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Construction of pBAD-clones using the TOPO TA Cloning System

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Previous studies have shown that conjugation efficiency decreases when the outer membrane protein, OmpA, was inactivated in *Escherichia coli.* To study the role of modulated OmpA expression in conjugation, construction of a pBAD24-*ompA* clone that would allow controlled gene expression under the regulation of arabinose-inducible promoter was attempted in the past but had no success. To assess whether problems arose due to the cloning design or other intrinsic difficulties of cloning *ompA,* the current investigation attempted to use an alternate TOPO TA cloning system to construct pBAD-*ompA.* Primers were designed to amplify *ompA* and restriction analysis verified that the PCR product was consistent with *ompA*'s expected banding pattern. Transformation results showed successful recovery of several clones carrying pBAD constructs. Construct pKT10W4 had a higher molecular weight which suggested that it carried a large insert worth further investigation. Due to time constraints, the identity and directionality of the insert had yet to be confirmed as *ompA.* Since no successful pBAD-*ompA* clones with correct orientation have been confirmed, results remained inconclusive as to whether any intrinsic difficulties associated with cloning *ompA* exist.

OmpA is an abundant outer membrane protein found in *Escherichia coli* and has been shown to reduce bacterial conjugation efficiency when inactivated (10). Previously, Hfr conjugation studies investigated the effect of cloning *ompA* into a plasmid and over-expressing the gene in an OmpA-deficient *E. coli* recipient strain called C156 (4, 5). Although the transformed and untransformed C156 strains had similar conjugation efficiency, it was speculated that the observation might be due to high levels of uncontrolled *ompA* expression (4).

To study the role of modulated OmpA expression in conjugation, past attempts were made to construct a pBAD-*ompA* clone that would allow controlled gene expression, under the regulation of arabinose-inducible (pBAD) promoter. These approaches involved using a pBAD24 vector which required generation of compatible sticky ends for both the *ompA* gene and plasmid in order for proper ligation and orientation of the construct (1, 7, 11). Specifically, pBAD vectors needed to be extracted from *E. coli* cells and digested first with *Pst*I, and then followed by *Eco*RI to generate two sticky ends compatible for directional ligation of the *ompA* insert. Then double-digested pBAD and double-digested *ompA* were incubated with T4 ligase for about an hour for the ligation reaction to occur. However, when the ligation mixture was used to transform *E. coli* C156, no transformants were recovered (7, 11). Troubleshooting was carried out by investigating whether different ligation reaction temperatures, incubation times and gel purification steps would optimize the transformation but efforts were unsuccessful (7). Possible reasons for not recovering transformants were proposed, such as having an inadequate amount of closed circular construct available for transformation (11), and potential lethality issues for introducing the *ompA* gene into strain C156 (7). A TOPO TA cloning system was used previously to generate a pCR2.1 construct (pCCK06-1) which has been shown to carry the *ompA* insert (4). However, actual OmpA expression has not been confirmed in the *E. coli* C156 strain complemented with pCCK06-1 and the orientation of the insert in pCCK06-1 has not been confirmed so that the gene might possibly be non-functional or not expressed properly (3).

In order to assess whether problems arose due to the previously described cloning design or other intrinsic difficulties of cloning *ompA,* this project attempted to construct a pBAD-*ompA* clone using an alternate TOPO TA cloning system instead of the previous attempt using restriction enzymes to generate the compound. The pBAD TOPO vector is supplied linearized with single 3'-thymidine overhangs, along with topoisomerase (TOPO) bound to the vector at both ends. By generating PCR products using *Taq* polymerase, single 3'-deoxyadenosine are added so that inserts could directly ligate with the plasmid. TOPO TA eliminates the need for post PCR procedures such as restriction digests to generate compatible sticky ends, and also eliminates the use of ligase (8). The overall aim of the project is to construct pBAD-clones and

select for plasmids which contain the functional *ompA* gene.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Escherichia coli (E. coli)* MG1655 strain was obtained from the Department of Microbiology and Immunology at the University of British Columbia and grown in Luria-Bertani (LB) broth (1% (w/v) tryptone (Bacto™, REF 211705), 0.5% (w/v) yeast extract (Bacto™, REF 212750), and 1% (w/v) NaCl (Fisher Chemicals, Cat. #S271-3), adjusted to pH 7.5). OneShot® TOP10 Chemically Competent *E. coli* strain was supplied by pBAD TOPO TA® Expression Kit (Invitrogen, Cat. #K4300-01) and was thawed on ice before direct use in transformation. After transformation of TOP10 *E. coli*, the mixture was spread on LB plates (1.5% (w/v) agar (Invitrogen, Cat. #30391-023)), supplemented with 100 µg/mL ampicillin (Sigma, Cat.# A-9518). Nine transformants selected for further screening were cultured on LB plates with 100 ug/mL ampicillin and also in 4 mL of LB broth with 100 ug/mL ampicillin for plasmid preparation. Cultured plates incubated at 37°C overnight or at room temperature for two to three days. Liquid cultures were grown overnight at 37°C in shaking incubator (200 rpm).

PCR primer design for *ompA*. New primers were designed to amplify *ompA* from MG1655 *E. coli* (NCBI GenBank, GI: 48994873) and generate *ompA* PCR products which would be suitable for cloning into pBAD-TOPO. Forward primer (5'- **TGA** GAG GAT TCC CCC C**AT G**AA AAA GAC AGC TAT CG -3') contained an in-frame stop codon and translation re-initiation sequence to remove the N-terminal leader and allow for native protein expression (IDT, Ref. #54816783). Reverse primer (5'– **TTA** AGC CTG CGG CTG AGT TAC AAC GTC TTT -3') included the stop codon of the native sequence of *ompA* to exclude the C-terminal expression of the V5 epitope and polyhistidine region (IDT, Ref. #54816784). Both primers were reconstituted with sterile distilled water to a final concentration of 100 µM.

Whole cell PCR amplification of *ompA*. PCR reactions consisted of 1x PCR buffer (10x stock, supplied by Invitrogen kit), 0.5 mM dNTP mix (50 mM stock, supplied by Invitrogen kit), and 1 µM each of forward and reverse *ompA* primers were made up to final volume of 24 µL using sterile water. Isolated *E. coli* MG1655 cells from a plate culture were directly transferred into the PCR reaction by picking up cells using the end of a sterile pipette tip. PCR mixture was then heated for 10 min at 94°C, placed on ice, and then 0.5 U of *Taq* polymerase (Invitrogen, Cat. #10342-053) was added to make a final volume of 25 µL. 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 62.1°C for 30 sec and then elongation at 72°C for 90 sec. For a final extension, the reaction was incubated for 10 min at 72°C, followed by a pause at 4°C or storage at -20°C. Optimal 62.1°C hybridization temperature was pre-determined by performing PCR using a temperature gradient program set at 55.1, 57.8, 62.1 and 66.0°C. PCR products were analyzed by 1.0% (w/v) agarose gel electrophoresis.

Restriction digest of *ompA*. Restriction digests of the PCR amplified *ompA* sample were prepared using 2 U of *Bgl* II (Gibco BRL, Cat. #15213-028), 1x REact3 (Gibco BRL, Cat. #Y90004), 4 µL PCR sample, and distilled water to make up to final volume of 10 µL. The reactions were incubated for one hour at 37°C and then stored at -20°C until analyzed by 1.0% (w/v) agarose gel electrophoresis.

DNA gel electrophoresis. To prepare DNA gels, 1.0% or 0.7% (w/v) agarose (Bio-Rad, Cat. # 161-3101) was dissolved 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). Molecular weight standards were prepared using 1 ug of GeneRuler™ 1 kb DNA Ladder (Fermentas, Cat. #SM0312), with 1x DNA loading dye (Fermentas, Cat. #R0611). Samples to be analyzed were prepared by adding DNA loading dye prior to being loaded into wells. Gels were run at a constant 100 V for approximately 45 minutes using 1x TAE buffer. After electrophoresis, gels were submerged in 0.5 ug/mL ethidium bromide bath for 20 minutes to stain the DNA. AlphaImager software v. 4.1.0 (Alpha Innotech Corp.) was used to visualize the DNA bands.

TOPO TA cloning of ompA into pBAD-TOPO vector. The *ompA* PCR product (4 µL) was cloned into the pBAD vector, as outlined by manufacturer's instructions, using the pBAD TOPO TA® Expression Kit (Invitrogen, Cat. #K4300-01).

Screening of pBAD insert by PCR amplification. Whole colony PCR was performed on the selected transformants to specifically amplify the insert region using pBAD primers (0.1 µg/ µL stock in TE buffer, supplied by Invitrogen kit). 9 µL of PCR reaction mix was prepared per sample, according to the recipe described for whole cell PCR amplification of *ompA* except that 0.02 ug each of pBAD forward and reverse primers were added instead of *ompA* primers. Samples containing cells were incubated at 94°C for 10 minutes before adding 1 µL of 0.2 U *Taq* polymerase. Then were tubes incubated in the Biometra® T Gradient machine at 94°C for 4 min; 35 cycles of amplification: 94°C for 45 sec, 55°C for 45 sec, 72°C for 90 sec; final extension at 72°C for 10 min.

PCR amplification was also performed using 0.5 µL of the cloning reaction (used to transform the TOP10 cells) to analyze the ligation of the insert and vector. To check for directionality of the insert, different combinations of pBAD primers and *ompA* primers were used in the PCR reaction. *Taq* polymerase was added directly to the tubes and samples incubated at the same PCR screening conditions.

Plasmid DNA preparation by 'toothpick miniprep' method. Refer to protocol 1.52 for the "Preparation of Plasmid DNA: Toothpick Miniprep" (12). Sterile pipet tips were used to transfer the cells instead of toothpicks. Bromophenol blue was not added to the samples before storing at -20°C. Samples were run on a 0.7% agarose gel to determine the presence and approximate size of isolated plasmids.

Plasmid DNA preparation by small-scale boiling lysis. Refer to protocol 1.44 for the "Preparation of Plasmid DNA by Small Scale Boiling Lysis" (12). Changes made to protocol: 3 µL of lysozyme (Sigma, Cat. #L-6876) was added instead of 25 µL.

Insert orientation analysis. Using the plasmids isolated by small-scale boiling lysis, 10 µL asymmetric restriction enzyme digests were carried out using 1 U of *Sph* I (NEB, #R0182S), 1x NEBuffer 2 (NEB, #B7002S), 5 µL of plasmid sample, and distilled water to make up to final volume of 10 µL. A linearized pUC19 DNA control was also performed by digesting 0.5 µg of pUC19 (Fermentas, #SD006.1) with *Sph* I. Control digest of a linearized plasmid sample was set up for pKT10W1 using 2 U of *Nco* I (NEB, #R0193S), 1x NEBuffer3 (NEB, #B7003S), 5 µL of plasmid sample, and distilled water to make up to final volume of 10 µL. At 37°C, *Sph* I digests incubated for 2 hrs while the *Nco* I digest incubated for 1 hour. Samples were analyzed by running on a 0.7% agarose gel.

RESULTS

PCR amplification of *ompA*. PCR amplification of *ompA* using a hybridization temperature gradient was performed and agarose gel results are shown in Figure 1. At 55.1, 57.8 and 62.1°C for hybridization, a faint DNA band was observed at approximately 1100 bp (calculated by log bp versus band distance travelled), which corresponds to the expected 1057 bp band size of *ompA* (lanes 2-4). At 66.0°C (lane 5), no PCR products

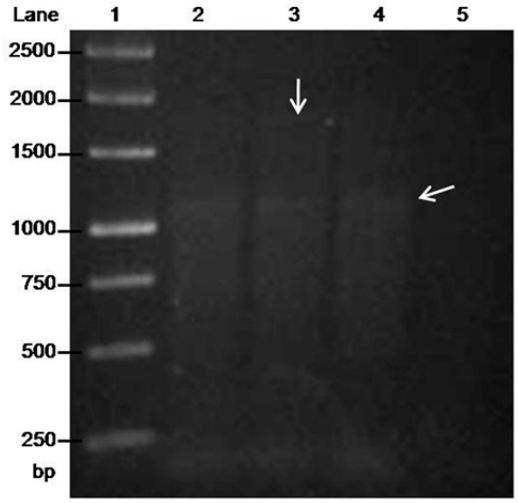

FIG. 1. Effect of hybridization temperatures on the specificity of PCR amplification of *ompA*. Lane 1: 1 kb DNA ladder; lanes 2-5: 55.1, 57.8, 62.1, 66.0°C hybridization temperatures in PCR cycle; top arrow: unspecific PCR product (1835 bp); lower arrow: desired PCR product (1062 bp) corresponding to expected band size of *ompA* (1057 bp).

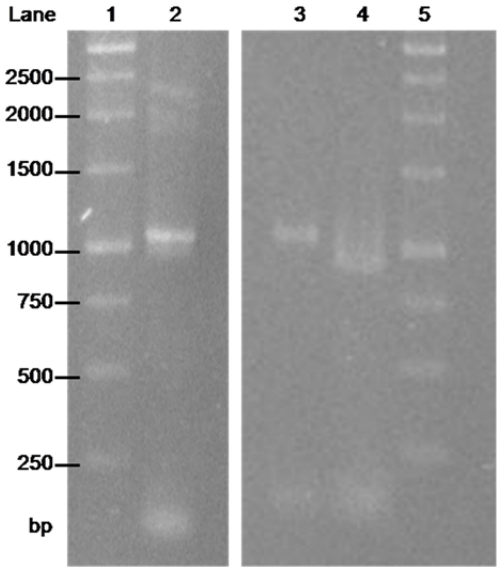

FIG. 2. Bgl II restriction enzyme digest analysis of PCR product to verify as *ompA*. Lane 1: 1 kb DNA ladder, lane 2: 10 ul of undigested PCR product, lane e: 4 ul of undigested PCR product, land 4: 4 ul of digested PCR product, land 5: 1 kb DNA ladder.

were observed. Low molecular weight (MW) unspecific PCR products (<230 bp) were observed at 55.1, 57.8 and 62.1°C. However, at 57.8°C, an additional unspecific DNA band (1835 bp) was observed, as indicated by the top white arrow in lane 3.

After the PCR amplification was repeated for *ompA* - with an additional 94°C boiling step for 10 minutes prior to adding *Taq* polymerase, the PCR cycles were increased from 30 to 35 and primers were bound at the optimized hybridization temperature of 62.1°C, the results showed an intense DNA fragment which corresponded to the desired *ompA* band size (Figure 2, lane 2). However, unspecific high and low MW PCR products were still present (as shown in lane 2). In order to verify the identity of the PCR product as *ompA*, a *Bgl* II restriction enzyme digest was performed (lane 4) and two bands were observed at 879 bp and 188 bp. The two bands corresponded well with the expected 896 bp and 161 bp fragments generated after *ompA* is cut with *Bgl* II.

Transformation of chemically competent TOP10 *E. coli.* Colonies recovered from spread plates were too numerous to count and it was suspected that the ampicillin used may not have been effectively selecting for transformants. After re-culturing selected colonies on fresh LB agar with ampicillin plates, the absence of bacterial growth confirmed that these cells were false positives. Nine new transformants labelled KT10W1 through KT10W9 were successfully recovered after a fresh spread plate was prepared from the remaining transformation reaction.

PCR screening of transformants and plasmids. Whole cell PCR screening using pBAD primers was performed for the selected colonies and an intense smear was observed on the DNA gel for clones KT10W1, 2 and 5 (data not shown). The PCR was repeated using fewer cells and then no bands or smears were apparent. It was unclear whether or not the PCR was working because no amplification of any specific PCR products was observed. PCR screening was also attempted for the cloning reaction but no amplification was detected.

Insert orientation analysis of plasmids. To determine whether the transformants carried the pBAD vector, a small scale plasmid preparation was performed and plasmids were observed by gel electrophoresis. Two bands were observed for each sample (2.6 kb and >10 kb) which may correlate to two conformations of plasmids (data not shown). Since the plasmids were not linearized prior to running on the gel, it was difficult to accurately calculate the molecular weight of the plasmids. The expected size of linearized pBAD vector is 4126 bp and pBAD-*ompA* is 5183 bp. For further analysis, a cleaner method of plasmid preparation was undertaken so that restriction digests using *Sph* I could be conducted. The pBAD vector has one *Sph* I site at position 3134 bp and the insert *ompA* has one *Sph* I site at 561 bp. For a pBAD-*ompA*

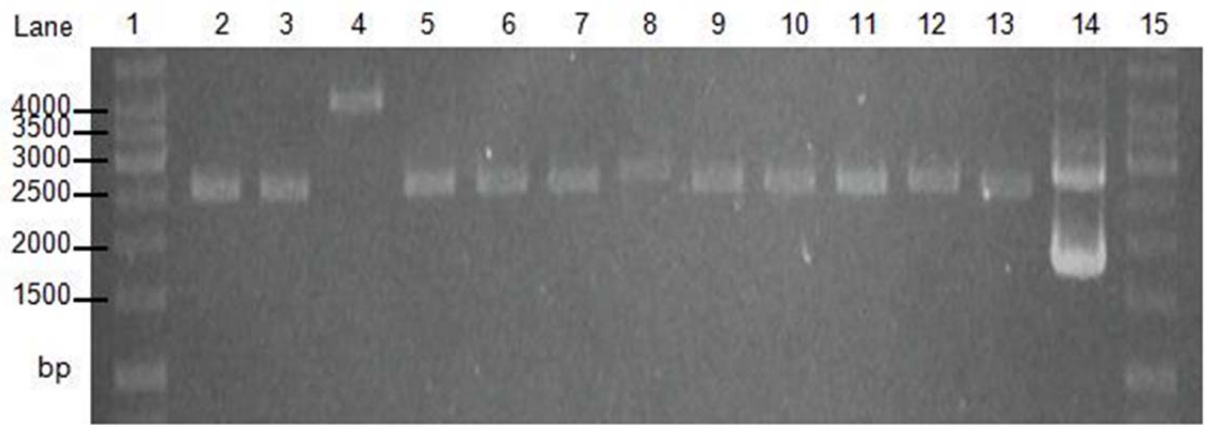

FIG. 1. *Sph* I asymmetrical restriction enzyme digest analysis of pKT10W1-9 to screen pBAD inserts. Lane 1: 1 kb DNA ladder, lane 2: pKT10W1 (uncut, not incubated), lane 3: pKT10W1 (uncut, incubated at 37°C), lane 4: pKT10W1 (cut with *Nco* I), lane 5-13: pKT10W1-9 (cut with *Sph* I), lane 14: pUC19 (cut with *Sph* I), lane 15: 1 kb DNA ladder.

construct with a correctly oriented gene, the expected DNA fragments observed should be 1941 bp and 3242. As a digest control, pUC19 plasmid was chosen because it contains one *SphI* restriction site which would produce a linearized plasmid with an expected size of 2686 bp. Digest results shown in Figure 3 suggest that no cutting occurred for the *Sph* I enzyme because the expected 2686 bp band for the pUC19 control was absent (lane 14). Also, no changes were observed between the uncut pKT10W1 samples in lanes 2-3 and the *Sph* I digested sample in lane 5. However, lane 4 shows pKT10W1 cut at a single *Nco* I site and a 4364 bp band was observed. The molecular weight of this band is consistent with the size of the pBAD vector (4126 bp) and a small non-specific PCR insert (<230 bp). Since the uncut bands of pKT10W1-3 and 5-8 (lanes 5-7, 9-12) migrated the same distance, it suggested that all of these plasmids were carrying a small <230 bp insert. For pKT10W9 (lane 13), the band migrated slightly farther down the gel which suggests that it has a lower molecular weight and may indicate an empty pBAD vector. An interesting observation is shown in lane 8 for pKT10W4 because the band is slightly higher than all the other uncut plasmids. This suggests that the higher molecular weight of this plasmid may contain a larger insert which has potential to be the desired *ompA* gene.

DISCUSSION

The *ompA* sequence was taken from a well-annotated strain of MG 1655 *E. coli* with a functional *ompA* gene. As an outer membrane integral protein, OmpA requires a vital signal peptide (first 21 amino acids from N-terminal) which affects its secretion, synthesis and assembly (9). For the proper expression and insertion of the native protein, primers were designed to exclude the optional N-terminal leader sequence and C-terminal V5 epitope and polyhistidine region, without modifying the *ompA* sequence itself. Results from the restriction digest and DNA gel verified that the amplified PCR product had the expected fragment sizes and banding pattern of *ompA*. Given that a pBAD-*ompA* clone is successfully recovered, the *ompA* insert should have the necessary components to be properly expressed and transported to the outer membrane.

Due to the use of new primers, a significant amount of time was spent designing and optimizing the PCR conditions to specifically amplify *ompA*. Temperature gradient was used to determine that 62.1°C was the more stringent temperature for optimal hybridization and any higher temperatures resulted in no products detected. Although unknown molecular weight PCR products were still present, gel purification of the verified *ompA* fragment was not carried out due to potentially diluting the DNA and introducing contaminants. The increased possibility of some pBAD constructs carrying the lower molecular weight insert was expected. This might have lowered the cloning frequency of the desired *ompA* fragment and given rise to the low molecular weight inserts observed in many of the pKT10W plasmids.

Due to parsimonious use of the pBAD TOPO vector, controls for the cloning reaction were not conducted so it was assumed that the PCR products ligated properly into the pBAD TOPO vector. PCR screening was unsuccessful because no specific DNA products were detected, which may be attributed to the use of new primers and non-optimized PCR conditions. Problems such as primer-dimer interactions, secondary structure

formations in *ompA* and primers are some possible reasons for poor amplification. In particular, stem loop formations in the *ompA* DNA fragments may be significant because previous studies have observed a 5' untranslated region in *ompA* mRNA which gives rise to a 5' terminal stem loop that was shown to stabilize the mRNA (2). During PCR, the presence of stem loops may also be formed by the *ompA* DNA template and this could make it difficult for primers to hybridize and for the polymerase to carry out DNA elongation. Finally, observed intense smearing gel patterns were likely due to using too many cells in the PCR reaction because the same effect was not present when the PCR was repeated using fewer cells.

From the insert orientation analysis, data showed that *Sph* I was not cutting the DNA at all and this problem can be explained by insufficient amounts of good quality *Sph* I available for carrying out the digest. Eight out of the nine transformants appeared to have pBAD constructs with lower molecular weights at a higher than expected cloning frequency. However, this observation makes sense because previous research has shown that at stationary phase, a correlation between increasing plasmid size and decreased *E. coli* cell viability exists so larger plasmids would be discriminated against (6). Since the eight transformants carried smaller plasmids, they possibly had a better advantage for survival, as demonstrated by its increased frequency over the clone carrying a larger plasmid.

The TOPO cloning system appears to be a viable approach to efficiently recover transformants carrying pBAD constructs. At this point, pKT10W4 is a plasmid worth further investigation because its larger molecular weight suggests that it carries a large insert which could potentially be *ompA*. Since no successful pBAD-*ompA* clone with correct orientation has been confirmed, results still remain inconclusive as to whether any intrinsic difficulties are associated with cloning *ompA*.

FUTURE DIRECTIONS

Restriction enzyme digest with *SphI* will reveal whether pKT10W4's digested fragments correspond with the expected band patterning of pBAD-*ompA*. It will also show whether or not *ompA* is oriented properly for gene expression. In addition, digests of pKT10W1-3 and 5-9 can be performed because all eight of these constructs appear to be 4364 bp or less and it would be worthwhile to verify that these plasmids do not actually carry the *ompA* insert.

Another cloning attempt may be considered so that a larger sample of transformants can be analyzed for the presence of pBAD-*ompA*. Restriction enzyme digest analysis appears to work but is more time-consuming and labour intensive. One suggestion is to pool 4-5 transformants per sample to be analyzed and then samples which show up positive for pBAD-ompA can be further investigated as individual transformants. If the restriction analysis shows that the vector contains *ompA*, an additional sequencing step can be considered by using pBAD sequencing primers (supplied by kit) to confirm the features of the construct.

Once a pBAD-*ompA* clone has been successfully verified, the plasmid construct can be used to transform the C156 (*ompA*) recipient *E. coli* strain. A successful C156 strain transformant which carries pBAD-*ompA* can then be induced with a range of L-arabinose to differentially express OmpA. Additional studies (e.g. by protein gel staining or Western blotting) will be needed to verify whether OmpA is being expressed properly and whether the OmpA levels correlate to the degree of induction. Finally, future studies may be conducted to investigate whether differential expression of OmpA of the recipient C156 *E. coli* plays a role in Hfr conjugation efficiency.

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