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ORIGINAL PAPER

A new Strategy for Gene Deletion in *Campylobacter jejuni*

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Abstract

Our protocol to generate a null mutant involved the replacement of the cytochrome P450 coding sequence with an antibiotic resistance gene (kanamycin) by homologous recombination. Two fragments, one upstream (F1 – 447 bp) and one downstream (F2 – 589 bp) of the P450 coding sequence were amplified separately by PCR and combined during the second PCR reaction giving a SmaI site at the junction point of the two DNA fragments. All the PCR reactions were performed using the proofreading polymerase for amplification (PFU). The final DNA fragment obtained (F3 – 1036 bp) was cloned into the SmaI site of the pBluescript SK- vector. This paper describes a new efficient strategy of gene knockout in Campylobacter jejuni via kanamycin gene replacement.

Keywords: bacterial pathogens, gene knockout, electron transport proteins

Introduction

In the last few years research on pathogenic bacteria increasingly involves genetic manipulation. Tompinks (1992) revealed that for pathogenic bacteria it is important to identify the potential virulence factors and especially to identify their precise role by using isogenic mutants. At the moment it is very common to produce mutants in *Campylobacter* either by transposom mutagenesis or by targeted gene inactivation. Introduction of the DNA into the organism may be achieved either by natural transformation or electro-transformation. The strategy used in this new approach is presented in figure 1 and the primers design in figure 2.

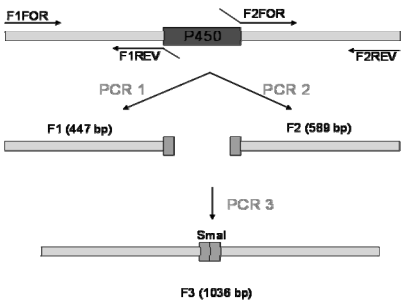

Fig. 1. Molecular strategy for gene knockout construction

Two fragments one upstream (F1) and one downstream (F2), were amplified separately by PCR 1 for F1 and PCR 2 for F2. In PCR 3 fragments F1 and F2 were combined forming the F3 fragment which has the *SmaI* restriction site at the junction between the two fragments.

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F2FOR                          TAACTCTTTTCACCCGGGATTTTTACCTTTTCTTCTAA
F1REV       CGCTTAAAATCAAGATTGAGAAAAGTGGGCCCTAAAAATGGAAA
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Fig. 2. Design of the proximal primers used to achieve the fusion PCR. The 30 nucleotide overlap is presented with the *SmaI* cleavage site in blue and the rest of the DNA sequence in black.

Materials and methods

Agarose Gel-Electrophoresis

Agarose gel electrophoresis of DNA fragments produced by restriction endonuclease digest or generated by PCR was typically performed in 0.8-1% (w/v) agarose gels. Gels were prepared by melting the appropriate amount of agarose in 1X TAE buffer and adding ethidium bromide to a final concentration of 0.1 μg/ml. DNA samples to be analyzed were mixed with 1X agarose gel loading buffer and water up to 14 μl and loaded directly onto the gel. The gel was placed in 1X TAE buffer and run at a constant current of 45 mA for 1 hour. To estimate the size of DNA fragments, 1Kb Plus DNA Ladder (Gibco BRL) was also loaded on the gel. The DNA could be visualized on a UV transilluminator.

1 X TAE Buffer (Tris-acetate): 0.04 M Tris – acetate, 0.001 M EDTA

Purification of DNA from agarose gels

As an alternative to the previous protocol, the DNA fragments produced by PCR or by restriction endonuclease digestion were purified after separation on an agarose gel. Using a clean razor blade, the band of interest was excised from the gel and further purified using the QIAquick gel extraction kit (Qiagen), following the manufacturer's guidelines. DNA was typically eluted in 20 μl of elution buffer (supplied with the kit) and stored at -20°C.

Ligation of DNA fragments

Ligations were typically performed in a total volume of 15 μl, containing 20-30ng of vector DNA, approximately 8 times this amount of insert DNA, 1X ligation buffer and 2 units of T4 DNA ligase (Promega or Novagen). The reactions were incubated at 16 ^{0}C overnight.

Restriction digests

Restriction endonuclease digestion of DNA was typically performed in volumes of 15-50 μl. These volumes contain the required quantity of DNA (usually 2-5 μg) and the appropriate restriction buffer (as supplied by the manufacturer) at 1X concentration. The restriction enzyme (typically 2-5 units) was added the last to the reaction taking care to keep the restriction enzyme volume below 10 % of the total volume. The restriction digest was incubated at the temperature recommended by the supplier for a period of 2-10 hours. For the double digest reaction, both enzymes were added concurrently providing that both would retain full activity in a suitable buffer. The products of the digestion were analyzed by agarose gel electrophoresis and isolated from the gel and purified.

Automated sequencing

A typical reaction mix was set up as follows: template DNA 200ng of plasmid DNA or 100-120ng of PCR product, primer 1.6pmol, terminator mix 4 μl, sterile and distilled water up to 10 μl. The following sequencing program was run for 25 cycles:

Step1: 96°C for 30 seconds
Step2: 50°C for 15 seconds
Step3: 60°C for 4 minutes

When was necessary DNA was precipitated from the reaction mix by adding 25μl of ice-cold ethanol (abs.) and 1μl of 3M NaOAc, pH 5.2, incubating on ice for 20 minutes followed by centrifugation (13,000rpm, 30 minutes, 4°C). The pellet was washed with 250 μl

of ice-cold 70% (v/v) ethanol and dried on bench for 1 hour. Samples were processed by the ICMB Automated Sequencing service on an ABI377 instrument.

Polymerase chain reaction (PCR)

Specific regions of DNA were amplified using the polymerase chain reaction (PCR). A PCR reaction mix was set-up in a 0.5ml Eppendorf tube as follows:

10X Polymerase Buffer	1X
Template DNA	10-500 ng of genomic DNA or 10-30 ng of plasmid DNA
Oligonucleotid primer 1	0.5 μM
Oligonucleotid primer 2	0.5 μM
dNTPs (dATP, dCTP, dGTP, dTTP)	200 μM each
$MgCl_2$ (when necessary)	1.5-6 mM
DNA polymerase[a]	1.25 units per 50 μl
Sterile distilled water	up to 50 μl

PCR reactions were carried in a PHC - 2 cycler from TECHNE programmed depending on the length of the desired product and the annealing temperature of oligonucleotide primers used.

A typical program contained the following steps:

Step 1 – Denaturation:	94°C	3 minutes
Step 2 – Denaturation:	94°C	1 minute
Step 3 – Annealing:	T_d°C[b]	1 minute
Step 4 – Extension:	72°C	1 minute[c]
32 cycles of Step 2 – Step 4		
Step 5 – Final extension	72°C	3 minute

[a]for most applications, *Pfu* DNA polymerase was used.

[b]T_d°C is the annealing temperature calculated for oligonucleotides up to 20 bases long with the formula:

$$T_d°C = \{4(G+C) +2(A+T)\} - 4$$

[c]One minute extension time per every kb of the length of the desired product.

Colony PCR

One bacterial colony was resuspended in 50 μl dH_2O and boiled for 5 minutes. 5 μl of the lysate was used to perform PCR reaction under the conditions described above.

Natural transformation

For natural transformation *C. jejuni 11168* was cultured on Muller-Hinton agar plates o/n at 42 ^{0}C. The cells were harvested and diluted to a concentration of 10^8 cfu / ml. To induce competence, 200 μl cells were incubated for 3 hours at 37 ^{0}C on 1 ml Muller-Hinton Broth in 2 ml micro test tube. Then the DNA was added and incubated for another 3 hours at 37 ^{0}C. Cells were harvested and plated on Muller-Hinton plates supplemented with Skirrow antibiotics and incubated at 42 ^{0}C for 48 hours (Wang, 1990).

Electroporation

For transformation by electroporation *C. jejuni 11168* was cultured on Muller-Hinton agar plates o/n at 42 ^{0}C. Cells were harvested in 1 ml EBP (272 mM sucrose, 15 % glycerol, 2.43 mM K_2HPO_4, 0.57 mM KH_2PO_4 at pH 7.4).

Cells were harvested again and washed twice in EBP. 1µg DNA was dissolved in water and mixed with 50 µl electrocompetent cells and in a pre-chilled 0.56 mm cuvette were pulsed at 0.7 kV, 25 µF and 600 Ω. The content of the cuvette was mixed with 0.2 ml Muller-Hinton Broth and plated on Muller-Hinton agar plates for 5 hours at 37 ^{0}C. At the end of incubation the cells were harvested and plated on Muller-Hinton agar plates supplemented with Skirrow antibiotics and 300 µg Km / ml to select transformants (Miller 1988).

Results and discussion

Primer design and PCR amplification of fragment F1

The F1 fragment (447 bp long) was amplified by PCR using as a template genomic DNA. The fragment was amplified using primers F1FOR (1 pmol / µl) and F1REV (1 pmol / µl) in reaction in which 0.5 µl genomic DNA was used. For a better amplification and to avoid the unexpected mutations *PFU* polymerase was used (0.5 µl / reaction).
A ≈ 447 bp long fragment was identified in agarose gel using a molecular weight marker and was sequenced by automated sequencing to identify possible inserted mutations which can be inserted during PCR reaction. The 447 bp long fragment (fig. 3) was purified from agarose gel and used as a template in the fusion PCR.

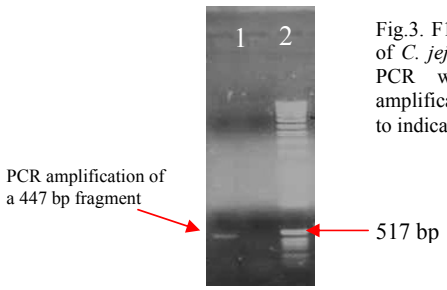

Fig.3. F1 PCR. Primers F1FOR and F1REV successfully initiated PCR amplification of *C. jejuni 11168* genomic DNA upstream of Cj1411c (P450 gene). The results of PCR were analysed using agarose gel electrophoresis that revealed the specific amplification of the expected 447 bp fragment (lane 1). 1 kb ladder (lane 2) was used to indicate the size of the PCR amplification products.

Primer design and PCR amplification of fragment F2

Using a similar protocol, as for F1 fragment, the F2 fragment (589 bp) was amplified by PCR using genomic DNA as template (fig. 4). In this case the primers used were F2FOR (1 pmol / µl of reaction) and F2REV (1 pmol / µl of reaction). The fragment was successfully amplified using *PFU* polymerase and purified from agarose gel. The purified DNA was the second template used in the fusion PCR. To identify possible mutations F2 was sequenced by automated sequencing with two point mutations detected. The thermocycle was programmed as with fragment 1.

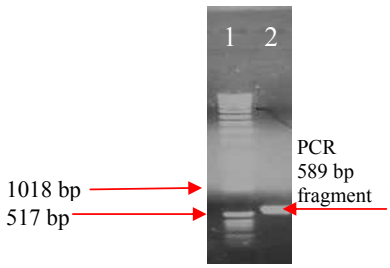

Fig. 4. F2 PCR. Primers F2FOR and F2REV successfully initiated PCR amplification of *C. jejuni 11168* genomic DNA upstream of Cj1411c (P450 gene). The results of PCR were analysed using agarose gel electrophoresis that revealed the specific amplification of the expected 589 bp fragment (lane 2). 1 kb ladder (lane 1) was used to indicate the size of the PCR amplification products.

To eliminate amplification of erroneous products during the final fusion PCR step, the desired 589 bp product was excised from a preparative agarose gel and purified. A high yield of the 589 bp PCR product was recovered to provide a suitable template for fusion PCR.

Amplification of fragment F3

The PCR products of the first two primary amplifications were used as a template DNA for the final fusion PCR amplification. The distal primers F1FOR and F2REV were used to amplify the gene-flanking DNA as a continuous sequence containing a *SmaI* cleavage site. High levels of the fusion PCR fragment at a size of 1036bp were obtained for each of the reactions with no secondary PCR products amplified (fig. 5).

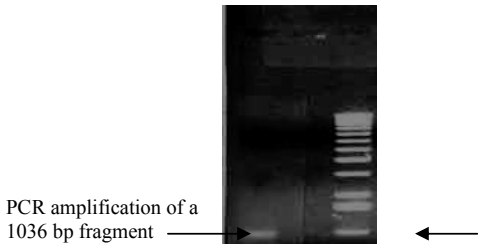

Fig. 5. F3 PCR. The distal primers F1FOR and F2REV initiated the fusion PCR of the gene-flanking genomic DNA sequences as a continuous fragment containing an *SmaI* cleavage site.

The DNA (0.02 μg) was purified from an agarose gel, ligated into the pBlueScript II SK$^-$ suicide vector and subsequently sequenced to check that no mutations were inserted during the PCR reaction.

Cloning of the F3 fragment into pBlueScript II SK$^-$ plasmid

The PCR template DNA generated by fusion PCR was inserted into pBlueScript II SK$^-$ (figure 6). This vector was chosen because of its previous use as a suicide vector to generate gene replacement in *Campylobacter* and because provides a unique *SmaI* cleavage site suitable for the insertion of the fusion PCR product by blunt-ended cloning necessitated by *PFU* amplification.

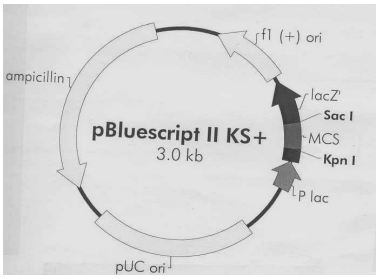

Fig.6. The map of pBlueScript II SK$^-$

A sample of pBlueScript II SK$^-$ plasmid DNA was cut with *SmaI* to generate blunt ends that were ligated to blunt ends of the fusion PCR amplified using *PFU* polymerase (fig. 8). Recombinant plasmid pBlueScript II SK$^-$ containing the homologous template DNA fusion PCR product was transformed into *E. coli* TG1. The transformants were screened for ampicillin resistance encoded on the plasmid DNA. On the *cells only* control plate no colonies

were found which indicated that the colonies on the transformation plates would all contains the pBlueScript II SK$^-$ plasmid. The recombinant plasmid has been called pBSF3 (fig. 7). Plasmid DNA was isolated from 7 single ampicillin resistant colonies and *SmaI* restriction digests were performed to identify those with the recombinant pBlueScript II SK$^-$plasmid. Cutting with *SmaI* indicated the presence of the *SmaI* restriction site inside the F3 fragment. Two of the colonies screened had the inserted fusion PCR into the *SmaI* site.

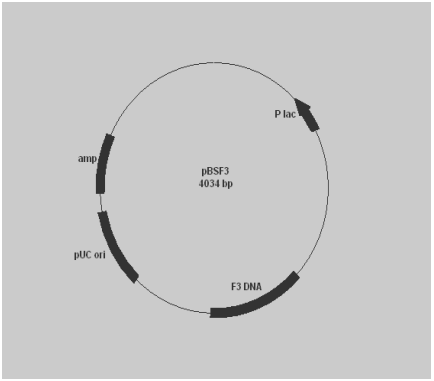

Fig. 7. Schematic representation of the pBSF3 plasmid

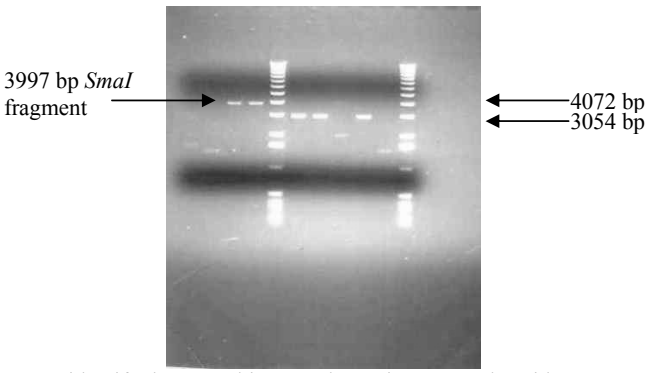

Fig. 8. Restriction digests to identify the recombinant pBlueScript II SK$^-$ plasmid
The homologous template DNA fragment was inserted in pBlueScript II SK$^-$ using blunt-end cloning at a *SmaI* cleavage site. Recombinant pBlueScript II SK$^-$ plasmid, named pBSF3, was identified using *SmaI* that cut the inserted homologous template DNA to give a single restriction fragment at 3997 bp (lanes 1 and 2). The presence of a single restriction band suggests that the F3 fragment has been inserted into the *SmaI* site of the pBlueScript II SK$^-$ plasmid with the designed *SmaI* site present in the middle of the F3 fragment. Lanes 4, 5, 6, 7 and 8 shows that this colonies had transformed only the pBlueScript II SK$^-$ plasmid without the fusion PCR product 1kb ladder was loaded into lanes 3 and 9.

The gene replacement fragment was a kanamycin cassette from *E. coli* transposom Tn5 provided as a 939 bp fragment from pSUP10121 plasmid. The *EcoRV* cleavage sites flanking the 5` and 3` termini of the cassette were digested to release the *EcoRV* kanamycin fragment from pSUP10121 plasmid. pBSF3 plasmid was digested with *SmaI* to generate blunt ended termini in order to ligate the kanamycin cassette. The recombinant plasmid was transformed into *E. coli* TG1 and transformants were screened for ampicillin and kanamycin resistance. *E. coli* TG1 *cells* are plasmid free bacteria which is why no growth was detected when kanamycin was added on the Agar plates. The number of colonies obtained from cells transformed with recombinant plasmid generated from blunt-ended ligation was sufficient in

order to perform the restriction digests for identification of insertion of the kanamycin cassette into the *SmaI* site of the pBSF3 plasmid. Following antibiotic selection, plasmid DNA was isolated from 3 single colonies and *EcoRI* restriction digests were used to verify insertion of the kanamycin cassette (fig.9).

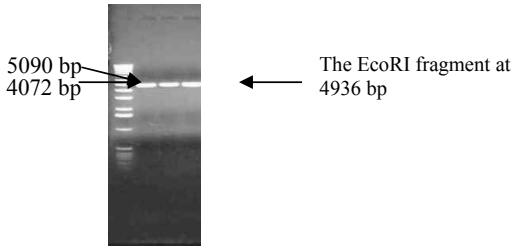

Fig. 9. Restriction digests to identify the insertion of the kanamycin cassette
The blunt-ended kanamycin cassette was inserted in the homologous template DNA of pBSF3 to form a kanamycin resistant gene replacement fragment. The new plasmid was named pBSF3-Km$^+$. Insertion of the kanamycin cassette into the *SmaI* site of the pBSF3 plasmid was verified using *EcoRI* restriction digests which cuts the plasmid in a single position without cutting into the homologous template DNA or inside the kanamycin cassette. The new plasmid (pBSF3-Km$^+$) has a size of 4936 bp. The restriction fragments of pBSF3-Km$^+$ plasmid DNA isolated from colonies 1-3 are shown in lanes 2-4. 1kb DNA ladder (lanes 1) was used to indicate that the EcoRI restriction fragments were of the correct size.

DNA transfer into *Campylobacter jejuni 11168* wild type

The pBSF3-Km$^+$ plasmid carrying the gene replacement fragment was transferred by electroporation and natural transformation into the wild type strain following the protocols described in material and methods. The pBSF3-Km$^+$ plasmid was introduced into *Campylobacter jejuni 11168* to allow homologous recombination to occur between the cloned gene replacement fragment and homologous DNA of the bacterial chromosome. Following the transformation the transformants were screened for kanamycin. *Campylobacters* are not resistant to kanamycin which explains why no growth was detected on kanamycin Agar plates. All the colonies resistant to kanamycin were than subjected to PCR screening to identify the replacement of the P450 gene sequence from the genome.

PCR screening to identify the replacement of the P450 gene

PCR screening was used to scan the transformants genomic DNA for the correct replacement of the P450 gene. Three types of colony PCR were performed:
PCR 1 – using Cj1411cFOR and Cj1411c REV primers on transformants genomic DNA and on wild type strain (fig.10, A and B); PCR 2 – using Tn5Km(L) and Tn5Km(R) which will amplify the Km cassette on transformants genomic DNA (fig.10C); PCR 3 – using primers F1FOR and F2REV which will amplify the homologous template DNA (1962bp fragment) from the transformants genomic DNA (fig 10 D)

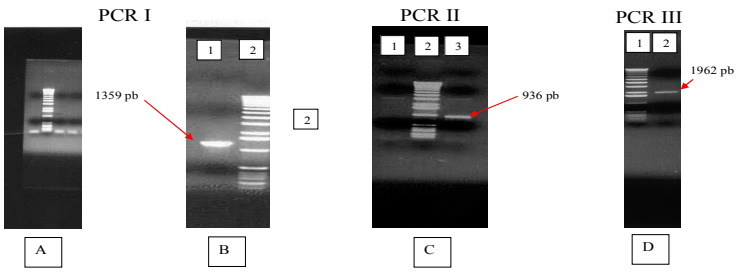

Fig. 10.PCR screening of transformants

A – PCR was performed using Cj1411cFOR and Cj1411cREV with transformants colonies and no amplification was detected by visualisation on agarose gel. B – this second PCR result was performed in conditions similar to PCR A but instead of transformants colonies the wild type colonies were used and 1359 bp fragment was identified in agarose gel which corresponds to the P450 gene (indicated by arrow in lane 1). In lane 2, 1 kb ladder is present. C – in order to detect the presence of the kanamycin cassette on the knockout strain chromosome Tn5Km(L) and Tn5Km(R) were used. Lane 1 shows that with the wild type colonies no amplification was detected and when the knockout colonies are used a band corresponding to 939 bp is amplified (lane 3). This PCR proves that the kanamycin cassette was inserted into the wild type chromosome. D – the third PCR was performed to identify the presence of the homologous template DNA (1962 bp) on the knockout chromosome. The same transformant colony used in PCR II was used for amplification of the desired fragment (1962 bp), in PCR III, which suggests that the homologous recombination was successfully performed (lanes 2). In lane one, 1 kb ladder is present.

In order to compare P450 expression into the wild type strain with the NCI strain (*Campylobacter jejuni* knockout strain, after P450 gene deletion) the cells were sonicated and the supernatant was tested for P450 expression. As presented in figure 11, there was no P450 expression detected in the NCI strain while the wild type shows that the P450 expression is present.

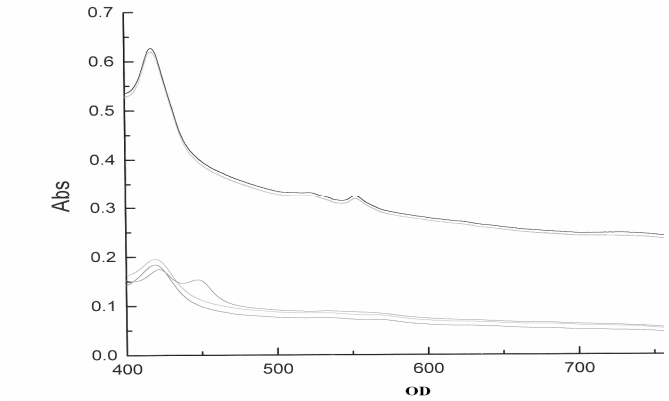

Fig. 11. NCI and wild type supernatant spectra for detection of the P450 expression

Conclusions

Despite years of research and a complete genome sequence (Parkhill *et al*., 2000), there is remarkably little understood about the molecular pathogenesis of *C. jejuni.* Considerable effort is being directed toward understanding the molecular basis of pathogenesis of this species. Genetic analysis of *C. jejuni* and its putative virulence factors has been difficult due to the absence of characterised systems for *in vivo* genetic manipulation. Techniques for DNA transformation or generalized transduction have not been described. This paper provides useful information for knockout construction in *Campylobacter spp.* The pBlueScript II SK^- was proven to be a suitable vector for gene replacing, in *Campylobacter jejuni* 11168, with a kanamycin resistance gene from *E. coli* Tn5. The successful deletion of the P450 gene from *Campylobacter jejuni 11168* genome offered the possibility to study the phenotypic differences between the mutant strain and the wild type strain.

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