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Rescuing the *ompA* Deletion Mutant *Escherichia coli* JW0940 by Reintroducing *ompA* in the TOPO Cloning Vector pBAD

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OmpA is a transmembrane protein that has an essential function in protecting the integrity of the outer membrane of *Escherichia coli*, and provides a docking point for the donor pilus during conjugation. The plasmid TOPO cloning vector pBAD containing the *ompA* insert, whose expression is induced by arabinose, was previously designed as a tool to study the relationship between *ompA* expression and conjugation. The purpose of this study was to introduce the expression system into an *ompA*-deletion *E. coli* strain to improve the controlled inducibility of expression of *ompA* at the outer membrane. The insert orientation in the plasmid was verified through a restriction digest containing EcoRV and HincII. An *ompA* expression assay resulted in increased *ompA* expression as increasing arabinose doses were added to the culture medium, and confirmed the presence of OmpA in the outer membrane. Differential *ompA* expression was not linked with a difference in resistance to sodium dodecyl sulfate. The effects of varying the levels of expression of the *ompA* gene on the integrity of the outer membrane, and on F conjugation remain yet to be fully elucidated.

Outer membrane protein A (OmpA), which consists of a β-barrel structure, is one of the main surface proteins in members of the Enterobacteriaceae family (9). OmpA plays a role in functions such as the maintenance of structural cell integrity, mammalian cell invasion, bacteriophage binding, and conjugation (14). Consequently, it has been suggested that *E. coli* K1 *ompA*-deletion mutants are significantly more sensitive than that of their parent strain to sodium dodecyl sulfate (SDS), cholate, acidic environment, high osmolarity, and pooled human serum. (13)

While previous research has shown that conjugation efficiency is reduced when OmpA is inactivated (6, 8), and when purified OmpA is added to the conjugation medium decreasing the available docking points for pili (12, 13), the details of varying its expression level on the membrane are uncertain, and efforts have been made by several groups to develop a tool to study this phenomenon. One of the first attempts consisted in introducing the pCCK06-1 plasmid containing the *ompA* gene under the control of the P_{lac} promoter into the *ompA*-mutation *E. coli* strain C156 (3). The results obtained by this group showed no significant difference in conjugation efficiency of the plasmid-complemented strains relative to the efficiency of the untransformed control (3). Because later groups failed to conclusively demonstrate the absence of an OmpA protein band in the gels of membrane fractions belonging to uninduced bacteria, a likely cause for the observed results was the competitive inhibition of baseline non-functional copies of OmpA synthesized by chromosomal DNA, with the OmpA produced by the plasmid (2). The *ompA* gene in the C156 strain was rendered inactive by point mutations in single amino acids, and is by no means a deletion strain (3). Therefore, the C156 strain may be a conjugation-deficient OmpA-mutant that expresses inactive OmpA protein at reduced levels. In addition, the P_{lac} promoter in the pCCk06-1 vector does not allow a controlled transcription of the inserted gene (10). Because one of the main goals of this project was to keep developing a tool that would allow the effects of differential expression of OmpA to be studied, a different approach was taken.

In this project, a plasmid TOPO TA cloning vector pBAD containing the *ompA* insert was used due to its properties that allow the upregulation of *ompA* transcription to take place in an arabinose-dependent manner (5). In the absence of arabinose, AraC stops *ompA* transcription at the pBAD TA promoter. In order to try to circumvent the competitive inhibition problem, the *ompA* deletion strain JW0940 was used as the recipient of the plasmid. After verifying the correct orientation of the insert, and transforming into the JW0940 cells, a protein assay was done in which different arabinose concentrations were present in the media. The results confirmed the expression of plasmid-derived *ompA* in the membrane in an arabinose-dependent fashion. Increasing arabinose

TABLE 1. Genotypes of the *E. coli* strains obtained from the Keio Collection at the Coli Genetic Stock Center that were used for this study

Strain	Host Genotype	Plasmid	Parental Strain
BW25113	F⁻*Δ(araD-araB)567, Δ lacZ4787*(::rrnB-3),*λ*⁻, *rph-1 Δ(rhaD-rhaB)568*, *hsdR514*	No plasmid	
LVYY11w-1	F⁻*Δ(araD-araB)567, Δ lacZ4787*(::rrnB-3),*λ*⁻, *rph-1 Δ(rhaD-rhaB)568*, *hsdR514, ΔompA*	pBAD TOPO	JW0940
LVYY11w-2	F⁻*Δ(araD-araB)567, Δ lacZ4787*(::rrnB-3),*λ*⁻, *rph-1 Δ(rhaD-rhaB)568*, *hsdR514, ΔompA*	pEA11w7 with *ompA* in pBAD TOPO	JW0940

doses were not linked to increased SDS resistance dissolved in the culture agar.

MATERIALS AND METHODS

Plasmid isolation and restriction digest. The plasmid pBAD-*ompA* and respective empty vector constructed by Hsieh and Williams (5) were isolated from the *E. coli* C156 freezer stocks using the Fermentas GeneJet Plasmid Miniprep Kit (Cat #K0502) and supplied protocol. Insert orientation and identity were verified by a double digest with the restriction enzymes HincII (NEB, Cat # R0103S) and EcoRV (NEB, Cat # R0195S). The digest was carried out in a 20 µl reaction with 2 µl NEB React 3 buffer, 10 µl (400 ng) plasmid DNA, 0.5 µl enzyme and dH_20 to volume and incubated at 37°C for 2 hours. Digests (20 µl) and the Fermentas 100 bp plus GeneRuler (10 µl) were run on a 0.8% agarose gel in 1x TAE buffer for 1 hour at 100 volts. The gel was post-stained in ethidium bromide (0.2 µg/ml) for 20 minutes and imaged using the AlphaImager Software (Alpha Innotech Corp., San Leandro, CA)

Transformation. Overnight cultures of the recipient strain (JW0940) grown in LB (10) were diluted to 0.2 OD_{600} and 1 ml was pelleted at 10,000 g for 5 minutes at 4°C. The supernatant was decanted and the pellet was resuspended in 50 µl ice cold 0.1M $CaCl_2$ and placed on ice. 10 ng of plasmid were added to each of two tubes for pBAD empty vector and pBAD-*ompA* before incubating on ice for 30 minutes. Competent cells were then heat shocked in a 42°C water bath for 30 seconds before immediate placement on ice for 2 minutes. LB (950 µl) was added to the tube before incubation at 37°C on a shaking platform for 1 hour. 50 µl of this culture was plated on LB agar containing 100 µg/ml ampicillin. Plates were incubated overnight at 37°C. The success of the transformation was verified by repeating the restriction digest described above.

Outer membrane protein isolation. Overnight cultures of the recipient strain (JW0940) and wild type control (BW25113) were diluted to 0.1 OD_{600} in a 200 mL culture before incubating them in the presence of the following concentrations of L-arabinose (w/v): 0%, 0002%, 0.002%, 0.02% and 0.2%. After 6 hours of incubation, the cells were harvested and processed as described in (2).

SDS viability assay variation 1. JW0940-*ompA* cells were inoculated into 200 ml of Luria Broth containing the following concentrations of L-arabinose (w/v): 0%, 0002%, 0.002%, 0.02% and 0.2% and incubated at 37°C. Parental and JW0940-vector only cells had 0.2% and 0% arabinose as controls. Growth was measured by taking turbidity readings (OD_{650}) at 0, 2, 4 and 6 hours. Turbidity readings were kept within a tight range throughout the experiment. From each flask, 0.5 ml of the sample was combined with 2X SDS (final concentration 2% w/v), incubated for 5 min, and subsequently diluted to appropriate levels (calculated via 1 Abs = $1x10^9$ cells/ml). These were then plated onto LB plates and allowed to incubate overnight.

SDS viability assay variation 2. Parental and JW0940-*ompA* were incubated in Luria broth and their optical densities were read via a spectrophotometer at 650 nm. Samples were diluted to appropriate levels and then plated onto a LB containing the following SDS concentrations: 0%, 0.5%, 1.0% and 2.0% and incubated overnight.

RESULTS

Agarose Gel of Digested Plasmids. After transformation of the JW0940 strains, a restriction enzyme digestion of isolated plasmids from presumptive transformants was performed to confirm the presence of the appropriate plasmids. The pBAD TOPO vector is known to have a size of around 4.1 kb. The *ompA* gene from *E. coli* MG1655, is known to have a size of 1027 bp. Using the two restriction enzymes EcoRV and HincII, the predicted fragment sizes and the direction of the insert relative to the vector was confirmed. EcoRV cut the pBAD TOPO vector once and did not cut the insert. Thus EcoRV linearized the plasmid. HincII cut the vector once and the insert once at 600 bp downstream from the 5' end. Thus HincII linearized a vector without an insert and gave different product lengths for vectors with an insert. The restriction enzymes were incubated at 37°C for two hours but this was not enough to fully digest the plasmids. This was shown in Figure 1 by the presence of bands other than those expected. Lane 1 and 2 showed undigested supercoiled plasmids. Lane 2 displayed 3 bands, likely the result of having three forms: supercoiled, open circular and linear. Lane 4 and 5 are plasmids digested by EcoRV. As expected, plasmid without an insert was 4.1 kb while the plasmid with the insert was slightly higher at 4.2 kb. Lane 6 and 7 are plasmids digested by HincII. Lane 6 contained the same band as lane 4, and also showed the undigested supercoiled band, and lane 7 displayed 3 bands. The largest band was plasmids digested once by HincII and the lower two bands were the result of the plasmid being digested twice. There was a 1.7 kb band and a 3.4 kb band, suggesting the plasmid was in the correct orientation.

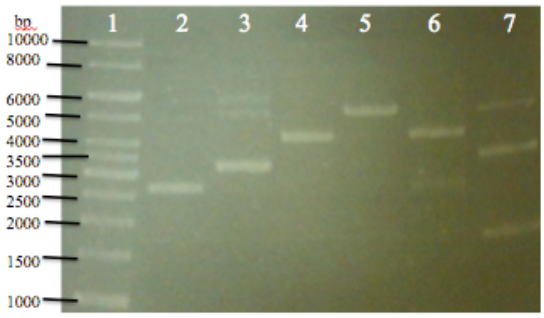

FIG. 1. HincII and EcoRV restriction digests of plasmid isolated from the JW0940 transformants. The undigested pBAD and pBAD-*ompA* plasmids are shown in lanes 2 and 3, respectively. Lanes 4 and 6 display pBAD plasmid digested with EcoRV and HincII, respectively. EcoRV and HincII digestions of pBAD-*ompA* are shown in lanes 5 and 7, respectively.

SDS-PAGE Gel. The protein of interest, OmpA, was expected to migrate between 30-35 kDa but was observed to migrate as a 27 kDa protein relative to the molecular weight standard (11). However, OmpA is known to run anomalously in SDS-PAGE gels as this is a characteristic of some membrane proteins (4). Unless fully denatured, OmpA remains a partially folded species that binds less SDS and migrates faster due to its compacted form (4). When more SDS binds OmpA, the protein becomes more linear and is able to migrate in proportion to the standards. Inadequate heat denaturation in the presence of SDS is a likely cause for the faster migration of OmpA displayed in this gel. While it is not possible to definitively determine the identity of this band as OmpA without anti-OmpA antibodies, we speculate its identity based on the approximate size of the band and its increasing intensity which is consistent with our prediction. The addition of varying amounts of arabinose to the media induced a proportional increase in OmpA expression and this shown clearly when comparing lanes 6 and 7 in Figure 2.This increase in band intensity is likely not a result of overloading the sample in lane 7 as the intensity is significantly higher for only OmpA while other bands in the lane are of comparable intensity to those in lane 6. Further, 4 ug of total protein was loaded in each lane to minimize lane to lane differences in loading. It is to be noted that in lanes 1 and 2, the negative controls with the pBAD vector in the JW0940 strain display bands observed at around the same size as OmpA. These may be background proteins of similar sizes and were masked by the presence of OmpA in the other lanes. Lane 8 was expected to have an equal or greater intensity for OmpA than in lane 7 due to the 10-fold increase in added arabinose to the media, however, during sample processing the cells may have been exposed to proteases for a prolonged period of time. Effectively, this would result in the same protein concentration determined by the BCA assay but there would be less protein present in the gel. Similarly, lane 9 was to serve as a wild type control but was unintentionally exposed to protease for a prolonged period of time. Lane 10 appeared to be under loaded or the basal level in the wild type positive control is lower relative to lane 7.

SDS Viability Assay. Culturing bacteria in medium with up to 4% SDS showed no correlation between OmpA induction levels and viability. Using the first variation of the experiment, as shown in Figure 3, the levels of viability did not follow any particular trend. Using the second variation, shown in Figure 4, there was a general trend that suggested that SDS has a negative correlation with viability. Nonetheless, the difference in viability between having no *ompA* expression at 0% arabinose and wild type expression of *ompA* from the parental strain was negligible.

DISCUSSION

After the transformation, the vectors were digested by restriction enzyme and the products were subsequently ran on an agarose gel. This confirmed that the obtained transformants had obtained the correctly oriented pBAD TOPO or pBAD TOPO ompA vectors. Additionally, the fragment sizes were also the correct sizes, determined via sequence analysis of the provided

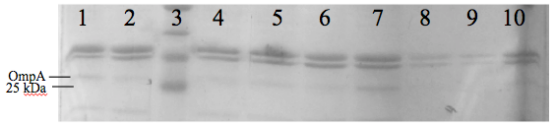

FIG. 2. 10% SDS-PAGE gel of isolated membrane proteins from the pBAD-*ompA* transformed JW0940 strain induced with 0-0.2% L-arabinose for 6 hours. Lanes 1 and 2 display OmpA expression in the pBAD -transformed JW0940 strain with 0% and 0.2% L-arabinose, respectively. Lane 3 is the Fermentas PAGE Ruler. Lanes 4-8 show OmpA expression in pBAD-*ompA* containing JW0940 cells following induction by 0%, 0.0002%, 0.002%, 0.02% and 0.2% L-arabinose, respectively. pBAD-*ompA* in the wild type strain (BW25113) induced by 0% and 0.2% L-arabinose is shown in lanes 9 and 10, respectively.

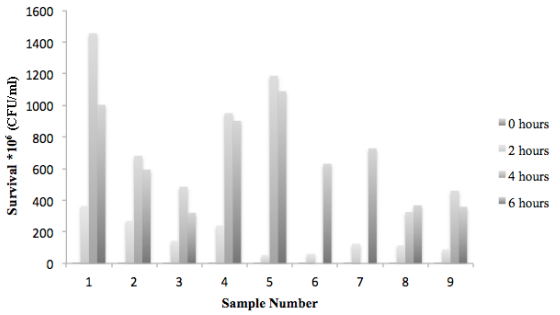

FIG. 3. SDS Viability Assay (variation 1). There is a lack of correlation between *ompA* expression levels and cell viability in the presence of SDS. Numbers 1 and 2 display survival in the pBAD -transformed JW0940 strain with 0% and 0.2% L-arabinose, respectively. Numbers 3 to 8 show survival in pBAD-*ompA* containing JW0940 cells following induction by 0%, 0.0002%, 0.002%, 0.02% and 0.2% L-arabinose, respectively. Survival of the pBAD-*ompA* in the wild type strain (BW25113) induced by 0% and 0.2% L-arabinose is shown in numbers 9 and 10, respectively.

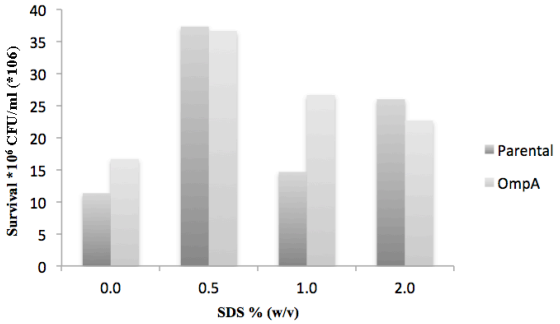

FIG. 4. SDS Viability Assay (Variation 2). The effect of SDS concentration in the medium on the survival of parental and pBAD -transformed JW0940 strain. There was no correlation between SDS in the medium and survival for any of the two strains.

vector sequence. These results bring confidence that the vectors in the transformants were the correct vectors.

The membranes of the transformed strains membranes were isolated via French press to reduce or eliminate cytosolic protein contamination and ran on SDS-PAGE. The staining with Coomassie Brilliant Blue G250 was sensitive enough to display the expected results. There was a trend that displayed an increasing band intensity from lane 4 to 7. This can be correlated with increasing protein concentration as a result of increasing arabinose concentrations in the JW0940 transformed with pBAD TOPO-*ompA*. However, the negative control strain transformed with pBAD TOPO vector had a high band intensity. This band intensity can be associated with the background expression of non OmpA proteins around the same size. This assay indicated that the vectors were expressing the insert. Comparing to the parental wild type controls at a concentration of 0.02% arabinose, the transformant overproduced OmpA. Induction by 0.002% arabinose yielded a band with intensity close to wild type levels of expression. Additionally, since the OmpA proteins are being transported to the membrane, this assay demonstrates that at the very least, the C-terminus of the OmpA protein is intact, as the membrane transport tag is located there (7).

None of the two versions of the SDS viability assays found conclusive evidence to support that increasing induction of *ompA* correlated with increased viability in the presence SDS. These data contradict Wang's findings (13) who demonstrated that as little as 0.3% SDS concentration can lead to a decreased membrane integrity and thus viability. The results of this experiment were definitely unexpected. Our strain JW0940, even without the induction of OmpA, did not seem to be significantly affected by SDS. Even when doing the same experiment Wang performed, where SDS was combined into the agar, the JW0940 strain grew on the agar at 4% concentration, 8X more than what Wang demonstrated as a threshold. Taking a look at sequenced *ompA* genes from several *E. coli* strains, the gene can significantly vary (addition or deletion of up to 100 bp (13). Perhaps, in the strain used by Wang (13), the *ompA* gene may have been selected to gain a protective role against harmful chemicals. However, in the JW0940 strain, no such role was observed.

Results from the restriction enzyme digestion demonstrate that the vector transformed into the recipient JW0940 *E. coli* cells were indeed the correct vectors. The results from the SDS-PAGE of membrane proteins support that the vectors respond to increased levels of arabinose concentration by expressing more insert (OmpA). Additionally, this gel indicated that OmpA was being incorporated into the membrane. The SDS assay results did not demonstrate a correlation between the increase of OmpA expression and viability against SDS. Consequently, while OmpA was being incorporated into the membrane, no conclusion can be drawn on whether or not the expressed ompA is functional.

FUTURE DIRECTIONS

Our results seem to indicate a lack of correlation between viability against SDS and presence of OmpA.

Consequently, this assay does not seem to be a good choice to assay for proper levels of OmpA. Instead, the best method to test the functionality of the expressed ompA may be to go ahead with a conjugation experiment. Plating of the cells after conjugation should be on two different medias. Firstly, a M9 media with lactose as the only carbon source and ampicillin will select for the transformants that receive the F'lacZ. Secondly, a rich media with X-gal as an additional screen may also be useful in examining the frequency. While X-gal is a great screening process, it only guarantees that a blue colony is blue but does not guarantee that a white colony is white. (in other words a white colony may be actually be lac positive but show up white.) It is expected that induction of ompA along with proper protein folding will correlate positively for conjugation.

The problem with going forward with the conjugation assay, is that proper folding cannot be guaranteed. As a result, failure to find a correlation between arabinose concentrations and conjugation frequency may be a result of a either the conjugation assay itself, or a problem with the proper protein folding. Going forward, it may still be better to find an alternative assay that can test for wild type conformations of OmpA and confirm for proper conformation before attempting the conjugation.

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