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Troubleshooting for the Proposed Construction of pBAD24-*ompA*

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A better understanding of the mechanisms controlling conjugation efficiency is imperative, as the horizontal transfer of DNA can have profound implications clinically and industrially. OmpA, an abundant outer membrane protein in *Escherichia coli*, has been demonstrated to have a crucial role during conjugation in gain-of function and loss-of-function experiments; however, the effects of varying the abundance of OmpA have not been demonstrated. In this experiment, the construction of a clone containing a pBAD24-*ompA* construct was attempted. Due to difficulties in the production of the construct, troubleshooting was carried out to determine the source of the quandary. It was hypothesized that the source of the problem could have stemmed from a form of "gene discrimination" of *ompA* for pBAD24 in either orientation, where the *ompA* gene preferentially inserted into another vector during ligation.

Bacterial conjugation involves cell-to-cell contact, recipient cell recognition, mating-pair stabilization, and DNA transfer (2). Measuring conjugation efficiency is imperative, as bacterial transfer of deoxyribonucleic acid (DNA) is an important process that can potentially lead to the emergence of antibiotic resistance strains of bacteria, making the process clinically relevant. Developing techniques to regulate conjugation efficiency can prove to be useful to limit the spread of antibiotic genes. On the contrary, enhancing conjugation efficiency can be beneficial for industrial uses, such as the manufacturing of antibiotics (3).

Bacterial conjugation has been studied extensively; however, the exact mechanisms controlling conjugation efficiency have not yet been demonstrated (2). Outer membrane protein A (OmpA), which makes up as much as fifty percent of the total outer membrane proteins in *Escherichia coli* (*E. coli*), is essential for conjugation. Strains of *E. coli* that are OmpA-deficient cannot act as recipients, due to OmpA's role of stabilizing mating pairs during conjugation (7). Conversely, restoring *ompA* expression in OmpA-deficient *E. coli* has been demonstrated to restore the strains' ability to act as a recipient during conjugation (5). That said, there have been limited studies on the effects of varying levels of OmpA.

The insertion of a gene into a plasmid usually does not allow for the modulation of gene expression. Incubation with the inducer of the plasmid's promoter can only restore that gene's expression to one level (4). In contrast, by adding different concentrations of the inducer arabinose, the expression of genes can be modulated under the control of the arabinose-inducible *araBAD* (pBAD) promoter, which is from the arabinose operon. Furthermore, expression can be reduced to extremely low levels when glucose is present, as catabolite repression occurs in the presence of glucose (6). The mechanism by which the pBAD promoter is controlled is similar to that of the *lac* operon. Arabinose binds to the repressor AraC, which in turn opens the DNA loop and allows RNA polymerase to bind; in the absence of glucose, the level of cytosolic cyclic adenosine monophosphate (cAMP) rises and the cAMP interacts with catabolite activator protein (CAP) (6). The CAP-cAMP binds at the pBAD promoter, which in turn enhances RNA polymerase binding and increases transcription levels (6). This allows for the controlled expression of the gene of interest, permitting the restoration, up-regulation, and differential expression of OmpA in a single strain.

The current investigation attempted to produce an OmpA-deficient clone containing a pBAD24-*ompA* construct, such that future experimentation could test the effects of varying *ompA* expression on conjugation efficiency. Previous researchers had attempted to achieve similar goals, but they had received the incorrect pBAD28 vector as opposed to pBAD24 (1). However, after numerous attempts failed to produce a pBAD24-*ompA* construct, troubleshooting resulted in the hypothesis that the source of the problem could have stemmed from a form of "gene discrimination" of *ompA* for pBAD24 in either orientation, where the *ompA* gene preferentially inserted into another vector during ligation. Further troubleshooting will have to be carried out to resolve the problem and successfully produce a clone containing a pBAD24-*ompA* construct.

TABLE 1. Primer sequences for *ompA* forward primer, *ompA* reverse primer, and pBAD24 reverse primer prepared by IDT Technology (Coralville, IA) and obtained from the Microbiology 421 frozen stock of reagents, Department of Microbiology and Immunology at the University of British Columbia. The primer sequences contain *Pst*I (*ompA* reverse primer) and *Eco*RI (*ompA* forward primer) cut sites at the 5' ends.

Primer	Sequence	Supplied Concentration
ompA forward primer	5'-GTGGAATTC ATCATGAAAA AGACAGCTAT CGCGATT-3'	100 μM
ompA reverse primer	5'-TTTCTGCAG TTAAGCCTGCG GCTGAGTTA-3'	100 μM
pBAD24 reverse primer	5'-GACCGCTTC TGCGTTCT GAT-3'	100 μM

MATERIALS AND METHODS

Bacterial strains. *E. coli* DH5α, *E. coli* B23, and *E. coli* TOP10 cells containing pCCK06-1 plasmid (pCR®2.1 TOPO-*ompA* construct), preparation described previously (4), were obtained from the Microbiology 421 frozen stock bacterial strain collection, Department of Microbiology and Immunology at the University of British Columbia. *E. coli* DH5α cells containing pBAD24 plasmid were obtained from the Hancock Laboratory (strain number C873) at the University of British Columbia.

Culture conditions. *E. coli* TOP10 cells containing pCCK06-1 plasmid and *E .coli* DH5α cells containing pBAD24 plasmid were grown in Luria-Burtani (LB) broth (1% (w/v) tryptone (BD; Cat. No. 211705), 0.5% (w/v) yeast extract (BD; Cat. No. 212750), 1% (w/v) NaCl (Fisher Scientific; Cat. No. 5271-3)) supplemented with 100 μg/μl ampicillin (Sigma; Cat. No. A-9518). *E. coli* B23 strain was incubated in LB broth for total chromosomal DNA isolation purposes. *E. coli* DH5α strain (without plasmid) was incubated in LB broth prior to transformation with the putative pBAD24-*ompA* construct. Post-transformation, *E. coli* DH5α cells were streaked onto LB plates (1.5% (w/v) agar (Invitrogen; Cat. No. 30391-023)) containing 100 μg/μl ampicillin or grown in LB broth containing 100 μg/μl ampicillin.

Chromosomal DNA isolation. The QIAamp DNA Mini kit (Qiagen; Cat. No. 51304) was utilized to isolate total chromosomal DNA from 3 ml of overnight culture of *E. coli* B23 as per the manufacturer's "bacterial culture" protocol from the QIAamp DNA Mini kit handbook.

PCR amplification of *ompA* and colony PCR. Refer to Table 1 for the *ompA* forward primer and *ompA* reverse primer sequences. The PCR reactions were prepared with 5 μl of 10x Taq Buffer (Fermentas; Cat. No. B16), 0.4 μl of 25 mM dNTP mix (Fermentas; Cat. No. R1121), 4 μl of 25 mM $MgCl_2$ (Fermentas; Cat. No. R0241), 1 μl of 10 μM *ompA* forward primer, 1 μl of 10 μM *ompA* reverse primer, and 36.35 μl of dH_2O. 2 μl of a 1-in-100 dilution (in dH_2O) of isolated *E. coli* B23 total chromosomal DNA or dH_2O (negative control) and 0.25 μl of 5 U/μl Taq polymerase (Fermentas; Cat. No. EP0402) were then added to each reaction tube. The reactions were placed in a BioRad Gene Cycler™ PCR machine and run under the following initial conditions: 4 minute denaturation at 94°C, 30 second annealing at 52°C, and 1 minute extension at 72°C. This was followed by 30 cycles each consisting of 30 seconds at 94°C, 30 seconds at 56°C, and 30 seconds at 72°C. After the 30 cycles, a final 15 minute extension at 72°C was run. PCR clean-up procedures were carried out with the PureLink™ PCR Purification Kit (Invitrogen; Cat. No. K3100-01) and the PCR products were then stored at -20°C. Following transformation using the ligation reaction of *Eco*RI-digested pCCK06-01 and pBAD24 plasmids, cells from fourteen of the colonies were picked for use in colony PCR. A colony of *E. coli* B23 was picked for the negative control reaction, and a colony of *E. coli* DH5α was picked for the positive control reaction. The *ompA* forward primer and pBAD24 reverse primer (Table 1) were used at a working concentration of 10 μM. PCR reactions were prepared and performed as above. PCR products were then prepared for agarose gel electrophoresis and run on a 1.0% (w/v) agarose gel, as described below (Materials and Methods: Agarose gel electrophoresis).

Plasmid extraction. The pBAD24 plasmid and pCCK06-1 plasmid were extracted from 3 ml of overnight cultures of *E. coli* DH5α and *E. coli* TOP10 cells, respectively, with the GeneJET™ Plasmid Mini Prep Kit (Fermentas; Cat. No. K0503) as per the manufacturer's instructions.

Restriction endonuclease digests of isolated pBAD24 vector, PCR amplified *ompA* fragment, and pCCK06-1 plasmid. To obtain DNA fragments containing the appropriate sticky ends for a direction-specific ligation reaction that would result in the pBAD24-*ompA* construct, the pBAD24 vector and *ompA* insert were each incubated for one hour in a 37°C dry incubator in solutions containing 4 μl of 10x React 2 buffer (Gibco BRL Life Technologies; Cat. No. 49004), 1 μl *Eco*RI (10 U/μl), 1 μl *Pst*I (10 U/μl), 31 μl of dH_2O, and 4 μl of DNA solution. Alkaline phosphatase (Biolabs; Cat. No. M0289S) was then added to the pBAD24 double-digest in an effort to prevent the vector's self-ligation, followed by an additional hour of incubation at 37°C in the dry incubator. After incubation, 4 ul of each of the digested pBAD24 and *ompA* were run on a 1.0% (w/v) agarose gel for gel purification. The remaining mixture was heat-shocked in an 80°C water bath for 20 minutes. In addition to this digest, to ensure that both restriction enzymes were digesting efficiently, sequential digests were performed. Digestions of pBAD24 and *ompA* with *Pst*I, which requires a greater number of base pairs between the end of its recognition site and the terminus of the fragment than *Eco*RI (Biolabs, as per manufacturer's description), were performed first for one hour at 37°C before *Eco*RI was added and incubated for another hour. Alkaline phosphatase (Biolabs; Cat. No. M0289S) was then added to the solution containing the pBAD24 vector, and the digest was incubated for an additional hour 37°C. In a separate strategy, the pBAD24 and pCCK06-1 plasmids were digested with *Eco*RI (Gibco BRL Life Technologies; Cat. No. 15202-013) in a solution containing: 4 μl of isolated plasmid, 4 μl of 10x React 3 buffer (Gibco BRL Life Technologies; Cat. No. 49004), 1 μl *Eco*RI (10 U/μl), and 31 μl of dH_2O. Incubation with alkaline phosphatase (Biolabs; Cat. No. M0289S), agarose gel electrophoresis, and heat-shock were done as indicated above.

Agarose gel electrophoresis. 1.0% (w/v) agarose (Bio-Rad Laboratories; Cat. No. 162-0100) was dissolved in 1x TAE buffer (40.0 mM Tris Base (Invitrogen; Cat. No. 15504-020), 0.1% (v/v) glacial acetic acid (Acros; Cat. No. 423220025), 1.3 mM EDTA (FisherBiotech; Cat. No. 6381-92-6), at pH 8). Gels were run to verify the success of the plasmid extractions and digestions, and for analyzing digested and undigested PCR products. 18 μl of each prepared sample were loaded onto the gel along with 4 μl of MassRuler™ Express Forward DNA Ladder (Fermentas; Cat. No. SM1283) or GeneRuler™ 1 kb DNA Ladder (Fermentas; Cat. No. SM0311). Gels were run at 100 V for 45 minutes in 1x TAE buffer. Following electrophoresis, the gel was submerged in a 0.5 μg/ml ethidium bromide bath for 20 minutes to stain DNA. The gel was then imaged immediately with AlphaImager Software v. 4.1.0 (Alpha Innotech Corp.).

TABLE 2. Optimization of various parameters to increase efficiency of ligation reactions and colony counts from transformation of chemically competent *E. coli* DH5α cells.

Ligation reaction contents	Gel-purified post-RE digest	Heat-shocked post-RE digest	Ligation reaction incubation temperature (°C)	Length of ligation reaction incubation (minutes)	Number of colonies after transformation into DH5α
pBAD24 and *ompA* double digests (*Eco*RI and *Pst*I)	Yes	No	23	5	0
			16	5	0
			4	5	0
			23	60	0
			16	60	0
			4	60	0
			23	Overnight	0
			16	Overnight	0
			4	Overnight	0
pBAD24 and *ompA* sequential digests (*Pst*I first, *Eco*RI second)	No	Yes	23	60	0
			16	60	0
			4	60	0
pCCK06-1 and *ompA* single digest (*Eco*RI)	No	Yes	23	60	450
Undigested pBAD24 vector (positive control)	No	No	Not applicable	Not applicable	>1000

Gel Purification. *Eco*RI and *Pst*I doubly-digested pBAD24 and *ompA* fragments were gel purified from agarose gels according to procedures outlined in the QIAEX II Gel Extraction Kit (Qiagen; Cat. No. 20021).

Ligation reactions of the *ompA* insert into pBAD24 vector. Ligation reactions were set up using the *Eco*RI- and *Pst*I-digested fragments of pBAD24 vector and PCR-amplified *ompA*. The ligation reactions contained 4 µl of 5x T4 DNA Ligase buffer (Fermentas Rapid DNA Ligation Kit; Cat. No. K1422), 1 µl of T4 Ligase (Fermentas Rapid DNA Ligation Kit; Cat. No. K1422), 4 µl of doubly-digested pBAD24 plasmid, 4 µl of doubly-digested *ompA*, and 7 µl of dH_2O and were carried out at 4, 16, or 23°C in five minute, one hour, or overnight incubations. Every possible combination of each temperature with each incubation period was performed, with all reactions resulting in zero colonies after transformation (Table 2). An alternative strategy was then used, where ligation reactions were set up using the *Eco*RI-digested pCCK06-1 plasmid and pBAD24. The ligation reactions consisted of 4 µl of 5x T4 DNA Ligase buffer (Fermentas Rapid DNA Ligation Kit; Cat. No. K1422), 1 µl of T4 Ligase (Fermentas Rapid DNA Ligation Kit; Cat. No. K1422), 4 µl *Eco*RI-digested pBAD24 plasmid, 2 µl *Eco*RI-digested pCCK06-1 plasmid, and 9 µl of dH_2O. The ligation was then incubated for one hour at 23°C.

Preparation of chemically competent *E. coli* DH5α cells. 50 µl of overnight culture (incubated aerobically at 37°C) were added to 20 ml of LB and incubated at 37°C while shaking at 200 rpm until an OD_{450} of 0.4 was reached. The cells were centrifuged at 4000 rpm in a JA-10 rotor (Beckman; Cat. No. 22686) for 10 minutes at 4°C. The supernatant was decanted and 2 ml of 50 mM ice cold $CaCl_2$ (Sigma Chemical Company; Cat. No. C-3881) were added. After a 10 minute incubation on ice, the solution was centrifuged at 4000 rpm in a JA-10 rotor for 10 minutes at 4°C, the supernatant was decanted, and 400 µl of 50 mM ice cold $CaCl_2$ and 15% (w/v) glycerol (Fisher Scientific; Cat. No. BP229-1) were added. 50 µl of cell solution were aliquoted to tubes placed on a dry ice and ethanol mix.

Transformation of *E. coli* DH5α cells. 5 µl of ligation reaction were added to 50 µl of chemically competent *E. coli* DH5α cells. The reaction was incubated on ice for 30 minutes, followed by a heat-shock at 42°C for 45 seconds. After being placed back on ice for two minutes, 1 ml of LB was added to the reaction and then incubated for one hour at 37°C in a dry incubator, on a shaking platform set to 200 rpm. The reaction solution was then centrifuged for 12 minutes at 6000 rpm (Eppendorf; Centrifuge 5424) and the supernatant removed, leaving 200 µl remaining. The pellet was resuspended by gently pipetting up and down, and 100 µl of a 1-in-10 dilution (in LB) and of an undiluted sample were spread plated onto LB-agar plates supplemented with ampicillin (100 µg/µl) (Sigma; Cat. No. A-9518). Plates were incubated overnight aerobically at 37°C.

RESULTS & DISCUSSION

The construction of a clone containing a pBAD24-*ompA* construct was not successful. Various troubleshooting procedures were performed to determine the source of the problem and attempts were made to resolve the alleged issues. The observed results were consistent over several replicates and troubleshooting returned analogous results. After troubleshooting, the source of the problems was hypothesized to have stemmed from the incompatibility of the *ompA* gene for the pBAD24 vector, potentially due to gene discrimination (8, 9) or a problem with the ligation reaction itself.

There were likely no problems with the isolation and digestion of the pBAD24 plasmid isolated from *E. coli* DH5α, as single digests of the isolated plasmid

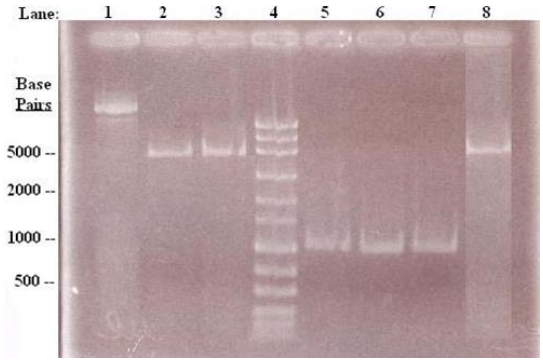

FIG. 1. Restriction endonuclease digests of pBAD24 and PCR amplified *ompA*. Lane 1, undigested pBAD24; lane 2, pBAD24 digested with *Eco*RI; lane 3, pBAD24 digested with *Pst*I; lane 4, MassRuler™ Express Forward DNA Ladder (Fermentas; Cat. No. SM1283); lane 5, *Eco*RI digested *ompA*; lane 6, *Pst*I digested *ompA*; lane 7; double digested (*Eco*RI and *Pst*I) *ompA*; lane 8, double digested (with *Eco*RI and *Pst*I) pBAD24.

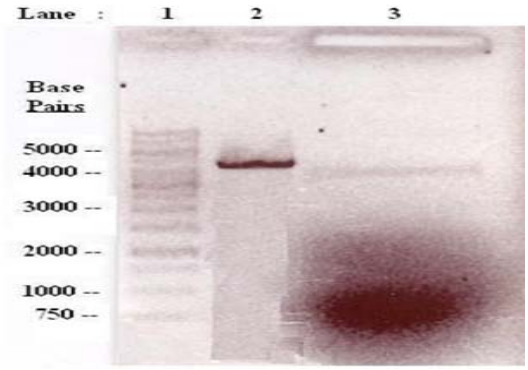

FIG. 2. *Eco*RI restriction endonuclease digests of pCCK06-1 and pBAD24 plasmid. Lane 1, GeneRuler™ 1 kb DNA Ladder (Fermentas; Cat. No. SM0311); lane 2, *Eco*RI digested pBAD24 vector; lane 3, *Eco*RI digested pCCK06-1 vector.

produced a single band, corresponding to the length of a linearized pBAD24 plasmid (~4.5 kb) as seen in lanes 2 and 3 (Fig. 1). This was expected, as pBAD24 contains only a single *Eco*RI and *Pst*I cut-site. Furthermore, the double digest of the isolated pBAD24 (Fig. 1, lane 8) did not produce an extra band in addition to the band observed at approximately 4.5 kb or cause the pBAD24 to migrate significantly faster; both results were expected as the fragment that was cut out due to the double digest should have been approximately 33 bp in length. The data were consistent for what would be expected for the pBAD24 vector.

It was also determined that it was unlikely to be a problem with the PCR performed on the 1041 bp *ompA* gene, as demonstrated by the approximately 1.1 kb band of the singly digested (with *Eco*RI or *Pst*I) PCR product in lanes 5 and 6 (Fig. 1). In addition to this, BLAST searches did not yield any results indicating significant binding of the *ompA* forward and reverse primers (refer to Table 1 for sequences) to any other regions in the *E. coli* genome.

It was possible that the digestion of the PCR product was not efficient as the difference between the undigested and digested PCR product is approximately 10 bp, which would not have been observable on the 1.0% agarose gel. The change in band size of the PCR product after double digestion with *Eco*RI and *Pst*I in lane 7 did not appear any different than the single digests in lanes 5 and 6 (Fig. 1).

It was hypothesized that there were contaminants present in the digestions that interfered with the ligation reactions. To reduce the potential of contaminants interfering with the ligation, doubly digested *ompA* and pBAD24 were run on a 1.0% (w/v) agarose gel and gel purified. In addition to this, doubly digested *ompA and* pBAD24 fragments that were heat-shocked post-digestion were processed with a PCR clean-up kit. Ligation reactions using the processed doubly digested *ompA* and pBAD24 were still unsuccessful, as indicated by the lack of DH5α *E. coli* transformants on LB and ampicillin, regardless of the conditions under which the ligation reactions were incubated (Table 2). The lack of transformants was not due to problems with the competent cell preparation or transformation protocol, as the transformation of chemically competent DH5α *E .coli* cells with undigested pBAD24 yielded greater than 1000 colonies, as was expected since pBAD24 confers ampicillin resistance.

It was then suspected that the problem may have arisen from the *Pst*I digestion of *ompA*, as it is known that *Pst*I is a less efficient enzyme than *Eco*RI (Biolabs, as per manufacturer's description). After attempting sequential double digestions, with *Pst*I added first, the ligation reactions with the sequentially cut pBAD24 and *ompA* were still unsuccessful (Table 2).

To remove the potential problem of inefficient cutting by *Pst*I, a different course of action was taken to eliminate its use. A pCCK06-1 vector (TOPO pCR2.1 vector with an *ompA* insert flanked by two *Eco*RI cut sites) was isolated from *E. coli* TOP10 cells and the plasmids were then digested with *Eco*RI (Fig. 2, lane 3). There was a substantial amount of RNA present on the gel and it could not have been ascertained that *ompA* had been successfully digested out of the plasmid because the broad RNA band was centred at approximately 1.1 kb, putatively containing the *ompA* insert. Nevertheless, the presence of the linearized pCCK06-1 plasmid at approximately 3.9 kb was indicative that the *ompA* had been successfully digested out of the plasmid. A pBAD24 vector was also digested

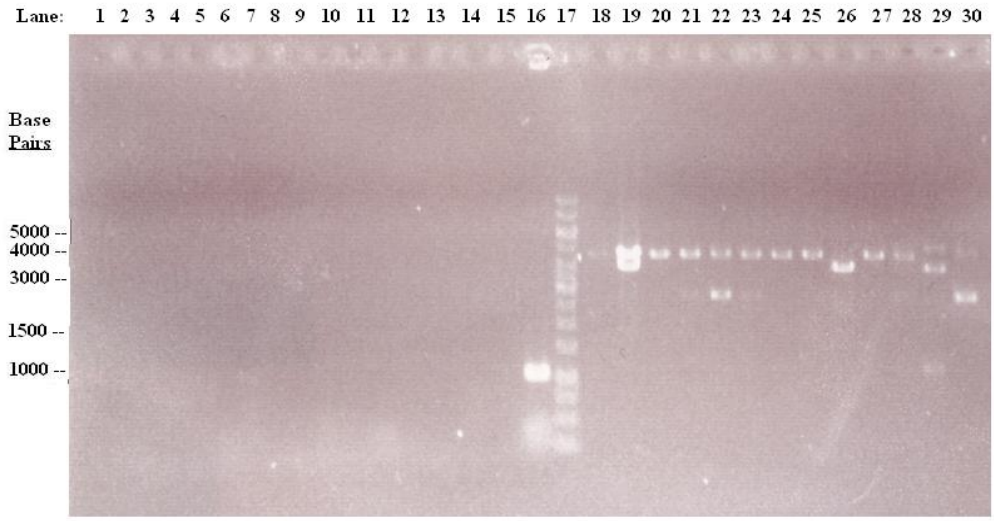

FIG. 3. Colony PCR and digestion of plasmids, from colonies obtained from *E. coli* DH5α transformed with the ligation reaction consisting of *Eco*RI digested pCCK06-1 and pBAD24 plasmids. Lanes 1-14 (inclusive), colony PCR reactions; lane 15, PCR negative control; lane 16, colony PCR positive control; lane 17, GeneRuler™ 1 kb DNA Ladder (Fermentas; Cat. No. SM0311); lanes 18-30 (inclusive), *Eco*RI digests of plasmid DNA isolated from overnight cultures of colonies chosen for colony PCR. In lanes 1-14 and 18-30, the colony chosen for colony PCR in lane 1 corresponds to the digested plasmid in lane 18, the colony chosen for colony PCR in lane 2 corresponds to the digested plasmid in lane 19, and so on.

with *Eco*RI and a ligation reaction was then performed with the products of the two digestions. The transformation of chemically competent DH5α *E .coli* cells with the ligation reaction yielded approximately 450 colonies (Table 2). Still, the colony PCR of the fourteen randomly selected colonies yielded no products, indicating that *ompA* had not inserted into the pBAD24 plasmid or that the *ompA* had inserted in the incorrect orientation into the pBAD24 plasmid. It should be noted that the lack of bands was not due to a problem with the PCR reaction, since the positive control (Fig. 3, lane 16) yielded a band at a size corresponding to *ompA*, which was expected as the primers utilized were the forward and reverse *ompA* primers.

When analyzing the digestion of the plasmids from overnight cultures of the colonies selected for the colony PCR, it was evident that there were no pBAD24-*ompA* constructs present, as none of the lanes had a band present at both 4.5 kb corresponding to linearized pBAD24 and 1 kb corresponding to the *omp*A insert (Fig. 3). An oddity to note was the presence of a band corresponding to the size of linearized pCCK06-1 (approximately 3.9 kb) in three lanes (Fig. 3, lanes 19, 26, and 29). The ligation reaction had two times the volume of pBAD24 than pCCK06-1 and the pBAD24 appeared to be twice as concentrated at an equal volume (Fig. 1, lanes 2 and 3). The chance of obtaining transformants with the pCCK06-1 plasmid should have been very slim, but the plasmid appeared to be present in 3 out of the 14 selected colonies. This may be suggesting that *ompA* was discriminating against pBAD24 in either orientation during the ligation reactions, as there should have been a high chance of the *ompA* ligation occurring with the pBAD24 vector. That is, a currently undetermined factor was discouraging the insertion of *ompA* into pBAD24. On the other hand, *ompA* appeared to have inserted into the pCR®2.1 TOPO vector, as seen in lane 29 (Fig. 3). This was indicated by the band at approximately 1 kb, 4 kb, and 5 kb, corresponding to the sizes of the *ompA* insert, the pCR®2.1 TOPO vector, and the uncut open circular form of pCCK06-1 (pCR®2.1 TOPO with the *ompA* insert), respectively. One possible mechanism leading to the preferential ligation of an insert into one vector over another is due to the sequence homology between the inserts and the vector ligation site (8). In support of this, the nucleotide sequence of the pCR®2.1 TOPO vector does resemble that of *ompA* more closely than does the pBAD24 vector (sources of nucleotide sequences from Invitrogen, as per manufacturer's description, EMBL-

EBI: X81837, and NCBI GenBank GI: 48994873). Statistically speaking, pCCK06-1 transformants should have not been present in the fourteen colonies. The fact that pBAD24 seemed to have self-ligated, as seen by the corresponding band at approximately 4.5 kb (Fig. 3, lanes 20 to 25, 27 to 28, and 30), was an oddity as well, as alkaline phosphatase was present in the digestion reactions. Alkaline phosphatase should have catalyzed the removal of the terminal phosphate groups on *Eco*RI cut ends, which should have prevented self-ligation, indicating that the alkaline phosphatase was not functional. The presence of a 3 kb band in lanes 22 and 30 was not expected and it was hypothesized that the digestion reactions did not go to completion and the band represented the supercoiled form of pBAD24 (Fig. 3). Finally, the presence of bands corresponding to linearized pBAD24 (approximately 4.5 kb) and pCCK06-1 (approximately 4 kb) in lane 20 (Fig. 3) was not expected, as a single strain appeared to have successfully taken up two plasmids which were both being replicated.

In the end, incompatibility of the *ompA* gene for the pBAD24 vector was thought to be possible due to a form of gene discrimination, as ligation of *ompA* into the pBAD24 vector was unsuccessful even in the presence of a relatively high amount of pBAD24. pCCK06-1 was preferentially undergoing self-ligation, even though it was present in substantially lower numbers in comparison to pBAD24. Alternatively, as no ligation controls were carried out, there was a potential that the ligation reactions were not optimized for the ligation of pBAD24 and *ompA* and further experimentation should be carried out to determine the source of the error.

FUTURE DIRECTIONS

Further experiments can be done to obtain a pBAD24-*ompA* clone and utilize it for future conjugation studies. There are a few strategies other than the ones used in the current experiment to clone the *ompA* gene into the pBAD24 vector. To determine whether the *ompA* PCR product is being properly and efficiently cleaved by *Eco*RI and *Pst*I, *ompA*, which has been amplified using primers containing *Eco*RI and *Pst*I cut sites, can be cloned into a pCR®2.1 TOPO vector using TOPO TA cloning. This linearized vector contains single 3'-thymidine overhangs with a bound Topoisomerase I enzyme. TOPO TA cloning allows efficient ligation of any PCR product amplified by Taq polymerase into the vector, since Taq polymerase adds a single deoxyadenosine to PCR products. After ligation, bacteria can be transformed, and plasmids can be isolated from positive transformants. Restriction digests using *Eco*RI and *Pst*I can then be performed. An insert corresponding to *ompA* that is liberated upon digestion indicates that *ompA* is being digested with the restriction enzymes, presumably generating the sticky ends necessary for directional cloning into pBAD24. It would also confirm that there are no problems with the ligation and transformation procedures and materials.

Once the pBAD24-*ompA* clone is constructed, it can used to study the effects of differential expression of *ompA* on conjugation efficiency. The construct can be transformed into *E. coli* C156 cells, which are *ompA*$^-$ mutants, and differential levels of *ompA* expression can be induced by growing the cells in different concentrations of arabinose. Since the *E. coli* C156 strain is a proline auxotroph, conjugation assays can be performed using these arabinose-induced strains, with the *E. coli* HfrC strain as the conjugation donor. Cells can be plated on media containing ampicillin but lacking proline to determine the extent of conjugation. Only recipient cells, which contain the pBAD24 vector and are thus ampicillin resistant, that have undergone conjugation and received the proline synthesis gene will survive on such media. Donor cells, which are not ampicillin resistant, will not grow. More colonies are expected to result from conjugation assays containing cells whose level of *ompA* expression is higher.

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