Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2015, 7(10):90-99

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Phenotypic and transcriptional analysis appear that bacterial cell wall assembly stop by conserved inner membrane protein (ElyC) gene disruption stimulates the enterobacterial common antigen (ECA) gene cluster transcription in *Escherichia coli*

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ABSTRACT

This study investigated the involvement of Conserved inner membrane protein (YcbC) in the regulation of Enterobacterial Common Antigen (ECA) synthesis operon and penicillin-binding protein (PBP1b) transcription and thus in thecell wall assembly of Escherichia coli. WT cells and ΔelyC, ΔmrcB and ΔrfE mutants were grown in LB medium. Cells were collected and RNA extraction and purification was achieved. Thereafter, transcriptional analysis by RT-PCR was performed on genes ycbC encoded for Polypeptide conserved inner membrane protein, wecA,wzzE,wecB, rffH, rffT, wzyE and wecG genes encoded for Enterobacterial Common Antigen (ECA) biosynthesis, colanic acid biosynthesis glycosyltransferase (wcaA) and undecaprenyl diphosphate synthase (uppS) genes. Overexpression of ycbC and rffH, rffT, wzzE, wzyE and uppS genes was observed in WT cells grown at 22°C. Furthermore, ΔelyC mutant grown at 37°C and 22°C revealed the overexpression of ECA biosynthesis genes (wecA, wzzE, wecB, rffH, rffT, wzyE, wecG), mrcB, wcaA genes and the high expression of uppS gene compared to WT. Overexpression of ECA cluster, uppS and wcaA genes was also observed in ΔmrcB. Furthermore, the absence of rfE gene displays the overexpression of uppS and mrcB genes. Our results show the coordination between ElyC (YcbC) factor and ECA polysaccharide, peptidoglycan (PG) and colonic acid synthesis. These results confirm the important role of YcbC protein in Gram-negative bacterial cell wall assembly. So, the characterisation of new envelope biogenesis factors important for Gram-negative bacteria will broaden our understanding of the bacterial cell envelope biogenesis and validate the new factors as antibacterial targets.

Keywords: cell wall, inner membrane, Envelope biogenesis, ElyC (YcbC) factor, ECA synthesis, PG synthesis, CPRG rapid test.

INTRODUCTION

Bacteria are covered by a complex envelope that delimits the cell and protects it against variations in osmotic pressure and environmental stresses. The envelope of Gram-negative bacterium is especially complex and contains two membranes with a thin layer of peptidoglycan (PG) exoskeleton sandwiched in between them [1]. These structures play a key role in maintaining cellular integrity and offer protection from external abuses [2]. PBPs are found in all free-living bacteria. Their ubiquity suggests an important role in cell physiology. They are known to synthesize and remodel cell wall peptidoglycan, which gives the cell its rigidity and shape, prevents osmotic lysis and resists toxins. PBPs are also the target of many antibiotics. PBP1B is the only PBP required for survival in competitive growth assays.

The dimer of PBP1B form could be divided into two classes. One class, which cofractionated with the cell wall fraction, could be artificially cross-linked to peptidoglycan. This class of PBP1B dimers was sensitive to beta-mercaptoethanol. The second class, like the monomeric form of PBP1B, could be isolated with the inner membrane

fraction(Zijderveld, 1995 #70). PBP1B has been found to be localized primarily to the inner membrane, but also to adhesion sites where the inner and outer membranes contact. Two important PG synthases were produced by *E. coli*. The bifunctional (Class A) PBPs, PBP1a and PBP1b, encoded by the *mrcA* (*ponA*) and *mrcB* (*ponB*) genes, respectively [3]. These factors have both peptidoglycan glycosyltransferase (PGT) activity to synthesize the glycan strands of PG and transpeptidase (TP) activity to crosslink the glycan chains via their attached peptide moieties [4].

Enteric bacteria synthesize and display a complex array of various cell surface polysaccharides: entebacterial communantigen (ECA), capsular polysaccharides (K-antigen), poly-1,6-N-acetyl-D-glucosamine(PNAG) (4), the -1,4-glucan polymer bacterial cellulose, and colanic acid (CA orM-antigen). The extracellular polysaccharide colanic acid (CA) is an exopolysaccharide common to many enterobacteria (*Enterobacter*and *Klebsiella* including *Raoultella* genera, it has been also isolated from *Escherichia coli, Salmonella typhimurium* and *Aerobacter cloacae* strains.

The CA polysaccharide repeat is assembled on the membrane lipid undecaprenolpyrophosphate (Und-PP) by a series of glycosyltransferases on the cytoplasmic face of the inner membrane, after which the single repeat is flipped to the periplasmic side and polymerized by the Wzy-dependent pathway Exopolysaccharide (EPS) production is required for development of *Escherichia coli* K-12 biofilm architecture. In each of these cases EPS has been shown to be required for cellular attachment to abiotic surfaces. Here, we undertook a genetic approach to examine the potential role of colanic acid, an EPS of Escherichia coli K-12, in biofilm formation. CA is a heteropolymer containing glucose, galactose, fucose, and glucuronic acid as monomers that appears to be involved in the protection of this bacterium against environment attacks.

Enterobacterial common antigen (ECA) is a family-specific surface antigen shared by all members of the Enterobacteriaceae and is restricted to this family. ECA is located in the outer leaflet of the outer membrane.

The majority of our best drugs such as penicillin and vancomycin block the biosynthesis of the bacterial envelope and cause cell lysis. Indeed, bacterial envelope biogenesis is one of the best sources of bacterial targets for antibacterial development because it involves factors that are unique to bacteria and are important for bacterial physiology [5]. We know very little about bacterial envelope assembly and a lot of factors and pathways need to be discovered because the control and coordination of these different processes remains unclear. Given that genes coding for envelope proteins constitute roughly one quarter of the *Escherichia coli* genome, and that over a third of these have an unknown or poorly understood function [6].

Actually, bacterial envelope biogenesis is one of the best sources of bacterial targets for antibacterial development. We know very little about bacterial envelope assembly and a lot of factors and pathways need to be discovered. Quantitative assay for mutants with envelope biogenesis defects was developed and used to screen an ordered single-gene deletion library of *Escherichia coli*. The screen was tough and correctly identified numerous mutants known to be involved in envelope assembly. Significantly, the screen also implicated 102 genes of unknown function as encoding factors in envelope biogenesis. One of these factors, ElyC (YcbC) was characterized further and shown to play a critical role in the metabolism of the essential lipid carrier used for the biogenesis of cell wall and other bacterial surface polysaccharides [7]. The discovery of the function of novel envelope assembly factors will open new avenues for the development of antibacterial agents against which resistance has not yet evolved.

To understand the link between ElyC factor and ECA, PG and CA synthesis and thus how ElyC factor affects the cell wall assembly, we investigated the importance role of *ycbC* (*elyC*) gene of unknown function in ECA polysaccharide, peptidoglycan (PG) and colonic acid synthesis and thus in cell wall biogenesis. We have measured the expression of ECA cluster genes (encoded by *wecA, wecB, wecG, rffH, rffT, wzzE* and *wzyE*) and genes encoded to enzymes implicated in peptidoglycan and colonic acid synthesis(encoded by *mrcB, uppS* and *wcaA*, respectively). To eliminate the background on LBagar, we perform aCPRG rapid test on LB liquid medium.

EXPERIMENTAL SECTION

Bacterial strains and growth conditions

One colonie of *Escherichia coli* WT and Δ*elyC*,Δ*mrcB* and Δ*rfE* mutants were picked from LB agar plates, inoculated into LB medium (10 g/L Peptone, 5 g/L Yeast extract, and 5 g/L NaCl, pH = 7), and incubated overnight at 37°C with shaking (250rpm). Five hundred micro-liter aliquots of overnight grown cell culture were inoculated into 100 mL LB medium to obtain OD600 ~ 0.05. Cells were grown at 37°C and 22°C with shaking (250rpm) till OD600 reached the OD~ 0.5. The cells were collected, centrifuged for 10 min at 10.000 rpm, and suspended in 1 ml of Tri-reagent and conserved at -80°C before RNA extraction and purification.

A rapid CPRG test in LB liquid and Efficiency test with ampicillin treatment

CPRG screen previously described by [7]was modified for LB liquid culture. *E. coli* WT and Δ*elyC*, Δ*mrcB* and Δ*rfE* mutants stored at −80°C was picked in LB agar plates, and grown overnight at 37°C.The resulting colonies was inoculated in LB (1%) NaCl and IPTG (100 µM) to induce the *lac* operon and incubated overnight at 37°C and 22°C. Inoculate200 µl of each cell culture in 96-well plate format and added the CPRG (40 µg/ml) and incubated at room temperature. For tested the treatment with ampicillin, we have inoculated 200µl of *E. coli* WT and Δ*elyC*cell culture at 37°C and in 96-well plate format. Ampicillin treatmentat different final concentrations(0, 4, 8, 16and32µg/ml) was performed. Treatments of cell cultures were incubated for 30min at room temperature to react. Next, the CPRG (40 µg/ml) was added and incubated at room temperature. The observed CPR color development on the plates was found to be directly proportional to incubation time. Finally, plates were monitored through time and were imaged both at the culture cells growth (~ T0, 5 min, 30 min, 1h and 2 hours).

Gene expression analysis

Primer design

The primers used are given in **Table 1**, it were disgned by NCBI/ **Primer-BLAST**. To evaluate the reproducibility of the method, three independent RNA samples were analyzed in parallel for three independent cultures performed at 37°C and 22°C. Samples were quantified using Rotor gene (Rotor-Gene 6000, Corbett RESEARCH) and standardized for two references gene *rrsA* encoding ribosomal RNA 16S (*rrsA*) and the geometric average of three genes (*cysG/idnT/hcaT*) [8]. The gene symbol and sequence for each candidate and reference gene was used to design two sets of primers for each target gene. The set of primers generated amplicons of ~200 bp which were used for tested the efficiency of each gene studied. The mRNA level changes of each gene were normalized to the mRNA level of the unregulated gene encoding 16S RNA and the average of *cysG/idnT/hcaT*, and quantified using the mathematical model established by Pfaffl [9].After that, we have considered the geometric average of three genes (*cysG/idnT/hcaT*) as a novel reference gene because it was highly invariant compared to *rrsA*.

RNA purification and cDNA synthesis

Total RNA from *E. coli* was prepared using Tri-reagent (Invitrogen) according to the manufacturer's instructions and purified by RNeasy Plus Mini Kit (Qiagen). Total RNA was collected from samples in triplicate at each growth condition for the WT and Δ*elyC*and Δ*mrcB* mutants. RNA concentration was quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific), and the 260/280 and 260/230 ratios were examined for protein and solvent contamination. The integrities of all RNA samples were confirmed by 2100 expert_Prokaryote Total RNA Pico (Analysis on the Bioanalyzer, Institut de Recherche en Immunologie et Cancérologie (IRIC), Université de Montréal) (Table 2). Two microliter of total RNA were reverse transcribed in a total volume of 20µL containing (5X VILO Reaction Mix, 10X SuperScript Enzyme Mix (Invitrogen), for 90 min at 42°C according to the manufacturer's instructions. The reaction was terminated by heating at 85°C for 5 min.

Quantitative real-time PCR

The cDNA levels were then analyzed using a Rotor gene Real-Time System (Rotor-Gene 6000, Corbett RESEARCH) with SYBR Green I detection. Each sample was measured in duplicate in a 0.1 ml in a reaction mixture (25µL final volumes) containing 1× Rotor-Gene SYBR Green PCR Master Mix (Qiagen), 1 µM primer mix, and 2 µL of cDNA. Real-time PCR was performed with an initial denaturation of 3 min at 95°C, followed by 40 cycles of 20 s at 95°C, 20 s at 60°C, and 20s at 72°C. Fluorescent detection was performed as previously described by [8].

RESULTS AND DISCUSSION

Rapid CPRG test and efficiency test of ampicillin treatment

The absence of membrane permeabilization, mutants impaired in envelope biogenesis could be identified by their enhanced LacZ activity over normal cells and the synthetic substrate CPRG was chosen over ONPG for screen development because of its increased sensitivity (Eustice, 1991 #80). The red color of CPRG cleavage product, CPR (chlorophenyl red) was detected on LB agar [7]. SO, the membrane of Gram-negative envelope is primarily responsible for preventing CPRG from entering cells. The outer membrane is a good candidate given the hydrophobic nature of the synthetic substrate and the well-known effectiveness of this layer at blocking uptake of other hydrophobic molecules. Thus, mutants capable of processing CPRG may either be defective in the permeability barrier of the outer membrane or possess a defect that results in an elevated frequency of cell lysis promoting the release of LacZ into the medium. In order to avoid the background in LBagar for long time of incubation, we have performed the rapid CPRG test on LB liquid medium. Our results were observed after 30 min of incubation at room temperature (Figure. 01A). Our rapid test was focused on the induction of LacZ activity during the over night culture cells grown at 37°C and 22°C and next the measure of CPRG product red color by a simple adds of CPRG.

Table 1 PCR primers used in this study

Primers	Function	Sequence (5'→3')[a]
rrsA	ribosomal RNA 16S	F:AGGCCTTCGGGTTGTAAAGT R: CGGGGATTTCACATCTGACT
cysG	uroporphyrin III C-methyltransferase	F: AGGCATGTTAACCCTCGTCG R: GCATAATAAAGCTGGCGGC
hcaT	HcaT MFS transporter	F: TGTTTATTGCAGGGGGCACA R:AGCATATCGCGTGCACTACA
idnT	L-idonate/5-ketogluconate/gluconate transporter	F:GCTTTATTGCCCTCGTTCTG R:CAATCAGCGTAGTGGCGATA
ycbC	Polypeptide: conservedinner membrane protein	F:GGCTGGCGCTCTTGTTATTG R: AGGCGTGGCAGACTGTTATT
wecA(rfE)	undecaprenyl-phosphate α-N-acetylglucosaminyltransferase	F: CCTCGCAATCTGGTGCTTTG R: GCTGATGGGATGGGTTTTGC
wecB (rffE)	UDP-*N*-acetylglucosamine 2-epimerase	F: CAAAACTTGCTGCGTGAAAA R: AAACTCTCACGCCTGTGACC
wecG(rffW)	UDP-N-acetyl-D-mannosaminuronic acid transferase	F:TTTAAACCCGAGCAGCGTCA R:CGTGACCGGTGAAAACATCG
rffH	dTDP-glucose pyrophosphorylase	F:GCT GGA ATA TGC CGA ACA R:GAT AGC CAA AAA CCG TCG
rffT	4-acetamido-4,6-dideoxy-D-galactose transferase	F:CGGTCATCGCGAAAGCAAAA R:CAGGCGACGTAGTGGGTAAA
wzzE	Enterobacterial Common Antigen (ECA) polysaccharide chain length modulation protein	F:TTTGCTCGTCAGGAGTGGAG R:ACGAGGCCAGTTGCATAACA
wzyE	predicted Wzy protein involved in ECA polysaccharide chain elongation	F: CATGTTCTGGCTGGCACTAA R: CACAGCCAGGAAGGGATAAA
mrcB(*ponB*)	penicillin-binding protein 1B	F: TCCAGCGAGCGTTCTTACTG R:CTTAGCTCTTCTACCGGGCG
ispU(*uppS*)	undecaprenyl diphosphate synthase	F: CAGCAAGGAAACCTGCAACC R:CATCGAAATCGGGCCAGAGA
wcaA	predicted colanic acid biosynthesis glycosyltransferase	F:GGCGAACCGTGGAAAGTAGA R: TGCGCCAGGTCATACGTTTA

a. *F. forward, R. reverse.*

Table 2 RNA Integrity Number RIN by Bioanalyser of RNAt purified by RNeasyPlus Mini Kit

Strains / **RIN**	Growth at 37°C	Growth at 22°C
WT1	6.2	8
WT2	8.5	8.1
WT3	7.5	8.9
Δ*elyC* 1	9.7	10
Δ*elyC* 2	8.9	10
Δ*elyC* 3	7.2	10
Δ*mrcB* 1	8.1	8.9
Δ*mrcB* 2	9	7.6
Δ*mrcB* 3	7.5	8.4
Δ*frE* 1	7.5	7.6
Δ*frE* 2	6.9	8.4
Δ*frE* 3	8.5	10

RNA Integrity Number (RIN) ~ 6 or more was considered as a good RNAt.

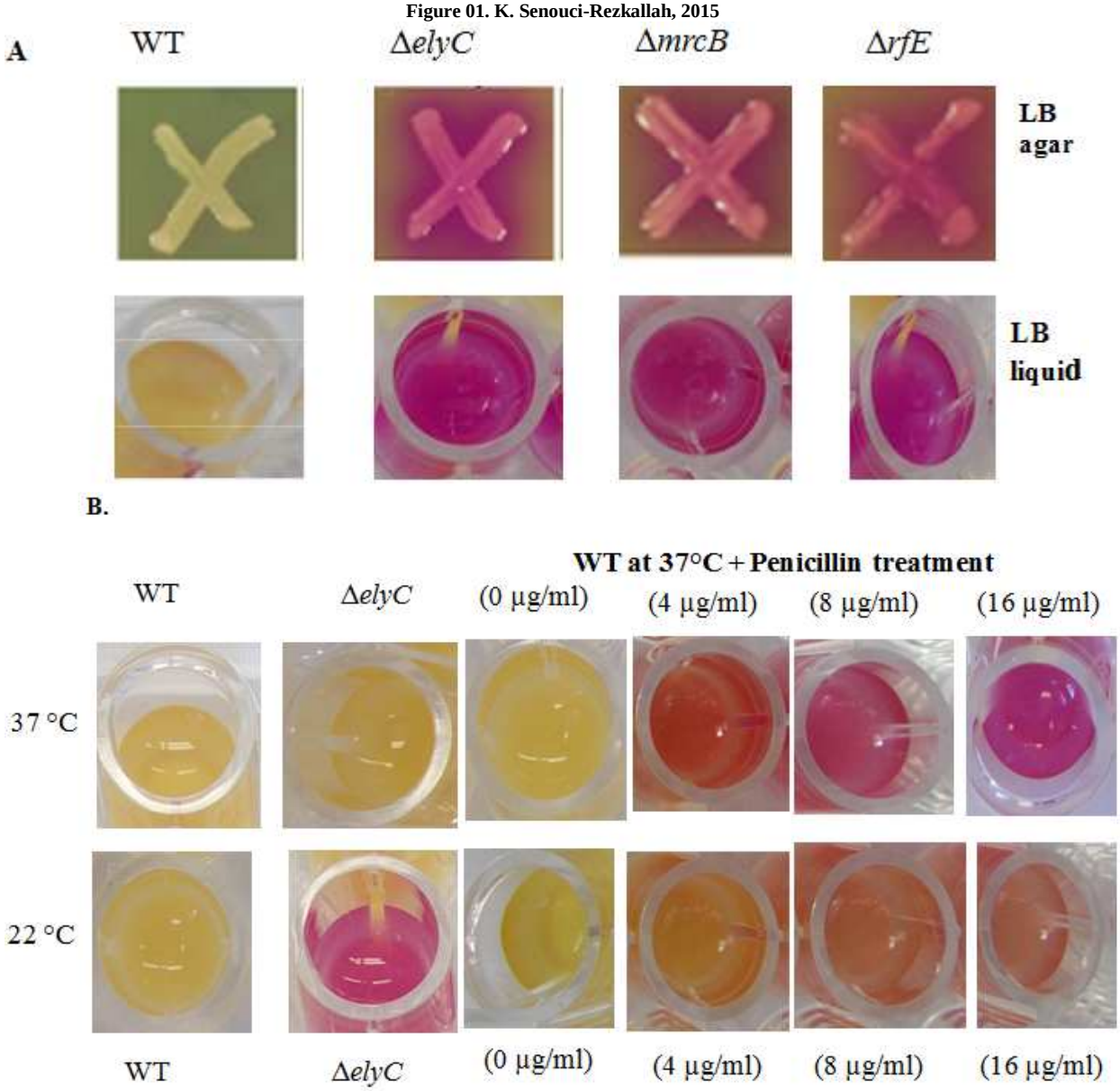
Our results show no color change in WT cells grown at 37°C and 22°C. In contrast, Δ*elyC* mutant cells grown at 22°C compared to cells grown at 37°C.

The second part of our experience is to study the efficiency of ampicillin treatment in *E.coli* wild-type and Δ*elyC* mutant. First, we have observed that WT at 37°C test was colored in the presence of penicilin between 4 an 16

$\mu g/ml$. The color observed in the presence of 16 $\mu g/ml$of penicillin show the same color of Δ*elyC* mutant grown at 22°C. The attaque of the envelope by penicillin as an antibiotic show that Δ*elyC* mutant cells have the envelope defected by the absence of ElyC protein. So the presence of ElyC protein was essential to envelope assembly function.

Figure 1. CPRG rapid test of: **A.**WT and Δ*elyC*, Δ*mrcB* and Δ*rfE*cells grown at 22°C in LB liquid (100 µM IPTG)and CPRG indicator liquid (40 µg/ml CPRG).The time CPR color development on the plates was observed after 30 min of incubation at room temperature. Cells patched onto CPRG indicator agar (20 µg/ml CPRG and 50 µM IPTG, upper panels), incubated at room temperature was compared with culture cells grown at 22°C. **B.**Efficiency test of WT and Δ*elyC*mutant grown at 37°Cin LB liquid (100 µM IPTG) treated with penicillin(at 0, 4, 8, 16 and32µg/ml) and added the CPRG indicator liquid (40 µg/ml CPRG).

Figure 01. K. Senouci-Rezkallah, 2015



Transcriptional analysis of Δ*elyC*, Δ*mrcB* and Δ*frE* mutants

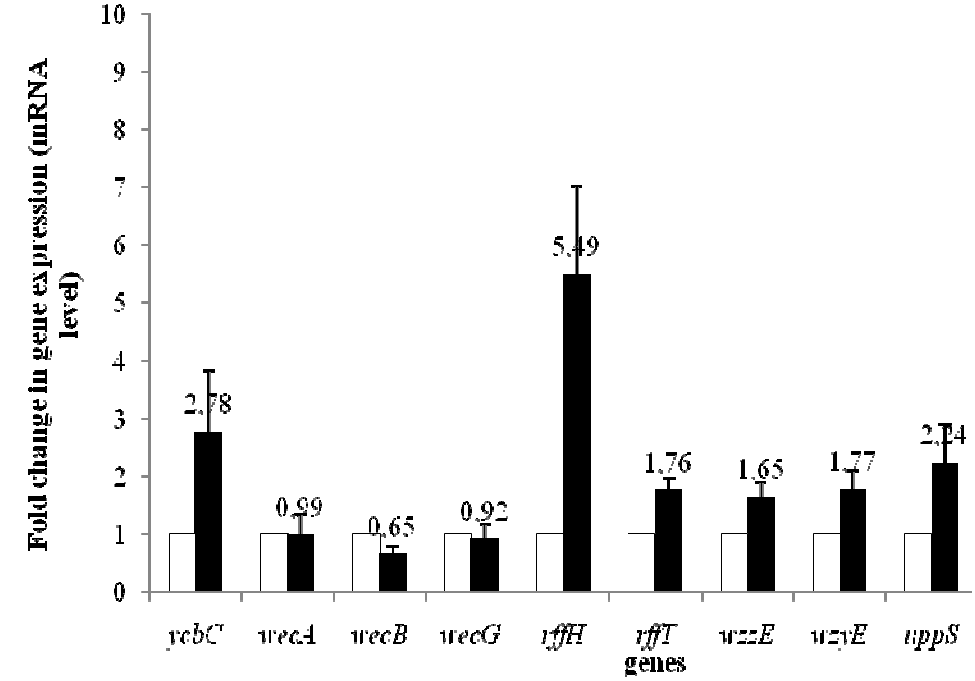
Null mutations within the *ycbC* gene stimulate the ECA gene cluster, PBP1b and *wecA* genes transcription

Real-time RT-PCR assays were conducted to determine the effect of growth temperature on genes expression. ECA gene cluster, transcripts showed 2-to 3-fold increase in WT cells grown at 22°C compared to cells grown at 37°C (Figure. 2). This result approves the little formation of the hydroxyl radical and the induction of oxidative stress defense systems at low temperature. Further, sodA, sodB, sodC and kactG genes was upregulated in Δ*elyC* and Δ*mrcB* cells (up to 3-fold) at growth temperature of 37°C compared to WT cells grown at 37°C (Figure. 2 A). These result shows that the loss of ElyC and PBP1b function induce SODs and catalase genes transcription, ECAcluster genes, PG and Colanic acid mRNA level increase at low temperature in Δ*elyC* mutant.

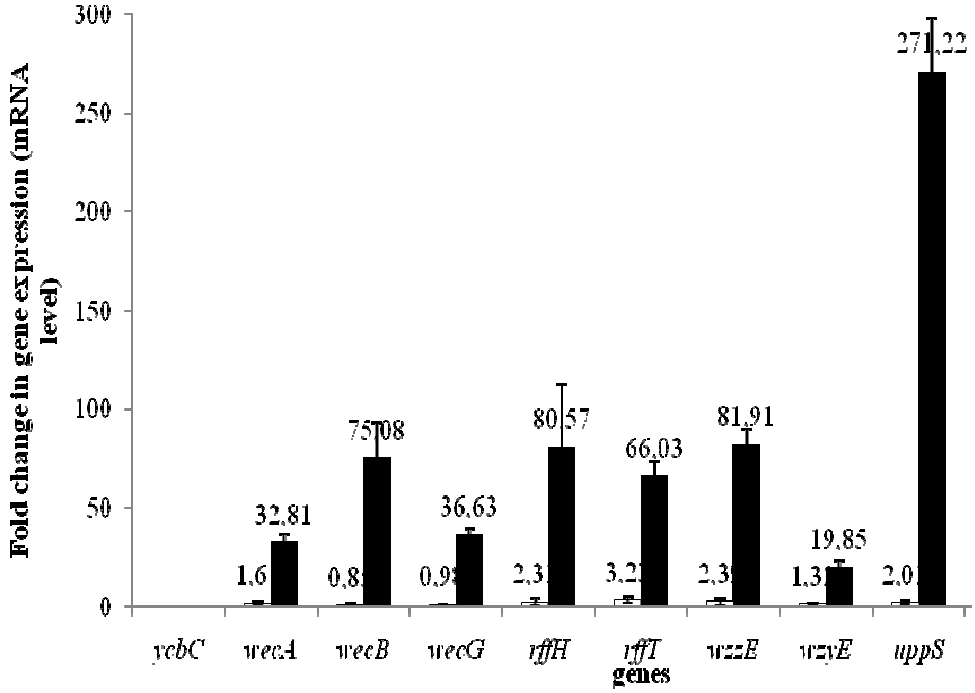
Figure 02. K. Senouci-Rezkallah, 2015

Figure 2. RT-PCR assays conducted on mRNA isolated in *E. coli***A.**WT cells grown at 37°C(⊡) and 22 °C(■). **B.**Δ*elyC* mutantgrown at 37°C(⊡) and 22 °C(■). **C.**Δ*mrcB* mutant grownat 37°C(⊡) and 22 °C (■). **D.** Δ*frE* mutant grown at 37°C (⊡) and 22 °C (■). The expression of *wecA(rfE), wecB (rffE), wecG(rffW), rffH, rffT, wzzE, wzyE, mrcB*(*ponB*, *ispU*(*uppS*), *wca*Agenes was measured. Fold changes in target gene expressions using reference gene normalized by the geometric mean of *idnT*, *cysG*, and *hcaT*. Relative gene expression of *E. coli* WT cells grown at 37°C was set at 1.0 (reference condition).

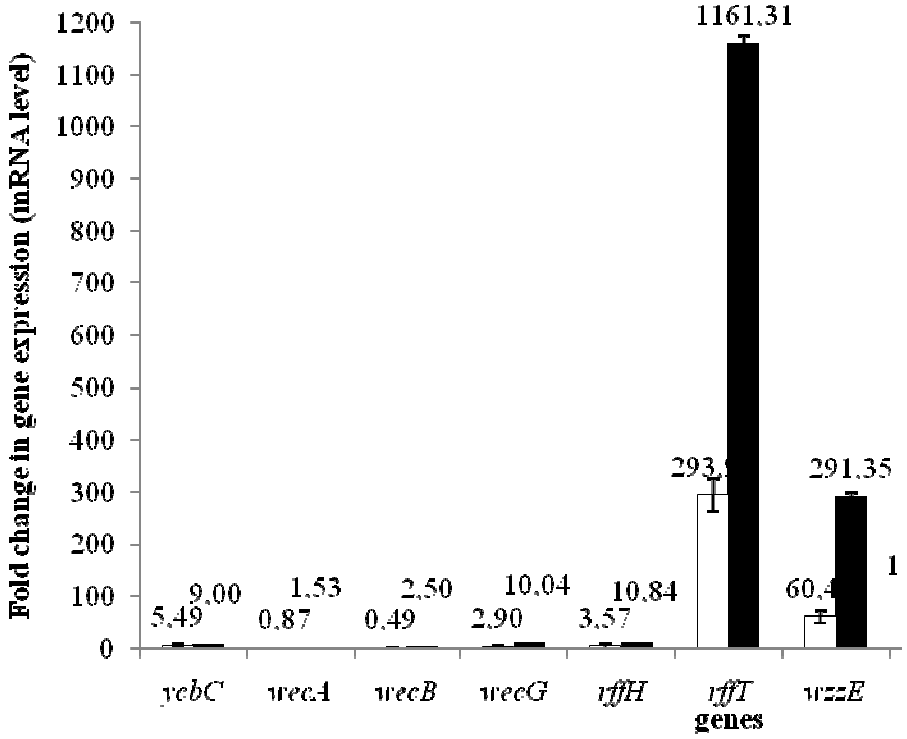
A



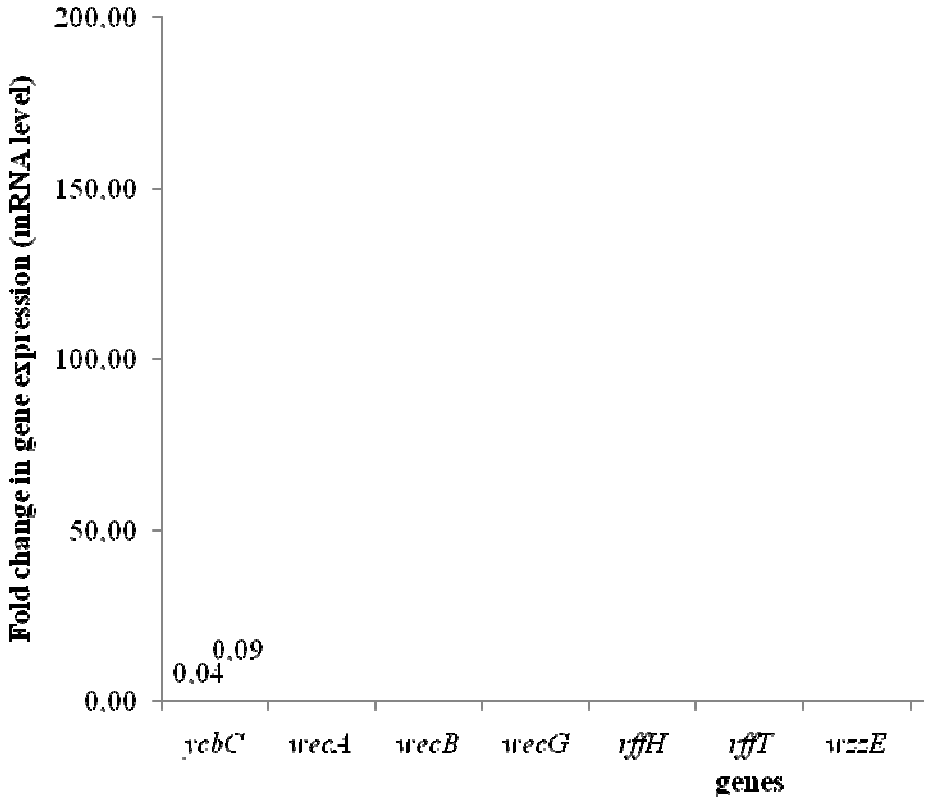
B



C


Fold change in gene expression (mRNA level)
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
1161,31
291,35
9,00
1,53
2,50
10,04
10,84
5,49
0,87
0,49
2,90
3,57
60,4
1
yebC
wecA
wecB
wecG
rffH
rffT
wzzE
genes

D


Fold change in gene expression (mRNA level)
200,00
150,00
100,00
50,00
0,00
0,09
0,04
yebC
wecA
wecB
wecG
rffH
rffT
wzzE
genes

In order to demonstrate the role of superoxide dismutase and catalases enzymes in oxidative stress response in Δ*elyC* and Δ*mrcB* mutants, *sodA*, *sodB*, *sodC* and *kactG* genes expression was also quantified at low temperature. Our results were shown the overexpression of *sodA* (800-fold), *sodB* (8-fold), *sodC* (115-fold) and *kactG* (15-fold) in Δ*elyC* mutant (Figure. 2 B). However, these genes show the same mRNA level in Δ*mrcB* mutant compared in WT cells grown at 22°C. Thus, the transcription of *sodA, sodB, sodC* and *kactG* genes expression was highly activated at low temperature in Δ*elyC* mutant compared to WT and Δ*mrcB* mutant.

Loss of ElyC function results in cell lysis and stimulates the ECA cluster, Und-pp and colonic acid genes transcriprtion. Although *E. coli* maintains 12 PBP genes, many of the gene products have overlapping biochemical functions and seem to be nonessential for exponential phase growth. In fact, an *E. coli* mutant lacking eight of 12 PBPs is viable[7].

PBP1b is vital for competitive survival of *E. coli* during extended stationary phase, but the other nine PBPs studied are dispensable. Loss of PBP1b leads to the stationary phase-specific competition defective phenotype and causes cells to become more sensitive to osmotic stress. Additionally, we present evidence that this protein, as well as AmpC, may assist in cellular resistance to beta-lactam antibiotics.

***uppS*gene is over expressed in Δ*elyC and* Δ*mrcB* mutants**

In order to determine the role of envelope biogenesis in oxidative stress response in *E. coli*, *murA*, *mrcB* and *uppS* genes expression was measured. Our results were shown that these genes were overexpressed at low temperature in WT cells, and highly expressed in Δ*elyC* and Δ*mrcB* mutants. These results show the role of PG and/or ECA synthesis at low temperature and correlate this overexpression with oxidative stress tolerance observed at low temperature. In addition, *uppS* gene was too up-regulated at 37°C and 22°C in Δ*mrcB* mutant. So, in the absence of PG synthesis, the lipid carrier Und-P can be produced for the cell wall or more precisely ECA or other polysaccharides biosynthesis. Prior results showed that the cells lacking either of these PBPs are viable, but the simultaneous inactivation of both factors results in rapid lysis and cell death [10]; [11]; [12]; [13].

We therefore observed that the ECA biosynthesis genes was expressed in the WT cells of *E. coli*at low temperature 22°C, and more expressed in Δ*elyC* and Δ*mrcB* mutants associated with the overexpression of *uppS* gene (data not shown). Similar results were recently observed that in *Yersinia enterocolitica*, the increase in the expression of the ECA biosynthetic cluster at low temperature was correlated with the higher production of ECA at 22°C relative to 37°C [14]. Taken together, these findings suggest that Δ*mrcB* mutant can increase the ECA biosynthesis in the absence of PG synthesis. It was recently demonstrated the induction of a number of stress regulators (as SoxS) and genes associated with the response to oxidative stress, membrane transporters and biosynthetic processes [15].Furthermore, our results showed also the overexpression of *ycbC* gene in the WT and Δ*mrcB* mutant at low temperature (data not shown). These results reveal the role of YcbC factor in the envelope biosynthesis correlated with oxidative stress response in *E. coli*. In addition, the overexpression of ECA biosynthetic cluster, *mrcB* and *uppS* genes Δ*elyC* mutant confirms the competition between the PG and ECA synthetic pathways for the lipid carrier Und-P.

Similar results describe the purification and quantification of a water-soluble cyclic form of enterobacterial common antigen (ECA (CYC)) from *Escherichia coli* K-12 as well as information regarding its subcellular location and the genetic loci involved in its assembly. Structural characterization of purified ECA (CYC) molecules obtained from *E. coli* K-12 revealed that they uniformly contained four trisaccharide repeat units, and they were substituted with from zero to four O-acetyl groups. Despite the body of evidence indicating that lipid II accumulation confers a host of envelope-associated defects, it is unclear why lipid II accumulation exerts these effects. While ECA is not essential for the viability of *E. coli*, the undecaprenyl carrier lipid that is used to synthesize the ECA trisaccharide is essential. **Transcriptional induction of *degP*.** Finally, we note that the lipid II-mediated induction of *degP* transcription is mediated by increases in both Cpx and sE activity ccumulation of the Enterobacterial Common Antigen Lipid II Biosynthetic Intermediate Stimulates *degP* Transcription in *Escherichia coli.*

The modality of enterobacterial common antigen polysaccharide chain lengths is regulated by the wec gene cluster of *Escherichia coli*. The Wzz(ECA)-mediated modulation of ECA polysaccharide chains is the first demonstrated example of Wzz regulation involving a polysaccharide that is not linked to the core-lipid A structure of lipopolysaccharide, *wzzE* has been shown to be required for the synthesis of cyclic ECA which contains 4 trisaccharide repeat units and is located in the periplasm.

We therefore observed that the ECA biosynthesis genes was expressed in the WT cells of *E. coli* at low temperature 22°C, and more expressed in Δ*elyC* and Δ*mrcB* mutants associated with the overexpression of *uppS* gene (data not shown). Similar results were recently observed that in *Yersinia enterocolitica*, the increase in the expression of the

ECA biosynthetic cluster at low temperature was correlated with the higher production of ECA at 22°C relative to 37°C [14]. Taken together, these findings suggest that Δ*mrcB* mutant can increase the ECA biosynthesis in the absence of PG synthesis. It was recently demonstrated the induction of a number of stress regulators (as SoxS) and genes associated with the response to oxidative stress, membrane transporters and biosynthetic processes [15]. Furthermore, our results showed also the overexpression of *ycbC* gene in the WT and Δ*mrcB* mutant at low temperature (data not shown). These results reveal the role of YcbC factor in the envelope biosynthesis correlated with oxidative stress response in *E. coli*. In addition, the overexpression of ECA biosynthetic cluster, *mrcB*and *uppS*genes Δ*elyC* mutant confirms the competition between the PG and ECA synthetic pathways for the lipid carrier Und-P.

Overexpression of *uppS*, *murA*, and *mrcB* genes in Δ*elyC* mutant and *uppS* and *murA* genes in Δ*mrcB* mutant support the role of these genes in restoring the phenotype of cell lysis and perhaps in oxidative stress response by cell wall biosynthesis. Overproduction of MurA, UppS, or PBP1b fully suppressed the CPRG+ phenotype of ElyC$^-$ cells and restored their growth at room temperature to normal. Our results confirm that the overproduction of MurA and UppS and PBP1b are both likely to suppress the ElyC defect by enhancing lipid-II^{PG} synthesis by increasing the flux through the PG and ECA synthesis pathways [7]. Interestingly, each of the enzymes with suppression activity functions at a major transition point in PG biogenesis [16]; [4]; [17]. Thus, overproduction of these factors may generally increase the flux through the pathway to alleviate the ElyC$^-$ defect.

The present work was interested by the observation of the role of ElyC factor in the competition between the PG and ECA synthetic pathways and in the oxidative stress response. The *elyC* reading frame encodes a protein with two predicted transmembrane domains and a large domain of unknown function (DUF) designated as a DUF218 domain in the Pfam database [18]. It was recently show that *elyC* (*ycbC*) and *mrcB* (*ponB*) mutants are cold-sensitive (CS) for growth due to cell lysis [7].

The ECA biogenesis pathway is oversensitive to competition for the lipid carrier from PG synthesis and potentially the synthesis of other surface polysaccharides that utilize Und-P. However, the absence of PBPs shows no difference in oxidative stress level in PBP1b$^-$ cells. Nevertheless, the overexpression of ECA biosynthetic cluster in ElyC$^-$ and PBP1b$^-$ cells demonstrates the role of ECA polysaccharide in the cell wall assembly and perhaps in oxidative stress response. Competition between PG and ECA synthesis in Δ*elyC* mutant is likely sensitive when the ECA pathway is impaired and its lipid intermediates accumulate [19], thus causing a greater drain on the Und-P pool and the observed synthetic lethal phenotypes. Therefore, ECA biosynthesis and/or other surfaces polysaccharides can have a positive impact on the envelope assembly and thus on oxidative stress defence. Sowe need to characterize whether the ECA cluster genes disruption shows an effect on oxidative stress.

CONCLUSION

In summary, ElyC factor play a major role in envelope assembly by ECA and/or other surface polysaccharides synthesis. To better clarify the role of YcbC protein on oxidative stress response, we need to study how this factor can regulate the biosynthesis of ECA and/or other surfaces polysaccharides and accordingly oxidative stress response systems (SODs and/or catalse enzymes). The characterisation of new envelope biogenesis factors important for Gram-negative bacteria will broaden our understanding of the bacterial cell envelope biogenesis and validate the new factors as antibacterial targets.

Acknowledgements
The authors would like to thank all members of Department of Microbiology, Infectiology and Immunology, Université de Montréal, CANADA, for their helpful material and comments. Financial support from Programme governmental Algerian is also acknowledged.

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