Journal of Experimental Microbiology and Immunology (JEMI) Vol. 17: 88 – 92

Induction of pBAD-*ompA* Fails to Enhance Conjugation in *ompA*-Deletion Mutant *Escherichia coli* Strains Containing the Plasmid

Byron Brook and Arella Wu
Department Microbiology & Immunology, University of British Columbia

Outer membrane protein A plays an essential role in horizontal gene transfer in *Escherichia coli* by acting as a docking protein for the pilus during conjugation. The purpose of this study was to assess the ability of plasmid-encoded and L-arabinose induced OmpA expression in a pBAD expression vector to restore mutant *ompA* phenotype back to wild type. The *Escherichia coli* recipient strains used were *ompA* wild-type strain BW25113, *ompA*-deletion mutants LVYY11w-1 with empty pBAD TOPO plasmid, LVYY11w-2 with pBAD TOPO inserted with *ompA*, and JW0940 without plasmid transformation. The F' donor used to test conjugation was *Escherichia coli* strain JCFLOxK1200. Relative conjugation efficiencies of mutant recipient strains were compared to the wild-type strain and with each other through statistical analysis. Our results showed that the wild-type BW25113 strain had the highest relative conjugation efficiency. All other *ompA*-deletion mutant recipients had reduced relative conjugation efficiencies when L-arabinose levels varied between 0% and 2% (w/v). Because there was no statistically significant difference in conjugation efficiency among strains LVYY11w-2, LVYY11w-1, and JW0940, we concluded that the plasmid-encoded OmpA might be misfolded, or falsely processed. Furthermore, since the *ompA* gene inserted in the plasmid was from *Escherichia coli* strain MG1655, its encoded protein might play different roles compared to OmpA protein in wild-type strain BW25113.

The process of cell-to-cell attachment in conjugation is encoded by a wide range of conjugative plasmids. During conjugation, donor and recipient bacterial cells are recognized and paired up by an extracellular filamentous organelle known as the pilus. The F factor from the donor cell is transferred to the recipient cell via the pilus and replicated to form a double-stranded circular DNA structure. The outer membrane protein A (OmpA) is a surface protein among *Enterobacteriaceae* species. It has been previously established that OmpA maintains structural integrity of the cell, promotes colicin susceptibility (1), and has a role in interactions with infectious phages (2). In addition, some studies suggested that OmpA stabilizes the mating pair during F-conjugation by acting as the docking protein for the pilus (3,4), and it has been demonstrated that *ompA* mutation decreases relative conjugation efficiency of *Escherichia coli* cells (3,4).

To confirm the role of OmpA during conjugation, *ompA* from *E. coli* strain MG1655 was cloned into a pBAD TOPO vector and transformed into two *ompA*-deletion *E. coli* strains, LVYY11w-1 and LVYY11w-2 (5, 6). Orientation of *ompA* was previously confirmed by EcoRV and HincII restriction enzyme digests, and OmpA expression level was controlled by varying L-arabinose concentration in the culture media(6). When L-arabinose induction was absent, *ompA* transcription was blocked at the pBAD TA promoter by AraC (7). Through SDS-PAGE gel analysis, it was shown that the OmpA protein was expressed from the *ompA*-containing plasmid and localized to the outer membrane (6). In addition, increasing L-arabinose induction in the media lead to increased OmpA expression (6). However, the functionality of plasmid-expressed OmpA in terms of conjugation efficiency was unknown. In the current study, F' conjugations were performed using various *E. coli ompA* mutant strains and a wild-type strain as recipients. The results concluded that the plasmid-restored OmpA did not seem to function properly to improve conjugation efficiency of LVYY11w-2, and there was no positive correlation between increasing L-arabinose induction level and closer level of conjugation efficiency compared to the *ompA* wild type.

MATERIALS AND METHODS

Bacterial strains and culture conditions. In this study *E. coli* strains JW0940 (*ompA*-deletion mutant), LVYY11w-1 (*ompA*-deletion with an empty pBAD TOPO plasmid), LVYY11w-2 (*ompA*-deletion with pBAD TOPO *ompA* insert) (6) and BW25113 (wild-type *ompA*) were used as recipient strains for the F' conjugation. *E. coli* strain JCFLOxk1200 was used as the F' donor (Table 1). All *E. coli* strains were obtained from the MICB 421 culture collection at the Department of Microbiology and Immunology at the University of British Columbia. All strains were cultured in 3 ml of Luria-Bertani (LB) broth as described (5), 1.5% (w/v) tryptone (Bacto™, Catalogue #211705), 0.50% (w/v) yeast extract (Bacto™, Cat. #212750), and 1.0% (w/v) NaCl (Fisher Chemicals, Cat.#S271-3), with adjustment to pH 7.5. The strains were incubated for 12 hours overnight at 37℃ in a shaking incubator at 100 rpm. All cultures except strain BW25113 were grown with L-arabinose at concentrations of 0% (w/v), 0.0020% (w/v), 0.020% (w/v), 0.10% (w/v), 0.20% (w/v) and 2.0% (w/v) for different experiments. Strain BW25113 was grown only with 0% (w/v) and 0.020% (w/v) L-arabinose. Cell density of individual overnight cultures was assessed by turbidity at OD_{660}(8). For subsequent conjugation experiments, all bacterial cultures were diluted with LB broth at a 1:20 ratio. The amount of bacterial stock added into the LB broth was adjusted so that the number of bacterial cells inoculated could be approximately equivalent across the strains.

Lactose utilization test with M9 minimal broth with 0.1% lactose and 0.1% glucose. M9 minimal broth with 0.68% (w/v)

TABLE 1 List of genotypes of *E. coli* strains used in the current study.

Strain		Genotype	Plasmid	Parental strain	*ompA* Origin
Recipients	JW0940 (6)	*DE(araD-araB)567, lacZ4787(del)(::rrnB-3), λ⁻, ompA772(del)::kan, rph-1, DE(rhaD-rhaB)568, hsdR514*	No plasmid	BW25113(6)	
	LVYY11w-1(6)	*F⁻Δ(araD-araB)567, Δ lacZ4787(::rrnB-3),λ⁻, rph-1 Δ(rhaD-rhaB)568, hsdR514, ΔompA*	pBAD TOPO without *ompA*	JW0940 (6)	
	LVYY11w-2(6)	*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 (6)	MG1655
	BW25113 (6)	*F⁻Δ(araD-araB)567, Δ lacZ4787(::rrnB-3),λ⁻, rph-1 Δ(rhaD-rhaB)568, hsdR514*	No plasmid		
Donor	JCFLOxK1200(11)	*F' lac, nal (resistant) lacΔ, U124Δ, nadA, aroG, gal, AttL, bio, gyrA*	No plasmid		

Na_2HPO_4 (anhydrous) (Fischer, Cat. #BP332-500), 0.30% (w/v) KH_2PO_4 (EM Science, Cat. #PX1565-1), 0.050% (w/v) NaCl (Fischer, Cat. #BP358-1), 0.10% (w/v) NH_4Cl (BDH Inc. Cat. #ACS048) was made with pH adjustment to 7.5 (9). The broth was separated into two equal portions, each supplemented with 0.10% (w/v) lactose (Bacto™, Cat. #0156-17) or 0.10% (w/v) glucose. Strains JCFLOxK1200, LVYY11w-1, LVYY11w-2, and JW0940 were individually inoculated into 3 ml of M9 minimal broth with either lactose or glucose supplement, and incubated at 37℃ in a shaking incubator at 120rpm for approximately 16 hours overnight for observation of growth.

Lactose utilization test with MacConkey agar plates. MacConkey agar plates were made with peptone (Cat. # unavailable), protease peptone (Difco, Cat. #0120-17-6), lactose (Bacto™, Cat.#0156-17), bile salts, NaCl (EMD Millipore, Cat.#SX0420-1), agar (Invitrogen,Cat.#30391-023), neutral red (Invitrogen, Cat.#N-6634), and crystal violet (Invitrogen, Cat. #C581-25) were made (10). Each of the JCFLOxK1200, LVYY11w-1, LVYY11w-2, and JW0940 strains were streaked onto individual MacConkey agar plates. The plates were incubated at 37℃ for 12 hours overnight.

Comparison of cell size based on OD readings and cell number. Nutrient agar plates were made with LB broth (as described above) and 1.5% reagent grade agar (Invitrogen, Cat. #30391-023).From the original cell stocks, each of strains LVYY11w-1, LVYY11w-2, JW0940, BW25113, and JCFLOxK1200 was inoculated into 3 ml of LB broth and incubated at 37℃ for 12 hours overnight. Cell turbidity was measured at OD_{660} (8). All broth cultures were diluted at ratios of 1:100,000, 1:1,000,000, and 1:10,000,000, and 100 to 200 µl of each diluted culture was spread onto a nutrient agar plate. Duplicates were prepared for each dilution ratio. The plates were incubated at 37℃ for 12 hours overnight. The colony numbers of each condition were used to calculate the total cell number of the original 3 ml culture broth, and colony morphology was recorded. The total cell number of each strain was then normalized based on their turbidity ratios to the strain with the smallest reading. Statistical difference in cell numbers was analyzed at 99.7% confidence level (three standard deviation).

Preparation of M9 selective agar.M9 minimal agar plates were made with M9 broth (as described previously) and1.5% (w/v) Select agar (Invitrogen, Cat. #30391-023), with pH adjusted to 7.5(9).Based on the genetic constructs of the donor and recipient strains (Table 1), 80 µg/ml kanamycin (Sigma, Cat.#K-4000) and 0.10% lactose (Fisher Scientific, Cat. #L5-500) were filter-sterilized and incorporated into the media to serve as selective markers for F' conjugation.

Conjugation. Bacterial cultures diluted 1:20 in LB broth were incubated at 37℃ in the shaking incubator at 120 rpm for two hours to approximately 0.1 OD_{660} (11). To 900 µl of each recipient strain, a standardized volume of donor cells was added to achieve a donor-to-recipient ratio of 1:9 based on their turbidity ratio (11). Each conjugation mixture was then incubated in a 37℃ shaking water bath at approximately six rpm/minute. After incubating for five minutes, the conjugation was interrupted by vortexing the conjugation mixture for 15 seconds. To assess the probability of spontaneous mutation of recipient cells utilizing lactose and donor cells resisting kanamycin, a control conjugation experiment with the same steps was carried out for each recipient and for the donor. Instead of mixing donor with recipients, sterile LB broth was used as a substitute in each control experiment.

Screening for successful conjugants. The conjugation mixture was diluted in 1X PBS at ratios of 1:500, 1:5,000, and1:50,000. Diluted conjugation mixture was briefly vortexed, and 100 µl of the mixture was dispensed onto an M9 selective agar plate and spread. The same steps were carried out for conjugation control samples. Each dilution was prepared in duplicate. The control and conjugant plates were incubated for 36 hours overnight at 37℃. Any large colony was recognized as from a successful conjugant cell. The total number of colonies on each plate was counted, and its statistical significance was analyzed using Chauvenet's Criterion (12). Relative conjugation efficiency of each *ompA*-deletion mutant strain was calculated by comparing the number of conjugant colonies to that of the wild-type strain.

RESULTS

Lactose utilization control test in M9 minimal broth. Equivalent levels of cloudiness were observed in M9 minimal broth with 0.1% glucose for strains LVYY11w-1, LVYY11w-2, JW0940, and JCFLOxK1200. As expected, no growth was observed for strains LVYY11w-1, LVYY11w-2, and JW0940 in M9 minimal broth with 0.1% lactose since these strains did not have functional *lacZ* for lactose utilization. Contrary to what was expected, donor strain JCFLOxK1200 did not show any growth in M9 minimal broth with 0.1% lactose.

Lactose utilization control test on MacConkey agar plates. Confluent colony growth was observed for the donor strain and all recipient strains. The colonies on the donor plate were all red but the colonies on the recipient plates were all white. The red colour of the colonies indicated drop in pH due to the acid produced by the donor *E. coli* strain as a result of lactose utilization (10). On the other hand, the recipient strains without functional *lacZ* could only utilize peptone in the media thus producing ammonia and raising the pH (10). As a result, the recipient strain colonies turned white.

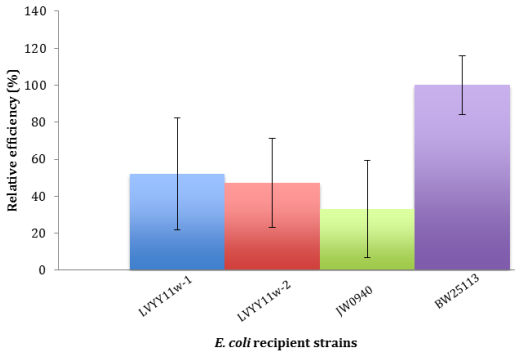

FIG 1 Relative conjugation efficiencies of *E. coli* recipient strains with 0.02% L-arabinose induction.The statistical significance of the data was analyzed using two standard deviations at a 96% confidence level.

Approximation of cell number and size. The correlation between *ompA* deletion and cell size was not clear. Since OmpA is a major outer membrane protein, the absence of OmpA could have an effect on cell size and therefore on turbidity readings. The *E. coli* strains used in this study were expected to have similar sizes. The total cell number for each strain was adjusted based on turbidity ratio when compared to the strain with the lowest OD at 0.549. Based on statistical analysis with 99.7% confidence, there was no significant difference in cell numbers among the *E. coli* strains at the same turbidity level. In addition, morphology observations showed that colonies of all strains appeared to be similar sizes, white and circularly shaped, with a smooth surface and raised elevation. The morphology observations and cell number analysis together indicated that all strains used in the current study were of similar sizes and morphology.

Conjugation with no L-arabinose induction. All donor- and recipient-only control plates had zero observable colony-forming units. With a 0% (w/v) L-arabinose induction level, strain BW25113 had at least 1.7 times more conjugation than the other recipient strains. No difference could be seen among the *ompA* mutant strains. This was determined at a one standard deviation level.

Conjugation with L-arabinose induction. The relative conjugation efficiencies of strains LVYY11w-1, LVYY11w-2 and JW0940 were also compared to strain BW25113 at L-arabinose induction levels of 0.002%, 0.02%, 0.1%, 0.2%, and 2% (w/v). It was expected that at 0.002% L-arabinose induction, LVYY11w-2 strain would have a similar level of relative conjugation efficiency as the BW25113 strain if OmpA function was fully restored(6). It was also expected that with increasing L-arabinose concentration in the media, OmpA expression in the LVYY11w-2 strain would proportionally increase, contributing to gradually higher relative conjugation efficiencies. On the other hand, due to the lack of functional *ompA*, the LVYY11w-1 and JW0940 strains were expected to have much lower conjugation efficiencies compared to the wild type and LVYY11w-2 strains.

Few data points were available for relative conjugation efficiencies at 0.002%, 0.1%, 0.2%, and 2% L-arabinose due to time constraint. However, at 0.02% L-arabinose, a more appreciable difference between the wild-type strain BW25113 and the mutant strains LVYY11w-1, LVYY11w-2, and JW0940 was observed (Fig 1).

DISCUSSION

Cell size was determined to be constant, as there was no significant difference between observed cell numbers among strains. This was investigated because OmpA, as a major outer membrane protein, had the potential to affect cell size and morphology. If OmpA did have a role in cell size, this would have changed the number of cells in relation to the observed level of turbidity, and a different standardization would have been performed. Since this did not occur, and since a constant donor to recipient ratio was maintained, any difference in CFU number was due to differences in the phenotypes of the recipient strains.

The lack of growth for strain JCFLOxK1200 in lactose M9 broth media was unexpected, but this could show that not all essential vitamins were included in the M9 minimal broth for this strain. For conjugation the cells were initially grown in LB broth, which had been shown to supported growth. Therefore it was not of high priority to determine what the missing vitamin was in the M9 minimal broth. As expected, the recipient strains in this study did not grow in M9 minimal broth with lactose because they have no functional *lacZ* for lactose degradation. This was confirmed with the presence of white colonies on MacConkey screening plates. Although the donor strain did not show any growth in the M9 minimal broth supplemented with lactose, it did show lactose degradation on the MacConkey plate as the colonies turned red. Together, the result indicated the effectiveness of using lactose as a selection marker for the recipient cells. The lack of growth for the donor strain JCFLOxK1200 on conjugation-selective agar was expected as it did not encode any kanamycin resistance gene. The lack of growth also confirmed that any probability of spontaneous mutation, which could generate Kan^+ mutants, was not high enough to negatively affect the total number of conjugant colonies or conjugation efficiency analysis. The ability to degrade lactose showed applicability of strain JCFLOxK1200 to be used in conjugation, while the issue of a missing vitamin was minor because these donor cells were grown in LB broth prior to conjugation. Together, only successful conjugants were able to survive on the M9 minimal agar plates with kanamycin and lactose.

Combining the results from the lactose utilization test and MacConkey plate test, it was suggested that the recipient cells must receive functional *lac* genes from the donor via conjugation to grow on the selective agar. This was supported as conjugant colonies were observed for all recipient strains after conjugation with the JCFLOxK1200 donor strain, whereas the recipient-only controls did not show any cell growth. Therefore, the probability of spontaneous mutation that generates lac^+ recipient mutants

was not significant to negatively affect the total number of conjugant colonies or conjugation efficiency analysis.

Since strains LVYY11w-1and JW0940 had *ompA* deletion mutation, it was hypothesized that they would have much lower conjugation efficiencies than the wild-type strain BW25113. The experimental results did show that the conjugation efficiencies of strain BW25113 were more than two folds higher than those of the *ompA*-deleted recipients. In the case of LVYY11w-2, *ompA* was restored by transformation with the pBAD TOPO plasmid. In addition, the orientation of the *ompA* insert was confirmed by restriction digest by HincII and EcoRV (6). Therefore, it was hypothesized that under an appropriate level of L-arabinose induction, strain LVYY11w-2 would have similar relative conjugation efficiency as the wild-type strainBW25113. However, the experimental result suggested that statistically the relative conjugation efficiency of strain LVYY11w-2 was indistinguishable from the other two *ompA*-deleted mutants, and the efficiency was over two folds lower than that of strain BW25113 under 0.02% L-arabinose induction.

Previously Chambers *et al* showed similar results with a different *E. coli* strain, as the plasmid-transformed mutants had similar conjugation efficiencies as their untransformed cultures, and these mutants all had much lower conjugation efficiencies when compared to the *ompA* wild-type control (9). In another study, Lal *et al* had isolated membrane proteins from *ompA*-restored cell samples after conjugation (6). Subsequent SDS-PAGE analysis showed that increasing L-arabinose concentration in the culture media induced increasing OmpA expression (6). In addition, Lal *et al* showed that at 0.002% L-arabinose, the band intensity of restored OmpA protein was close to that of wild-type OmpA protein band, and at 0.02% L-arabinose, *ompA* was overexpressed in transformed cells (6). They also showed that OmpA was localized to the membrane; therefore another issue must be responsible for preventing the reversion to wild-type phenotype from the *ompA* mutant.

An earlier study by Manoil *et al* had suggested that a conjugation pathway without OmpA involvement is possible in recipient cells (4). This pathway showed better efficiency when conjugation was performed on a surface of solid media(4).It is possible that an OmpA-independent conjugation pathway caused similar numbers of conjugant colonies for *ompA*-deletion mutants JW0940, LVYY11w-1, and LVYY11w-2 as the conjugation mixtures were plated on solid agar media in our experiment. However, we speculated that wild-type OmpA would facilitate more efficient conjugation than the OmpA-independent pathway, even though OmpA might not play as important of a role as expected. A liquid conjugation and culturing system should be developed to study this further.

Manoil *et al* suggested that conjugation and reception of incoming phages involve different sites of the OmpA protein (4). Incomplete processing of the site specific for pilus reception could have caused a defect in functions of the plasmid-delivered OmpA, yielding the observed phenotype.

From another study, Achtman *et al* had suggested that OmpA is involved in stabilization of mating aggregation, and the stabilization process requires close interaction between OmpA and surface lipopolysaccharide (LPS) (3). Due to time constraints, interaction of OmpA in the LVYY11w-2 strain with LPS and the functional role of such interaction were not tested. However, if plasmid-expressed OmpA had weaker interaction with LPS than wild-type OmpA, relative conjugation efficiency of LVYY11w-2 would, in this case, be affected, as the mating pairs could be comparatively less stable than those of the BW25113 strain. In addition to this, the *ompA* gene inserted into the pBAD TOPO plasmid was originally from *E. coli* strain MG1655, which was different from the wild-type *ompA* investigated in this study. It is unknown whether the MG1655 and JW0940 strains have the same requirements for the OmpA protein. To investigate the MG1655 strain a specific cell selection process in conjugation would need to be developed.

The current study did not show significant improvement in relative conjugation efficiency for mutant strain LVYY11w-2 containing the *ompA*-inserted plasmid, even when L-arabinose level was increased from 0% to 2%. Although OmpA might be overexpressed in LVYY11w-2 as Lal *et al* had demonstrated (6), its functional role in conjugation could not be confirmed because the relative conjugation efficiency of the *ompA*-restored mutant strain was not demonstrably different from the *ompA*-deletion strains JW0940 and LVYY11w-1. Furthermore, relative conjugation efficiency of the LVYY11w-2 strain failed to match that of the wild type. This could be caused by protein misfolding, misprocessing, or different modes of actions for the encoded genes from their strain origins.

FUTURE DIRECTIONS

Further study should be performed to compare *ompA* in the wild-type control (BW25113) and *ompA* inserted in the pBAD TOPO plasmid (strain MG1655), as there might be different roles of OmpA protein in these two strains. This study used an F' conjugation plasmid on solid media, but it may be beneficial to test this in broth with gentle aeration to remove the chance of secondary conjugation mechanisms. An Hfr conjugation could also be beneficial to test by increasing the amount of time required for conjugation to occur.

ACKNOWLEDGEMENTS

We thank the Department of Microbiology and Immunology of the University of British Columbia for the financial support throughout the study. We thank Dr. William Ramey for his patient guidance, support, and advice. We also thank Mr. Nick Cheng and Mr. Lando Robillo for their technical support.

REFERENCES

1. **Manning PA, Pugsley AP, Reeves AP.** 1977. Defective growth functions in mutants of *Escherichia coli* K12 lacking a major outer membrane protein. J. Mol. Biol. **116:** 285-300.
2. **Morona R, Klose M, Henning U.** 1984. *Escherichia coli* K12 outer membrane protein (OmpA) as a bacteriophage receptor: analysis of mutant genes expressing altered proteins. J. Bacteriol. **159**: 570-578.

3. **Achtman M, Schwuchow S, Helmuth R, Morelli G, Manning PA.** 1978. Cell-cell interaction in conjugating *Escherichia coli*: Con$^-$ mutants and stabilization of mating aggregates. Mol. Gen. Genet. **164**: 171-183.
4. **Manoil C, Rosenbusch JP.** 1982. Conjugation-deficient mutants of *Escherichia coli* distinguish classes of functions of the outer membrane OmpA protein. Mol. Gen. Genet. **187**: 148-156.
5. **Hsieh A, Williams E.** 2012. Genetic compensation of *E. coli* C156 with pBAD-*ompA*for the investigation of conjugation efficiency. J. Exp. Microbiol. Immunol. **16**: 113-118.
6. **Lal A, Vela J, Yee J, Yuan E.** 2012. Rescuing the *ompA* deletion mutant *Escherichia coli* JW0940 by reintroducing *ompA*in the TOPO cloning vector pBAD. J. Exp. Microbiol. Immunol. **16**: 108-112.
7. **Life Technologies Inc.** 2009. pBAD TOPO® TA Expression Kit: five-minute cloning of *Taq* polymerase amplified PCR products for regulated expression in *E. coli.* Life Technologies Inc., Burlington, ON.
8. **Carrie J, Decker M, Galac L, Hird T.** 2005. The effects of extraneous autoinducer-2 addition to a young *Escherichia coli* B23 culture. J. Exp. Microbiol. Immunol. **7**: 20-30.
9. **Chambers C, Chang B, Fehrmann K, Quan G.** 2007. Investigating the sex life of *Escherichia coli*: the effect of OmpA expression on Hfr conjugation efficiency. J. Exp. Microbiol. Immunol. **11**: 98-102.
10. **BD Diagnostics.** 2009. MacConkey agars: formulae, p 331. *In*Zimbro MJ, Power DA, Miller SM, Wilson GE, Johnson JA (ed), Difco™ & BBL™ manual: manual of microbiology culture media, 2nd ed, Sparks, MD.
11. **Cho J, Jenneson S, Lane M, MacFadyen A, van Rietschoten S.** 2003. The effects of altering autoinducer-2 concentration on transfer efficiencies of the F and RPI plasmids to the quorum sensing recipient *Escherichia coli* strain AB1157. J. Exp. Microbiol. Immunol. **3**: 8-14.
12. **Taylor JR.** 1997. Rejection of data: Chauvenet's Criterion, p 166-167. *In* McGuire A (ed), An introduction to error analysis: the study of uncertainties in physical measurements, 2nd ed, Sausalito, CA.