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) phenoxo-3H-xanthene-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 envelope 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 ΔelyC and ΔmrcB 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 colony of Escherichia coli WT and ΔelyC and ΔmrcB 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 ΔelyC and ΔmrcB 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. At the same time, 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 Na2HPO4 and 1.8 mmol/l KH2PO4 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 designed 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/idnThcaT) (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**

<table>
<thead>
<tr>
<th>Primers</th>
<th>Function</th>
<th>Sequence (5’→3’)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>rrsA</td>
<td>ribosomal RNA 16S</td>
<td>F: AGGCCTTCGGGTTGTTAAGGT&lt;br&gt;R: CCGGGATTTTCACATCTGACT</td>
</tr>
<tr>
<td>cysG</td>
<td>uroporphyrin III C-methyltransferase</td>
<td>F: AGGCATGTTAACCCTCGTCG&lt;br&gt;R: GCATAAATAAGCTGGCGGC</td>
</tr>
<tr>
<td>hcaT</td>
<td>HcaT MFS transporter</td>
<td>F: TGTTTATTGCGAGGGGGGCAACA&lt;br&gt;R: AGCATACGCGTCGACTACA</td>
</tr>
<tr>
<td>idnT</td>
<td>L-Idonate/5-ketogluconate/glucor Jonate transporter</td>
<td>F: GCTTTATTGCGACTCCTGTTCG&lt;br&gt;R: CAATCAGCCTGATGGCGATA</td>
</tr>
<tr>
<td>elyC (ycbC)</td>
<td>Polypeptide: conserved inner membrane protein</td>
<td>F: GCGTGCGCTCTGTATTTAG&lt;br&gt;R: AGGCCTTCGGGTTGTTATT</td>
</tr>
<tr>
<td>sodA</td>
<td>Superoxide dismutase (Mn)</td>
<td>F: GAAAGCGGCTATCGAAGATGT&lt;br&gt;R: CCATAATCGGGAAGC CGGAA</td>
</tr>
<tr>
<td>sodB</td>
<td>Superoxide dismutase (Fe)</td>
<td>F: CGCAGTTTACTGATGCAGCG&lt;br&gt;R: AGGACGTGCATTCGAGT</td>
</tr>
<tr>
<td>sodC</td>
<td>Superoxide dismutase (Cu-Zn)</td>
<td>F: GTCTTGAGGTTTTCGCCCGAT&lt;br&gt;R: GCAGGCAGATCGCCTAAATG</td>
</tr>
<tr>
<td>katG</td>
<td>Hydroperoxidase I (HPI)</td>
<td>F: GCAAGAACCGGTCTTCCTGACG&lt;br&gt;R: CCAGCATGACGAGCTAGAA</td>
</tr>
<tr>
<td>murA</td>
<td>UDP-N-acetylglucosamine enolpyruvoly l transferase</td>
<td>F: TATGATGCCCGCGAGCTTGA&lt;br&gt;R: TGATGTCGCCCTAATTGT</td>
</tr>
<tr>
<td>mrcB (ponB)</td>
<td>penicillin-binding protein 1B</td>
<td>F: TCCAGCGACGTTCTA CAGG&lt;br&gt;R: CTTAGCTTCTACCAGGCG</td>
</tr>
<tr>
<td>ispU (uppS)</td>
<td>undecaprenyl diphosphate synthase (Und-P)</td>
<td>F: CAGCAAAGGAAACCTGCAACCC&lt;br&gt;R: CATCGAAATCGGCCGAGA</td>
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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 RNAeasy plus Mini Kit (Qiagen). Total RNA was collected from samples in triplicate at each growth condition for the WT and ΔelyC and ΔmrcB 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 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 Bioanlyser of RNA purified by RNeasyPlus Mini Kit**

<table>
<thead>
<tr>
<th>Strains</th>
<th>Growth at 37°C</th>
<th>Growth at 22°C</th>
</tr>
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<tbody>
<tr>
<td>WT1</td>
<td>6.2</td>
<td>8</td>
</tr>
<tr>
<td>WT2</td>
<td>8.5</td>
<td>8.1</td>
</tr>
<tr>
<td>WT3</td>
<td>7.5</td>
<td>8.9</td>
</tr>
<tr>
<td>∆elyC 1</td>
<td>9.7</td>
<td>10</td>
</tr>
<tr>
<td>∆elyC 2</td>
<td>8.9</td>
<td>10</td>
</tr>
<tr>
<td>∆elyC 3</td>
<td>7.2</td>
<td>10</td>
</tr>
<tr>
<td>∆mrcB 1</td>
<td>8.1</td>
<td>8.9</td>
</tr>
<tr>
<td>∆mrcB 2</td>
<td>9</td>
<td>7.6</td>
</tr>
<tr>
<td>∆mrcB 3</td>
<td>7.5</td>
<td>8.4</td>
</tr>
</tbody>
</table>

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

**Quantitative real-time PCR**
The cDNA levels were then analyzed using a Rotor gene Real-Time System (RotorGene 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 ∆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 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 ∆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 ∆elyC mutant but not of ∆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 ∆elyC mutant.

**Loss of ElyC function results in cell lysis and oxidative stress at low temperature**
Using the dye hydroxycoumarine 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 ∆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 ΔelyC 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.
PBP1b is required for proper peptidoglycan biosynthesis but not for oxidative stress defense

In order to establish the formation of hydroxyl radical in ΔmrcB mutant, we have measured hydroxyl radical formation under the same growth conditions of WT and ΔelyC mutant. Our results showed no difference of hydroxyl radical level between ΔmrcB 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 pnpb 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 ΔelyC and ΔmrcB mutants

sodA, sodB, sodC and katG mRNA level increase at low temperature in ΔelyC mutant

Real-time RT-PCR assays were conducted to determine the effect of growth temperature on genes expression. sodA, sodB, sodC and katG 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 kacG genes was up regulated inΔelyC andΔmrcB 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 PBPIb function induce SODs and catalase genes transcription.

In order to demonstrate the role of superoxide dismutase and catalases enzymes in oxidative stress response inΔelyC andΔmrcB mutants, sodA, sodB, sodC and kacG 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 kacG (15-fold) inΔelyC mutant (Figure. 2 B). However, these genes show the same mRNA level inΔmrcB mutant compared in WT cells grown at 22°C. Thus, the transcription of sodA, sodB, sodC and kacG genes expression was highly activated at low temperature inΔelyC mutant compared to WT andΔmrcB mutant. Superoxide dismutases (SODs) catalyze the detoxification 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 inE. 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 inE. coli, the KatG enzyme is the bifunctional hydroperoxidase I (HPI) (Loewen and Switala 1986); (Farr and Kogoma 1991). Induction of katG, abpFC, 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 protect cells against oxidative stress by Superoxide dismutases (SODs) and catalase systems activation.

**upps gene is over expressed in ΔelyC and ΔmrcB mutants**

In order to determine the role of envelope biogenesis in oxidative stress response inE. 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 inΔelyC andΔmrcB 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 inΔmrcB 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 ofE. coli at low temperature 22°C, and more expressed inΔelyC andΔmrcB mutants associated with the overexpression ofuppS gene (data not shown). Similar results were recently observed that inYersinia 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 thatΔmrcB 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 ofycbC gene in the WT andΔmrcB mutant at low temperature (data not shown). These results reveal the role of YcbC factor in the envelope biosynthesis correlated with oxidative stress response inE. coli. In addition, the overexpression of ECA biosynthetic cluster, mrcB anduppS genesΔelyC mutant confirms the competition between the PG and ECA synthetic pathways for the lipid carrier Undecaprenyl-diphosphate phosphatase (Und-P).
Fig. 2: RT-PCR assays conducted on mRNA isolated in *E. coli* WT cells (□) and ΔelyC mutant cells ( ), and ΔmrcB 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 showed 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 PBPlb 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 PBPlb 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 dismutate 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 multigulation 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 posttranscriptional 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 ΔelyC mutant and parental strain.

Overexpression of uppS, murA, and mrcB genes in ΔelyC mutant and uppS and murA genes in ΔmrcB 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 PBPlb 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 PBPlb are both likely to suppress the ElyC defect by enhancing lipid-IPP 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 PBPlb cells. Nevertheless, the overexpression of ECA biosynthetic cluster in ElyC− and PBPlb 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 ΔelyC 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
YecC 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 catalase 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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