Creation of an *endA* Mutant Strain in *Pseudomonas aeruginosa* PAO1 using Gene Replacement

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Abstract

Endonuclease I is an enzyme encoded by the *endA* gene. This nuclease degrades double stranded DNA. Many *Escherichia coli* common laboratory strains contain a mutation in the *endA* gene that inactivates the DNA-specific endonuclease I. A mutation in this gene greatly increases plasmid DNA yields in such *E. coli* strains as well as improves the quality of DNA that is isolated. The purpose of this research is to create an *endA* mutant strain in *Pseudomonas aeruginosa* PAO1 using gene replacement, thereby leading to the development of a useful laboratory *Pseudomonas* strain for use as a cloning strain. To accomplish this, chromosomal DNA from *P. aeruginosa* PAO1 was isolated, and the *endA* gene was then amplified by PCR using specific primers designed to the flanking upstream and downstream sequence of the *endA* coding region. The resulting amplified 1100 bp DNA fragment containing the *endA* gene was cloned into pCR2.1. This newly created plasmid was named pCR2.1-*endA*. In order to create an insertionally inactivated *endA* gene, a Gm^R encoding cassette from pPS856 needed to be inserted into the *Sal*I sites of the cloned *endA* gene. The pCR2.1-*endA* plasmid was digested using *Sal*I restriction enzyme. A 4500 bp *Sal*I fragment of pCR2.1-*endA* was isolated and then religated by T4 DNA ligase. The new plasmid created was called pCR2.1-*endA*Δ*Sal*ID. This plasmid was digested with *Sal*I, and blunt ends were created with T4 DNA polymerase. Inactivation of the *endA* gene was accomplished by insertion of a blunt-ended, Gm^R encoding gene into the blunt-ended *Sal*I site of the *endA* coding sequence. The resulting recombinant plasmid was called pCR2.1-*endA*Δ*Sal*ID(Gm1). A 1700 bp *Hind*III x *Pst*I DNA fragment from pCR2.1-*endA*Δ*Sal*ID(Gm1), containing the insertionally inactivated *endA* gene, was isolated and cloned into the similarly digested pEX18Ap plasmid. The pEX18Ap plasmid is a mobilizable suicide plasmid used in gene replacement. The new recombinant plasmid was called pEX18Ap-*endA*(Gm1). In future experiments, the pEX18Ap-*endA*(Gm1) containing the *endA* gene knockout will be conjugally transferred into a wild-type *P. aeruginosa* PAO1 strain. This will allow for the creation of a new mutant *endA Pseudomonas* strain by gene replacement.

Introduction

The *endA* gene of *Escherichia coli* encodes for the protein Endonuclease I. It is a 26kDa DNA-specific endonuclease that degrades double stranded DNA. Many common *E. coli* laboratory strains used for molecular studies contain a mutation in the *endA* gene that will inactivate the Endonuclease I. The genotypes of common laboratory *E. coli* strains that contain an *endA* mutation include:

DH5 strains [F⁻ *endA1 recA1 hsdR17* (r_k^- m_k^+) *supE44 thi-1* λ⁻ *gyrA96 relA1*]

DH5αF' strains [F' φ80d*lacZ*ΔM15 *endA1 recA1 hsdR17* (r_k^- m_k^+) *supE44 thi-1* λ⁻ *gyrA96 relA1*Δ(*lacZYA-argF*)U169]

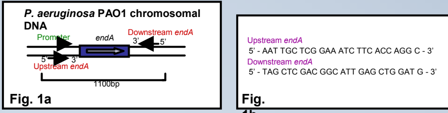
A mutation in the *endA* gene greatly increases plasmid DNA yields in such *E.coli* strains as well as improving the quality of the DNA that is isolated.

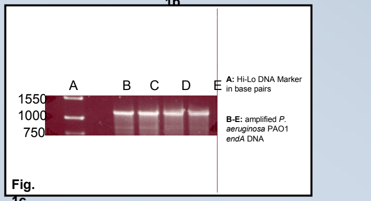
Pseudomonas aeruginosa PAO1 is the most common laboratory strain used by researchers doing *Pseudomonas* research. The creation of a *P. aeruginosa* PAO1 strain that has a mutation in the *endA* gene would lead to a useful laboratory strain, for use as a cloning strain. As a result of the mutation, it will be possible for laboratories involved with *Pseudomonas* research to have a mutant strain available to use in plasmid isolations, thereby causing a decrease in DNA degradation during the isolation procedure.

Objective

The purpose of this research is to create an *endA* mutant strain in *Pseudomonas aeruginosa* PAO1 using gene replacement, thereby leading in the future, to the development of a useful laboratory *Pseudomonas* strain for employment as a molecular cloning strain.

Experimental Design and Results





Figures 1a, b, c: PCR amplification of *endA* gene from *P. aeruginosa* PAO1

Chromosomal DNA from *P.aeruginosa* PAO1 was isolated using the IsoQuick DNA Extraction Kit. The extracted chromosomal DNA was used in a PCR reaction. The *endA* gene was amplified using the designed Upstream *endA* and Downstream *endA* primers that will amplify the flanking DNA sequence – upstream and downstream – of the *endA* coding region (Fig. 1a,1b). The PCR machine was set up for a 3-step process: 30 cycles of 95 °C for one minute, 58 °C for 45 seconds and 72 °C for 1.5 minutes. Following the PCR, a 1.2% agarose gel was run with the amplified products and then stained with ethidium bromide. Analysis of the gel showed a fragment at approximately 1100 bp when compared to the Hi-Lo DNA Markers (Fig. 1c). This is the predicted product size of the amplified *endA* gene based on sequence analysis information.

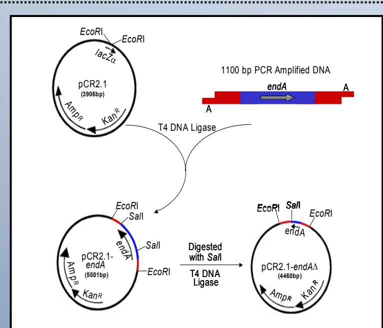


Figure 2: Construction of the pCR2.1-endAΔ plasmid

The amplified PCR DNA was isolated from the agarose gel using a QIAquick Gel Extraction Kit. The amplified (1100 bp) DNA fragment containing the *endA* gene was cloned into the pCR2.1 vector plasmid using T4 DNA Ligase, then the ligation mixture was transformed into One Shot *E. coli* TOP10F' cells. Transformed cells were selected on Luria Broth (LB) Ampicillin (Ap) containing plates. Plasmid DNA was isolated from the transformed cells using the QIAprep Spin Miniprep Kit. The correct construct was identified from the transformants based on restriction digests. The new plasmid was called pCR2.1-*endA* (Fig. 2). Initially, the pCR2.1-*endA* plasmid contained two *Sal*I sites within the *endA* gene. The pCR2.1-*endA* plasmid was digested using the *Sal*I restriction enzyme. Agarose gel electrophoresis of the digestion showed a fragment at ~4000 bp and a fragment at ~600 bp. The ~4000bp DNA fragment was isolated from the gel by the QIAquick Gel Extraction Kit. The ~4000bp DNA fragment was self-ligated at its single *Sal*I site using T4 DNA Ligase. This plasmid was transformed into *E. coli* DH5αF' competent cells, and transformants were selected on LB_{Ap100} containing plates. Plasmid DNA was isolated from the transformants, and the correct construct was identified through restriction digests. The resulting plasmid was called pCR2.1-*endA*Δ.

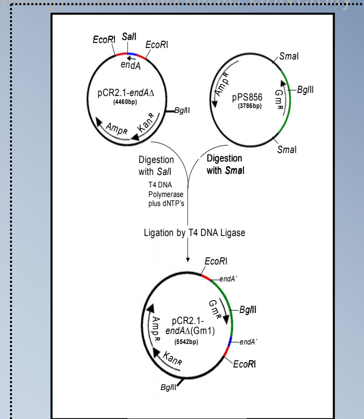


Figure 3: Construction of the pCR2.1-*endA*Δ(Gm1) plasmid

In order to create an insertional inactivation of the *endA* gene, a Gm^R cassette from the pPS856 plasmid was inserted into the single *Sal*I site of the *endA* coding sequence on the pCR2.1-*endA*Δ plasmid. The pCR2.1-*endA*Δ vector was digested with *Sal*I, and the pPS856 plasmid was digested with *Sma*I. The *Sal*I cut vector was made blunt with T4 DNA Polymerase and dNTPs so it would be compatible with the blunt ends created by the *Sma*I digestion of the pPS856 Gm^R cassette. The *Sma*I, 1077 bp Gm^R cassette and the pCR2.1-*endA*Δ blunt ended vector were gel isolated, and then ligated using the Fast-Link DNA Ligation Kit. The ligation mixture was then transformed into highly competent *E. coli* DH5α cells, and the transformed cells were selected on LB_{Gm15} containing plates (Fig. 3). Plasmid DNA was isolated from the transformants with the QIAprep Spin Miniprep Kit. A restriction digest was performed on transformants using *Bgl*II to confirm the presence of the recombinant plasmid. The new recombinant was called pCR2.1-*endA*Δ(Gm1).

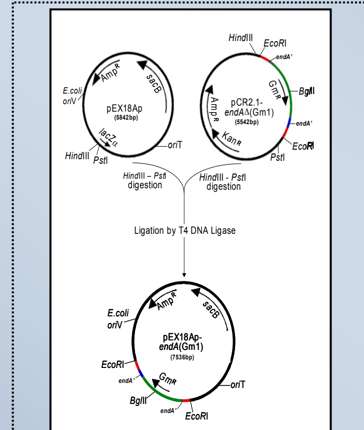


Figure 4: Construction of the pEX18Ap-*endA*(Gm1) plasmid

The insertionally inactivated gene segment from the pCR2.1-*endA*D(Gm1) plasmid could now be cloned into the mobilizable pEX18Ap suicide plasmid. This plasmid is an important feature of the gene replacement strategy. A double digestion was performed on the two plasmids. The pEX18Ap plasmid was digested by *Hind*III x *Pst*I enzymes, and the pCR2.1-*endA*D(Gm1) plasmid was digested using *Hind*III x *Pst*I enzymes, and the correct DNA fragments were isolated from an agarose gel by the QIAquick Gel Extraction Kit. The two DNA fragments were ligated together using the Fast-Link DNA Ligation Kit, and were then transformed into highly competent *E. coli* DH5a cells. The transformed cells were selected on LB_{Ap100} containing plates. The plasmid DNA was isolated from transformants using a QIAprep Spin Miniprep Kit, and the correct construct was identified based on restriction digests. The new plasmid is called pEX18Ap-*endA*(Gm1).

Conclusions

1. The *endA* gene of *Pseudomonas aeruginosa* PAO1 was amplified by PCR using primers. The names of the two primers were Upstream *endA* and Downstream *endA*, which were designed to flanking sequences upstream and downstream of the *endA* coding sequence. An 1100 bp amplified fragment containing *endA* was cloned into pCR2.1. The resulting plasmid was re-named pCR2.1-*endA*.
2. Insertional inactivation of the *endA* gene in the pCR2.1-*endA*Δ plasmid was accomplished by insertion of a Gm^R cassette from the pPS856 plasmid into a single *Sal*I site of the *endA* coding sequence. This resulted in the creation of pCR2.1-*endA*Δ(Gm1).
3. The insertionally inactivated *endA* segment from the pCR2.1-*endA*Δ(Gm1) plasmid was re-cloned into the mobilizable pEX18Ap suicide plasmid. The resulting recombinant plasmid was called pEX18Ap-*endA*(Gm1). This plasmid is now available in the future to create an *endA* mutant *P. aeruginosa* PAO1 strain by gene replacement strategies.

Future Work

In future experiments, the pEX18Ap-*endA*(Gm1) containing the *endA* gene knockout will be conjugally transferred into a wild-type *P. aeruginosa* PAO1 strain. This will allow for the creation of a new mutant *endA Pseudomonas* strain by gene replacement. Molecular verification will be performed through PCR analysis to confirm that a *P. aeruginosa* PAO1 *endA* mutant strain was created. Biochemical testing must then be done to confirm that the mutant strain lacks Endonuclease I activity verses that of the original *P. aeruginosa* PAO1 strain.

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