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Genetic Compensation of *E. coli* C156 with pBAD-*ompA* for the Investigation of Conjugation Efficiency

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OmpA is a membrane protein known to serve a stabilizing function in mating aggregates of *Escherichia coli*. The disruption of OmpA function has been shown to reduce conjugation efficiency. Currently, little progress has been made in creating a reliable construct that allows *ompA* expression to be differentially controlled or over expressed. Such a construct would allow researchers to further elucidate the precise role of *ompA* expression in F-conjugation. To this effort, the current study generated a pBAD TOPO-*ompA* construct using the TOPO TA cloning technology in an attempt to transform into an *ompA*$^-$ *E. coli* C156 strain. The presence and orientation of pBAD TOPO-*ompA* was verified using PCR screening with novel pBAD TOPO primers as well as *Hinc* II restriction digest. Secondary transformation of the new construct into *E. coli* C156 was performed, and pBAD clones were isolated. A 10 kb band of undigested plasmid shown in the plasmid preparation of a potential transformant supports the presence of pBAD TOPO-*ompA* in the *E. coli* C156 clone.

Conjugation is a process by which bacterial cells horizontally transfer genetic material via a physical attachment of two cells. There are many proteins involved in the attachment process, which ultimately leads to fusion of the two cells' membranes for a brief period of time (3). Initiation and stability of the membrane fusion is not fully understood, but several candidate proteins are expected to play a role. Specifically, the 1047 base pair *ompA* gene codes for the abundant outer membrane protein A (OmpA) in *Escherichia coli*. This 47 kDa protein has suspected importance in the conjugation process (5).

Previous work has shown that *ompA*$^-$ *Escherichia coli* C156 have greatly reduced conjugation efficiency (2). Furthermore, deficient strains that have been complemented with a vector containing the *omp*A gene have recovered wildtype conjugation abilities. Other investigations have explored cloning the *omp*A gene into a pCR2.1 vector (2), but expression was neither inducible nor quantifiable. To date, no studies have been performed involving varying expression levels or the over expressing of *omp*A. Further experimentation on *ompA* expression and its effects required an inducible construct.

Past attempts at using the TOPO TA cloning system (4) to achieve this construct have shown limited success in screening the transformants (8). The cloning system avoided lengthy and troublesome restriction digest incubations by using the available 3'-guanidine overhangs left on the PCR product by *Taq* polymerase. These ends were paired to overhanging thymidines on the pBAD vector, where ligation was completed by the associated topoisomerase. No cleanup of the PCR reaction was necessary, and the specificity yielded high cloning efficiency.

Previous experiments have used *E. coli* C156 as the final recipient strain, as it is *omp*A$^-$ and selectable by streptomycin (1,2). This experiment successfully cloned and screened the desired pBAD-*ompA* product and created *E. coli* C156 transformants that potentially harbour the correct clone. Further screening of the created clones to verify the *omp*A insert and its directionality remain to be accomplished.

MATERIALS AND METHODS

Bacterial *s*trains and culture conditions. MG1655 and *Escherichia coli* (*E. coli*) C156 (*ompA*$^-$) strains were obtained from the Department of Microbiology and Immunology at the University of British Columbia. Luria-Bertani (LB) broth (1% (w/v) tryptone (Bacto™, Cat. #211705), 0.5% (w/v) yeast extract (Bacto™, Cat. #212750), and 1% (w/v) NaCl (Fisher Chemicals, Cat. #S271-3), adjusted to pH 7.5) was used to culture MG1655. All bacterial liquid cultures were grown for 16 hours in a shaking incubator at 37˚C (200 rpm). TOP10 OneShot Chemically Competent *E. coli* cells were obtained from the pBAD TOPO TA® Expression Kit (Invitrogen, Cat. #K4300-01) for transformation. Transformed TOP10 cultures were spread on LB agar (1.5% (w/v) agar (Invitrogen, Cat. #30391-023)) plates containing 100 ug/ml ampicillin (Sigma, Cat. #A-9518) and grown at 37˚C for 16 hours overnight. Eight TOP10 clones selected from the transformation were inoculated in 5 ml of LB broth containing 100 ug/ml ampicillin for plasmid preparation. *E. coli* C156 cultures were grown in LB broth containing 100 ug/ml streptomycin (Sigma, Cat. #S6501-50G) before they were made chemically

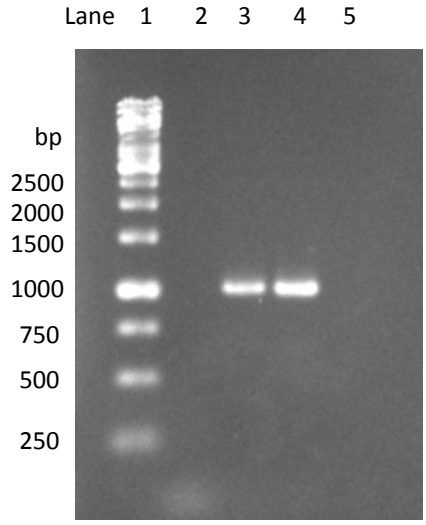

FIG. 1. PCR Amplification of 1057 bp *ompA* gene. Lane is a negative PCR control; Lanes 2 and 3 contain replicate PCR reactions with a strong band representing *ompA*.

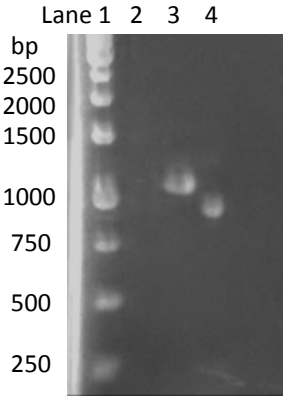

FIG. 2. *Bgl* II restriction digest of PCR amplified *ompA*. Lane 3 contains uncut PCR product; Lane 4 is the digested DNA giving a band at 900 bp.

competent. Transformed *E. coli* C156 were streaked on LB agar containing 100 ug/ml Ampicillin, and six clones were inoculated in 5 ml of LB containing 100 ug/ml ampicillin and 100 ug/ml Streptomycin for plasmid preparations.

Colony PCR amplification of *ompA*. The forward (5'- **TGA** GAG GAT TCC CCC C**AT G**AA AAA GAC AGC TAT CG -3') and reverse (5'– **TTA** AGC CTG CGG CTG AGT TAC AAC GTC TTT -3') *ompA* primers were previously designed (8) to remove the N-terminal leader sequence via an in-frame stop codon and translation re-initiation sequence that allows for native expression of *ompA* (NCBI GenBank, GI: 48994873). The PCR reaction (reagents supplied in Invitrogen kit) included 1X PCR Buffer, 100 uM dNTPs (50 mM stock), 1 mM of each *ompA* primer and 150 mM $MgCl_2$. The final reaction volume was 24 ul, which was incubated at 94˚C for 10 minutes before adding 0.2 U of Taq polymerase and a section of a single bacterial colony. Reactions were incubated in Biometra® T Gradient (Software version 4.15) PCR machine at 94°C for 4 min, followed by 35 cycles of amplification: denaturing at 94°C for 45 sec, annealing at 55°C for 30 seconds and then elongation at 72°C for 90 sec. The final extension consisted of 10-min incubation at 72°C. PCR products were analyzed using 1.0% (w/v) agarose gel electrophoresis.

***Bgl* II restriction digest of *ompA*.** Amplified *ompA* was digested using 2 U of *Bgl* II (GibcoBRL, Cat. #15213-028) in a reaction containing 1x REact3 (Gibco BRL, Cat. #Y90004), 4 ul PCR sample, and distilled water with a 10 final volume. The digestion occurred over a 37ºC incubation for one hour. Reaction products were stored at -20ºC and analyzed using 1.0% (w/v) agarose gel electrophoresis.

DNA gel electrophoresis. DNA gels consisted of 1.0% (w/v) agarose (Bio-Rad, Cat. # 161-3101) in 1X TAE buffer (40 mM Tris Base (Fisher Bioreagents, BP152-1), 0.1% (v/v) glacial acetic acid (Across, Cat. #42322-0025), 1.3 mM EDTA (Fisher Chemical, Cat. # BP120-1), adjusted to pH 7.5). 1 ug of Generuler™ 1 kb DNA Ladder (Fermentas, Cat. #SM0312) was used as a molecular weight standard, and 1X loading dye (Fermentas, Cat. #R0611) was included with each sample. The solidified gel was placed in 1X TAE electrode buffer for one hour at 108 volts. A 0.5 ug/ml ethidium bromide bath was used to post-stain the gels overnight. Bands were visualized and saved using AlphaImager software (v. 4.1.0, Alpha Innotech Group).

TOPO TA cloning of *ompA* into pBAD TOPO vector. As described in the manufacturer's protocol, 4 ul of *ompA* PCR amplicon was used in the pBAD TOPO TA® Expression Kit cloning reaction (Invitrogen, Cat. #K4300-01) to transform *ompA* into the pBAD vector.

Alkaline lysis plasmid preparation. Alkaline lysis and phenol-chloroform extraction were performed according to Protocol 1.1, "Preparation of Plasmid DNA by Alkaline Lysis with SDS: Minipreparation" protocol (7) on a 4.5 ml liquid culture of selected clones. The final plasmid resuspension volume was 50 ul.

PCR primer design for pBAD TOPO-*ompA* constructs. Primers provided by Invitrogen to amplify the cloning site in pBAD TOPO constructs are optimized for sequencing, and were not appropriate for PCR screening of insert orientation (Invitrogen Technical Support, 1-800-831-6844). New primers were designed to amplify the cloning site of pBAD TOPO. Forward primer (5'- GAT CCG GTG ATG ACG ATG AC -3') (IDT, Cat. #80468649) and reverse primer (5'- AGA CCG AGG AGA GGG TTA GG -3') (IDT, Cat. #80468648) were designed using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). Before use, the primers were reconstituted with distilled water to 100 uM.

PCR screening of pBAD TOPO-*ompA* constructs. The insert region of pBAD from alkaline lysis plasmid preparation of TOP10 clones were screened using PCR. Both newly designed pBAD primers and *ompA* (8) were used in combination in this screen. *OmpA*-*ompA*, pBAD (forward)-*ompA* (reverse), and *ompA* (forward)-pBAD (reverse) primer combinations were used to screen each clone to verify insert directionality. PCR reactions contained 1mM of each primer and 0.5 ul (approximately 500 ng) of neat plasmid preparation. With the exception of the different primer combinations, the PCR reaction was identical to the composition described previously in the PCR amplification of *ompA*.

Secondary transformation of pBAD-*ompA* into *E. coli C156*. *E. coli* C156 cells were made chemically competent and transformed with pBAD-*ompA* construct through a procedure described in protocol 1.25, "Preparation and Transformation of Competent *E. coli* Using Calcium Chloride" (7). Six clones that survived both

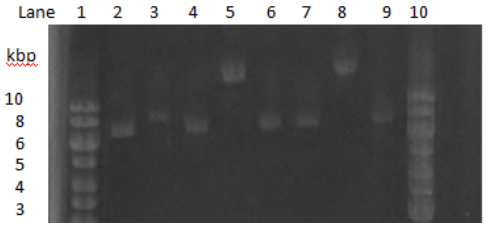

FIG. 3. Agarose gel analysis of plasmids prep TOP10 transformants. The plasmid DNA from clones EA11W1-8 are shown in Lanes 2-9 respectively. Lanes 5 and 8 display higher molecular weight plasmid.

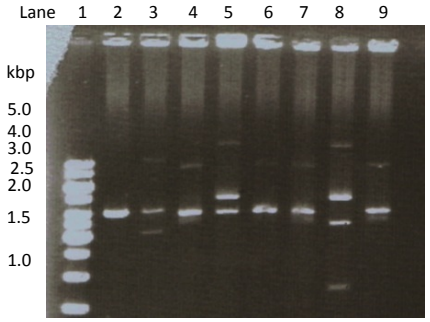

FIG. 4. *Hinc* II restriction digest of plasmid preparations. The isolated and *HinC* II digested plasmid DNA from clones EA11W1-8 are shown in Lanes 2-9 respectively. Lanes 5 and 8 display banding patterns that match those expected for forward and reverse *ompA* inserts

ampicillin and streptomycin selection were screened via *Hinc* II digestion.

***OmpA* insert orientation analysis by *Hinc* II restriction digest.** Both TOP10 and *E. coli* C156 transformants were screened using *Hinc* II restriction digest. The 10 ul digest reaction was comprised of 8.5 ul of neat plasmid DNA, 1 ul of 10X NEBuff 3 (NEB, Cat. #B7003) and 0.5 units of *Hinc* II enzyme (NEB, Cat. #R0103). Reactions were incubated at 37˚C for 1.5 hours. Reaction products were stored at -20^{o}C and analyzed using 1.0% (w/v) agarose gel electrophoresis.

RESULTS

Amplification and analysis of *ompA*. The two PCR reactions for the amplification of *ompA* were analyzed by agarose gel electrophoresis, and the results are shown in Figure 1. Both samples produced a strong band which migrated at 1046 bp band, which corresponds with the expected size of 1057 bp for the *ompA* gene. There was no non-specific amplification and the negative control was free of any contamination. To confirm the identity of the amplified product, the *Bgl* II restriction digest of the *ompA* product was analyzed by agarose gel electrophoresis, and the results are shown in Figure 2. The uncut *ompA* in Lane 3 migrated at 1050 bp as expected, while the *Bgl* II digested product in Lane 4 migrated with one band at 900 bp. Although two bands were expected for this digest at 861 bp and 161 bp, the lower band was too faint to be seen by this analysis.

Transformation of TOP10 *E. coli* with pBAD-*ompA*. There were 5 colonies recovered from the 10 ul plating and 50 colonies recovered from the 50 ul plating of transformed chemically competent TOP10 *E. coli*. Furthermore, the untransformed negative controls showed no colonies as expected, confirming that the ampicillin selection was effective. The number of positive colonies was significantly lower than the optimum transformation efficiency suggested by the manufacturer, however it did yield sufficient transformants to move forward with. From the 50 ul plating, 8 colonies were selected and designated EA11W-1 through EA11W-8. These selected colonies were cultured in liquid media for further experiments.

Insert size and orientation analysis. The plasmids isolated by alkaline lysis from the selected transformants were analyzed by agarose gel electrophoresis and the results are shown in Figure 3. Lanes 2 through 9 show the whole plasmids from clones EA11W-1 through EA11W-8 in the respective order. Clones 1, 3, 5, 6, and 8 migrated equal distances, while clones 2, 4, and 7 appeared to migrate slower which suggests a larger total size. Since the actual sizes cannot be estimated from the uncut plasmid in unknown physical conformation, a restriction digest was then performed with *Hinc* II.

For the *ompA* insert in the correct direction, the expected band sizes after *Hinc* II digestion were 3423 bp and 1760 bp, while the expected fragment sizes for a reverse insert were 4114 bp and 1069 bp. The digested plasmids were analyzed by gel electrophoresis and the results are shown in Figure 4. Lanes 2 through 9 show *Hinc* II digested plasmid from clones EA11W-1 through EA11W-8 in the respective order, however all samples retained an uncut plasmid band in an unknown physical conformation at high molecular weight. Clones 1, 3, 5, 6 and 8 display a single band migrating at 4125 bp which corresponds to the expected size of an empty linear vector (4100 bp). Clone 2 (Lane 3) shows one band at approximately 3500 bp, and the linearized construct only slightly higher than the empty vector samples. This does not match the expected sizes for an *ompA* insert in either direction. Clone 4 (Lane 5) had two bands that migrated at 4104 bp and the 1015 bp

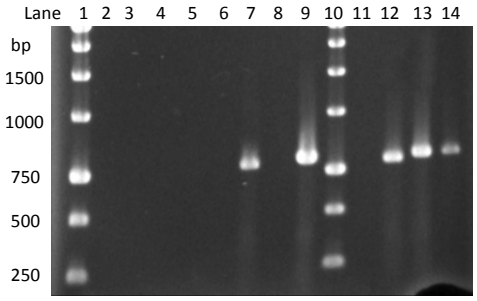

FIG. 5. PCR analysis of pBAD-*ompA* constructs for orientation. Lanes 3-6 contain an empty vector control for all primer combinations; Lanes 7-9 and 12-14 contain the PCR reactions with the following primer sets (in order) for clones EA11W-4 and -7 respectively: *ompA*-forward + *ompA*-reverse, pBAD-forward + *ompA* reverse, and pBAD forward + *ompA* forward. Lane 9 shows detection of a reverse insert in clone 4, while Lane 13 shows detection of a forward insert in clone 7.

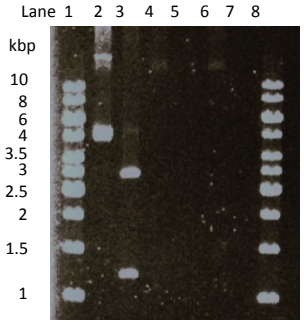

FIG. 6. *Hnc* II digest of plasmid preps from *E. coli* C156 transformants. Lanes 2 and 3 contain undigested and digested positive (forward insert) control, displaying the expected banding pattern. Lanes 4 and 6 contain the plasmid DNA isolated from a transformants containing forward and reverse inserts respectively. Lanes 5 and 7 contain those plasmids digested with *Hnc* II, but no bands are visible

band of the standard, suggesting that it contained a reverse *ompA* insert, as well as a band at approximately 5200 bp standard which could be the linearized construct. Clone 7 (Lane 8) had the same linearized construct band at 5200 bp, and had two more bands which migrated at 3486 bp and 1720 bp, suggesting a forward *ompA* insert.

PCR screening of pBAD-*ompA* constructs. In order to confirm the identity and orientation of the inserts, the PCR reaction using newly designed primers for the pBAD vector combined with *ompA* primers was analyzed by gel electrophoresis. The results are shown in Figure 5. Lanes 3 through 5 are the products of using Clone 1 as a negative control (empty vector) template in the PCR reaction, while Lanes 7-9 and 12-14 show the reaction with clones 4 and 7 respectively as the template. Lanes 3-5 confirm that the proposed empty vector did not amplify with pBAD and *ompA* primers, suggesting that there is no insert of visible size, as well as no *ompA* present.

For the reverse insert construct (Clone 4) Lanes 7 shows that *omp*A primers do amplify a 1048 bp fragment as expected for an *ompA* insert. Lane 8 shows that a combination of pBAD forward and *omp*A reverse does not produce a band at 1120 bp, however a combination of pBAD forward and *ompA* forward primers does produce a band, as is expected for a reverse insert. Similarly for the forward insert, the *ompA* primers amplified an 1048 bp fragment in Lane 12, and the pBAD forward and *omp*A reverse primers amplified an 1120 bp fragment in Lane 13, suggesting a forward insert. However, there was also a faint band in Lane 14, meaning some reverse insert template was available.

Transformation into *ompA*-deficient *E. coli* C156. Controls for ampicillin and streptomycin selection were successful, showing no colonies from the plating of cells transformed with no DNA. Plating 50 ul of *E. coli* C156 transformed with 50, 500 and 5000 ng of target plasmid resulted in colonies only on the 5000 ng transformations. Specifically, the forward insert construct transformation gave 25 colonies on both ampicillin and streptomycin selection, while the reverse insert construct transformation yielded 32 colonies. A single reverse insert transformant was selected, and 5 proposed forward insert transformants were chosen for further screening.

Analysis of pBAD TOPO-*ompA* transformation into *E. coli* C156. Plasmid preparations by alkaline lysis of the 6 selected transformants were digested with *Hinc* II as before, and analyzed by gel electrophoresis alongside the undigested constructs. The results are shown in Figure 6. The positive control in Lanes 2 and 3 are a Hinc II digest of the forward insert construct from EA11W-7 digested (Lane 2) and undigested (Lane 3). Lanes 4 and 6 represent the undigested plasmids (forward insert and reverse insert respectively), which migrate a similar distance to the uncut pBAD TOPO-*ompA* constructs in Figure 3 (at >10 kb), suggesting that they are the correct size for the target construct. However, the digested samples in Lanes 5 and 7 have no visible bands. Similar results were obtained for

repeated attempts of *Hinc* II digestion with more transformants (data not shown).

DISCUSSION

The PCR amplification of *ompA* used primers that were previously designed with proper assessment of gene function (8). This experiment proceeded on the assumption that the amplified product could be expressed from the pBAD TOPO vector and that the product would be properly located on the outer membrane of the cell.

The *Hinc* II restriction digest in Figure 4 shows that the plasmid from EA11W-7 (Lane 8) had bands with good resolution that were of the correct size to indicate a forward *ompA* insert. There was no indication of contamination with other constructs in this analysis. However, PCR analysis of this plasmid showed that there was in fact some reverse-insert construct contaminating the sample. The band in Lane 14 of Figure 5 is the result of the PCR reaction with pBAD forward and *omp*A forward primers, which should only give a band of approximately 40 bp with a forward-insert template, and would not appear on the gel. The reverse-insert band was significantly fainter after 35 PCR cycles, whereas the other bands are clearly saturated. Since the contaminating construct did not saturate, it was probably at a much lower concentration in the plasmid preparation (9). Due to the low transformation efficiency, there was no risk of overlapping colonies and therefore the only assumption is that the EA11W-7 clone was harbouring two different constructs. The pBR322 origin on the pBAD vector maintains a low copy number (4), so it is possible that multiple constructs at varying copy numbers were maintained in this strain. The sequence of the insert has potential to affect the physical conformation of the plasmid (6) which may impact the replication efficiency and could explain the difference in observed levels.

The *Hinc* II digestion of the plasmids isolated from the transformed *E. coli* C156 resulted in no visible bands, with several possible explanations. First, the undigested samples are already faint on this gel, so a cut plasmid of the same mass and concentration would be even fainter. However, previous experiments using the same digest contained samples of similar concentration which were visible after digestion (Figure 4). Another possible reason is that other endonucleases present in the *E. coli* C156 strain were not removed by the alkaline lysis procedure, and they were active during the restriction digest incubation at 37°C, which is the optimal temperature for many nucleases. Since the alkaline lysis procedure is performed mostly on ice, the undigested plasmids were not affected by this problem. In comparison, the first transformation into TOP10 *E. coli* would not have suffered from this problem because the TOP10 strain has the majority of the *E. coli* endonuclease genes knocked out. Usually the phenol-chloroform step in the alkaline lysis procedure should have removed the majority of the protein in the sample (7), though it may not have been effective in this experiment.

There is significant evidence that the constructs contained in clones EA11W-4 and EA11W-7 are the pBAD vector containing the *ompA* gene in reverse and forward orientation respectively. Although expression analysis was not reached in the scope of this study, this work concludes that these constructs have been successfully created and potentially cloned into the *ompA* deficient *E. coli* C156.

FUTURE DIRECTIONS

Further analysis of the *E. coli* C156 transformants is needed, as they likely carry the desired pBAD-*ompA* construct. A more specialized alternative method for isolating the plasmids at a higher concentration or purer yield may offset the difficulties encountered by this study to quantify the transformed plasmid. Upon confirmation of the constructs, expression analysis of *ompA* in the deficient *E. coli* C156 strain can begin.

Analysis of membrane associated proteins such as OmpA requires robust isolation methods, which should be the aim of future studies. A comparison between outer membrane protein preparations of *E. coli* C156 ($ompA^-$) and a comparable $ompA^+$ strain can be performed to validate such an isolation method. Once outer membranes can be isolated, the inducible expression of *ompA* by L-arabinose can be studied. Varying arabinose concentrations or induction times could yield measurable differences leading to the verification of a reliable *ompA* inducible construct. Finally, the connection between OmpA abundance in the outer membrane of either the donor or the recipient can be correlated with F-conjugation efficiency in further experiments.

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