1777*

Double stranded region of the construct pUC19-pf1776-PdaD-pf1778:

```

      10              30              50
TCGCGCGTTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCT
AGCGCGCAAAGCCACTACTGCCACTTTTGGAGACTGTGTACGTCGAGGGCCTCTGCCAGTGTGGAACAGA
70

      80              100             120
GTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGGGCTGG
CATTGCGCTACGGCCCTCGTCTGTTGCGGCAGTCCCGCGCAGTCGCCCACAACCGCCACAGCCCCGACC
140

      150             170             190
CTTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCATATGCGGTGTGAAATACCGCACAGAT
GAATTGATACGCCGTAGTCTCGTCTAACATGACTCTCACGTGGTATACGCCACACTTTATGGCGTGTCTA
210

      220             240             260
GCGTAAGGAGAAAATACCGCATCAGGCGCCATTGCCATTAGGCTGCGCAACTGTTGGGAAGGGCGATC
CGCATTCTCTTTTATGGCGTAGTCCGCGGTAAGCGGTAAAGTCCGACGCGTTGACAACCTTCCCGCTAG
280

      290             310             330
GGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTA
CCACGCCCCGAGAAGCGATAATGCGGTCGACCGCTTCCCCCTACACGACGTTCCGCTAATTCAACCCAT
350

      360             380      pUC_M13F_UpTransp_F      400
ACGCCAGGGTTTTCCAGTCACGACGTTGTAAAACGACGGCCAGTCCGCTTCAAGTTGATTCATTCTCCG
TGGGTCCCAAAGGGTCAGTGCTGCAACATTTTGTGCGGGTCAGGCGAAGTTCAACTAAGTAAGAGGC
420

                                Ampl_pUC R
      430             450             470
CCACCCCATCTTAAGTTAACAAAAATTTGTATAAATGAGTTTCGGAAAGGTTTGGAAAGAAAGCAAAGAA
GGTGGGGTAGAATTCAATTGTTTTTAAACATATTTACTCAAAGCCTTTCAAACCTTCTTTTCGTTTCTT
490

      500             520             540
GTCTTCGAAATCCTTAAAAACCCATTAGGAATCATCGTAAGTGGGAAAGGGCCCGTGGTCTAGACTGGTT
CAGAAGCTTTAGGAATTTTGGGTAATCCTTAGTAGCATTACCCCTTTCGGGGCACCAGATCTGACCAA
560

      570             590             610
ATGACGCCGCCCTCACAAGGCGGAGGTCCGGGGTTCAAATCCCCGCGGGCCACCAAAAGCGCAGAGATG
TACTGCGGCGGGAGTGTTCCGCCTCCAGGCCCAAGTTTAGGGGCGCCGGGTGGTTTTTCGCGTCTCTAC
630

      640             660      Ampl_Trafo_Transp_F      680
ATGACCGGTTTCGGATCCGATGGATGAGGACATCAGCTATCGCTGATCTCTATTCTATTCTAATTCCCC
TACTGGCCAAAGCCTAGGCTACCTACTCCTGTAGTCGATAGCGACTAGAGAGTAAGATAAGATTAAGGGG
700

      710             730             750
AAAATCCAATCTGTGACCTGGCTCCAGCTGAGGGGAATGGAGTATGTTTGATAAAGTCTTATGGAAAAC
TTTTAGGTTGAGACACTGGACCGAGGTCGACTCCCCTTACCTCATACAACTATTTGAGAATACCTTTTG
770

      780             800             820
AAAATAAGCGCTGCTCTTTTCCATCCTCTTTGCAGCCACACTTATAAACTCCCTCTTAAGCTCTGAATTT
TTTTATTGCGGACGAGAAAAAGGTAGGAGAAACGTCGGTGTGAATATTTGAGGGAGAATTCGAGACTTAAA
840

      860             870             890
TTGTGGAACCTTTATAAGTGCTGAAAACCTTCTCTTGTGTCTAGGTCTTTGAGTGTTCCCTAGGTATAAAG
AACAGCTTGAAATATTCACGACTTTTGAAGAGAAGACACAGATCCAGAACTCACAAGGATCCATATTTT
910
```

920 940 960
 AAGGCGTCCCATATGCAAATCCAACAACATCAATTCTTTTTGGAGGATTAACCTCCCTTTTCATTTTTTC 980
 TTCCGCAGGGTATACGTTTAGGTTGTTGTAGTTAAGAAAAACCTCCTAATTGGAGGGAAAAAGTAAAAAG
 990 1010 1030
 TGTGATAAAGCTGTATACACCCTCTCCTATTTTTGAGTTGTACTTGATTTCTTTCTTTACAACCTATTCCC 1050
 ACACTATTTTCGACATATGTGGGAGAGGATAAAAACTCAACATGAATAAAGAAAGAAATGTTGATAAGGG
 1060 1080 1100
 TCAAGGGTGTTAAATATCTCCAGCCTACTAACCATCAACTTCGGATATTTTTTCGATTATACTATAAATT 1120
 AGTTCCCACAATTTATAGAGGTCGGATGATTGGTAGTTGAAGCCTATAAAAAAGCTAATATGATATTTAA
 1150 1170 1190 Agma_Rk-F
 TTTCCGAAAATTTTTGACAAATTGTTAAACGAGTTAATTACCTCATATGCAGAACCC**TCCTACAAAACA** 1190
 AAAGGCTTTTAAAAAAGTGTAAACAATTG**CTCAATTAATGGAGTATACGCTTTGGGAGGATGTTTTGT**
 Agma_Upst_Transp_R
 1200 1220 1240
CCGCAGATTAAACTTCTCCTACTTACGAAAACTTTAAAAAGATAATAAATTGGTATCCGTTTAGCGAGAG 1260
GGCGTCTAATTTGAAGAGGATGAATGCTTTTGAAATTTCTATTATTTAAACCATAGGCAAAATCGCTCTC
 1270 1290 1310
 AATATAGGAGGGGAGAAGAATGAGCTGGATAACTCCTAAAAAGGCCATAATGCTTGCAGCGGCAGCTGAAG 1330
 TTATATCCTCCCTCTTCTTACTCGACCTATTGAGGATTTTTCCGGTATTACGAACGTGCGCGTCGACTTC
 1340 1360 1380
 GCGGGACAAAGCTTAATGCTTTTCGATAATGCTCTATTAAAGATGGGAATTGGAAATGTGAACCTAGTAAA 1400
 CGCCCTGTTTCGAATTACGAAAGCTATTACGAGATAATTTCTACCCTTAACCTTTACACTTGATCATT
 1410 1430 1450
 GCTCAGCAGTGTTATACCGGCTCATATAGAGTGGCTGGATGAGTTGCCAAAGAACATTCTATAGGGATG 1470
 CGAGTCGTCACAATATGGCCGAGTATATCTCACCGACCTACTCAACGGTTTTCTTGTAAGGATATCCCTAC
 1480 1500 1520
 TTGTTACCAACAGTCTACACCCACATAGAGAGCGATGAGCCAGGTTCAACGATAAGCGCAGCTTTAGGTA 1540
 AACAATGGTTGTCAGATGTGGGTGTATCTCTCGCTACTCGGTCCAAGTTGCTATTGCGCTCGAAATCCAT
 1550 1570 1590
 TTGGGCTT**AGCGAAGGCAATGAAGGTGGGCTAATCTATGAATATTCAGGATACTGCAAGAAGGAAGAGGC** 1610
AACCCGAATCGCTTCCGTTACTTCCACCCGATTAGATACTTATAAGTCTATGACGTTCTTCTTCTCCG
 1620 1640 1660
 TGAGGAAATGGTTAAGAAGATGGTTGAGGAAGTTTTAGGGTTAGAGGATGGAACTCAAGGAAATAAAG 1680
 ACTCCTTTACCAATTCTTCTACCAACTCCTTCCAAAATCCCAATCTCTACCTTTGAGTTCCTTTATTTT
 1690 1710 1730
 ATAGTCTCCGCTGAAATAACGGTTAAGGACAAGCCAGCGGCTGCAGTTGCTGCAGTAGTTATGTTCCCAT 1750
 TATCAGAGGCGACTTTATTGCCAATTCCTGTTTCGGTCGCGGACGTCAACGACGTCATCAATAAAGGGTA
 1760 1780 1800
 ACTAGCAAGAGCTTTTCTTACCTCTTCTATTACCTCATCATTGCAATCCCAACACATAACCTCAAAGCTG 1820
 TGATCGTTCTCGAAAAGAATGGAGAAGATAATGGAGTAGTAACGTTAGGGTTGTGTATTGGAGTTTCGAC
 1830 1850 1870 Agma_DowTransp_F
 GGAACCTTACACTAATCCTCACTTTTCTCTTTCTT**GCTATTTTGCTCACTTCATAACAGTATCCAGGAG** 1890
 CCTTGAGAATGTGATTAGGAGTGAAAAGAG**AAAGAACGATAAAACGAGTGAAGTATT**GTTCATAGGTCCTC
 Agma_Rk-R
 1900 1920 1940
GATTAGGTAACGTTTATATCTTTTTTTGAATGTCATTTTTCCAGGGGAGAAAGATGGAAGTAGAAAAGCTT 1960
 CTAATCCATTGCAAATATAGAAAAAAGTTACAGTAAAAAGGTCCCCTCTTCTACCTTCATCTTTTCGAA
 1970 1990 2010
 GCACTCAAGTACGCTCTAATAAATGCAATAGAGCACGACGGTAAAGCTAATCCAAAGGCAGTCATAGGGA 2030
 CGTGAGTTCATGCGAGATTATTTACGTTATCTCGTGCTGCCATTTGATTAGGTTTCCGTCAGTATCCCT
 2040 2060 2080
 AAATACTCGGAGAAAACCCCGAGCTCAGAAAGAAGGCCAGAGAAATTGTTCCAATAGTTAATAAAATTGT 2100
 TTTATGAGCCTCTTTTGGGGCTCGAGTCTTTCTCCGGTCTCTTTAACAAGGTTATCAATTATTTTAACA

2110	2130	2150	
AGAGGAAGTTAATACCCTCTCACTGGAAGAGCAGAAAAAGAACTTGAAGAAATTTATCCAGAGTACTTC			2170
TCTCCTTCAATTATGGGAGAGTGACCTTCTCGTCTTTTCTTTGAACTTCTTTAAATAGGTCTCATGAAG			
2180	2200	2220	
ATGGAAGGCACTAAAAAGAAAAAGAGGAGAAAAAGCAACTCCCCCACTACCTAAAGCAGAAAAGGGTA			2240
TACCTTCCGTGATTTTTCTTTTTCTCTCTTTTTCGTTGAGGGGGGTGATGGATTCGTCTTTTCCCAT			
2250	2270	2290	
AGGTTGTAACAAGATTTGCTCCAAATCCAGACGGTGCTTTTCACCTGGGGAATGCTAGAGCAGCAATATT			2310
TCCAACATTGTTCTAAACGAGGTTTAGGTCTGCCACGAAAAAGTGGACCCCTTACGATCTCGTCGTTATAA			
2320	2340	2360	
AAGCTATGAGTATGCCAAAATGTACGATGGAAAGTTTATTCTAAGGTTTCGATGACACGGATCCAAAGGTA			2380
TTCGATACTCATACGGTTTTACATGCTACCTTTCAAATAAGATTCCAAGCTACTGTGCCTAGGTTTCCAT			
			Ampl_Trafo_Transp_R
2390	2410	2430	
AAGAGGCCAGAGCTAATATTCTACGACATGATAATAGAAGACTTAGAATGGTTAGGGATAAAACCAGACG			2450
TTCTCCGGTCTCGATTATAAGATGCTGTACTATTATCTTCTGAATCTTACCAATCCCTATTTTGGTCTGC			
2460	2480	2500	
AAATTGTTTATGCAAGTGATAGGCTTGAAATTTATTACAAATACGCAGAAGAGCTCATAAAAATGGGAAA			2520
TTTAACAAATACGTTCACTATCCGAACCTTTAAATAATGTTTATGCGTCTTCTCGAGTATTTTTACCTTT			
2530	2550	2570	
GGCATATGTATGTACTTGCCCAACCAGATGAGTTTAGGAAGTTAAGGGATCAAGGAATTGCTTGCCCCCAT			2590
CCGTATACATACATGAACGGGTGGTCTACTCAAATCCTTCAATTCCTAGTTCCTTAACGAACGGGGTA			
2600			
AGAGATGAACCCGGGTCATAGCTGTTTCCTGTGTG			pUC_Amp_F
TCTCTACTTGGGCCAGTATCGACAAAGGACAC...			2660
			pUC_M13R_DowTransp_R

After position 2625, the sequence of the vector pUC19 follows with the same sequence as in the double stranded sequence of the construct pUC19-*pf0559*-PdaD-*pf0561* at page 77. (corresponding sequence at position: 4004-4626)

Double stranded sequence of the insert pf1776-PdaD-pf1778:

10 **AmpI_Trafo_Transp_ F** 30 50
GGATGAGGACATCAGCTATCGCTGATCTCTCATTCTATTCTAATTCCTCCAAAATCCAACCTCTGTGACCTG 70
CCTACTCCTGTAGTCGATAGCGACTAGAGAGTAAGATAAGATTAAGGGGTTTTAGGTTGAGACACTGGAC
80 100 120
GCTCCAGCTGAGGGGAATGGAGTATGTTTGATAAAGTCTTATGGAAAACAAAATAAGCGCTGCTCTTTTC 140
CGAGGTGCGACTCCCCTTACCTCATACAACTATTTTCAAGAATACCTTTTGTTTTATTTCGCGACGAGAAAAG
150 170 190
CATCCTCTTTGCAGCCACACTTATAAACTCCCTCTTAAGCTCTGAATTTTTGTGCGAACTTTATAAGTGCT 210
GTAGGAGAAACGTCGGTGTGAATATTTGAGGGAGAATTCGAGACTTAAAAACAGCTTGAAATATTCACGA
220 240 260
GAAAACTTCTTTCTGTGTCTAGGTCTTTGAGTGTTCCTAGGTATAAAGAAGGCGTCCCATATGCAAATC 280
CTTTTGAAGAGAAGACACAGATCCAGAACTCACAAAGGATCCATATTTCTTCCGCAGGGTATACGTTTAG
290 310 330
CAACAACATCAATTCTTTTTGGAGGATTAACCTCCCTTTTCATTTTTCTGTGATAAAGCTGTATACACC 350
GTTGTTGTAGTTAAGAAAAACCTCCTAATTGGAGGGAAAAGTAAAAAAGACACTATTTTCGACATATGTGG
360 380 400
CTCTCCTATTTTTGAGTTGTACTTGATTTCTTTCTTACAACCTATTCCTCAAGGGTGTTAAATATCTCC 420
GAGAGGATAAAAACTCAACATGAATAAGAAAGAAATGTTGATAAGGGAGTCCCACAATTTATAGAGG
430 450 470
AGCCTACTAACCATCAACTTCGGATATTTTTTCGATTATACTATAAATTTTTCCGAAAATTTTTTGACAA 490
TCGGATGAT TGGTAGTTGAAGCCTATAAAAAAGCTAATATGATATTTAAAAAGGCTTTTAAAAAACTGTT
500 520 **Agma_RK-F** 540
ATTGTTAAACGAGTTAATTACCTCATATGCAGAACCC**TCCTACAAAACACCGCAGATTAAAC**TTCTCCTA 560
TAACAATTTGCTCAATTAATGGAGTATACGTCTTGGGAGGATGTTTTGTGGCGTCTAATTTG AAGAGGAT
570 590 610
CTTACGAAAACTTTAAAAGATAATAAATTTGGTATCCGTTTAGCGAGAGAATATAGGAGGGAGAAGAATG 630
GAATGCTTTTGAAATTTCTATTATTTAAACCATAGGCAAATCGCTCTCTTATATCCTCCCTCTTCTTAC
640 660 680
AGCTGGATAACTCCTAAAAAGGCCATAATGCTTGACGCGCAGCTGAAGGCGGGACAAAGCTTAATGCTT 700
TCGACCTATTGAGGATTTTCCGGTATTACGAACGTCGCCGTCGACTTCGCCCTGTTTCGAATTACGAA
710 730 750
TCGATAATGCTCTATTAAAGATGGGAATTGGAAATGTGAACCTAGTAAAGCTCAGCAGTGTTATACCGGC 770
AGCTATTACGAGATAATTTCTACCCTTAACCTTTACACTTGATCATTTTCGAGTCGTACAATATGGCCG
780 800 820
TCATATAGAGTGGCTGGATGAGTTGCCAAAGAACATTCTATAGGGATGTTGTTACCAACAGTCTACACC 840
AGTATATCTCACCGACCTACTCAACGGTTTTCTTGTAAGGATATCCCTACAACAATGGTTGTCAGATGTGG
850 870 890
CACATAGAGAGCGATGAGCCAGGTTCAACGATAAGCGCAGCTTTAGGTATTGGGCTTAGCGAAGGCAATG 910
GTGTATCTCTCGCTACTCGGTCCAAGTTGCTATTCGCGTCGAAATCCATAACCCGAATCGCTTCCGTTAC
920 940 960
AAGGTGGGCTAATCTATGAATATTCAAGATACTGCAAGAAGGAAGAGGCTGAGGAAATGGTTAAGAAGAT 980
TTCCACCCGATTAGATACTTATAAGTCCTATGACGTTCTTCCTTCTCCGACTCCTTACCAATTCTTCTA
990 1010 1030
GGTTGAGGAAGGTTTTAGGGTTAGAGGATGGAACTCAAGGAAATAAAGATAGTCTCCGCTGAAATAACG 1050
CCAACCTCCTTCCAAAATCCCAATCTCCTACCTTTGAGTTCCTTTATTTCTATCAGAGGCGACTTTATTGC
1060 1080 1100
GTTAAGGACAAGCCAGCGGCTGCAGTTGCTGCAGTAGTTATGTTCCATACTAGCAAGAGCTTTTCTTAC 1120
CAATTCCTGTTTCGGTCGCCGACGTCAACGACGTCATCAATACAAGGGTATGATCGTTCTCGAAAAGAATG

1130	1150	1170	
CTCTTCTATTACCTCATCATTGCAATCCCAACACATAACCTCAAAGCTGGGAACTCTTACACTAATCCTC			1190
GAGAAGATAATGGAGTAGTAACGTTAGGGTTGTGTATTGGAGTTTCGACCCTTGAGAATGTGATTAGGAG			
1200	1220	1240	
ACTTTTCTCTTTCTTGCTATTTTGCTCACTTCATAACAGTATCCAGGAGGATTAGGTAACGTTTATATCT			1260
TGAAAAGAGAAAGAACGATAAAACGAGTGAAGTATTGTCATAGGTCCTCCTAATCCATTGCAAATATAGA			
Agma_RK-R			
1270	1290	1310	
TTTTTGAATGTCATTTTTCCAGGGGAGAAAGATGGAAGTAGAAAAGCTTGCACTCAAGTACGCTCTAATA			1330
AAAAAATTACAGTAAAAAGGTCCCCTCTTTCTACCTTCATCTTTTCGAACGTGAGTTCATGCGAGATTAT			
1340	1360	1380	
AATGCAATAGAGCACGACGGTAAAGCTAATCCAAAGGCAGTCATAGGGAAAATACTCGGAGAAAACCCCG			1400
TTACGTTATCTCGTGCTGCCATTTTCGATTAGGTTCCGTCAGTATCCCTTTTATGAGCCTCTTTTGGGGC			
1410	1430	1450	
AGCTCAGAAAGAAGGCCAGAGAAATTGTTCCAATAGTTAATAAAATTGTAGAGGAAGTTAATACCCTCTC			1470
TCGAGTCTTTCTTCCGGTCTCTTTAACAAGTTATCAATTATTTTAACATCTCCTTCAATTATGGGAGAG			
1480	1500	1520	
ACTGGAAGAGCAGAAAAAGAACTTGAAGAAATTTATCCAGAGTACTTCATGGAAGGCACTAAAAAAGAA			1540
TGACCTTCTCGTCTTTTTCTTTGAACTTCTTTAAATAGGTCTCATGAAGTACCTCCGTGATTTTTTCTT			
1550	1570	1590	
AAAGAGGAGAAAAAGCAACTCCCCCACTACCTAAAGCAGAAAAGGGTAAGGTTGTAAACAAGATTTGCTC			1610
TTTCTCCTCTTTTTCGTTGAGGGGGGTGATGGATTCGTCCTTTTCCATTCCAACATTGTTCTAAACGAG			
1620	1640	1660	
CAAATCCAGACGGTGCTTTTCACCTGGGGAATGCTAGAGCAGCAATATTAAGCTATGAGTATGCCAAAAT			1680
GTTTAGGTCTGCCACGAAAAGTGGACCCCTTACGATCTCGTCGTTATAATTGATACTCATACGGTTTTA			
1690	1710	1730	
GTACGATGGAAAGTTTATTCTAAGGTTTCGATGACACGGATCCAAAGGTAAAGAGGCCAGAGC			1750
CATGCTACCTTTCAAATAAGATTCCAAGCTACTGTGCCTAGGTTTCATTTCTCCGGTCTCG			
Ampl_Trafo_Transp_ R			

Single stranded sequence of the insert pf1776-PdaD-pf1778:

pf1776 (upstream of pf1777):	1-527
pf1623 (PdaD):	528-1226
pf1778 (downstream of pf1777):	1227-1742

GGATGAGGACATCAGCTATCGCTGATCTCTCATTCTATTCTAATTCCCCAAAATCCAACCTCTGTGACCTGGCTCC
AGCTGAGGGGAATGGAGTATGTTTGATAAAGTCTTATGGAAAACAAAATAAGCGCTGCTCTTTTCCATCCTCTTT
GCAGCCACACTTATAAACTCCCTCTTAAGCTCTGAATTTTTGTGCAACTTTATAAGTGCTGAAAACCTTCTCTTCT
GTGTCTAGGTCTTTGAGTGTTCTAGGTATAAAGAAGGCGTCCCATATGCAAATCCAACAACATCAATTCTTTTT
GGAGGATTAACCTCCCTTTTCATTTTTTCTGTGATAAAGCTGTATACACCCTCTCCTATTTTTGAGTTGTACTTG
ATTTCTTTCTTTACAACCTATTCCCTCAAGGGTGTTAAATATCTCCAGCCTACTAACCATCAACTTCGGATATTTT
TTCGATTATACTATAAATTTTTCCGAAAATTTTTTGACAAAATTGTTAAACGAGTTAATTACCTCATATGCAGAAC
CCTCTACAAAACACCGCAGATTAACTTCTCCTACTTACGAAAACCTTTAAAAGATAATAAATTTGGTATCCGTT
TAGCGAGAGAATATAGGAGGGAGAAGAATGAGCTGGATAACTCCTAAAAAGGCCATAATGCTTGACGCGGCAGCT
GAAGGCGGGACAAAGCTTAATGCTTTTCGATAATGCTCTATTAAGATGGGAATTGGAAATGTGAACCTAGTAAAG
CTCAGCAGTGTTATACCGGCTCATATAGAGTGGCTGGATGAGTTGCCAAAGAACATTCCTATAGGGATGTTGTTA
CCAACAGTCTACACCCACATAGAGAGCGATGAGCCAGGTTCAACGATAAGCGCAGCTTTAGGTATTGGGCTTAGC
GAAGGCAATGAAGGTGGGCTAATCTATGAATATTCAGGATACTGCAAGAAGGAAGAGGCTGAGGAAATGGTTAAG
AAGATGGTTGAGGAAGGTTTTAGGGTTAGAGGATGGAACTCAAGGAAATAAAGATAGTCTCCGCTGAAATAACG
GTTAAGGACAAGCCAGCGGCTGCAGTTGCTGCAGTAGTTATGTTCCCATACTAGCAAGAGCTTTTCTTACCTCTT
CTATTACCTCATCATTGCAATCCCAACACATAACCTCAAAGCTGGGAACCTTACACTAATCCTCACTTTTCTCT
TTCTTGCTATTTTGCTCACTTCATAACAGTATCCAGGAGGATTAGGTAACGTTTATATCTTTTTTGAATGTCATT
TTTCCAGGGGAGAAAGATGGAAGTAGAAAAGCTTGCACTCAAGTACGCTCTAATAAATGCAATAGAGCACGACGG
TAAAGCTAATCCAAAGGCAGTCATAGGGAAAATACTCGGAGAAAACCCGAGCTCAGAAAAGAAGGCCAGAGAAAT
TGTTCCAATAGTTAATAAAATTGTAGAGGAAGTTAATACCCTCTCACTGGAAGAGCAGAAAAAGAACTTGAAGA
AATTTATCCAGAGTACTTCATGGAAGGCACTAAAAAAGAAAAAGAGGAGAAAAAGCAACTCCCCCACTACCTAA
AGCAGAAAAGGGTAAGGTTGTAACAAGATTTGCTCCAAATCCAGACGGTGCTTTTCACCTGGGGAATGCTAGAGC
AGCAATATTAAGCTATGAGTATGCCAAAATGTACGATGGAAAGTTTATTCTAAGGTTGATGACACGGATCCAAA
GGTAAAGAGGCCAGAGC

Construct for the deletion of *pf1018* (1. approach)

Double stranded sequence of the construct pUC19-pf1017-PdaD-pf1020:

```

      10              30              50
TCGCGCGTTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCT 70
AGCGCGCAAAGCCACTACTGCCACTTTTGGAGACTGTGTACGTCGAGGGCCTCTGCCAGTGTCGAACAGA

      80              100             120
GTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGGGCTGG 140
CATTCGCCTACGGCCCTCGTCTGTTTCGGGCAGTCCCGCGCAGTCGCCCACAACCGCCACAGCCCCGACC

      150             170             190
CTTAAGTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCATATGCGGTGTGAAATACCGCACAGAT 210
GAATTGATACGCCGTAGTCTCGTCTAACATGACTCTCACGTGGTATACGCCACACTTTATGGCGTGTCTA

      220             240             260
GCGTAAGGAGAAAATACCGCATCAGGCGCCATTGCCATTTCAGGCTGCGCAACTGTTGGGAAGGGCGATC 280
CGCATTCTCTTTTATGGCGTAGTCCGCGGTAAGCGGTAAGTCCGACGCGTTGACAACCCCTCCCGCTAG

      290             310             330
GGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAAGGGGGATGTGCTGCAAGGCGATTAAAGTTGGGTA 350
CCACGCCCCGAGAAGCGATAATGCGGTGACCGCTTTCCCCCTACACGACGTTCCGCTAATTCAACCCAT

      360             380             400
ACGCCAGGGTTTTCCAGTCACGACGTTGTAAACGACGGCCAGTGCCTCTTTCTTCTCCTACTCTATC 420
TGCGGTCCCAAAAGGGTCAGTGCTGCAACATTTTGTGTCGGGTACGCGGAGAAAGAAGAGGATGAGATAG

                                AmpL_pUC_R
      430             450             470
TGCAGTTATTATATATACCCTGTTTTCAAGAGCCCTTATGGGCATGGCTCTAGGAGCATAGGGCATAACT 490
ACGTCAATAATATATATGGGACAAAAGTTCTCGGGAATACCCGTACCGAGATCCTCGTATCCCGTATTGA

      490             520             540
AAATTGGCTGGATGAGCTATAATATCCGCTCCTTTCAAGGCAAGAGTTCTGGCAGATTTCAGGGAAAAACC 560
TTTAACCGACCTACTCGATATTATAGGCGAGGAAAGTTCCGTTCTCAAGACCGTCTAAGTCCCTTTTTTG

      570             590             610
AATCGAAGCATATCATAATTCCTACTCTTATAAACCCAGGTCAAATACATTAAAGCCAAGATCCCCCGG 630
TTAGCTTCGTATAGTATTAAGGATGAGAATATTTGGGGTCCAGTTTATGTAATTTTCGGTTCTAGGGGGCC

      640             660             680
GTCAAAGAATACCTTCTCCCTATAAAAGAGGTGGATCTTCTATATTTTCCAGGTAGCCAGTGGGACCT 700
CAGTTTCTTATGGAAGAGGGATATTTTCTCCACCTAGAAAGATATAAAAGGGTCCATCGGTACCCTGGA

      710             730             750
ACGAGAACTGCTGAATTGTATAAGCGATCGTTATATTTTTCAGCAGTACCTGCCACAATGTAGACCTCTA 770
TGCTCTTGACGACTTAACATATTCGCTAGCAATATAAAAAAGTCGTCATGGACGGTGTTACATCTGGAGAT

      780             800             820
AATCTCCAGCAAGGTCCATGAGAAAGGTAGTTGTTTCTCCTTCAGGTATTCTTTGAGCTATTTCAAATAC 840
TTAGAGGTGCTTCAGGTACTCTTCCATCAACAAAGAGGAAGTCCATAAGAACTCGATAAAGTTTATG

      850             870             890
TTCATCTCTGCTTTCAAATTTGTATCCAGTGTCAAAAAGCTCAGGGAGAACTATGAGTTTAGCTTCTCTC 910
AAGTAGAGACGAAAGTTTAAACATAGGTCACAGTTTTTCGAGTCCCTCTTGATACTCAAATCGAAGAGAG

      920             940             960
TTTGCTGCCTCTTTTACTAATTTTTTCAGCCTTTGAGAGATTCTTGTCAAGTTCAAGTATTTTAGGTTCCA 980
AAACGACGGAGAAAATGATTAAAAAGTCGGAACTCTCTAAGAACAGTTCAAGTTCATAAAATCCAAGGT

```

990 1010 1030
TTTGCACATAGGCAACCTTAACCATGATAACCCCCATATCTAGTTGCTTATTTAGTATTTCAATGTTTA 1050
AAACGTGTATCCGTTGGAATTGGTACTATTGGGGGTATAGATCAACGAATAAATCATAAAGTTACAAAT

1060 1080 1100
GATTATAAGAAAACTCTCAGATCTCCTAAGATCACATTTTTTCAATTCTGTTAGTTGCCTTGCCAATAA 1120
CTAATATTCTTTTTGAGAGTCTAGAGGATTCTAGTGTAAAAAGTTAAGACAATCAACGGAACGGTTATT

1130 1150 1170
AATAGTTTTAAGATGACAAAAGATTGAAAATTTTTGTGTCTGTATCGCATTCAATGATTATAATCAAAT 1190
TTATCAAAATTCTACTGTTTTCTAACTTTTAAAAACACAGACATAGCGTAAGTTACTAATATTAGTTTA

1200 1220 1240 Agma_Rk_F
AACCAGCCAAI I AAGCAAAATGTCCTAAGAACTTGGGGAAAATGTATCTACAAAACACCGCAGATTAAA 1260
TTGGTCGGTTAATTCTGTTTTAGTCTAAGAACTTGGGGAAAATGTAAGGATGTTTTGTGGCGTCTAATTT
Agma_Up_L41e_R

1270 1290 1310
CTTCTCCTACTTACGAAAACCTTTAAAAGATAATAAATTTGGTATCCGTTTAGCGAGAGAATATAGGAGGG 1330
GAAGAGGATGAATGCTTTTGAAATTTTCTATTATTTAAACCATAGGCAAATCGCTCTCTTATATCCTCCC

1340 1360 1380
AGAAGAATGAGCTGGATAACTCCTAAAAAGGCCATAATGCTTGACGCGGCAGCTGAAGGCGGGACAAAGC 1400
TCTTCTTACTCGACCTATTGAGGATTTTTCCGGTATTACGAACGTCGCCGTCGACTTCCGCCCTGTTTCG

1410 1430 1450
TTAATGCTTTCGATAATGCTCTATTAAGATGGGAATTGGAAATGTGAACCTAGTAAAGCTCAGCAGTGT 1470
AATTACGAAAGCTATTACGAGATAATTTCTACCTTAACCTTTACACTTGGATCATTTTCGAGTCGTCACA

1480 1500 1520
TATACCGGCTCATATAGAGTGGCTGGATGAGTTGCCAAAGAACATTCTATAGGGATGTTGTTACCAACA 1540
ATATGGCCGAGTATATCTCACCGACCTACTCAACGGTTTCTTGTAAGGATATCCCTACAACAATGGTTGT

1550 1570 1590
GTCTACACCCACATAGAGAGCGATGAGCCAGGTTCAACGATAAGCGCAGCTTTAGGTATTGGGCTTAGCG 1610
CAGATGTGGGTGTATCTCTCGCTACTCGGTCCAAGTTGCTATTTCGCGTCGAAATCCATAACCCGAATCGC

1620 1640 1660
AAGGCAATGAAGGTGGGCTAATCTATGAATATTCAGGATACTGCAAGAAGGAAGAGGCTGAGGAAATGGT 1680
TTCCGTTACTTCCACCCGATTAGATACTTATAAGTCCTATGACGTTCTTCTTCTCCGACTCCTTTACCA

1690 1710 1730
TAAGAAGATGGTTGAGGAAGGTTTTAGGGTTAGAGGATGGAAGCTCAAGGAAATAAAGATAGTCTCCGCT 1750
ATTCTTCTACCAACTCCTTCCAAAATCCCAATCTCTACCTTTGAGTTCTTTATTTCTATCAGAGGCGA

1760 1780 1800
GAAATAACGGTTAAGGACAAGCCAGCGGCTGCAGTTGCTGCAGTAGTTATGTTCCCATAGCAAGAGC 1820
CTTTATTGCCAATTCCTGTTTCGGTCGCCGACGTCAACGACGTCAATACAAGGGTATGATCGTTCTCG

1830 1850 1870
TTTTCTTACCTCTTCTATTACCTCATCTTGAATCCCAACACATAACCTCAAAGCTGGGAACCTTTACA 1890
AAAAGAATGGAGAAGATAATGGAGTAGTAACGTTAGGGTTGTGTATTGGAGTTTCGACCCTTGAGAATGT

1900 1920 1940 Agma_Down_L41e_F
CTAATCCTCACTTTTCTTTCTTGCTATTTTGCTCACTTCATAACTAATCAACGATCTATATAGAATAC 1960
GATTAGGAGTGAAAAGAGAAAGAACGATAAAACGAGTGAAGTATTGATTAGTTGCTAGATATATCTTATG
Agma_Rk_R

19970 19990 2010
TGAGCTATATATTTTCTATAGCGGTAAAATATTTATTTGGAGGGAAACACATGCAACTGGGGTTCGCCAT 2030
ACTCGATATATAAAAGATATCGCCATTTTATAAATAAACCTCCCTTTGTGTACGTTGACCCCAAGCGGTA

2040 2060 2080
GCTTGCTGATCCAATTTTGTAAATTACGATACTTTTGGGATTTGCAATGGCCTGGGCAATAGGTGCAAAT 2100
CGAACGACTAGGTTAAACAATTAATGCTATGAAAACCTAAACGTTACCGGACCCGTTATCCACGTTTA

2110 2130 2150
GATGCTGCTAATTCCATGAGCACCGCCGTAGGTGCAGGAGCAATAACACCCAGACAGGCCGTGATAATTG 2170
CTACGACGATTAAGGTACTCGTGGCGGCATCCACGTCCTCGTTATTGTGGGTCTGTCCGGCACTATTAAC

2180	2200	2220	
CTGGAGTTCTTGAATTTATGGGAGCTTATTTCTTTGGAAAGACAGTTACAGAGACAATTAGAAAAGGCAT			2240
GACCTCAAGAACTTAAATACCCTCGAATAAAGAAACCTTTCTGTCAATGTCTCTGTTAATCTTTTCCGTA			
2250	2270	2290	
CATCGATCCCTCAAAAATTACAGATCCAAATGTCTTAATATTTGGCTCAATAGCGGCATTAATTGGTGCA			2310
GTAGCTAGGGAGTTTTTAATGTCTAGGTTTACAGAATTATAAACCGAGTTATCGCCGTAATTAACCACGT			
2320	2340	2360	
ACAATATGGCTAGTAATCGCAACAAAATATGGACTTCCAGTATCTACTACTCACTCAATAATAGGGGGGA			2380
TGTTATACCGATCATTAGCGTTGTTTTATACCTGAAGGTCATAGATGATGAGTGAGTTATTATCCCCCT			
2390	2410	2430	
TAGTGGGTTATGGAATTGTTTATGGAGGAATGTCAATAGTAAATTGGGATAAGATGATTAAAGTAGTTCT			2450
ATCACCCAATACCTTAACAAATACCTCCTTACAGTTATCATTTAACCTATTCTACTAATTTTCATCAAGA			
2460	2480	2500	
CAGTTGGATACTCTCCCCAATCGTCGGAGCAATCTTTCATACCTAGTATTCAGAGCGCTAAGTAAAACG			2520
GTCAA CCTATGAGAGGGGTTAGCAGCC TCGTTAGAAACGTATGGATCATAAGTCTCGCGATTCAATTTGC			
AmpI_Trafo_L41e_R			
2530	2550	2570	
GTTCTTCAAAGCAAGGACCCTGTGAAAAGTGCAAAGAGATGGTCTCCATTCTGGATAGGGCTAGCATTTG			2590
CAAGAAGTTTCGTTCCCTGGGACACTTTTTCACGTTTCTCTACCAGAGGTAAGACCTATCCCGATCGTAAAC			
2600	2620	2640	
TGGTCATTGGAACAATGTTCTACATAAAGGTTCTACACGGTAATTCATTACTTGAAGGATTTCTCAAATA			2660
ACCAGTAACCTTGTTACAAGATGTATTTCCAAGATGTGCCATTAAGTAATGAACCTCCTAAAGAGTTTAT			
2670	2690	2710	
CGGAATGCCAGCTGGAATTCTAACATTCATAGTAGTCTCCTTAATAATGGAAAAGAGATTTCCAGCCACT			2730
GCCTTACGGTCGACCTTAAGATTGTAAGTATCATCAGAGGAATTATAACCTTTTCTCTAAAGGTCGGTGA			
2740	AmpI_pUC_F		
GATCCCTATCTTGGGTCATAGCTGTTTCCTGTGTG			2800
CTAGGGATAGAACCCAGTATCGACAAAGGACACAC...			
pUC_M13R_Down_L41e_R			

After position 2765, the sequence of the vector pUC19 follows with the same sequence as in the double stranded sequence of the construct pUC19-*pf0559*-PdaD-*pf0561* at page 77. (corresponding sequence at position: 2426-4626)

Double stranded sequence of the insert of the construct pf1017-PdaD-pf1020:

10	Ampl_Trafo_L41e_ F	30	50	
GCAGTACCTGCCACAATGTAGACCTCTAAATCTCCAGCAAGGTCCATGAGAAAGGTAGTTGTTTCTCCTT				70
CGTCATGGACGGTGTTACATCTGGAGATTTAGAGGTCGTTCCAGGTACTCTTCCATCAACAAAGAGGAA				
80		100	120	
CAGGTATTCTTTGAGCTATTTCAAATACTTCATCTCTGCTTTCAAATTGTATCCAGTGTCAAAAAGCTC				140
GTCCATAAGAACTCGATAAAGTTTATGAAGTAGAGACGAAAGTTTTAACATAGGTCACAGTTTTTCGAG				
150		170	190	
AGGGGAACTATGAGTTTAGCTTCTCTCTTGTGCTCTTTTACTAATTTTCAGCCTTTGAGAGATTC				210
TCCCTCTTGATACTCAAATCGAAGAGAGAAACGACGGAGAAAATGATTAAAAAGTCGGAACTCTCTAAG				
220		240	260	
TTGTCAAGTTCAAGTATTTTAGGTTCCATTTGCACATAGGCAACCTTAACCATGATAACCCCCCATATCT				280
AACAGTTCAAGTTCATAAAATCCAAGGTAAACGTGTATCCGTTGGAATTGGTACTATTGGGGGGTATAGA				
290		310	330	
AGTTGCTTATTTAGTATTTCAATGTTTAGATTATAAGAAAACTCTCAGATCTCCTAAGATCACATTTTT				350
TCAACGAATAAATCATAAAGTTACAAATCTAATATCTTTTTGAGAGTCTAGAGGATTCTAGTGTA AAAA				
360		380	400	
TCAATTCTGTTAGTTGCCTTGCCAATAAAATAGTTTTAAGATGACAAAAGATTGAAAATTTTTTGTGTCT				420
AGTTAAGACAATCAACGGAACGGTTATTTTATCAAAATTCTACTGTTTTCTAACTTTTAAAAACACAGA				
430		450	470	
GTATCGCATTCAATGATTATAATCAAATAACCAGCCAATTAAGCAAAATGTCCTAAGAACTTGGGGAAAA				490
CATAGCGTAAGTTACTAATATTAGTTTATTGGTCGGTTAATTCGTTTTACAGGATTCCTGAACCCCTTTT				
500	Agma_RK-F	520	540	
TGTATCTTACAAAACACCGCAGATTAACTTCTCCTACTTACGAAAACCTTTAAAAGATAATAAATTTGGT				560
ACATAGGATGTTTTGTGGCGTCTAATTTGAAGAGGATGAATGCTTTTTGAAATTTTCTATTATTTAAACCA				
570		590	610	
ATCCGTTTAGCGAGAGAATATAGGAGGGGAGAAGAATGAGCTGGATAACTCCTAAAAAGGCCATAATGCTT				630
TAGGCAAATCGCTCTCTTATATCCTCCCTCTTCTACTCGACCTATTGAGGATTTTCCGGTATTACGAA				
640		660	680	
GCAGCGGCAGCTGAAGGCGGGACAAAGCTTAATGCTTTCGATAATGCTCTATTAAAGATGGGAATTGGAA				700
CGTCGCCGTCGACTTCCGCCCTGTTTCGAATTACGAAAGCTATTACGAGATAATTTCTACCCCTTAACCTT				
710		730	750	
ATGTGAACCTAGTAAAGCTCAGCAGTGTTATACCGGCTCATATAGAGTGGCTGGATGAGTTGCCAAAGAA				770
TACACTTGGATCATTTGAGTCGTCACAATATGGCCGAGTATATCTCACCGACCTACTCAACGGTTTCTT				
780		800	820	
CATTCTATAGGGATGTTGTTACCAACAGTCTACACCACATAGAGAGCGATGAGCCAGGTTCAACGATA				840
GTAAGGATATCCCTACAACAATGGTTGTCAGATGTGGGTGTATCTCTCGCTACTCGGTCCAAGTTGCTAT				
850		870	890	
AGCGCAGCTTTAGGTATTGGGCTTAGCGAAGGCAATGAAGGTGGGCTAATCTATGAATATTCAGGATACT				910
TCGCGTCGAAATCCATAACCCGAATCGCTTCCGTTACTTCCACCCGATTAGATACTTATAAGTCCTATGA				
920		940	960	
GCAAGAAGGAAGAGGCTGAGGAAATGGTTAAGAAGATGGTTGAGGAAGGTTTTAGGGTTAGAGGATGGAA				980
CGTTCTTCCTTCTCCGACTCCTTTACCAATTCTTCTACCAACTCCTTCCAAAATCCCAATCTCTACCTT				
990		1010	1030	
ACTCAAGGAAATAAAGATAGTCTCCGCTGAAATAACGGTTAAGGACAAGCCAGCGGCTGCAGTTGCTGCA				1050
TGAGTTCTTTTATTTCTATCAGAGGCGACTTTATTGCCAATTCCTGTTTCGGTCGCCGACGTCAACGACGT				
1060		1080	1100	
GTAGTTATGTTCCCATAGCAAGAGCTTTTTCTTACCTCTTCTATTACCTCATCATTGCAATCCCAACA				1120
CATCAATACAAGGGTATGATCGTTCTCGAAAAGAATGGAGAAGATAATGGAGTAGTAACGTTAGGGTTGT				

1130	1150	1170	
CATAACCTCAAAGCTGGGAACTCTTACACTAATCCTCACTTTTCTCTTTCTTGCTATTTTGCTCACTTCA			1190
GTATTGGAGTTTCGACCCTTGAGAATGTGATTAGGAGTGAAAAGA			
		AAAGAACGATAAAACGAGTGAAGT	
		Agma_RK-R	
1200	1220	1240	
TAACTAATCAACGATCTATATAGAATACTGAGCTATATATTTTCTATAGCGGTAAAATATTTATTTGGAG			1260
ATTGATTAGTTGCTAGATATATCTTATGACTCGATATATAAAAGATATCGCCATTTTATAAATAAACCTC			
1270	1290	1310	
GGAAACACATGCAACTGGGGTTCGCCATGCTTGCTGATCCAATTTTGTTAATTACGATACTTTTGGGATT			1330
CCTTTGTGTACGTTGACCCCAAGCGGTACGAACGACTAGGTTAAAACAATTAATGCTATGAAAACCTAA			
1340	1360	1380	
TGCAATGGCCTGGGCAATAGGTGCAAATGATGCTGCTAATTCCATGAGCACCGCCGTAGGTGCAGGAGCA			1400
ACGTTACCGGACCCGTTATCCACGTTTACTACGACGATTAAGGTACTCGTGGCGGCATCCACGTCTCTCGT			
1410	1430	1450	
ATAACACCCAGACAGGCCGTGATAATTGCTGGAGTTCTTGAATTTATGGGAGCTTATTTCTTTGGAAAGA			1470
TATTGTGGGTCTGTCCGGCACTATTAACGACCTCAAGAACTTAAATACCCTCGAATAAAGAAACCTTTCT			
1480	1500	1520	
CAGTTACAGAGACAATTAGAAAAGGCATCATCGATCCCTCAAAAATTACAGATCCAAATGTCTTAATATT			1540
GTCAATGTCTCTGTTAATCTTTTCCGTAGTAGCTAGGGAGTTTTTAATGTCTAGGTTTACAGAATTATAA			
1550	1570	1590	
TGGCTCAATAGCGGCATTAATTGGTGCAACAATATGGCTAGTAATCGCAACAAAATATGGACTTCCAGTA			1610
ACCGAGTTATCGCCGTAATTAACCACGTTGTTATACCGATCATTAGCGTTGTTTTATACCTGAAGGTCAT			
1620	1640	1660	
TCTACTACTCACTCAATAATAGGGGGGATAGTGGGTTATGGAATTGTTTATGGAGGAATGTCAATAGTAA			1680
AGATGATGAGTGAGTTATTATCCCCCTATCACCCAATACCTTAACAAATACCTCCTTACAGTTATCATT			
1690	1710	1730	
ATTGGGATAAGATGATTAAAGTAGTTCTCAGTTGGATACTCTCCCCAATCGTCGG			1750
TAACCCTATTCTACTAATTTTCATCAAGAGTCAA			
		CCTATGAGAGGGGTTAGCAGCC	
		Ampl_Trafo_L41e_R	

Single stranded sequence of the insert pf1017-PdaD-pf1020:

pf1017 (upstream of *pf1018*):

1-494

pf1623 (PdaD):

495-1193

pf1019/pf1020 (downstream of *pf1018*):

1194-1735

GCAGTACCTGCCACAATGTAGACCTCTAAATCTCCAGCAAGGTCCATGAGAAAGGTAGTTGTTTCTCCTTCAGGT
ATTCTTTGAGCTATTTCAAATACTTCATCTCTGCTTTCAAATTGTATCCAGTGTCAAAAAGCTCAGGGAGAAGCT
ATGAGTTTAGCTTCTCTCTTTGCTGCCTCTTTTACTAATTTTTTCAGCCTTTGAGAGATTCTTGTCAAGTTCAAGT
ATTTTAGGTTCCATTTGCACATAGGCAACCTTAACCATGATAACCCCCCATATCTAGTTGCTTATTTAGTATTTT
AATGTTTAGATTATAAGAAAACTCTCAGATCTCCTAAGATCACATTTTTTCAATTCTGTTAGTTGCCTTGCCAA
TAAATAGTTTTAAGATGACAAAAGATTGAAAATTTTTGTGTCTGTATCGCATTCAATGATTATAATCAAATAA
CCAGCCAATTAAGCAAAATGTCCTAAGAACCTGGGGAAAATGTATCCTACAAAACACCGCAGATTAACTTCTCC
TACTTACGAAAACCTTTAAAGATAATAAATTTGGTATCCGTTTAGCGAGAGAATATAGGAGGGAGAAGAATGAGC
TGGATAACTCTAAAAAGGCCATAATGCTTGCAGCGGCAGCTGAAGGCGGGACAAAGCTTAATGCTTTCGATAAT
GCTCTATTAAGATGGGAATTGGAATGTGAACCTAGTAAAGCTCAGCAGTGTATACCGGCTCATATAGAGTGG
CTGGATGAGTTGCCAAGAACATTCTATAGGGATGTTGTTACCAACAGTCTACACCCACATAGAGAGCGATGAG
CCAGGTTCAACGATAAGCGCAGCTTTAGGTATTGGGCTTAGCGAAGGCAATGAAGGTGGGCTAATCTATGAATAT
TCAGGATACTGCAAGAAGGAAGAGGCTGAGGAAATGGTTAAGAAGATGGTTGAGGAAGGTTTTAGGGTTAGAGGA
TGGAACTCAAGGAAATAAAGATAGTCTCCGCTGAAATAACGGTTAAGGACAAGCCAGCGGCTGCAGTTGCTGCA
GTAGTTATGTTCCATACTAGCAAGAGCTTTTCTTACCTCTTCTATTACCTCATCATTGCAATCCCAACACATAA
CCTCAAAGCTGGGAACCTTACACTAATCCTCACTTTTCTCTTCTTGCTATTTTGCTCACTTCATAACTAATCA
ACGATCTATATAGAATACTGAGCTATATATTTTCTATAGCGGTAAAATATTTATTTGGAGGGAAACACATGCAAC
TGGGGTTCGCCATGCTTGCTGATCCAATTTTGTTAATTACGATACTTTTGGGATTTGCAATGGCCTGGGCAATAG
GTGCAATGATGCTGCTAATTCCATGAGCACCGCCGTAGGTGCAGGAGCAATAACACCCAGACAGGCCGTGATAA
TTGCTGGAGTTCTTGAATTTATGGGAGCTTATTTCTTTGGAAAGACAGTTACAGAGACAATTAGAAAAGGCATCA
TCGATCCCTCAAAAATTACAGATCCAAATGTCTTAATATTTGGCTCAATAGCGGCATTAATTGGTGCAACAATAT
GGCTAGTAATCGCAACAAAATATGGACTTCCAGTATCTACTACTCAATAATAGGGGGGATAGTGGGTTATG
GAATTGTTTATGGAGGAATGTCAATAGTAAATTGGGATAAGATGATTAAAGTAGTTCTCAGTTGGATACTCTCCC
CAATCGTCGG

Construct for the deletion of *pf1018* (2.approach)

Double stranded region of the construct pUC19-pf1017-PdaD-Ppf1018-pf1019/pf1020:

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      10              30              50
TCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCT
AGCGCGCAAAGCCACTACTGCCACTTTTGGAGACTGTGTACGTCGAGGGCCTCTGCCAGTGTGGAACAGA
80              100              120
GTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTGGCGGGTGTGCGGGCTGG
CATTGCGCTACGGCCCTCGTCTGTTCGGGCAGTCCCGCGCAGTCGCCCACAACCGCCACAGCCCCGACC
140
      150              170              190
CTTA ACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCATATGCGGTGTGAAATACCGCACAGAT
GAATTGATACGCCGTAGTCTCGTCTAACATGACTCTCACGTGGTATACGCCACACTTTATGGCGTGTCTA
210
      220              240              260
GCGTAAGGAGAAAATACCGCATCAGGCGCCATTGCCATTACAGGCTGCGCAACTGTTGGGAAGGGCGATC
CGCATTCTCTTTTATGGCGTAGTCCGCGGTAAGCGGTAAGTCCGACGCGTTGACAACCCCTCCCGCTAG
280
      290              310              330
GGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAAGGGGGATGTGCTGCAAGGCGATTAAAGTTGGGTA
CCACGCCCCGAGAAGCGATAATGCGGTGACCGCTTTCCCCCTACACGACGTTCCGCTAATTCAACCCAT
350
      360              380      pUC_M13F_Up_L41e_F              400
ACGCCAGGGTTTTCCAGTCACGACGTTGTAAACGACGGCCAGTGCCCTCTTTCTTCTCCTACTCTATC
420
      430              450      AmpL_pUC_R              470
TGCAGTTATTATATATACCCTGTTTTCAAGAGCCCTTATGGGCATGGCTCTAGGAGCATAGGGCATAACT
490
      500              520              540
AAATTGGCTGGATGAGCTATAATATCCGCTCCTTTCAAGGCAAGAGTTCTGGCAGATTACAGGGAAAAACC
TTTAACCGACCTACTCGATATTATAGGCGAGGAAAGTTCCGTTCTCAAGACCGTCTAAGTCCCTTTTTGG
560
      570              590              610
AATCGAAGCATATCATAATTCTACTCTTATAAACCCAGGTCAAATACATTAAAGCCAAGATCCCCCGG
TTAGCTTCGTATAGTATTAAGGATGAGAATATTTGGGGTCCAGTTTATGTAATTCGTTCTAGGGGGCC
630
      640              660              680
GTCAAAGAATACCTTCTCCCTATAAAAGAGGTGGATCTTCTATATTTTCCAGGTAGCCAGTGGGACCT
700
      690              710              730      AmpL_Trafo_L41e_F
ACGAGAACTGCTGAATTGTATAAGCGATCGTTATATTTTCAAGCAGTACCTGCCACAATGTAGACCTCTA
770
      740              760              780
TGCTCTTGACGACTTAACATATTGCTAGCAATATAAAAAAGTCGTCATGGACGGTGTACATCTGGAGAT
790
      780              800              820
AATCTCCAGCAAGGTCCATGAGAAAGGTAGTTGTTTCTCCTTCAGGTATTCTTTGAGCTATTTCAAATAC
TTAGAGGTGCTTCCAGGTACTCTTCCATCAACAAAGAGGAAGTCCATAAGAACTCGATAAAGTTTATG
840
      830              850              870              890
TTCATCTCTGCTTTCAAATTTGTATCCAGTGTCAAAAAGCTCAGGGAGAAGTATGAGTTTAGCTTCTCTC
AAGTAGAGACGAAAGTTTAAACATAGGTCACAGTTTTTCGAGTCCCTCTTGATACTCAAATCGAAGAGAG
910
      880              900              920              940              960
TTTGCTGCCTCTTTTACTAATTTTTCAGCCTTTGAGAGATTCTTGTCAGTTCAAGTATTTTAGGTTCCA
AAACGACGGAGAAAATGATTAATAAAGTCGGAAGTCTCTAAGAACAGTTCAAGTTCATAAAATCCAAGGT
980
      970              990              1010              1030
TTTGCACATAGGCAACCTTAACCATGATAACCCCCCATATCTAGTTGCTTATTTAGTATTTCAATGTTTA
AAACGTGTATCCGTTGGAATTGGTACTATTGGGGGTATAGATCAACGAATAAATCATAAAGTTACAAAT
1050

```

1060 1080 1100
GATTATAAGAAAACTCTCAGATCTCCTAAGATCACATTTTTTCAATTCTGTTAGTTGCCTTGCCAATAA 1200
CTAATATTCTTTTTGAGAGTCTAGAGGATTCTAGTGTAAAAAAGTTAAGACAATCAACGGAACGGTTATT

1130 1150 1170
AATAGTTTTAAGATGACAAAAGATTGAAAATTTTTGTGTCTGTATCGCATTCAATGATTATAATCAAAT 1190
TTATCAAAATTCTACTGTTTTCTAACTTTTAAAAACACAGACATAGCGTAAGTTACTAATATTAGTTTA

1200 1220 1240 Agma_Rk_F
AACCAGCLAAI | AAGCAAAATGTCCTAAGAACTTGGGGAAAATGTATCTACAAAACACCGCAGATTAAA 1260
TTGGTCGGTTAATTCTGTTTTAGTCTAAGAAGCTTGGGGAAAATGTAAGGATGTTTTGTGGCGTCTAATTT

Agma_Up_L41e_R
1270 1290 1310
CTTCTCCTACTTACGAAAACCTTTAAAAGATAATAAATTTGGTATCCGTTTAGCGAGAGAATATAGGAGGG 1330
GAAGAGGATGAATGCTTTTGAAATTTTCTATTATTTAAACCATAGGCAAATCGCTCTCTTATATCCTCCC

1340 1360 1380
AGAAGAATGAGCTGGATAACTCCTAAAAAGGCCATAATGCTTGACGCGGCAGCTGAAGGCGGGACAAAGC 1400
TCTTCTTACTCGACCTATTGAGGATTTTTCCGGTATTACGAACGTCGCCGTCGACTTCCGCCCTGTTTCG

1410 1430 1450
TTAATGCTTTTCGATAATGCTCTATTAAAGATGGGAATTGGAAATGTGAACCTAGTAAAGCTCAGCAGTGT 1470
AATTACGAAAGCTATTACGAGATAATTTCTACCCTTAACCTTTACACTTGGATCATTTTCGAGTCGTCACA

1480 1500 1520
TATACCGGCTCATATAGAGTGGCTGGATGAGTTGCCAAAGAACATTCTATAGGGATGTTGTTACCAACA 1540
ATATGGCCGAGTATATCTCACCGACCTACTCAACGGTTTCTTGTAAGGATATCCCTACAACAATGGTTGT

1550 1570 1590
GTCTACACCCACATAGAGAGCGATGAGCCAGGTTCAACGATAAGCGCAGCTTTAGGTATTGGGCTTAGCG 1610
CAGATGTGGGTGTATCTCTCGCTACTCGGTCCAAGTTGCTATTTCGCGTCGAAATCCATAACCCGAATCGC

1620 1640 1660
AAGGCAATGAAGGTGGGCTAATCTATGAATATTCAGGATACTGCAAGAAGGAAGAGGCTGAGGAAATGGT 1680
TTCCGTTACTTCCACCCGATTAGATACTTATAAGTCTTATGACGTTCTTCTTCTCCGACTCCTTTACCA

1690 1710 1730
TAAGAAGATGGTTGAGGAAGGTTTTAGGGTTAGAGGATGGAACTCAAGGAAATAAAGATAGTCTCCGCT 1750
ATTCTTCTACCAACTCCTTCCAAAATCCCAATCTCTACCTTTGAGTTCTTTATTTCTATCAGAGGCGA

1760 1780 1800
GAAATAACGGTTAAGGACAAGCCAGCGGCTGCAGTTGCTGCAGTAGTTATGTTCCCATAGCAAGAGC 1820
CTTTATTGCCAATTCCTGTTTCGGTCGCCGACGTCAACGACGTCAATACAAGGGTATGATCGTTCTCG

1830 1850 1870
TTTTCTTACCTCTTCTATTACCTCATCATTGCAATCCCAACACATAACCTCAAAGCTGGGAACTCTTACA 1890
AAAAGAATGGAGAAGATAATGGAGTAGTAACGTTAGGGTTGTGTATTGGAGTTTCGACCCTTGAGAATGT

1900 1920 1940 AgmaRK_PromL41ePhospho_F
CTAATCCTCACTTTTCTTTCTTTGCTATTTTGCTCACTTCATAAAAAACCAATAAACTTAAAAATTGA 1960
GATTAGGAGTGAAAAGAGAAAGAACGATAAAACGAGTGAAGTATTTTTGGTTATTTTGAATTTTAACT

Agma_Rk_F
19970 1990 PromL41e_Phosphoester 2010
CTTAGAAGGTAAAAATTGGGAAAATAAGGAGGTGATGATCATGGCTTTTTTGAAGATTTTTCCCTTTAT 2030
GAATCTTCCATTTTTTAAACCTTTTATTCTCCACTACTAGTACCGAAAAAACTTCTAAAAAGGGAAATA

2040 2060 2080
TCTTTAGAATTTGAAACATCTTTAGGAAAACTCTAATTTTTGCAGATCCTCATCTCGGTTTTGAAATAG 2100
AGAAATCTTAACTTTGTAGAAATCTTTTTGAGATTAAAAACGTCTAGGAGTAGAGCCAAAACCTTTATC

2110 2130 2150
CTAAAGGTGTTAGATTAGGTCGAGGTTAGAAGAAAACTTGCAAATTTTATAAAATCCAAGAATCCAGA 2170
GATTTCCACAATCTTAATCCAGCTCCAATCTTCTTTTGAACGTTTAAAATATTTTAGGTTCTTAGGTCT

2180	2200	2220	
TGCAATAATAATATTAGGAGATATAAAGGAGGAAATTGGACTGAGCAGTTTTACTCGAAAAGATTTTGATG			2240
ACGTTATTATTATAATCCTCTATATTTCTCTCTTAACCTGACTCGTCAAAATGAGCTTTCTAAACTAC			
2250	2270	2290	
TCGTTCTTTTTAGAGCTTAAAGATTACATAGTAATAATCACAAAGGGAAATCATGATGGGAGAATAGAAG			2310
AGCAAGAAAAATCTCGAATTTCTAATGTATCATTATTAGTGTTCCTTTAGTACTACCCTCTTATCTTC			
2320	2340	2360	
AAGTCACTAAAACTTCGATAACATAAATGTTGTCGAATACTATTTGCTCGATGACGTTCTATTTTTTCA			2380
TTCAGTGATTTTTGAAGCTATTGTATTTACAACAGCTTATGATAAACGAGCTACTGCAAGATAAAAAAGT			
2390	2410	2430	
TGGTCACAAAGTTGCACCTCCAATAGAGTTTAAGATTGCATTTTTAGGCCATGCTCACCCTGTTATTTAT			2450
ACCAGTGTTCACGTGGAGGTTATCTCAAATCTAACGTAAAAATCCGGTACGAGTGGGACAATAAATA			
2460	2480	2500	
GTAAACGTTAAAGGAATTAAGAAAAAAGCAAAATGTTTCATAAAAGCTGGAAGGTATGTAGTTTTGCCAA			2520
CATTTGCAATTTCTTAATTCTTTTTTCGTTTTACAAAGTATTTTCGACCTTCATACATCAAAACGGTT			
2530	2550	2570	
CAATAAATCCCTTTTACGAGGGAATTGAGACCAACAAGCGATAAAAAATGATTCCTACGCTCAAAGAAGT			2590
GTTATTTAGGGAAAATGCTCCCTTAACCTCTGGTTTGTTCGCTATTTTTACTAAGGATGCGAGTTTCTTCA			
2600	2620	2640	
AAAAACAGTTGATGTAGTTATTCCCCCTGGGATTTACCTAGGCCAATATACCATAGATATCTAATCAACG			2660
TTTTTGTCAACTACATCAATAAGGGGGACCTAAATGGATCCGGTTATATGGTATCTATAGATTAGTTGC			
2670	2690	2710	
ATCTATATAGAATACTGAGCTATATATTTTCTATAGCGGTAAAAATATTTATTTGGAGGGAAACACATGCA			2730
TAGATATATCTTATGACTCGATATATAAAAGATATCGCCATTTTATAAATAAACCTCCCTTTGTGTACGT			
2740	2760	2780	
ACTGGGGTTCGCCATGCTTGCTGATCCAATTTTGTTAATTACGATACTTTTGGGATTTGCAATGGCCTGG			2800
TGACCCCAAGCGGTACGAACGACTAGGTTAAACAATTAATGCTATGAAAACCCTAAACGTTACCGGACC			
2810	2830	2850	
GCAATAGGTGCAAATGATGCTGCTAATTCATGAGCACCGCCGTAGGTGCAGGAGCAATAACACCCAGAC			2870
CGTTATCCACGTTTACTACGACGATTAAGGTACTCGTGGCGGCATCCACGTCCTCGTTATTGTGGGTCTG			
2880	2900	2920	
AGGCCGTGATAATTGCTGGAGTTCTTGAATTTATGGGAGCTTATTTCTTTGGAAAGACAGTTACAGAGAC			2940
TCCGGCACTATTAACGACCTCAAGAACTTAATACCCTCGAATAAAGAAACCTTTCTGTCAATGTCTCTG			
2950	2970	2990	
AATTAGAAAAGGCATCATCGATCCCTCAAAAATTACAGATCCAAATGTCTTAATATTTGGCTCAATAGCG			3010
TTAATCTTTTCCGTAGTAGCTAGGGAGTTTTTAATGTCTAGGTTTACAGAATTATAAACCGAGTTATCGC			
3020	3040	3060	
GCATTAATTGGTGCAACAATATGGCTAGTAATCGCAACAAAATATGGACTTCCAGTATCTACTACTCACT			3080
CGTAATTAACCACGTTGTTATACCGATCATTAGCGTTGTTTTATACCTGAAGGTCATAGATGATGAGTGA			
3090	3110	3130	
CAATAATAGGGGGGATAGTGGGTTATGGAATTGTTTATGGAGGAATGTCAATAGTAAATTGGGATAAGAT			3150
GTTATTATCCCCCTATCACCCAATACCTTAACAAATACCTCCTTACAGTTATCATTTAACCCCTATTCTA			
3160	3180	3200	
GATTAAAGTAGTTCTCAGTTGGATACTCTCCCCAATCGTCGGAGCAATCTTTCATACCTAGTATTCAGA			3220
CTAATTTTCATCAAGAGTCAACCTATGAGAGGGGTTAGCAGCCTCGTTAGAAACGTATGGATCATAAGTCT			
Ampl_Trafo_L41e_R			
3230	3250	3270	
GCGCTAAGTAAAACGGTCTTCAAAGCAAGGACCCTGTGAAAAGTGCAAAGAGATGGTCTCCATTCTGGA			3290
CGCGATTCATTTTGCCAAGAAGTTTCGTTCTGGGACACTTTTCACGTTTCTCTACCAGAGGTAAGACCT			
3300	3320	3340	
TAGGGCTAGCATTTGTGGTCATTGGAACAATGTTCTACATAAAGGTTCTACACGGTAATTCATTACTTGA			3360
ATCCCGATCGTAAACACCAGTAACCTTGTTACAAGATGTATTTCCAAGATGTGCCATTAAGTAATGAAC			
3370	3390	3410	
AGGATTTCTCAAATACGGAATGCCAGCTGGAATTCTAACATTCATAGTAGTCTCCTTAATATTGGAAAAG			3430
TCCTAAAGAGTTTATGCCTTACGGTCGACCTTAAGATTGTAAGTATCATCAGAGGAATTATAACCTTTTC			

3440 3460 AmpLpUC_F 3500

AGATTTCACGCCACTGATCCCTATCTTGGGGTCATAGCTGTTTCCTGTGTG
TCTAAAGGT CGGT GACTAGGGATAGAACCCAGTATCGACAAAGGACACAC...

pUC_M13R_Down_L41e_R

After position 3481, the sequence of the vector pUC19 follows with the same sequence as in the double stranded sequence of the construct pUC19-*pf0559*-PdaD-*pf0561* at page 77. (corresponding sequence at position: 2426-4626)

Double stranded sequence of the insert pf1017-PdaD-Ppf1018-pf1019/pf1020:

10 Ampl_Trafo_L41e_ F 30 50
GCAGTACCTGCCACAATGTAGACCTCTAAATCTCCAGCAAGGTCCATGAGAAAGGTAGTTGTTTCTCCTT 70
CGTCATGGACGGTGTTACATCTGGAGATTTAGAGGTCGTTCCAGGTACTCTTCCATCAACAAAGAGGAA
80 100 120
CAGGTATTCTTTGAGCTATTTCAAATACTTCATCTCTGCTTTCAAAATTGTATCCAGTGTCAAAAAGCTC 140
GTCCATAAGAACTCGATAAAGTTTATGAAGTAGAGACGAAAGTTTAAACATAGGTCACAGTTTTTCGAG
150 170 190
AGGGAGAAGTATGAGTTTAGCTTCTCTCTTTGCTGCCTCTTTTACTAATTTTTTCAGCCTTTGAGAGATTC 210
TCCCTCTTGATACTCAAATCGAAGAGAGAAACGACGGAGAAAATGATTA AAAAGTCGGAAACTCTCTAAG
220 240 260
TTGTCAAGTTCAAGTATTTTAGGTTCCATTTGCACATAGGCAACCTTAACCATGATAACCCCCCATATCT 280
AACAGTTCAAGTTCATAAAATCCAAGGTAACGTGTATCCGTTGGAATTGGTACTATTGGGGGTATAGA
290 310 330
AGTTGCTTATTTAGTATTTCAATGTTTAGATTATAAGAAAACTCTCAGATCTCCTAAGATCACATTTTT 350
TCAACGAATAAATCATAAAGTTACAAATCTAATATTCTTTT GAGAGTCTAGAGGATTCTAGTGTA AAAA
360 380 400
TCAATTCTGTTAGTTGCCTTGCCAATAAAATAGTTTTAAGATGACAAAAGATTGAAAATTTTTTGTGTCT 420
AGTTAAGACAATCAACGGAACGGTTATTTTATCAAATCTACTGTTTTCTAACTTTTAAAAAACACAGA
430 450 470
GTATCGCATTCAATGATTATAATCAAATAACCAGCCAATTAAGCAAAATGTCCTAAGAACTTGGGGAAAA 490
CATAGCGTAAGTTACTAATATTAGTTTATTGGTCGGTTAATTCGTTTTACAGGATTCTTGAACCCCTTTT
500 Agma_RK-F 520 540
TGTATCCTACAAAACACCGCAGATTAAACTTCTCCTACTTACGAAAACCTTTAAAGATAATAAATTTGGT 560
ACATAGGATGTTTTGTGGCGTCTAATTTGAAGAGGATGAATGCTTTTGAAATTTTCTATTATTTAAACCA
570 590 610
ATCCGTTTAGCGAGAGAATATAGGAGGGAGAAGAATGAGCTGGATAACTCCTAAAAAGGCCATAATGCTT 630
TAGGCAAATCGCTCTCTTATATCCTCCCTCTTCTACTCGACCTATTGAGGATTTTCCGGTATTACGAA
640 660 680
GCAGCGGCAGCTGAAGGCGGGACAAAGCTTAATGCTTTGATAATGCTCTATTAAAGATGGGAATTGGAA 700
CGTCGCCGTCGACTCCGCCCTGTTTCGAATTACGAAAGCTATTACGAGATAATTTCTACCCTTAACCTT
710 730 750
ATGTGAACCTAGTAAAGCTCAGCAGTGTTATACCGGCTCATATAGAGTGGCTGGATGAGTTGCCAAAGAA 770
TAACTTGGATCATTTGAGTCGTCACAATATGGCCGAGTATATCTACCGACCTACTCAACGGTTTCTT
780 800 820
CATTCCTATAGGGATGTTGTTACCAACAGTCTACACCCACATAGAGAGCGATGAGCCAGGTTCAACGATA 840
GTAAGGATATCCCTACAACAATGGTTGTCAGATGTGGGTGTATCTCTCGCTACTCGGTCCAAGTTGCTAT
850 870 890
AGCGCAGCTTTAGGTATTGGGCTTAGCGAAGGCAATGAAGGTGGGCTAATCTATGAATATT CAGGATACT 910
TCGCGTCGAAATCCATAACCCGAATCGCTTCCGTTACTTCCACCCGATTAGATACTTATAAGTCCTATGA
920 940 960
GCAAGAAGGAAGAGGCTGAGGAAATGGTTAAGAAGATGGTTGAGGAAGGTTTTAGGGTTAGAGGATGGAA 980
CGTTCTTCTCTCCGACTCCTTTACCAATTCTTCTACCAACTCCTTCCAAAATCCCAATCTCTACCTT
990 1010 1030
ACTCAAGGAAATAAAGATAGTCTCCGCTGAAATAACGGTTAAGGACAAGCCAGCGGCTGCAGTTGCTGCA 1050
TGAGTTCTTTATTTCTATCAGAGGCGACTTTATTGCCAATTCCTGTTTCGGTCGCCGACGTCAACGACGT

1060 1080 1100
 GTAGTTATGTTCCCATAGCAAGAGCTTTTCTTACCTCTTCTATTACCTCATCATTGCAATCCCAACA 1120
 CATCAATACAAGGGTATGATCGTTCTCGAAAAGAATGGAGAAGATAATGGAGTAGTAACGTTAGGGTTGT
 AgmaRK_PromL41ePhospho_F
 1130 1150 1170
 CATAACCTCAAAGCTGGGAACTCTTACCTAATCCTCACTTTTCTCTTTCTT **GCTATTTTGCTCACTTCA** 1190
 GTATTGGAGTTTCGACCCCTTGAGAATGTGATTAGGAGTGAAAAGA **AAAGAACGATAAAACGAGTGAAGT**
 Agma_RK-R
 1200 1220 1240 PromL41e_Phosphoesterase_F
TAAAAACCAATAAACTTAAAAATTGACTTAGAAGGTAAAAATTGGGAAATAAGGAGGTGATGATCAT 1260
ATTTTTTGGTTATTTGAATTTTAACTGAATCTTCCATTTTAA **CCCTTTATTCCTCCACTACTAGTA**
 Promotor L41e R
 1270 1290 1310
GGCTTTTTTTGAAGATTTTCCTTTATTCTTTAGAATTTGAAACATCTTTAGGAAAACTCTAATTTTT 1330
 CCGAAAAAACTTCTAAAAAGGGAAATAAGAAATCTTAACTTTGTAGAAATCCTTTTTGAGATTAAAA

1340 1360 1380
 GCAGATCCTCATCTCGGTTTTGAAATAGCTAAAGGTGTTAGAATTAGGTCGAGGTTAGAAGAAAACTTG 1400
 CGTCTAGGAGTAGAGCCAAAACCTTTATCGATTTCCACAATCTTAATCCAGCTCCAATCTTCTTTTTGAAC
 1410 1430 1450
 CAAATTTTATAAAATCCAAGATCCAGATGCAATAATAATATTAGGAGATATAAAGGAGGAAATTGGACT 1470
 GTTTAAAATATTTTAGGTTCTTAGGTCTACGTTATTATTATAATCCTCTATATTTCTCCTTTAACCTGA
 1480 1500 1520
 GAGCAGTTTTACTCGAAAGATTTTGATGTGCTTTTCTTTTAGAGCTTAAAGATTACATAGTAATAATCACA 1540
 CTCGTCAAATGAGCTTTCTAAACTACAGCAAGAAAAATCTCGAATTTCTAATGTATCATTATTAGTGT
 1550 1570 1590
 AAGGGAAATCATGATGGGAGAATAGAAGAAGTCACTAAAACTTCGATAACATAAATGTTGTGCGAATACT 1610
 TTCCCTTTAGTACTACCTCTTATCTTCTTCAGTGATTTTGAAGCTATTGTATTACAACAGCTTATGA
 1620 1640 1660
 ATTTGCTCGATGACGTTCTATTTTTTTCATGGTCACAAAGTTGCACCTCCAATAGAGTTTAAGATTGCATT 1680
 TAAACGAGCTACTGCAAGATAAAAAAGTACCAGTGTTTCAACGTGGAGGTTATCTCAAATCTAACGTAA
 1690 1710 1730
 TTTAGGCCATGCTCACCTGTTATTTATGTAAACGTTAAAGGAATTAAGAAAAAGCAAAATGTTTCATA 1750
 AAATCCGGTACGAGTGGGACAATAAATACATTTGCAATTTCTTAAATTCTTTTTTCGTTTTACAAAGTAT
 1760 1780 1800
 AAAGCTGGAAGGTATGTAGTTTTGCCAACAATAAATCCCTTTTACGAGGGAATTGAGACCAACAAGCGA 1820
 TTTTCGACCTTCATACATCAAAACGGTTGTTATTTAGGGAAAATGCTCCCTTAACTCTGGTTTGTTGCT
 1830 1850 1870
 TAAAAATGATTCTACGCTCAAAGAAGTAAAAACAGTTGATGTAGTTATTCCCCCTGGGATTTACCTAGG 1890
 ATTTTTACTAAGGATGCGAGTTTCTTCATTTTTGTCAACTACATCAATAAGGGGGACCCTAAATGGATCC
 1920 1920 1940
 CCAATATACCATAGATATCTAATCAACGATCTATATAGAATACTGAGCTATATATTTTCTATAGCGGTAA 1960
 GGTTATATGGTATCTATAGATTAGTTGCTAGATATATCTTATGACTCGATATATAAAAGATATCGCCATT
 1970 1990 2010
 AATATTTATTTGGAGGGAACACATGCAACTGGGGTTCGCCATGCTTGCTGATCCAATTTTGTTAATTAC 2030
 TTATAAATAAACCTCCCTTTGTGTACGTTGACCCCAAGCGGTACGAACGACTAGGTTAAAACAATTAATG
 2040 2060 2080
 GATACTTTTGGGATTTGCAATGGCCTGGGCAATAGGTGCAATGATGCTGCTAATTCCATGAGCACCGCC 2100
 CTATGAAAACCCTAAACGTTACCGGACCCGTTATCCACGTTTACTACGACGATTAAGGTACTCGTGGCGG
 2110 2130 2150
 GTAGGTGCAGGAGCAATAACACCCAGACAGGCCGTGATAATTGCTGGAGTTCTTGAATTTATGGGAGCTT 2170
 CATCCACGTCCTCGTTATTGTGGGTCTGTCCGGCACTATTAACGACCTCAAGAAGTTAAATACCCTCGAA

2180	2200	2220	
ATTTCTTTGGAAAGACAGTTACAGAGACAATTAGAAAAGGCATCATCGATCCCTCAAAAATTACAGATCC TAAAGAAACCTTTCTGTCAATGTCTCTGTTAATCTTTCCGTAGTAGCTAGGGAGTTTTTAATGTCTAGG			2240
2250	2270	2290	
AAATGTCTTAATATTTGGCTCAATAGCGGCATTAATTGGTGCAACAATATGGCTAGTAATCGCAACAAAA TTTACAGAATTATAAACCAGATTATCGCCGTAATTAACCACGTTGTTATACCGATCATTAGCGTTGTTTT			2310
2320	2340	2360	
TATGGACTTCCAGTATCTACTACTCAATAATAGGGGGGATAGTGGGTTATGGAATTGTTTATGGAG ATACCTGAAGGTCATAGATGATGAGTGAGTTATTATCCCCCTATCACCCAATACCTTAACAAATACCTC			2380
2390	2410	2430	
GAATGTCAATAGTAAATTGGGATAAGATGATTAAAGTAGTTCTCAGTTGGATACTCTCCCAATCGTCGG CTTACAGTTATCATTTAACCCATTCTACTAATTTTCATCAAGAGTCAACCTATGAGAGGGGTTAGCAGCC			2450

Ampl_Trafo_ L41e_ R

Single stranded sequence of the insert pf1017-PdaD-Ppf1018-pf1019/pf1020:

pf1017 (upstream of pf1018):	1-494
pf1623 (PdaD):	495-1193
Promotor pf1018:	1936-1999
pf1019:	2000-2654
pf1020 (downstream of pf1018):	2655-3192

GCAGTACCTGCCACAATGTAGACCTCTAAATCTCCAGCAAGGTCCATGAGAAAGGTAGTTGTTTCTCCTTCAGGT
ATTCTTTGAGCTATTTCAAATACTTCATCTCTGCTTTCAAAATTGTATCCAGTGTCAAAAAGCTCAGGGAGA
ATGAGTTTAGCTTCTCTCTTTGCTGCCTCTTTACTAATTTTTTCAGCCTTTGAGAGATTCTTGTCAAGTTCAAGT
ATTTTAGGTTCCATTTGCACATAGGCAACCTTAACCATGATAACCCCCCATATCTAGTTGCTTATTTAGTATTT
AATGTTTAGATTATAAGAAAACTCTCAGATCTCCTAAGATCACATTTTTTCAATTCTGTTAGTTGCCTTGCCAA
TAAATAGTTTTAAGATGACAAAAGATTGAAAATTTTTGTGTCTGTATCGCATTCAATGATTATAATCAAATAA
CCAGCCAATTAAGCAAAATGTCCTAAGAAGCTTGGGAAAAATGTATCTACAAAACACCGCAGATTAACTTCTCC
TACTTACGAAAACTTTAAAGATAATAAATTTGGTATCCGTTTAGCGAGAGAATATAGGAGGGAGAAGAATGAGC
TGGATAACTCTAAAAAGGCCATAATGCTTGCAGCGGACGTGAAGGCGGGACAAAGCTTAATGCTTTCGATAAT
GCTCTATTAAAGATGGGAATTGGAAATGTGAACCTAGTAAAGCTCAGCAGTGTTATACCGGCTCATATAGAGTGG
CTGGATGAGTTGCCAAAGAACATTCTATAGGGATGTTGTTACCAACAGTCTACACCCACATAGAGAGCGATGAG
CCAGGTTCAACGATAAGCGCAGCTTTAGGTATTGGGCTTAGCGAAGGCAATGAAGGTGGGCTAATCTATGAATAT
TCAGGATACTGCAAGAAGGAAGAGGCTGAGGAAATGGTTAAGAAGATGGTTGAGGAAGTTTTAGGGTTAGAGGA
TGGAAACTCAAGGAAATAAGATAGTCTCCGCTGAAATAACGGTTAAGGACAAGCCAGCGGCTGCAGTTGCTGCA
GTAGTTATGTTCCCACTAGCAAGAGCTTTTCTACCTCTTCTATTACCTCATCATTGCAATCCCAACACATAA
CCTCAAAGCTGGGAACCTTACACTAATCCTCACTTTTCTCTTTCTTGCTATTTTGCTCACTTCATAA
AAAAACCA
ATAAACTTAAAAATTGACTTAGAAGGTAAAAATTGGGAAAAATAAGGAGGTGATGATCATGGCTTTTTTTGAAGA
TTTTTCCCTTTATTCTTTAGAATTTGAAACATCTTTAGGAAAACTCTAATTTTTGCAGATCCTCATCTCGGTTT
TGAAATAGCTAAAGGTGTTAGAATTAGGTCGAGGTTAGAAGAAAACTTGCAAATTTTATAAAATCCAAGAATCC
AGATGCAATAATAATATTAGGAGATATAAAGGAGGAAATTGGACTGAGCAGTTTTACTCGAAAGATTTTGATGTC
GTTCTTTTTAGAGCTTAAAGATTACATAGTAATAATCACAAAGGGAAATCATGATGGGAGAATAGAAGAAGTCAC
TAAAAACTTCGATAACATAAATGTTGTCGAATACTATTTGCTCGATGACGTTCTATTTTTTCATGGTCACAAAGT
TGCACCTCCAATAGAGTTTAAAGATTGCATTTTTAGGCCATGCTCACCTGTTATTTATGTAAACGTTAAAGGAAT
TAAGAAAAAAGCAAAATGTTTCATAAAAGCTGGAAGGTATGTAGTTTTGCCAACATAAATCCCTTTTACGAGGG
AATTGAGACCAACAAGCGATAAAATGATTCCTACGCTCAAAGAAGTAAAAACAGTTGATGTAGTTATTCCTCC
TGGGATTTACCTAGGCCAATATACCATAGATATCTAATCAACGATCTATATAGAATACTGAGCTATATATTTTCT
ATAGCGGTAAAATATTTATTTGGAGGGAAACACATGCAACTGGGGTTCGCCATGCTTGCTGATCCAATTTTGTTA
ATTACGATACTTTTGGGATTTGCAATGGCTGGGCAATAGGTGCAAATGATGCTGCTAATTCATGAGCACCGCC
GTAGGTGCAGGAGCAATAACACCCAGACAGGCCGTGATAATTGCTGGAGTTCTTGAATTTATGGGAGCTTATTT
TTTGGAAAGACAGTTACAGAGACAATTAGAAAAGGCATCATCGATCCCTCAAAAATTACAGATCCAATGTCTTA
ATATTTGGCTCAATAGCGGCATTAATTGGTGCAACAATATGGCTAGTAATCGCAACAAAATATGGACTTCCAGTA
TCTACTACTCACTCAATAATAGGGGGATAGTGGGTTATGGAATTGTTTATGGAGGAATGTCAATAGTAAATTGG
GATAAGATGATTAAAGTAGTTCTCAGTTGGATACTCTCCCCAATCGTCGG