The Role of Elyc Protein in Protection Against Oxidative Stress in *E. Coli* Cells

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ABSTRACT
Bacteria are covered by a complex envelope that delimits the cell and protects it against variations in osmotic pressure and environmental stresses. The goal of our study was the characterization of the importance role of Conserved inner membrane protein ycbC (elyC) gene in cell wall assembly and oxidative stress response in Escherichia coli. E. coli WT cells, ΔelyC and ΔmrcB mutants were grown in LB medium 37°C and 22°C. After that, the hydroxyl radical level was measured by the Flow cytometry (FACS). RNA extraction and purification was achieved and transcriptional analysis by RT-PCR was performed. Our results demonstrate the increase of Hydroxyl radical (oxidative stress) in ΔelyC mutant compared to WT and ΔmrcB mutant. Our data show the up-regulation of genes encoding for ycbC gene, the genes encoded for the enzymes implicated in PG synthesis and oxidative stress response in WT cells grown at 22°C. Furthermore, ΔelyC and ΔmrcB mutants grown at 37°C and 22°C revealed the overexpression of genes encoding enzymes involved in PG synthesis compared to WT cells. Moreover, gene expression of genes encoded for enzymes implicated in oxidative stress response in ΔelyC mutant was over-expressed but not changed in ΔmrcB mutant. These results approve the significant role of ElyC protein in Gram-negative bacterial envelope assembly and report a possible connection between cell wall biogenesis by ElyC factor and oxidative stress defense in Escherichia coli.

Keywords: *Envelope biogenesis, Enterobacterial Common Antigen (ECA), peptidoglycan (PG) synthesis, ElyC (YcbC) factor, flow cytometry (FACS)*

INTRODUCTION

Every free-living bacterium is 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 (Lemaux PG, *et al.*) exoskeleton sandwiched in between them (Silhavy *et al.* 2010). These structures play a key role in maintaining cellular integrity and offer protection from external abuses (Henderson *et al.* 2010). 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 (Breidenstein *et al.* 2011).

The external membrane of Gram-negative proteobacteria offers the essential resistance to antibiotics (Delcour, 2009). Pathogenic bacteria are becoming more and more resistant to antibiotics, and we need to identify new bacterial targets for the development of new antibacterial agents (Taubes, 2008). Remarkable progress has been made in our comprehension of Gram-negative envelope assembly over the last two decades (Ruiz *et al.* 2006). Most bacteria surround themselves with a peptidoglycan (PG) exoskeleton synthesized by polysaccharide polymerases called penicillin-binding proteins (PBPs). PBP accessory proteins play a central role in PG biogenesis and, like the PBPs they work with, these factors are attractive targets for antibiotic development. Bacteria typically encode two varieties of PBPs: class A and class B (Sauvage *et al.* 2008).

Two important PG synthases was produced by *E. coli*. The bifunctional (Class A) PBPs, PBP1a and PBP1b, encoded by the *mrcA* (*ponA*) and *mrcB* (*ponB*) genes, respectively (Typas *et al.* 2012). These factors have both peptidoglycan glycosyltransferase (PGT) activity to synthesize the glycan strands of PG and trans peptidase (TP) activity to crosslink the glycan chains via their attached peptide moieties (Sauvage *et al.* 2008). *E. coli*, like many bacteria, encodes multiple class A PBPs: PBP1a, PBP1b, and PBP1c (Sauvage *et al.* 2008).

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 (Hu *et al.* 2009). So, the assembly of this multilayered structure is still poorly understood. In an effort to shed light on this biological process, genetics screens for envelope biogenesis mutants were performed many years ago taking advantage of the release of periplasmic RNase from defective cells (Lopes *et al.* 1972); (Lazzaroni and R. Portalier 1979). Though, These "periplasmic leaky" screens were performed in the pre-genomic era and only identified some mutants were never precisely mapped (Fung *et al.* 1978); (Lazzaroni and Portalier 1981). Recently, quantitative assay for mutants with envelope biogenesis defects was developed and used to screen an ordered single-gene deletion library of *E. 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 (Paradis-Bleau *et al.* 2014). 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.

Oxidative stress, through the production of reactive oxygen species, is a natural consequence of aerobic metabolism. Oxidative stress may be induced in bacteria by exogenous biocidal agents and is involved in endogenous metabolism leading to cell death from the wide range of bactericidal antibiotics (Dwyer *et al.* 2008); (Dwyer *et al.* 2009); (Paulander *et al.* 2014). Recent progress on the genetics and molecular biology of the cellular responses to oxidative stress, primarily in *Escherichia coli* and *Salmonella typhimurium*, is studied. Bacteria respond to oxidative stress by two distinct stress responses, the peroxide stimulon and the superoxide stimulon, depending on whether the stress is mediated by peroxides or the superoxide anion (Farr and Kogoma, 1991).

Flow cytometric analysis in live bacteria is limited in part by the cell wall, which impairs penetration of vital peroxides and imposes a need for permeabilization procedures. After that, flow cytometry functional assay was tested in some *Escherichia coli* strains B WP2 strains (Herrera *et al.* 2002); (Herrera *et al.* 2003). hydroxyphenyl fluorescein: 2-[6-(4_-hydroxy) phenoxy- 3*H*-xanthen-3-on-9-yl] benzoic acid (HPF)**,** a reactive oxygen species (ROS) indicator show limited non-selective reactivity and relatively high resistance to light-induced oxidation. This fluorescein derivative is non fluorescent until they react with the hydroxyl radical (OH) or peroxynitrite anion (Setsukinai *et al.* 2003).

Our initial goal was to identify the role of *ycbC* and *mrcB* genes in envelop assembly and in oxidative stress defense of *E. coli*. In the present work, we have studied the role of YcbC and PBP1b function in PG and/or ECA biosynthesis and in the oxidative stress response pathways. *Escherichia coli* WT cells and Δ*elyC* and Δ*mrcB* mutants were grown aerobically and the oxidative stress (Hydroxyl radical OH^-) was measured by the flow cytometry assay through the hydroxyphenyl fluorescein (HPF) fluorescein probe. Next, we have measured the expression of genes encoding both enzymes that catalyzes the assembly of peptidoglycan (encoded by *murA*, *mrcB* and *uppS*) and enzymes implicated in the response of oxidative stress (encoded by *sodA*, *sodB*, *sodC* and *katG* genes).

MATERAILS AND METHODS

Bacterial strains and growth conditions

One colonie of *Escherichia coli* WT and Δ *elyC* and Δ *mrcB* 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 (250 rpm). 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 (room temperature) with shaking (250 rpm) till OD600 reached the OD ~ 0.5. The cells were collected, centrifuged for 10 min at 10.000 rpm, and prepared for the flow cytometry essay. Instead, the cells were suspended in 1 ml of Tri-reagent and conserved at -80°C before RNA extraction and purification.

Flow Cytometry analysis (Hydroxyl Radical Experiments)

Bacterial cells (WT and Δ*elyC* and Δ*mrcB* mutants) grown at 37°C or 22°C to reached an optical density (OD) OD~ 0.5 were diluted into 10 ml of fresh medium (LB) to obtain 10^6/ml. After that, cells were centrifuged 5 min at 5000 rpm and washed twice with PBS buffer solution (137 mmol/l NaCl, 2.7 mmol/l KCl, 10 mmol/l Na_2HPO_4 and 1.8 mmol/l KH_2PO_4 in water filtered through a 0.2-µm-pore-size syringe filter) and then suspended in PBS buffer. An untreated sample was used as negative control and treated cells with potassium dichromate at 0.5 µM was used as an oxidizing agent to induce oxidative stress and considered as positive control.

Cells from 1 ml of bacterial culture were collected at different conditions by centrifugation, and samples were directly stained. For staining of live cells, bacteria were washed once with PBS and then suspended and incubated for 30 min at 37°C in 1 ml HPF-staining solution (10 µM HPF in buffer solution). Bacteria were washed once and then suspended in PBS buffer solution after staining. Finally, cells were collected using a flow cytometer (FAC Sort; Becton Dickinson) with a 488-nm blue laser and FL1 (530/30-nm). Fluorescence excitation an emission maxima are 490 and 515 nm, respectively as described above by (Rengglia *et al.* 2013)

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*) (Zhou *et al.* 2011). 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 **(Pfaffl, 2001).** After that, we have 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*.

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
elyC (*ycbC*)	Polypeptide: conserved inner membrane protein	F:GGCTGGCGCTCTTGTTATTG R: AGGCGTGGCAGACTGTTATT
sodA	Superoxide dismutase (Mn)	F: GAAAGCGGCTATCGAACGTG R: CCATAATCGGGAAGCCGGAA
sodB	Superoxide dismutase (Fe)	F: CGCAGTTTACTGATGCAGCG R: AGGACGTGCATTGCGATAGT
sodC	Superoxide dismutase (Cu-Zn)	F:GTCTGGAGTTTTCGCCCGAT R:GCAGGCAGATCGCCTAAATG
katG	Hydroperoxidase I (HPI)	F: GCAAAAACGGCGTCTTCACT R: CACGCAGGACGGAGTTAGAA
murA	UDP-*N*-acetylglucosamine enolpyruvoyl transferase	F: TATTGATGCCCGCGACGTTA R: TGATGGTCGCGCCTAATTGT
mrcB(*ponB*)	penicillin-binding protein 1B	F: TCCAGCGAGCGTTCTTACTG R: CTTAGCTCTTCTACCGGGCG
ispU(*uppS*)	undecaprenyl diphosphate synthase (Und-P)	F: CAGCAAGGAAACCTGCAACC R: CATCGAAATCGGGCCAGAGA

a. F. forward, R. reverse.

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 Nano Drop 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 Super Script 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.

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

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

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 (Zhou *et al.* 2011).

RESULTS

Role of *elyC* (*ycbC*) and *mrcB* (*ponB*) genes on oxidative stress tolerance

Previous study indicated that ElyC (YcbC) factor play a key role in the metabolism of the essential lipid carrier used for cell wall synthesis. The phenotypes displayed by a Δ*elyC* mutant are likely to be the result of competition between the PG and ECA synthetic pathways (Paradis-Bleau *et al.* 2014). The discovery of ElyC as a new envelope biogenesis factor opens new opportunities to study this factor in more detail. To better characterise the role of ElyC in PG and ECA biosynthesis and thus in oxidative stress response, we have interested to determine whether oxidative damage is associated with the disruption of ElyC factor or the PG synthase PBP1b in cell wall biogenesis of *E. coli*. Thus, the disruption of *elyC* (*ycbC*) or *mrcB* (*ponB*) genes may cause a modification of ECA biogenesis or the architecture of peptidoglycan, reducing the ability of the cell to protect against oxidative stress.

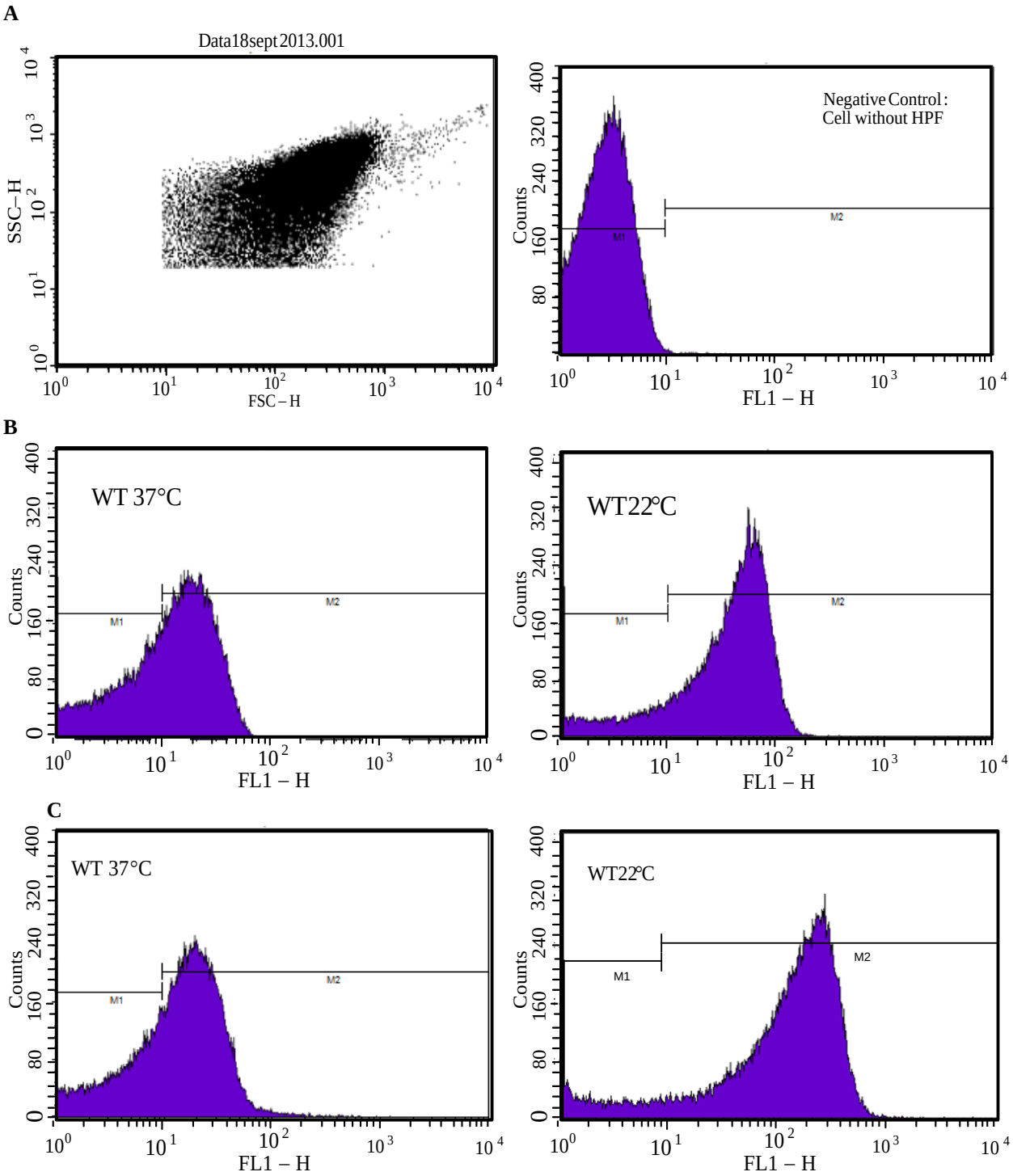
We have first observed that the loss of ElyC and PBP1b function results in cell lysis at low temperature by Screening of transposon library for envelope defective mutants with LB (0.5% NaCl) agar supplemented with CPRG (20 µg/ml) and IPTG (50 µM) (Paradis-Bleau *et al.* 2014). cell lysis, but no experiments are shown to prove that Aerobic conditions cause the production of reactive oxygen species, as a natural consequence of aerobic metabolism and thus results the oxidative stress (Chiang and Schellhorn, 2012). Hydroxyl Radical Formation in *E. coli* cells involves the Fenton Reaction and Intracellular Iron. Fenton reaction leads to the formation of hydroxyl radicals through the reduction of hydrogen peroxide by ferrous iron (Imlay *et al.* 1988). We next sought to directly block the damaging effects of hydroxyl radicals generated via the Fenton reaction by adding 2, 2'-dipyridyl to cells cultures. We found that Fenton reaction results a significant reduction in cell lysis in Δ*elyC* mutant (data not shown). Similar results showed that bactericidal Antibiotics Induce Hydroxyl Radical Formation These results imply that intracellular ferrous iron is a key source for Fenton-mediated hydroxyl radical formation by bactericidal drugs (Kohanski *et al.* 2007). Thus, ElyC^{-} cells have a severe defect in ECA and/or PG biogenesis at low temperatures and may be result the oxidative stress. We conclude a possible connection between cell wall biosynthesis and oxidative stress defense in *E. coli*. Our results indicate that hydroxyl radical formation by the Fenton reaction play a critical role in effective oxidative stress and cell lysis of Δ*elyC* mutant but not of Δ*mrcB* mutant. The Association between the low temperature and oxidative stress was evidenced by increase in the catalase and SOD activities and also in the amount of free radicals generated in *E. coli* cells (Chattopadhyay *et al.* 2011). This result provides a likely explanation for the temperature-dependent nature of oxidative stress phenotype displayed by Δ*elyC* mutant.

Loss of ElyC function results in cell lysis and oxidative stress at low temperature

Using the dye hydroxyphenyl fluorescein (HPF), this is oxidized by hydroxyl radicals with high specificity (Setsukinai *et al.* 2003). The concentration of hydrogen peroxide known to induce hydroxyl radical formation via Fenton chemistry was examined by (Bakker, 1979, Imlay *et al.* 1988) and (Kohanski *et al.* 2007). Cellular death was observed with 1 mM hydrogen peroxide and accompanied by an increase in HPF fluorescence.

To determine whether oxidative stress was observed in ElyC^{-} cells, Hydroxyl Radical was measured by flow cytometry through HPF fluorescein probe (Fig. 1). In WT cells, we have observed that the Hydroxyl Radical is less formed at 22°C compared to cells grown at 37°C. However, the amount of HPF fluorescence markedly increased in Δ*elyC* mutant cells grown at low

temperature compared to cells grown at 37°C. So, hydroxyl radical formation was induced or accumulated at low temperature. This result confirms the correlation between the cell lysis and hydroxyl radical increase and thus the oxidative stress in Δ*elyC* mutant at low temperature. Thus, ElyC factor play an important defensive role against hydrogen peroxide and superoxide radicals. An alternative hypothesis is to view ElyC factor as part of a chain of interacting elements that can induce a protective system defending the cell against environmental stresses.



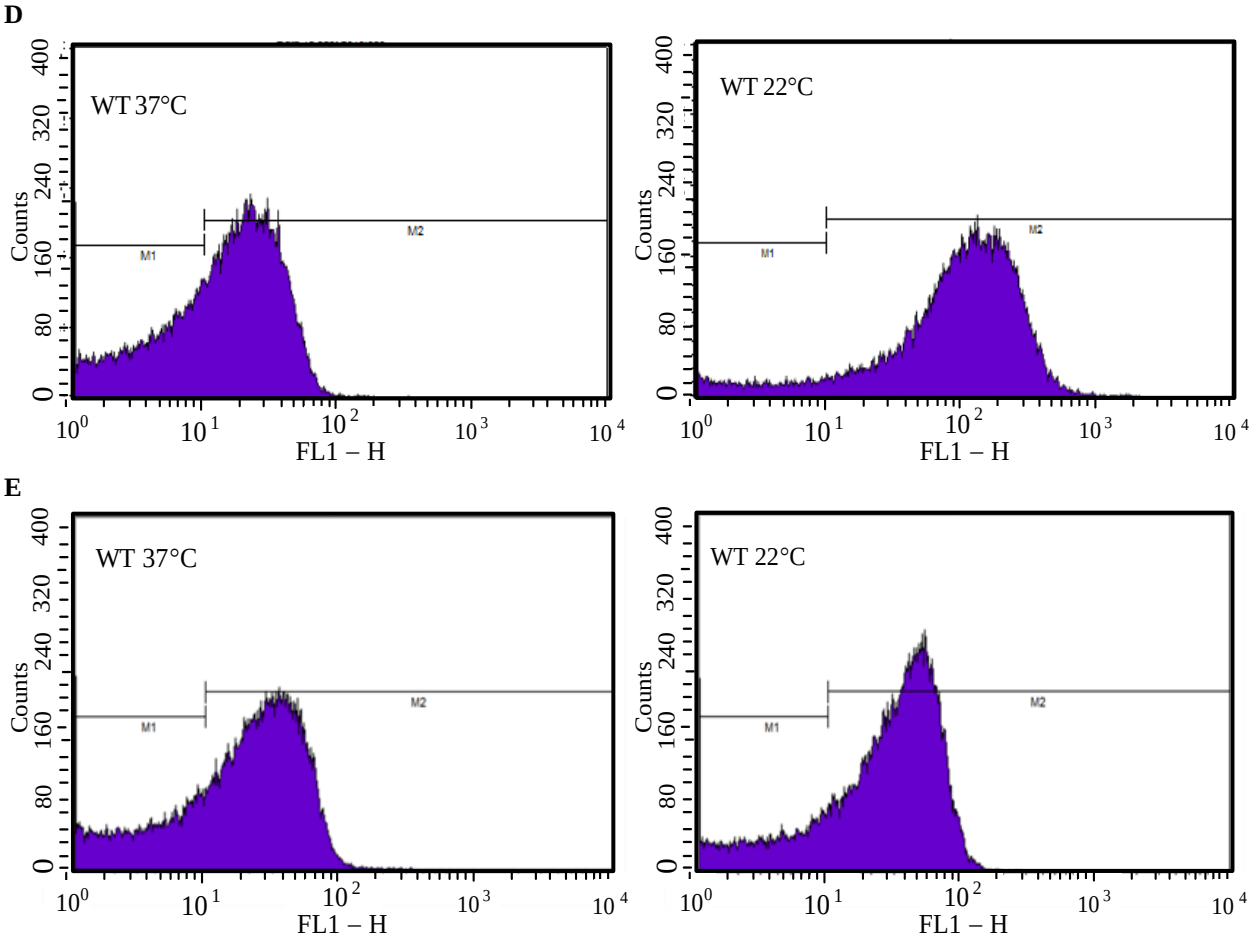


Fig. 1: Hydroxyl radical generation by Fluorescence of HPF probe in *E. coli* WT grown at 37°C and 22°C (B) and Δ*elyC* mutant grown at 37°C and 22°C (D) and Δ*mrcB* mutant grown at 37°C and 22°C (E). As positive control, cells were grown in the presence of potassium dichromate at 0.5 µM (C), and untreated cells with the HPF probe were used as negative control (A), cell. The data correspond to a representative result of 3 independent trials.

PBP1b is required for proper peptidoglycan biosynthesis but not for oxidative stress defense

In order to establish the formation of hydroxyl radical in Δ*mrcB* mutant, we have measured hydroxyl radical formation under the same growth conditions of WT and Δ*elyC* mutant. Our results showed no difference of hydroxyl radical level between Δ*mrcB* mutant and WT cells. The slight level of hydroxyl radical formation was also observed in cells grown at 22°C. Thus, disruption of *mrcB* (*ponB*) gene may cause a modification of the architecture of the peptidoglycan, but not reducing the ability of the cell to protect against oxidative stress. A biological function of peptidoglycan was demonstrated in determination of cell shape, in phage resistance, in induction of capsule synthesis, and in regulation of autolysis. (Young, 2001). Mutations in *mrcB* (*ponB*) gene may reduce the number of cross-links between glycan chains; lessen the degree of elongation of the chains, but not have a major role in oxidative stress defense. Though, previous results demonstrate the role of *rodA* and *pbp2b* genes in oxidative stress response and report a possible connection between peptidoglycan (Lemaux PG, *et al.*) synthesis and oxidative stress defense in *Streptococcus thermophilus* CNRZ368. (Thibessard *et al.* 2002). So, PG biosynthesis has a major role in oxidative stress defense in Gram-positive bacteria. Conversely, our results indicate that the PBPs or their peptidoglycan product does' not have significant role in oxidative stress defense in *E. coli* cells.

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

sodA, sodB, sodC **and** ***katG*** **mRNA level increase at low temperature in Δ*elyC*** **mutant**

Real-time RT-PCR assays were conducted to determine the effect of growth temperature on genes expression. *sodA, sodB, sodC* and *kactG* transcripts showed 2-to 3-fold increase in WT cells grown at 22°C compared to cells grown at 37°C (Fig. 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 up regulated 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.

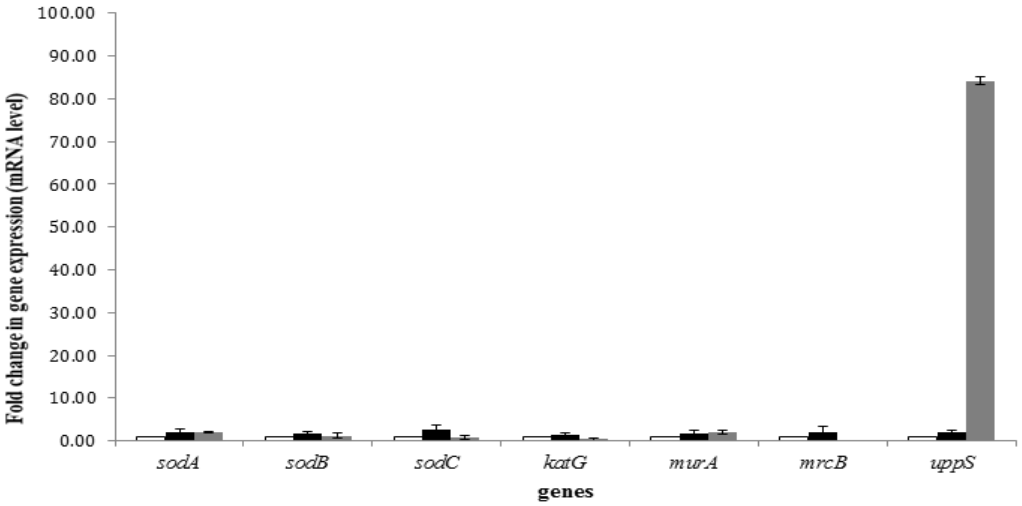
In order to demonstrate the role of superoxide dismutase and catalases enzymes in oxidative stress response in Δ*elyC* and Δ*mrcB* mutants, *sodA*, *sodB*, *sodC* and *kactG* genes expression was also quantified at low temperature. Our results were shown the overexpression of *sodA* (800-fold), *sodB* (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. Superoxide dismutases (SODs) catalyze the de toxification of superoxide. Preceding results demonstrate that FeSOD (SodB) and MnSOD (SodA) enzymes are not functionally equivalent; MnSOD is more effective than FeSOD in preventing damage to DNA, while FeSOD seems more effective in protecting a cytoplasmic superoxide-sensitive enzyme (Hopkin *et al.* 1992). Mn and FeSOD are adapted to different antioxidant roles in *E. coli* (Brown *et al.* 1995). SodC is the only periplasmic enzyme, the three enzymes differ in their metal cofactor requirement (Benov *et al.* 1995); (Miller, 2012). There are two distinct catalases in *E. coli*, the KatG enzyme is the bifunctional hydro peroxidase I (HPI) (Loewen and Switala 1986); (Farr and Kogoma 1991). Induction of katG, ahpFC, and perhaps other genes prevented the accumulation of oxidatively modified lipids but may not have protected DNA (González-Flecha and Demple 1997). Overexpression of SODs and catalase genes can have a protective role of the ElyC$^-$ cells against oxidative stress. Consequently, these results agree that the loss of ElyC function disturbs the cell wall assembly and thus increases the stress level in cell. Therefore, ElyC factor can protects cells against oxidative stress by Superoxide dismutases (SODs) and catalase systems activation.

***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 Undecaprenyl-diphosphate phosphatase (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 (Yousif *et al.* 1985),; (Kato *et al.* 1985); (Typas *et al.* 2010); (Paradis-Bleau *et al.* 2010)

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 (Muszynski *et al.* 2013). 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 (Molina-Quiroz *et al.* 2013). 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 Undecaprenyl-diphosphate phosphatase (Und-P).

A



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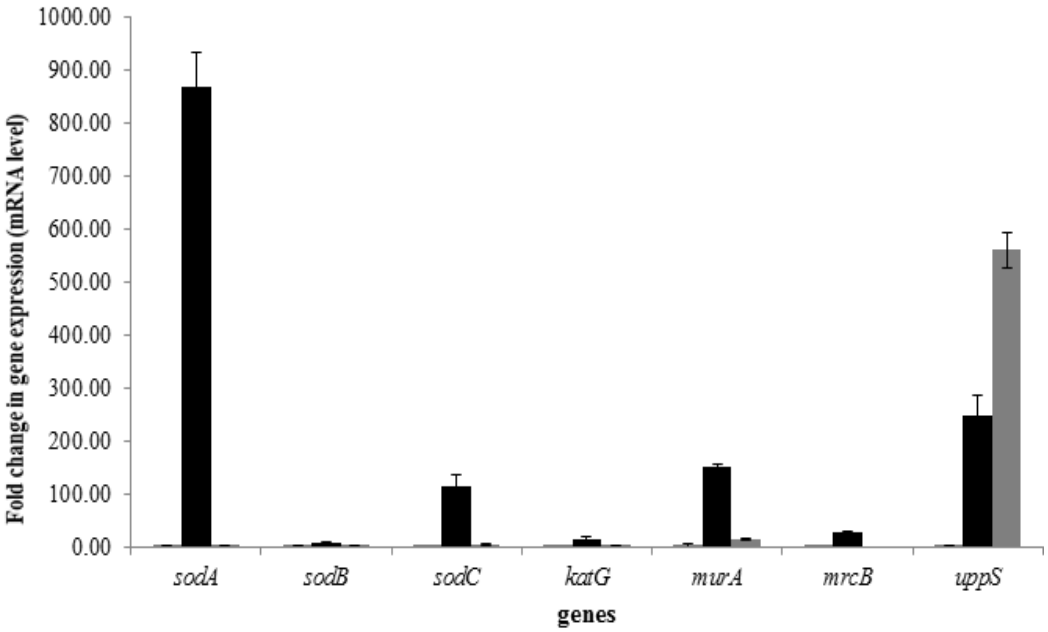

Fig. 2: RT-PCR assays conducted on mRNA isolated in *E. coli* WT cells (□) and Δ*elyC* mutant cells ( ), and Δ*mrcB* mutant ( ) grown at 37°C (A) and 22°C (B). The expression of *sodA, sodB, sodC, katG, murA, mrcB, and uppS genes* was measured. Fold changes in target gene expressions using reference 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).

DISCUSSION

Bacterial envelope biogenesis is one of the best sources of bacterial targets for antibacterial development. The cell envelope of Gram-negative bacteria is a rigid barrier that is difficult for antimicrobial drugs to penetrate. Thus, with the increase of new resistance mechanisms possessed by *Enterobacteriaceae*, a lot of treatments against these organisms decrease quickly (McKenna, 2013). To counter the problem of antibiotic resistance, we need to identify new bacterial targets for develop new antibacterial factors of bacterial cell wall biosynthesis in *E. coli*. The identification of bacterial response network targets can be exploited to combat the rise of resistant bacteria.

Oxidative stress may be induced in bacteria by exogenous biocidal agents and is involved in endogenous metabolism. As noted earlier, antibiotic-induced ROS formation, leading to cell death from the wide range of bactericidal antibiotics (Dwyer *et al.* 2008). Assumed the observation that ROSs (i.e. ·OH) and oxidative stress that results from antimicrobial exposure are generally associated with the lethal effects of bactericidal antimicrobials (Dwyer *et al.* 2009). It was also recently displayed that increased

hydroxyphenyl fluorescein (HPF) signals in antibiotics-exposed bacterial cells are clarified by fluorescence linked with increased cell size, and do not reflect reactive oxygen species (ROS) concentration and thus present the oxidative stress in cells (Paulander *et al.* 2014). Our initial approach was to identify new envelope biosynthesis factors can be implicated in antibiotic resistance by the study of oxidative stress response system in *E. coli*.

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 trans membrane domains and a large domain of unknown function (DUF) designated as a DUF218 domain in the Pfam database (Finn *et al.* 2008). It was recently show that *elyC* (*ycbC*) and *mrcB* (*ponB*) mutants are cold-sensitive (CS) for growth due to cell lysis (Paradis-Bleau *et al.* 2014). However, we have observed that ElyC$^-$ cells show the accumulation of Hydroxyl radical at low temperature contrary to PBP1b$^-$ cells. Therefore, ElyC factor can induce a protective system defending the cell against environmental stresses. So, the lack of ElyC protein would then result in the breakage of such a signalling pathway and lead to the loss of oxidative stress resistance. Mutation of *mrcB* (*ponB*) gene shows cell lysis but not oxidative stress formation in PBP1b$^-$ cells. So, PG synthase can play a key role in PG synthesis and growth normally at low temperature but not in oxidative stress response. We suggested therefore that YcbC factor have a most important role in oxidative stress response.

Our finding confirms the role of ElyC factor in oxidative stress response by SODs and catalase enzymes activation in *E. coli*. Our results confirm that the MnSOD is highly induced in response to oxidative stress while the FeSOD activity remains at approximately constant levels as previously demonstrated by Steinman et al., (1994). Besides, the association of MnSOD with DNA localizes dismutase activity near a target of oxidative stress and increases protection of DNA from oxidative damage (Steinman *et al.* 1994). However, other results show the transcriptional and posttranscriptional regulation of MnSOD biosynthesis in *E. coli*. The Induction of the Mn-superoxide dismutase is independent of the SOS system (Hancock and Hassan 1985). Overproduction of superoxide dismutase delays with the induction of the soxR and soxQ regulons and thus prevents a balanced adaptation and responds to an unknown environmental signal (Liochev and Fridovich 1991). Mn2+ causes activation of sodA transcription, implying a posttranscriptional or most likely a posttranslational modulation of enzyme activity via metal ions suggest the multiregulation of MnSOD (Touati 1988); (Schrum and Hassan 1993). *E. coli* requires several major regulators activated during oxidative stress, including OxyR, SoxRS, and RpoS. OxyR and SoxR undergo conformation changes when oxidized in the presence of hydrogen peroxide and superoxide radicals, respectively (Chiang and Schellhorn 2012). Previous work propose that the aconitase proteins of *E. coli* serve as a protective buffer against oxidative stress by modulating translation of the sodA transcript (Tang *et al.* 2002). These results support the assessment that ElyC factor can play a key role in posttranscriptional regulation of superoxide dismutase biosynthesis in *E. coli*. To investigate whether ElyC-mediated post-transcriptional regulation contributes to the changes in SodA, SodB and/or SodC content, revealed by the proteomic and *sodA, B, C–lacZ* fusion studies, and compared the amounts and half-lives of *sodA, B* and *C* mRNA in Δ*elyC* mutant and parental strain.

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 (Paradis-Bleau *et al.* 2014). Interestingly, each of the enzymes with suppression activity functions at a major transition point in PG biogenesis (Brown *et al.* 1995); (Sauvage *et al.* 2008); (Barreteau *et al.* 2009). Thus, overproduction of these factors may generally increase the flux through the pathway to alleviate the ElyC$^-$ defect.

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 Undecaprenyl-diphosphate phosphatase 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 (Danese *et al.* 1998), thus causing a greater drain on the Undecaprenyl-diphosphate phosphatase (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. So we need to characterize whether the ECA cluster genes disruption shows an effect on oxidative stress.

In summary, ElyC factor play a major role oxidative stress defense 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.

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