Cloning and Overexpression of Imp/OstA, an essential *Escherichia coli* outer membrane protein with a possible role in lipid transport

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Imp (increased membrane permeability) is an outer membrane (OM) protein with a possible role in lipidpolysaccharide (LPS) transport across the outer membrane of *Escherichia coli*. Attempts were made to replace the chromosomal copy of *imp* with a kanamycin resistant cassette by transforming *E. coli* cells with the kan^R cassette PCR product. These attempts to knock out the chromosomal copy of *imp* were unsuccessful. Biochemical experiments including overexpression of Imp, membrane separation by a sucrose gradient and solubilizaion were also conducted. Results indicate that Imp can easily be overexpressed and that the Imp protein co-fractionates with the outer membrane but remains recalcitrant to solubilization.

INTRODUCTION

The cell envelope of Gram-negative bacteria contains an inner membrane (IM) composed of a phospholipid bilayer, the periplasm, and a highly asymmetric outer membrane (OM). The OM protects the cell from harmful compounds such as antibiotics and detergents and acts as a semipermeable layer. The inner leaflet of this OM contains phospholipids. Lipopolysaccharide (LPS) is found in the outer leaflet of the OM and is anchored to the OM by lipid A (Raetz, Whitfield, 2002). LPS is also known as endotoxin because it causes an inflammatory immune response in mammals, which results from activation of the TLR4 (toll-like receptor 4) signal transduction pathway. In addition to lipid A, LPS also possesses an oligosaccharide core region and, in some species, O-antigen (Raetz, Whitfield, 2002).

All components of the OM are synthesized in the cytoplasm of the cell and must cross the inner membrane and the periplasm before reaching their final location in the cell. LPS must additionally be transported across the OM. Newly synthesized lipid A is transported across the IM by MsbA, which is an essential IM protein belonging to the ABC transporter superfamily (Doerrler, et. al., 2001). The O-antigen is transported across the IM by several different "flipping" mechanisms and is ligated to the lipid A on the periplasmic side of the IM (Raetz, Whitfield, 2002). The periplasm lacks an obvious energy source, such as ATP. For this reason, it is hard to imagine exactly how this assembly process takes place (Wu, et. al., 2005). Little is known about the transport of the fully assembled LPS across the OM; however, recent studies have implicated an OM protein called Imp (*i*ncreased *m*embrane *p*ermeability). There is supporting evidence that Imp plays a role in membrane biogenesis of Gram-negative bacteria. *Imp* is co-transcribed with *surA* which encodes a periplasmic protein with chaperone activity, and it is regulated by σ^E that controls genes that combat periplasmic stress. In addition to this, *imp* is an essential gene and associated with a large OM protein complex. Imp depleted cells quickly lose viability and have OMs of altered composition. Imp is also highly conserved among Gram-negative bacteria (Braun, Silhavy, 2002). All of these factors are expected of a protein involved in membrane biogenesis. More recently, it was shown that loss of *imp* from *Neisseria meningitidis*, an organism that does not require LPS for viability and where *imp* is not essential, resulted in loss of surface expression of LPS (Bos, et. al. 2004).

Many enzymes have been identified in LPS biogenesis and the pathway across the IM by MsbA is well understood (Bos, et. al. 2004). Discovering the mechanism of LPS transport across the OM is important for many reasons.

Because the OM is the first line of defense between Gram-negative bacteria and the external environment, knowing how the OM components arrive at their location could lead to new antibiotics. Imp is a promising target for new antibiotics for several reasons including its high conservation, cell surface localization, and its essential role in most Gram-negative bacteria.

MATERIALS AND METHODS

Replacing chromosomal copy of *imp* with Kan^R cassette

Amplification of the Kan^R cassette was achieved using primers with 50 nucleotides that were homologous to the *imp* gene on either side, including a ribosomal binding site. PCR conditions included one cycle at 94°C for 2min followed by twenty-five sets of 45sec at 94°C, 45sec at 50°C and 1min at 72°C, and finally ending with 10min at 72°C. A vent polymerase was added after the first step. An agarose gel confirmed that the correct Kan^R cassette was created.

An intact *imp* gene was also amplified by PCR and then digested with the restriction enzymes *Bam*HI and *Nde*I. A pET23a vector was also digested with the same enzymes and the imp insert was ligated into the pET23a vector. Both were incubated at 37°C for 2 hours. The enzyme Antarctic phosphatase was also added during this incubation time.

This plasmid was then again digested with the *Bam*HI and *Nde*I enzymes and an agarose gel was ran to confirm both the pET23a vector and the correct intact *imp* were present. The plasmid was electroporated into *E. coli* cells and plated. After 24 hrs at 37°C incubation six colonies were selected from the plates and purified using a Miniprep procedure. This DNA was then digested with the *Bam*HI and *Nde*I enzymes and an agarose gel was ran to confirm both the pET23a `vector and the correct insert were present.

After this, the *imp* insert was cut out of the pET23a vector using the restriction enzymes *Xba*I and *Bam*HI. This insert was then ligated into two different vectors, pMAK705 and pACYC184, which were digested with the same enzymes. Digestion was done sequentially, first with *Xba*I followed by heat inactivation and then with *Bam*HI. Once this plasmid was created, it was electroporated into *E. coli* cells. The cells were allowed to outgrow at 37°C with shaking for 2 hours before being plated.

A digestion of pACYC, pACYC-imp, pMAK, and pMAK-imp with *Xba*I and *Bam*HI was conducted and then the DNA was ran on an agarose gel to confirm that the vectors did indeed contain the correct insert.

DY330 *E. coli* cells were transformed with pACYC-*imp*, allowed to outgrow for 2 hours at 37°C and then plated to ampicillan plates. After 24 hours of incubation at 37°C , an overnight culture of the transformed DY330 cells was prepared. One ml of the overnight culture was inoculated into 50 ml of LB and grown at 32C until the O.D.600 = 0.5. Induction was achieved by taking 10 ml of the culture in a 125 ml flask and shaking in a waterbath at 42°C. The flask was placed into an ice slurry and shaken by hand to cool. Next, the culture was spun at 3500 rpm for 10 min at 4°C. The pellet was washed four times with ice cold water and resuspended in 200ml of ice cold water. 1.5ml of the Kan^R cassette DNA was pippetted into a cold electroporation cuvette and 100 µl of cold competent cells were added and pulsed immediately. The cells were allowed to outgrow for 2 hours before being plated on kanamycin plates.

Overexpression of Imp

Biochemical experiments were also conducted. C41 cells were transformed with pET23a (empty vector) and pET23a-*imp* plasmid. The cells were grown until O.D.600 = 0.50. At this time, they were induced with 1 ml of IPTG and allowed to grow for 3 hours at 37°C. After this time, the cells were centrifuged for 10 min at 4°C. The pellet was resuspended in 6ml of 50mM Hepes (pH 7.5) and then the cells were lysed using a French Press. After lysing, the cells were spun at 4000 rpm, 4°C for 10 min. The supernatent was transferred to a tube and then ultracentrifuged for 1 hr. This pellet was rinsed using a solution at 50mM Hepes (pH 7), 10% glycerol, and .5M NaCl and ultracentifuged again for one hour. Once this pellet was washed and a protein assay was performed following the BCA Protein Assay Kit protocol. SDS-PAGE was then done, after which the gel was washed 3 times with water and the proteins stained with Coomassie Blue.

Separation of the inner and outer membranes

A sucrose gradient was then set up. C41/pET23a and C41/pET23a-*imp* were added,

each to one sucrose gradient tube. Ultracentrifugation took place using a swinging bucket rotor overnight. A hole was poked into the bottom of each tube and 1 ml fractions were collected. A BCA Protein Assay was performed on each fraction and then an SDS-PAGE gel was run using the fractions.

Solubilization of Imp

Finally solubilization of Imp was attempted using two different detergents, LDAO and TritonX-100. 50 ml of EDTA was added to 15 ml of the membrane preparations. To 7 ml of this membrane fractions 0.8ml of 20% LDAO was added and to another 7ml of the membrane fractions 1.7ml of 10% TX-100 was added. These were allowed to sit on ice for 1 hour. An SDS-PAGE was then run using the membrane fractions.

RESULTS

An agarose gel of the pET23a-*imp* with *Bam*HI and *Nde*I confirmed that the insert was successfully litgated into the vector (Figure 1). An agarose gel of the pACYC, pACYC-*imp*, pMAK, and pMAK-*imp* shows that the pACYC vector contained the correct insert; however, the pMAK vector and pMAK vector-*imp* lanes do not show any DNA present (Figure 2). An agarose gel of the PCR products, testing the potential knockout mutants shows that the procedure was unsuccessful. Knockout lanes were expected to have a band around 800 base pairs and the original to have bands at 2300 bp (Figure3). The knockout of the chromosomal copy of imp was unsuccessful.

Overexpression of Imp was successful (Figure 4). The graph of the sucrose gradient (Figure 5-A) as well as the SDS-PAGE of the sucrose gradients fractions (Figure 5-B) both indicate that Imp co-fractioned with the outer membranes.

The SDS-PAGE of the solubilization attempts shows that Imp was not solubilized – it remained in the pellet (Figure 6).

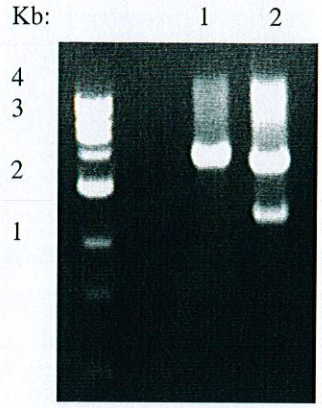


Figure 1: Cloning of Imp:
E. Coli Imp (2.3 kb) was amplified from genomic DNA by PCR and cloned into expression vector pET23 (*NdeI*/*Bam*HI).

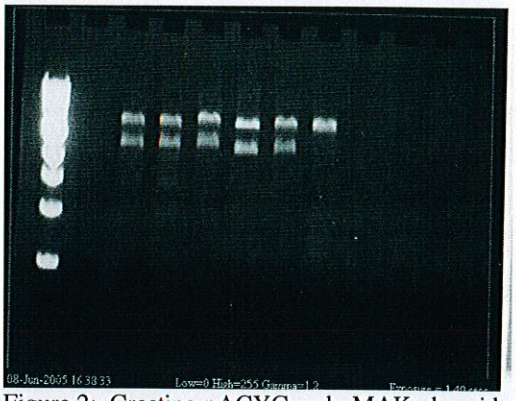


Figure 2: Creating pACYC and pMAK plasmids

Figure 3: Unsuccessful knockout of chromosomal copy of *imp*.

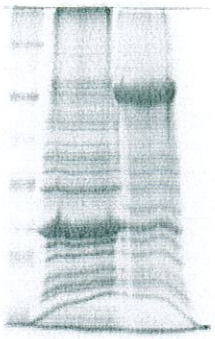
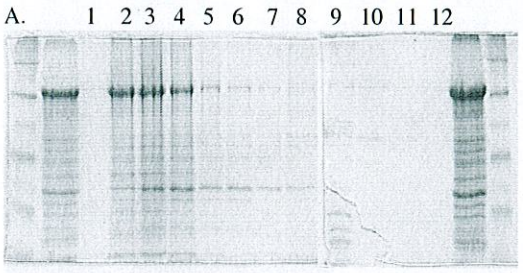


Figure 4: Overexpression of Imp:C41(DE3) cells were transformed with pET23a, pET23-Imp plasmid DNA and protein expression was induced with 0.5 mM IPTG. Membranes were isolated and proteins were separated on a 10% SDS-PAGE gel. Lanes 1 and 2 contain membranes from cells transformed with the empty pET23a vector and pET23-Imp respectively. The heavy band at ~80 kDa indicates overexpression of Imp.

A.



B.

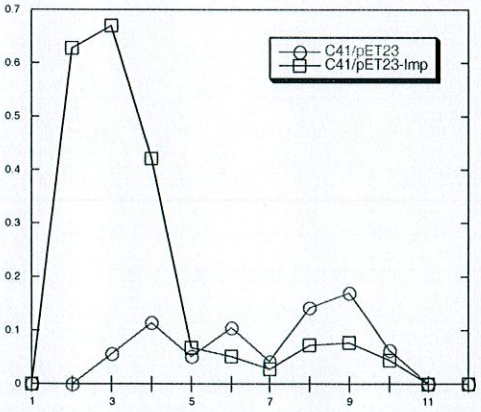


Figure 5: Overexpressed Imp is correctly localized at the outer membrane:
The inner and outer membranes were separated by sucrose-gradient centrifugation to determine where Imp was located. Twelve 1.0 mL fractions were collected after centrifugation from both C41-pET23 and C41/pET23-Imp membranes, and the protein concentration was determined for each fraction. A. The graph indicates the highest concentration of proteins in C41/pET23-Imp were found in the early fractions. B. The SDS-PAGE gel also shows overexpression of Imp in lanes 2-4 indicating Imp co-fractioned with the outer membranes.

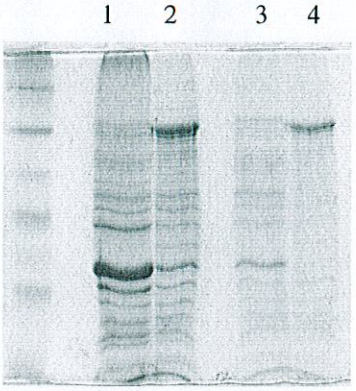


Figure 6: Solubilization of Imp:
Attempts were made to solubilize Imp using the detergents LDAO and Triton X-100; however, these were unsuccessful. The gel shows the empty C41/pET23 vector membranes (lane 1), C41/pET23-Imp membranes (lane 2), and both the supernatent (lane 3) and pellet (lane 4) of C41/pET23-Imp membranes following solubilization. Despite the fact that most membrane proteins were solubilized under these conditions including the outer membrane porins, Imp remained in the pellet, indicating that it was not solubilized.

DISCUSSION

The Kan^R cassette was used to replace the chromosomal copy of *imp* so the mutated strain could easily be selected for. The cells were also transformed with a plasmid containing an intact *imp* gene. This was done to provide the cell with Imp, an essential protein. DY330 is a strain of *E. coli* that has been engineered to contain deletions in genes involved in the degradation of linear DNA. With these genes missing, it becomes more likely that linear DNA can undergo recombination and insertion into the chromosome. Since the Kan^R cassette was linear, a strain with these mutations was needed.

The results indicate that the pACYC vector contained the correct *imp* insert and pMAK did not. pMAK is a temperature sensitive vector with a high copy number and

promoters; however, Imp is a low copy number protein, so the cell does not need many copies of *imp* to survive. pACYC is a low copy number vector and it is almost promoter free. The problem with pACYC is it is not temperature sensitive. A temperature sensitive plasmid was required so the plasmid could be "turned off," essentially shutting down Imp production. The agarose gel with the two vectors and the insert (Figure 3) shows no DNA present in the pMAK lanes. The pMAK and *imp* might have recombined. Time was an issue, so pACYC was selected as the vector of choice; however, pACYC is not temperature sensitive, so it could not be used in any temperature controlled experiments. pACYC was used to see if the DY330 cells would take up the Kan^R cassette. Had this step been successful, further attempts at creating a temperature sensitive plasmid that had a low copy number and the normal Imp promoter would have been made.

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