

Construction of pBAD24-*OmpA* for Modulating *OmpA* Expression in *Escherichia coli* to Assess Role in Conjugation

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OmpA is an abundant outer membrane protein in *Escherichia coli* that has been shown to play an important role in bacterial conjugation by maintaining membrane stability. Previous experiments have looked at the effect of high-level *ompA* expression on conjugation efficiency but these vector systems were limited to uncontrolled and non-differential increases in expression. The proposed pBAD24-*OmpA* construct was a more suitable system because it would allow for a range of *ompA* to be expressed and be tested in conjugation efficiency studies. In this study, the proposed construct was created but transformants from electroporation could not be recovered possibly because an insufficient quantity of the construct in the conformation that was favourable for efficient transformation. However, it is likely that by using an elevated quantity of the construct to increase the likelihood of generating a circular construct and by improving the transformation efficiency of the system, transformants carrying the pBAD24-*OmpA* can be recovered to assess conjugation efficiencies over a range of *ompA* levels.

Bacterial conjugation is an important process that describes the horizontal transfer of genes responsible for antibiotic resistance, virulence factors, and metabolic processes (2). DNA transfer by conjugation is the unidirectional transfer of DNA from a donor cell to a recipient, which involves cell-to-cell contact mediated by a pilus (2). While many aspects of conjugation remain unclear, the outer membrane protein (*ompA*) is thought to be required in mediating F-dependent conjugation and for maintaining the integrity of the outer membrane (3,8,10). *OmpA* is a well-characterized and abundant protein in the outer membrane of *Escherichia coli* that has been implicated in surface interactions and biofilm formation (3,10). The role of *ompA* in conjugation has been investigated in studies where the gene was cloned into a plasmid and expressed in high levels in *E. coli* (4,5). However, it was difficult to resolve the effect of *ompA* on conjugation efficiencies since these studies used pCCK06-1 and pCR2.1-TOPO expression systems where the gene was expressed at high and uncontrolled levels (4,5).

Researchers first proposed the construction of a pBAD24-*ompA* vector system to regulate the differential expression of the *ompA* gene to assess the effects of varying levels of *ompA* in bacterial conjugation studies (1). The pBAD family of plasmids contain the *araC* gene which acts to activate pBAD transcription in the presence of L-arabinose while repressing the promoter activation in the absence of the sugar (7,9). The pBAD24 vector is an ideal system as it

would allow simple modulation of *ompA* expression levels over a wide range of arabinose concentrations (9).

In this study, the proposed pBAD24-*ompA* vector was constructed in an attempt to test a range of *ompA* expression levels on conjugation efficiency in *E. coli*. However, transformants carrying the putative construct could not be recovered possibly because an inadequate amount of the closed circular construct was used in the transformation reactions.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *E. coli* strains C149 and C156 (refer to table 1 for genotypes) were obtained from the Department of Microbiology and Immunology at the University of British Columbia and were grown in Luria-Bertani (LB) broth (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl). The DH5 α *E. coli* containing the pBAD24 plasmid provided by the Hancock lab at UBC was grown in LB broth supplemented with 100 μ g/mL ampicillin. All liquid cultures were grown overnight at 37°C in a shaking incubator (200 rpm).

Genomic DNA isolation. Chromosomal DNA was isolated from 1 mL C149 bacteria overnight culture using the QIAamp DNA Mini Kit protocol for bacterial suspension cultures (Qiagen; 51304). DNA quantity and quality was assessed using A₂₆₀ and A₂₈₀ readings obtained from Thermo Scientific ND 1000 spectrophotometer.

PCR amplification of *ompA* gene. PCR reactions were prepared from 10 μ L 10x PCR Buffer (Invitrogen; 46-0128), 0.2 μ L 50 mM dNTP mix (Invitrogen; 46-0122), 2.5 μ L of isolated C149 DNA, 1.25 μ L of 10 mM forward primer 5'-GTGGAATTCATCATGAAAAAGACAGCTATCGCGATT-3' (IDT Technology), 1.25 μ L of 10 mM reverse primer 5'-TTTCTGCAGTTAAGCCTGCGGCTGAGTTA-3' (IDT Technology), 0.1 μ L 5 U/ μ L *Taq* polymerase (Invitrogen; 18038-043)

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

Strain	Genotype
C149	F- <i>proc</i> -24 <i>aroA</i> -357 <i>his</i> -53 <i>purE</i> -41 <i>ilv</i> -277 <i>met</i> - 65 <i>lacY</i> -29 <i>xyl</i> -14 <i>rps</i> -97 <i>cycA</i> -1 <i>cycB</i> -2? <i>Tsx</i> -63 lambda
	F- <i>proc</i> -24 <i>ompA</i> -256 <i>his</i> -53 <i>purE</i> -4 <i>ilv</i> -277 <i>met</i> 65
C156	<i>lacY</i> -29 <i>xyl</i> -14 <i>rpsL</i> -97 <i>cycA</i> -1 <i>cycB</i> -2? <i>Tsx</i> -63 lambda

and sterile H₂O was added for a final volume of 25 µL. Reactions were incubated initially on a BioRad Gene Cyclor PCR machine at 94°C for 3 minutes followed by 30 cycles, each consisting of 45 seconds at 94°C, 30 seconds at 55°C, and 90 seconds at 72°C. This was followed by a 10 minute incubation at 72°C and samples were stored at -20°C. Identity of PCR product was assessed with a 1% agarose gel to correlate PCR product size and fragment sizes following a restriction digest reaction of 4.5 µL PCR product, 1 µL 10x REact3 buffer (Invitrogen; Y90004), 0.1 µL of 10U/µL BamH1 (Gibco; 15201-O23) and 4.3 µL distilled H₂O incubated for 1 hour at 37°C. The gel was run at 90 V for 1 hour in TAE buffer followed by post-staining in a 0.2 µg/mL ethidium bromide bath for 15 minutes.

pBAD24 extraction. Plasmid extractions were isolated from an overnight culture of 4 mL DH5α *E. coli* containing pBAD24 using the PureLink HQ Mini Plasmid Purification Kit (Invitrogen; Cat. No. K2100-01) and also from a 100 mL overnight culture of DH5α *E. coli* containing pBAD24 using the Maxiprep protocol of the PureLink HQ Plasmid purification kit (Invitrogen; Cat. No. K2100-07). Plasmid quantity and quality was assessed using A₂₆₀ and A₂₈₀ readings obtained from a Beckman DU 530 Spectrophotometer.

Cloning of *ompA* into pBAD24. Individual double digest reactions of 4.5 µL *ompA* PCR product or 4.5 µL pBAD24 plasmid were completed with 0.1 µL 10U/µL EcoR (Invitrogen; 1117342), 0.1 µL 10U/µL Pst1 (Invitrogen; 1115758), 1 µL 10x REact 2 buffer (Invitrogen; 251104), and 4.3 µL distilled H₂O at 37°C for 1 hour. A ligation reaction followed, containing 4 µL digested plasmid, 2 µL digested PCR product, 4 µL 5x T4 ligase Buffer (Invitrogen; Y90001), 2 µL T4 DNA ligase (Invitrogen; 15224-017) and 8 µL distilled H₂O.

Transformation of C156 cells. Electrocompetent C156 cells were prepared for transformation. 1.5 mL overnight C156 *E. coli* cultures were transferred to microcentrifuge tubes and spun at 16100 x g for 2 minutes at 4°C. Supernatant was removed and the pellet was resuspended in 1.5 mL ice-cold 10% glycerol. This wash step was repeated three times. 40 µL of the electrocompetent cells were added to 1-2 µL of control pBR332 (Fermentas; SD0041) or pBAD24 containing *ompA* and allowed to incubate for 1 minute over ice. The mixture was then transferred to a chilled cuvette and pulsed once at 2.5 kV with a BioRad GenePulser. The cells were transferred to a microcentrifuge tube and incubated for 1 hour at 37°C in shaking incubator (200 rpm). Following recovery, 100 µL of cells were spread on LB agar plates supplemented with 100 µg/mL ampicillin for selective growth of transformants in triplicates. Colonies were counted and the average was used to derive the transformation efficiency of each reaction.

RESULTS

PCR amplification of *ompA*. The amplification of *ompA* was successful as indicated by the band size of the PCR product in the 1000 bp range and consistent with the expected 1041 bp fragment observed in lane 2

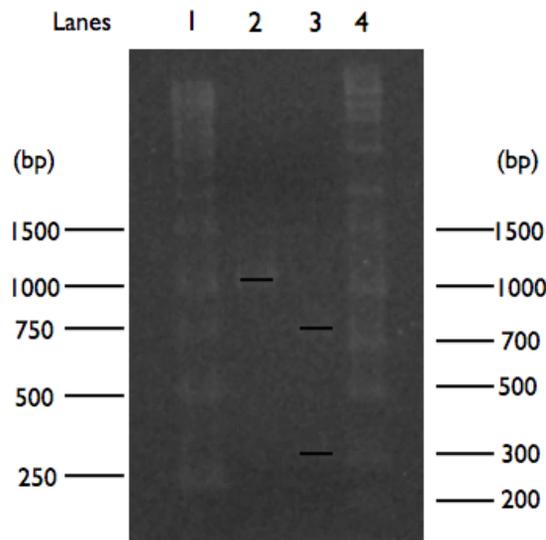


FIG. 1. PCR amplification of the *ompA* gene. Lane 1, GeneRulerTM 1 kb DNA Ladder (Fermentas; SM0311); Lane 2, undigested *ompA* PCR product; Lane 3, BamH1 digested *ompA* PCR product; Lane 4, MassRulerTM Express Forward DNA Ladder Mix (Fermentas; SM1283)

of Figure 1. Also, the fragment sizes visualized in Lane 3 of Figure 1 are similar to the expected 748 and 293 bp fragment sizes following BamH1 restriction digest of the *ompA* gene.

Cloning of *ompA* gene into pBAD24. Agarose gel electrophoresis was used to assess the outcome of the restriction digest and ligation reactions in cloning the *ompA* gene into the plasmid. Lanes 2 and 8 contain the unmodified pBAD24 plasmid in various relaxed plasmid conformations as indicated by the low motility of the banding patterns. The prominent band in lane 3, which represents *EcoR*I and *Pst*I double digested pBAD24 plasmid, has an approximate size of 4.5 kb. This might suggest that the identity of the plasmid was correct and the double digest reactions were successful, resulting in the observed band representing the cut plasmid in a linear conformation. Lanes 4 and 5 contain the *EcoR*I and *Pst*I digested products of the PCR amplification of the *ompA* gene. As the approximate 1.1 kb size is maintained in the bands of these two lanes, there is additional confirmation that the identity of the PCR product was correct and the integrity of the gene was preserved during the double digest reactions. Lanes 6 and 7 contain the ligation reactions of the digested *ompA* gene and pBAD24. While the bands observed near the wells might indicate low motility of some sufficiently ligated plasmids in a relaxed conformation, whether or not the *ompA* gene was successfully inserted in these plasmids cannot be

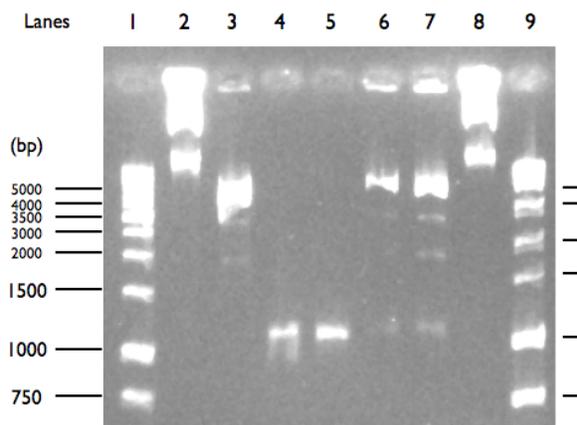


FIG. 2. Restriction digest and ligation reactions. Lane 1, GeneRuler 1 kb DNA Ladder (Fermentas; SM0311); Lanes 2 and 8, undigested pBAD24 plasmid; Lane 3, *EcoRI* and *PstI* digested pBAD24; Lanes 4 and 5, *EcoRI* and *PstI* digested *ompA* PCR product; Lanes 6 and 7, ligation reaction of *EcoRI* and *PstI* digested *ompA* PCR product and pBAD24; Lane 9, MassRuler Express Forward DNA Ladder Mix (Fermentas; SM1283)

confirmed. In relation to the band in lane 3, the position of the conspicuous bands in lane 6 and 7 have a higher molecular weight above 5 kb which might suggest the partial ligation of the PCR product at either end of the plasmid in a linear conformation.

Transformation efficiency of C156 cells. The calculated transformation efficiencies describe the number of transformants produced from 1 ng of plasmid DNA. In the first transformation reaction, C156 *E. coli* cells were successfully transformed with pBR322 control plasmid carrying ampicillin resistance while no transformants were generated from either the unmodified pBAD24 plasmid or the putative pBAD24-*ompA* construct. The transformants obtained from the pBR322 plasmid suggests that the C156 cells were electrocompetent and were not killed during electroporation. The relatively low quantities of isolated pBAD24 plasmid could account for the failure of the subsequent electroporation reactions. In the second transformation experiment, the positive control

demonstrates that the transformation system was functional. A higher pBAD24 concentration was obtained and its quantity was sufficient to produce ampicillin resistant C156 cells. However, transformants could not be generated from the pBAD24 construct containing the *ompA* gene, possibly due to the low plasmid quantity. In the third series of transformation experiments, the pBAD24 construct was assembled as described previously except the *ompA* PCR product was omitted in the ligation step. In this instance, transformants were successfully generated from the pBR322 plasmid, the pBAD24 plasmid, and the digested and ligated pBAD24 plasmid.

DISCUSSION

In creating a vector system to modulate *ompA* expression intended for assessing its role in bacterial conjugation, transformants carrying the putative pBAD24-*ompA* plasmid could not be recovered. This failure was probably due to an insufficient quantity of the construct in a closed circular conformation.

It is likely that the construction of the putative plasmid was successful as indicated by electrophoresis analysis of the ligation reaction, which gave evidence of linear pBAD24 plasmid ligated with the *ompA* gene in addition to closed circular plasmids that might have contained the *ompA* gene. The observed fragment sizes also confirmed the identity of the pBAD24 plasmid and *ompA* gene and also demonstrated that these two components were not cut at unexpected sites during the digest reactions.

As transformants were successfully generated from the pBR322 plasmid control and the isolated pBAD24 plasmid, the C156 cells were electrocompetent and viable. Although 4 pg was the minimum amount of the pBR322 plasmid required for successful *E. coli* transformation by electroporation in a high efficiency system developed by Dower *et al.* (8), it is unlikely that the transformation system described in the experiment shares the same effectiveness since transformants could not be recovered from a higher quantity of unmodified plasmid.

TABLE 2. Transformation efficiencies and plasmid quantity. In transformation experiments 1, 2, and 3, pBR322 was the positive control and the pBAD24 was unmodified. In transformation experiments 1 and 2, the pBAD24 construct contained *ompA* PCR product in the final ligase reaction while in transformation experiment 3, pBAD24 construct contains the digested and ligated pBAD24 without *ompA* insertion.

	pBR322		pBAD24		pBAD24 construct	
	Transf. Efficiency (Trans./ng)	Plasmid Quantity (ng)	Transf. Efficiency (Trans./ng)	Plasmid Quantity (ng)	Transf. Efficiency (Trans./ng)	Plasmid Quantity (ng)
<i>Transf. Exp. 1</i>	45.2	1000	0	345	0	30.5
<i>Transf. Exp. 2</i>	68.9	500	13.8	1232	0	120
<i>Transf. Exp. 3</i>	56.9	500	11.4	1232	1.5	140

Plasmid conformation greatly affects transformation efficiencies (6). It has been shown that closed circular plasmids have significantly higher transformation efficiency than nicked or open plasmids (6). The agarose gel banding patterns in lanes 6 and 7 of Figure 2 indicate that following the ligation reactions, the majority of the DNA plasmid remained in a linear form though a smaller population representing closed circular plasmid is also present. Although, the linear conformation of the plasmid construct can be repaired by the C156 cells, the transformation efficiency was still reduced. The difference in transformation efficiencies between the undigested pBAD24 and pBR322 plasmids can be explained by their respective topologies. The undigested pBAD24 plasmid was mostly in a relaxed conformation as indicated by banding patterns in Figure 2 whereas 90% of the commercially available and undigested pBR322 plasmid exists in the supercoiled conformation, which was more favorable for efficient transformation.

In the third set of transformation reactions, there was a 10-fold decrease in transformation efficiency of the digested and ligated pBAD24 without *ompA* in comparison to the unmodified pBAD24 plasmid. This may be in part due to the residual components of the digest and ligase reactions that can decrease transformation efficiency. However, it is more likely that the majority of the double digested pBAD24 population following ligation remained in a linear form following ligation as the two restriction cut sites are incompatible. Nonetheless, the small population of correctly ligated circular pBAD24 was sufficient to transform cells. However, when attempting to insert the *ompA* gene into the vector in accordance with the design of the first two transformation experiments, the likelihood of a correct ligation for a closed circular confirmation carrying the *ompA* gene may not be sufficient to generate transformants. This may explain why transformants could be generated from the digested and ligated pBAD24 in the third transformation experiment while the previous transformation reaction involving the construct containing *ompA* did not work, despite having similar DNA quantities. An inadequate quantity of the putative construct in a closed conformation is the probable cause as to why transformants were not generated.

FUTURE EXPERIMENTS

To achieve successful transformation, it would be necessary to obtain a higher pBAD24 concentration to increase the likelihood of generating a pBAD24-*ompA* construct in a closed conformation that is sufficient for transformation. Furthermore, to improve the efficiency

of the transformation system, specialized media such as SOC medium could be used in lieu of LB for incubating competent cells following electroporation. Once transformants have been isolated, the identity of the pBAD24-*ompA* construct needs to be verified by isolating the plasmid and performing a restriction digest map to ensure that the vector contains the *ompA* gene. When this has been verified, *ompA* expression can be differentially expressed over a range of L-arabinose concentrations to assess its effect on conjugation. It would also be necessary to ensure that *ompA* levels are indeed varied and are found in the outer membrane in response to different inducer concentrations. This can be verified by running the proteins extracted from the outer membrane on an SDS-PAGE gel and then performing a Western blot using an anti-*ompA* antibody to quantify relative changes in *ompA*.

AWKNOWLEDGEMENTS

This work was funded for by the UBC Department of Microbiology and Immunology. I thank Dr. Bill Ramey for his valuable guidance and insight, Robillo Lando and Nick Cheng for their friendly technical support, and Shaan Gellatly for providing the DH5a *E. coli* strain with the pBAD24 plasmid.

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