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Proposed Construction of pBAD24-OmpA for the Differential Expression of OmpA in Conjugation Efficiency Studies

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A better understanding of the mechanisms behind bacterial conjugation may allow for selectively enhancing or repressing conjugation; this has possible profound implications, both clinically and industrially. OmpA, an abundant outer membrane protein of *Escherichia coli*, has been shown to be necessary for conjugation and mating-pair formation, although the details of this interaction have not been fully elucidated. Although an o*mpA* gene containing pCR2.1-TOPO plasmid has been developed, a system allowing for the controlled and differential expression of OmpA would be more appropriate for investigating the effect of increasing OmpA levels on conjugation efficiency. The current investigation attempted to clone *ompA* into a pBAD24 vector in order to produce a pBAD24-OmpA construct. Although *ompA* was amplified and the plasmid was isolated, no transformants were observed as pBAD28 was accidentally received in place of pBAD24. Despite this error, the reported investigation describes appropriate protocols for the correct construction of the pBAD24-OmpA vector system.

Bacterial transfer of DNA by conjugation is an extremely important process for the promiscuous transfer of genetic elements between bacterial cells/species, including the clinically relevant transfer of resistance genes, and is thus an important factor in bacterial evolution (1, 7, 11). Conjugation has also been exploited in the laboratory; mainly for the interspecies transfer of plasmids as in the industrial manufacture of antibiotics (3, 7). It follows, therefore, that being able to selectively enhance or repress conjugation might have profound implications in clinical and industrial applications including providing a potential target for disrupting bacterial resistance gene transfer (7).

Although bacterial conjugation has been extensively studied, the mechanism and regulatory factors behind this process are still not fully understood (11). The mechanism of bacterial conjugation involves primarily: recipient cell recognition, pilus retraction/cell-to-cell contact, mating-pair stabilization, and DNA transfer (1). Studies with outer membrane protein A (OmpA) deficient or mutant strains as conjugation recipients have shown this protein to be necessary for conjugation, due to its essential role in the stabilization of mating-pairs (1, 10). OmpA is an abundant outer membrane (OM) protein of *Escherichia coli* involved in maintaining the structural stability of the OM; OmpA can also serve as a bacteriophage or colicin receptor (10).

While loss-of-function OmpA strains have been extensively studied and described in the literature, gain-of-function and de-repressed OmpA strains have not been sufficiently studied to understand the implications of introducing or up-regulating OmpA expression on conjugation efficiency (1, 10, 11). Previous researchers have attempted to introduce pCCK06-1, a pCR2.1-TOPO plasmid containing a cloned o*mpA* gene, into wild type and OmpA deficient *E. coli* strains in order to investigate conjugation efficiency with increased OmpA expression (4, 5).

The pCCK06-1 plasmid is not controllable and can only restore (in OmpA deficient) or increase (in wild type) expression at one level (4, 5). Also, a major problem with overexpressing any protein, particularly highly expressed OM proteins, is overexpression lethality (2). Conversely, the pBAD vectors provide controllable expression systems which are often used to generate differential expression levels of a particular gene by partial induction of the promoter (6, 8, 9). As a controllable expression system, these plasmids allow for the restoration, up-regulation, and differential expression of OmpA in a single strain.

The current investigation attempted to clone the *ompA* gene into the pBAD24 vector system in order to produce a pBAD24-OmpA vector capable of inducing and regulating differential levels of OmpA expression for the study of increasing/decreasing OmpA levels on conjugation efficiency. Although the plasmid was isolated and *ompA* was amplified, the pBAD24-OmpA vector was not generated. The digestion/ligation reactions were likely unsuccessful as the wrong initial starting vector, pBAD28, was received. Despite this error, the pBAD24-OmpA vector system should be appropriate for the analysis of OmpA levels on conjugation efficiency and could

be properly made using the described conditions and methods.

MATERIALS AND METHODS

Bacterial Strains. The C149 (wildtype) and C156 (OmpA$^-$) *E. coli* strains were obtained from the MICB 421 bacterial strain collection in the Department of Microbiology and Immunology at the University of British Columbia. Refer to Table 1 for the complete genotypes of these strains. *E. coli* DH5α cells containing pBAD plasmid were obtained from the Hancock laboratory at the University of British Columbia.

TABLE 1. Genotypes of C156 and C149 *Escherichia coli* strains

Strain	Genotype
C149	F$^-$ *pro*C-24 *aro*A-357 *his*-53 *pur*E-41 *ilv*-277 *met*-65 *lac*Y-29 *xyl*-14 *rps*-97 *cyc*A-1 *cyc*B-2? *Tsx*-63 lambda
C156	F- *pro*C-24 *omp*A-256 *his*-53 *pur*E-4 *ilv*-277 *met*65 *lac*Y-29 *xyl*-14 *rps*L-97 *cyc*A-1 *cyc*B-2? *tsx*-63 lambda

Culture Conditions. C149 and C156 strains were grown in Luria-Bertani (LB) broth (1% (w/v) tryptone (Bacto; Cat. No. 211701), 0.5% (w/v) yeast extract (Bacto; Cat. No. 212730), 1% (w/v) NaCl (Fisher; Cat. No. 642-500)). *E. coli* DH5α cells containing pBAD plasmid were grown in LB broth containing 100 µg/mL ampicillin (Sigma; Cat. No. A9393). All cultures were incubated at 37ºC in a shaking water bath or tube roller.

Primer Design for *ompA* gene. The *ompA* gene sequence was taken from *E. coli* K-12 MG1655 (1018236…1019276; NCBI GenBank GI: 48994873). Primers were designed using PrimerQuest (2007; IDT technology) software. *Pst*I and *Eco*RI cut sites were introduced into primers to allow for directional ligation of *ompA* into plasmid. These cut site sequences were blasted (using NEBcutter V2.0) against *ompA* and pBAD24 to ensure their absence from the *ompA* gene and the vector. Primers were ordered from IDT technology (Coralville, IA). Forward primer: 5'-GTGGAATTCATCATGAAAAAGACAGCTATCGCGATT-3'. Reverse primer: 5'-TTTCTGCAGTTAAGCCTGCGGCTGAGTTA-3'. Primers were suspended with dH_2O at 100 µM and diluted to a working concentration of 10 µM.

Chromosomal DNA isolation. A Qiagen QIAamp DNA Mini kit (Cat. No. 51304) was used to isolate total chromosomal DNA from a 50 mL overnight culture of E. coli C149 cells according to the "bacterial culture" protocol from the Qiagen QIAamp DNA Mini kit handbook. A Beckman DU 530 Life Science UV/Vis Spectrophotometer was used to take A_{260} and A_{280} readings to evaluate DNA concentration and purity.

PCR amplification of *ompA* gene. PCR reactions were prepared with 5 µL 10X Taq Buffer (Fermentas; Cat. No. B16), 0.4 µL of 25 mM dNTPs (Fermentas; Cat. No. R0181), 4 µL of 25 mM $MgCL_2$ (Fermentas; Cat. No. RO971), and 1 µL *ompA* forward primer (10 µM), and 1 µL *ompA* reverse primer (10 µM). 1.6 µL isolated E. coli C149 chromosomal DNA and 0.2 µL Taq polymerase (Fermentas; Cat. No. EP042) were added to each reaction tube and reactions were run on a BioRad Gene Cycler PCR machine. The initial cycle involved: 4 minute denaturation at 94ºC, followed by 30 second annealing at 52ºC, and 1 minute extension at 72ºC. This was followed by 30 cycles each consisting of 30 seconds at 94ºC, 30 seconds at 56ºC, and 30 seconds at 72ºC. After the 30 cycles, a final 15 minute extension at 72ºC was run. PCR product was stored at -20ºC.

pBAD vector systems. Expression in the pBAD vectors is controlled by the P_{BAD} promoter and *araC*. Transcription is induced with arabinose, but P_{BAD} has very low level basal transcription, further reduced in the presence of glucose (8). The pBAD24 vector, 4.5 kb, has a Shine-Dalgarno sequence for cloning genes lacking initiation sequences as well as a strong *rrnB* transcription terminator, a ColE1 origin of replication for regulating copy number, an M13 intragenic region for phage packaging and production of single stranded DNA, and an ampicillin resistance gene for selection (8).

pBAD plasmid extraction. pBAD plasmid was isolated from 10 mL overnight cultures of DH5α *E. coli* cells using a PureLink HQ Mini Plasmid purification kit (Invitrogen; Cat. No. K2100-01), according to the manufacturer's instructions. A Beckman DU 530 Life Science UV/Vis Spectrophotometer was used to take A_{260} and A_{280} readings to evaluate DNA concentration and purity.

Agarose gel electrophoresis. 0.7% Agarose (Invitrogen; Cat. No.15510-027) gels were used to verify PCR amplication of *ompA*, extraction of PCR plasmid, and the ligation of the *ompA* amplicon into the pBAD plasmid. Gels were run at 100 V for 45 minutes in TAE buffer (40.0 mM Tris Base (Invitrogen; Cat. No.15504-020), 0.1% (v/v) glacial acetic acid (Multipharm; Cat. No. 000056), 1.3 mM EDTA (Fisher; Cat. No. 703452), pH 8). Fermentas 6X loading dye (Cat. No. R0611) was used to track migration of samples and MassRuler^{TM} Express Forward DNA Ladder (Fermentas, Burlington, ON, Ca. No. SM1283) was used to estimate band sizes. Following electrophoresis, gels were stained in a 0.2 µg/mL ethidium bromide bath for approximately 25 minutes. Gel photographs were taken immediately after removal from ethidium bromide bath using AlphaImager Software v. 4.1.0 (Alpha Innotech Corp).

Cloning of ompA into pBAD vector. 4.5 µL *ompA* PCR product and 4.5 µL isolated pBAD plasmid were both digested with *Eco*RI (Gibco BRL; Cat. No. 15202-013) and *Pst*I (Gibco BRL; Cat. No. 15215-015). Double digests were carried out for 1 hour at 37ºC with 1 µL React 2 (Gibco BRL; Cat. No. 16302-010) or React 3 buffer (Gibco BRL; Cat. No. 16303-018), 4.3 µL dH2O, and 0.1 µL of each restriction enzyme (10 U/µL). Digested *ompA* PCR product and pBAD plasmid were ligated for one hour at room temperature (21°C). Ligation reaction consisted of 8 µL dH2O, 4 µL 5X T4 DNA Ligase buffer (Invitrogen; Cat. No. 46300-018), 4 µL digested pBAD, 2 µL digested PCR product, and 2 µL T4 DNA Ligase (Invitrogen; Cat. No. 15224-025).

Transformation of C156 cells. C156 cells were made electrocompetent and transformed with putative pBAD plasmid containing *ompA* via electroporation. To make the C156 cells electrocompetent, 5 mL overnight cultures were split into four 1.5 mL microfuge tubes, and each was centrifuged using an Eppendorf 5415D centrifuge for two minutes at 16100 × g. The supernatants were then discarded, and the pellets were resuspended in 1.5 mL ice cold water. This wash step was repeated three times. 2 µL putative ligations, pBAD with *ompA* insert, and 40 µL electrocompetent C156 *E. coli* cells were added to a well chilled cuvette. A Biorad Micropulser set to the EC2 setting (2.5 kV) was used to expose the mixture to a single electric pulse. 0.96 mL LB was then added immediately to the cuvette and the resulting solution was transferred to a 5 mL test tube and incubated at 37ºC for one hour in a tube roller, before being plated as 100 mL aliquots on LB plates containing 100 µg/mL ampicillin for selection of transformants.

RESULTS

PCR amplification of *ompA*. Genomic DNA from *E. coli* C149 was isolated for amplification of *ompA* and 5 identical replicates of the PCR reaction were carried out. Figure 1 shows the successful amplification of *ompA* in all 5 reactions; the tilt of the comb in the gel caused duplicate bands but the expected 1050 bp band (*ompA* itself is 1041 bp) can be seen in lanes 2 to 6. The isolated plasmid DNA (lane 7) yielded a clear band at around 4.0 kb as measured by the linear ladder. Although this

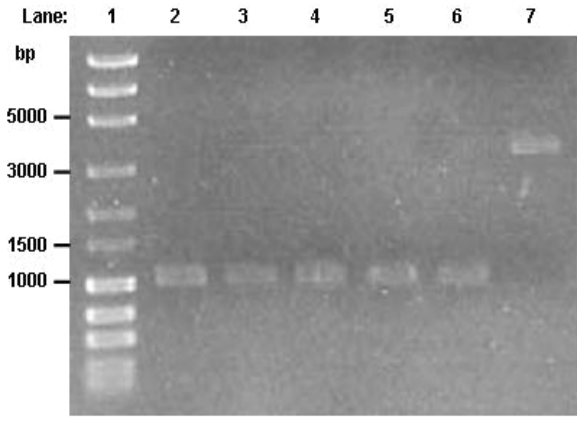

FIG. 1. PCR amplification of *ompA* from *E. coli* C149. Lane 1, MassRuler™ Express Forward DNA Ladder (Fermentas; Cat. No. SM1283); lanes 2-6, PCR products from reaction tubes 1-5; lane 7, undigested pBAD plasmid.

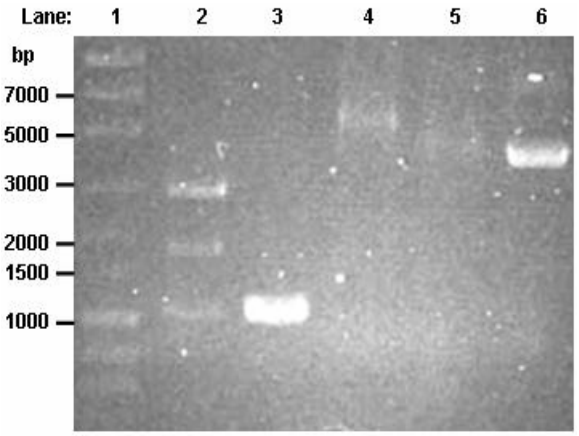

FIG. 2. Restriction enzyme double digests (*Eco*RI and *Pst*I) and ligation reaction of the *ompA* PCR product and the pBAD plasmid. Lane 1, MassRuler™ Express Forward DNA Ladder (Fermentas; Cat. No. SM1283); lane 2, double digest of pBAD plasmid; lane 3, double digest of *ompA* PCR amplification product; lane 4, ligation reaction with digested *ompA* PCR product and digested pBAD plasmid; lane 5, empty; lane 6, undigested pBAD plasmid control.

confirms the presence of DNA, the exact size cannot be determined since the plasmid conformation is unknown.

Restriction digest and ligation of pBAD plasmid and *ompA*. The isolated pBAD plasmid and *ompA* PCR product were double digested with the restriction endonucleases *Eco*RI and *Pst*I to achieve directional insertion of the *ompA* gene into the vector. Although a 1.1 kb band can be seen in lane 3, confirming the presence of PCR product, it cannot be determined with certainty whether the digestion reaction was successful or whether it went to completion (FIG. 2). The difference in fragment size between undigested and digested PCR product (~10 bp) would not be observable on the gel (FIG. 2). The ligation reaction (lane 4) yielded a faint band at approximately 6 kb, which was larger than the undigested pBAD control in lane 6. Thus, the band in lane 4 may represent a successful ligation, although not necessarily in the correct orientation or conformation. The intensity of the band in lane 4 is comparable to the bands of the ladder (lane 1), suggesting the ligation reaction in lane 4 contained approximately 0.05-0.1 μg/μL of DNA. As can be seen in figure 2, the digested plasmid yielded three distinguishable bands at approximately 1.1, 1.8 and 2.9 kb (lane 2). This is inconsistent with what would be expected for pBAD24, and suggests the incorrect plasmid may have been used.

Transformation of *E. coli* C156. *E. coli* C156 cells were transformed with putative pBAD-*ompA* ligated vector via electroporation. No pBAD-*ompA* transformants were isolated (Table 2). Confluent growth of electroporated cells on amp^- LB confirmed that the electroporation procedure was not killing all the cells. The formation of colonies on amp^+ LB plates after transformation with the pBR322 control plasmid confirmed that the electroporation procedure was sufficient to allow *E. coli* C156 cells to take up plasmid DNA.

TABLE 2. *E. coli* C156 transformants grown on selective and non-selective LB plates

Electroporation condition	Selection	Growth[a]
No plasmid control	Amp^-	Confluent
No plasmid control	Amp^+	No growth
pBR322 control	Amp^-	Confluent
pBR322 control	Amp^+	85 colonies
pBAD ligation	Amp^-	Confluent
pBAD ligation	Amp^+	No growth

[a]Each reaction was plated in triplicate.

***Eco*RI and *Pst*I restriction digest of isolated vector.** Control digestion reactions were carried out in an attempt to explain the unexpected banding pattern observed in figure 2 and to provide a possible hypothesis as to why transformation was unsuccessful. Double digestion of pBAD vector with *Eco*RI/*Pst*I, as seen in figure 3 lane 3, reproduced the unexpected results from figure 2. Single digests of the vector with *Eco*RI in React 2 and React 3 buffers (lanes 4 and 6 respectively) produced identical high intensity bands at approximately 2.9 kb, whereas the single *Pst*I digest yielded bands at 4.7 and 1.1 kb (lane 5). No *Eco*RI star activity was noted with the use of React 3. The 2.9 kb band generated by *Eco*RI (lane 4 and 6) is similar to that seen in the double digest from lane 3. However, the 2.9 kb band is comparably thicker and more intense in the single *Eco*RI digests than the *Eco*RI/*Pst*I double digest. Furthermore, the 1.1 kb band seen in lanes 3 and 5 is

absent from the *Eco*RI single digest, suggesting *Pst*I was responsible for producing this fragment, while the 1.8 kb band in lane 3 was likely produced by a combination of *Eco*RI and *Pst*I (FIG. 3).

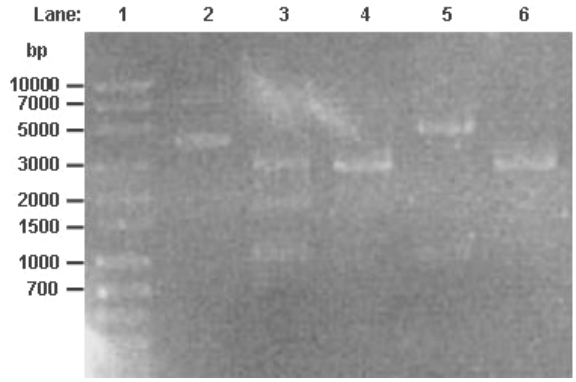

FIG. 3. Restriction enzyme digests of isolated pBAD plasmid with *Eco*RI and *Pst*I. Lane 1, MassRuler™ Express Forward DNA Ladder (Fermentas, Burlington, ON, Ca. No. SM1283); lane 2, undigested isolated pBAD plasmid; lane 3, double digest of isolated pBAD plasmid with *Eco*RI and *Pst*I in React 2 buffer; lane 4-5, *Eco*RI and *Pst*I single digests of isolated pBAD plasmid in React 2 buffer, in that order; lane 6, *Eco*RI single digest of isolated pBAD plasmid in React 3 buffer.

DISCUSSION

The matter of whether the pBAD system was a cogent method for elucidating questions pertaining to the effect of OmpA levels on conjugation efficiency is still unresolved. Despite the inability to generate the pBAD24-OmpA vector, the system appears to be well designed without obvious flaws. The observed results were consistent over several replicates and troubleshooting invariably returned comparable results. The apparent cause of error was at the interface between the stages of the restriction digest and ligation protocols.

There were likely no problems with the PCR performed on the 1041 base pair *ompA* gene as demonstrated by the appropriately sized PCR product bands in figure 1, although sequencing or digest mapping would be needed for complete confirmation. Spacer regions in the primers were designed in frame with the gene and extra nucleotide residues were added to the ends of the primers to optimize restriction enzyme activity. BLAST searches indicated no significant binding of the primer sequences to any other regions of the *E. coli* genome.

With only a small number of base residues to remove at the end of each PCR product, it is possible, but unlikely, that the digestion of PCR product did not work at a high efficiency. The banding patterns on the gels before and after digestion of putative 1041 base pair *ompA*, however, would not be observably different. Since a surfeit of terminal residues were used compared with previous successful experimental protocols, the PCR product digestion was not a likely source of error (6, 9).

Figures 2 and 3 show that the most likely cause of the unsuccessful construct generation was the plasmid itself. Assuming the plasmid isolated from carrier bacteria was indeed pBAD24, as requested by the experimenters, there should be only one 4.5 kb linear DNA fragment produced by single digestion with either *Eco*RI or *Pst*I (8, Fig. 3). A double digest of pBAD24 should not have produced a band varying by more than 33 bp from the 4.5 kb of a single digest (8). Lane 2 in figure 2 shows a double digest that returns fragments of approximately 1.1, 1.8, and 2.9 kb. These values are strikingly similar to the 1077, 1808 and 2872 base pair bands that would be expected upon concurrent digestion of pBAD28 with *Eco*RI and *Pst*I (8). This result is duplicated in lane 3 of figure 3, and was replicated in every other attempt at digestion (results not shown). The relative band intensities were also consistent for each replicate, lending support to the hypothesis that this is the real banding pattern rather than an artifact. The pBAD28 plasmid is the only other pBAD vector available in the Hancock lab. Also intriguing, are the banding patterns in lanes 4 and 5 of figure 3. Instead of the single linear 4.5 kb band expected with one cut site, there appear to be bands at just under 3.0 kb in lane 4 (*Eco*RI digest) and at 4.7 and 1.1 kb in lane 5 (*Pst*I digest). While the smaller band in lane 5 is likely less intense due to less available DNA for the ethidium bromide to bind to, the apparent solo band in lane 4 seems to have close to double the intensity of the similarly sized bands in neighboring lanes; this is likely due to it containing double the amount of DNA. *Eco*RI digestion of pBAD28 is expected to give linear DNA bands of 2928 and 2872 base pairs since it cuts after base 1306 and base 4234 in a circular 5.8 kb plasmid (8). With the gel conditions used for figure 3 it is likely that these two bands appear as one large band at around 2.9 kb in lane 4. *Pst*I digestion of pBAD28 is expected to give cuts after base pair 1349 and 2426, generating 4723 and 1077 linear DNA fragments in a 1:1 ratio (8). The bands located at approximately 4.7 and 1.1 kb in lane 5 of figure 3 are consistent with this.

The possibility that pBAD28 was isolated from cells thought to contain pBAD24 agrees with the restriction digest patterns in figures 2 and 3 and is supported by band sizes as well as the overall outcome: the failure to transform C156 *E. coli* with pBAD24 containing *ompA*. Furthermore, it is in accord with the multiple trials and troubleshooting performed in the present study. It is therefore most logical to conclude that through some errant act, pBAD28 was being utilized instead of pBAD24.

Assuming that pBAD28 was in fact used, it is important to note that the digestion protocols were appropriate.

Ligations in this study were carried out using standard procedures that have worked previously under a multitude of conditions (6, 9). The most likely problems with the ligation were the lack of expected fragments present to be ligated. Digested with both *Eco*RI and *Pst*I, pBAD28 would be cut into four fragments of 2872, 1808, 1077 and 43 base pairs (8). It is unlikely that these four fragments would come together in the proper order in sufficient quantity to incorporate the *ompA* PCR product and regenerate important replication origins and antibiotic resistance markers during ligation. Very little of the DNA in the ligation reactions (if any) would be fit to transform the C156 cells with ampicillin resistance. This limitation may account for the lack of transformants observed in the current investigation.

The ligation reaction run in figure 2, lane 4 interestingly appeared as a single band at approximately 5.7 kb on the linear ladder. If all the fragments, the four pBAD28 and one *ompA* PCR amplicon, had not ligated together, a combination of ligated and non-ligated fragments would be expected. The single band seems to show that all the pieces present joined together, although not necessarily in the correct order. Interestingly, the size, approximately 6 kb, is lower than expected, 6.9 kb, for the sum of our fragments in a linear conformation. Therefore, this ligation product may be missing one of the fragments. The ligation product size, approximately 6 kb, is consistent with a regenerated linear plasmid missing the PCR gene product. This could have been produced if the PCR product was present in too low a concentration relative to the plasmid. As the ligation reaction band is not very intense, a 1 kb band may not be visible, explaining the size discrepancy. As with the digestion, the ligation protocols appear appropriate as some ligation occurred.

Successful ligation does not guarantee successful transformation; DNA concentration is an important factor. Approximately 0.1 to 0.2 μg of ligated DNA were added to transformation reactions. This is significantly lower than the 1 μg of control pBR322 plasmid DNA added to the successful transformations (Table 2). Thus, the minimum threshold of appropriately ligated plasmid construct required to yield transformants may not have been surpassed given the number of replica plates used.

Control transformations done with pBR322 demonstrate that the electroporation protocol used was a viable means of generating ampicillin resistant transformants. Moreover, there were viable C156 *ompA* negative cells in the transformation reactions, as evidenced by the growth on non-selective media (Table 2). C156 cells were probably not efficiently transformed with an appropriate pBAD construct as desired and this is why they failed to grow on ampicillin containing media.

The pBAD24-OmpA vector system would be beneficial for analyzing the effect of OmpA level on conjugation efficiency and should be exploited for this purpose. In constructing this system, there is no reason to doubt the effectiveness of the described protocols which should be repeated using the correct pBAD vector.

FUTURE EXPERIMENTS

Primarly, this protocol should be repeated with the appropriate plasmid (pBAD24 instead of pBAD28) to produce pBAD24-OmpA and successful transformants. Once a transformant has been obtained, it can be grown on different levels of arabinose to modulate OmpA expression. In order to confirm differential expression of OmpA, OmpA expression can be measured directly by various methods. Ideally, membrane proteins would be isolated, run on a SDS-PAGE gel, and exposed to anti-OmpA antibody (Western blot). If an anti-OmpA antibody is unavailable, the SDS-PAGE gel could be stained with Coomassie Blue and the relative intensity of the putative OmpA bands could be compared. Additionally, bacteriophage with OmpA target receptors can be used to confirm the proper location of OmpA in the OM of the *E. coli* cells (4). The conjugation assay described by Chambers *et al.* (4) can then be used to determine the effect of OmpA expression on conjugation efficiency. This procedure would help elucidate the extent to which changes in the level of OmpA expression affect conjugation efficiency.

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