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BRIEF NOTES

A study on prophagic and chromosomal *sod*C genes involvement in *Escherichia coli* O157:H7 biofilm formation and biofilm resistance to H_2O_2

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Abstract

Introduction. *Escherichia coli* O157:H7 possesses one chromosomal and two prophagic *sod*C genes encoding for Cu,Zn superoxide dismutases. We evaluated the contribution of *sod*C genes in biofilm formation and its resistance to hydrogen peroxide.

Methods. The biofilm of *sod*C deletion mutants has been studied, in presence or absence of hydrogen peroxide, by crystal violet in 96-well plates and Scanning Electron Microscopy on glass coverslips.

Results. Deletion of prophagic *sod*C genes had no effect on biofilm construction, in contrast to the chromosomal gene deletion. Hydrogen peroxide treatment showed higher cell mortality and morphological alterations in *sod*C deletion mutants respect to wild type. These effects were related to the biofilm development stage.

Conclusion. The role of the three SodCs is not redundant in biofilm formation and the resistance to oxidative damage. The stage of biofilm development is a crucial factor for an effective sanitization.

Key words

- biofilm
- oxidative stress
- hydrogen peroxide
- scanning electron microscopy
- superoxide dismutase

INTRODUCTION

A biofilm is a structured community of bacterial cells enclosed in a self-producing exopolysaccharide matrix able to adhere to a biotic or abiotic surface. Biofilm plays a crucial role in the pathogenesis of many chronic human infections, and it has been estimated that more than 60% of all microbial infections are associated with biofilm presence [1].

Escherichia coli O157:H7 is a human pathogenic bacterium responsible for hemorrhagic colitis and the Hemolytic-Uremic Syndrome. The Center for Disease Control and Prevention reported 1994 cases of illness caused by *E. coli* O157:H7 in USA in the past 4 years, resulting in 782 cases of hospitalization (www.cdc.gov/foodnet/data/trends/tables/2013/table2a-b.html#table-2a).

E. coli O157:H7 shows the ability to form biofilm on a variety of surfaces. Biofilm allows a broad spread of disease caused by this organism, due to its higher resistance to external stresses, sanitizers and antibiotics [2]. Knowledge of factors that can affect biofilm formation may be useful to control persistent infections [3, 4]. *E. coli* O157:H7, like the most of enteric pathogens, may undergo oxidative stress caused by reactive oxygen species derived from aerobic metabolism, environmental sources and host immune response [5, 6]. Antioxidant enzymes, as peroxidases and superoxide dismutases, mediate the resistance to reactive oxygen species. Proteomic studies on bacterial communities show that proteins involved in oxidative stress are highly expressed in natural microbial biofilms [7-9].

E. coli O157:H7 possesses three *sod*C genes encoding for Cu,Zn superoxide dismutase (SodC): one identical to the gene present in the non-pathogenic K12 strain (chromosomal *sod*C), and the other located in sequences (*sod*C-F1 and *sod*C-F2) derived from lambdoid prophages. In a previous work, we demonstrated that the proteins of prophagic origin have different structural/functional features with respect to the enzyme encoded by the chromosomal *sod*C copy [10].

The purpose of this study was to compare the ability of *sod*C mutants and the wild type strains to form biofilm and to resist to hydrogen peroxide (H_2O_2). In particular, we used the RG101 strain lacking the chromosomal *sod*C gene, the RG104 strain lacking both the prophagic *sod*C genes and RG105 strain lacking all three *sod*C genes.

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MATERIALS AND METHODS

Strains and growth conditions

E. coli O157:H7 strains used in this work originated from a clinical isolate ED597 identical to EDL 933 reference strain ATCC 43895 for the *sodC* gene sequences [10].

We analyzed the ability of *E. coli* O157:H7 to form biofilm in modified M9 minimal medium, hereafter named modM9 (0.6% w/v of Na_2HPO_4, 0.3% w/v of KH_2PO_4, 0.1% w/v of NH_4Cl, 0.5% w/v casamino acid, 0.1% w/v $MgSO_4$ and 0.2% w/v glucose). The cells were grown at 28 °C for 48 h without shaking in polypropylene 96-well plates (Greiner) or on glass coverslips degreased with acetone before using.

In all experiments we employed a bacterial inoculum of 10^6 cells ml^{-1}. In particular, 200 µl was added to the wells of 96-well plates and 6 ml to contact plates of 5 cm diameter (LP italiana), containing the glass coverslips.

Biofilm assay

To analyze the ability of wild type and *sodC* deletion mutants to form biofilm under stressful conditions, we used the Zhang *et al.* method [11]. After an initial cell growth at 28 °C for 6 h in modM9, the medium was replaced by fresh modM9 or modM9 media supplemented with 250 µg l^{-1} H_2O_2 and incubated for an additional 42 h. The biofilm grown on the coverslips was processed as described in Scanning Electron Microscopy (SEM) section, while the biofilm grown in the 96-well plates was stained with the crystal violet (CV) modified method [12].

Briefly, biofilm was washed three times with PBS, dried at 37 °C for 5 min, fixed at 60 °C for 1 h and stained with 0.1% CV. After 20 min at RT, CV staining was removed. The biofilm was rinsed in tap water for three times and the dye was solubilized in dimethylsulphoxide (DMSO) for 15 min [13]. The optical density was determined at 595 nm by a 96-well plate reader (Biotek Instrument mod. ELX808). Each experiment was carried out in a single 96-well plate where eight wells were used for each strain grown in the presence or absence of H_2O_2.

Hydrogen peroxide assay

To test the *sodC* deletion mutants susceptibility to the oxidative stress, we treated biofilms with H_2O_2. The biofilm was formed at 28 °C for 48 h in modM9 and, after three washing with PBS, the 96-well plates were incubated at 37 °C for 1 h with PBS or PBS supplemented with 1.5 mg l^{-1} H_2O_2. The challenged cells were washed in PBS and detached by treating with 200 µl of Trypsin-EDTA at 37 °C for 5 min. The number of viable cells was determined by plating on LB agar. The survival percentage was calculated for each strain by the ratio of the colony-forming unit (CFU) obtained after incubation with H_2O_2 and the CFU obtained after incubation with PBS. Each experiment was carried out in a single 96-wellplate, where four wells were used for each strain in presence or absence of H_2O_2.

After coverslips treatment with 250 µg l^{-1} H_2O_2 at 37 °C for 1 h, the effects of H_2O_2 on the morphology of a 48 h-biofilm were evaluated.

Scanning Electron Microscopy (SEM)

Biofilms formed on coverslips of 12 mm diameter were fixed with 2.5% glutaraldehyde in 0.1 g l^{-1} sodium cacodylate buffer, pH 7.4 at room temperature for 30 min. The fixed cells were then washed three times with the same buffer and post fixed with 1% osmium tetroxide for three weeks at 4 °C. These samples were washed twice with cacodylate buffer and then dehydrated using a graded alcohol series. After the passage in 100% ethanol, the samples were critical point-dried in CO_2 (CPD 030 Balzers device, Bal-Tec, Balzers) and gold coated by sputtering (SCD 040 Balzers device, Bal-Tec). The samples were examined with a Cambridge Stereoscan 360 scanning electron microscope (Cambridge Instruments, Cambridge, United Kingdom).

Statistical analysis

Data analysis was performed with Excel software (Microsoft Office Excel 2007). The level of significance was calculated by Student's t test.

RESULTS

Ability to form biofilm in modM9 and in stressful condition

CV staining of bacteria grown in modM9 (*Figure 1*) showed that wild type, as RG104 mutant, produced more biofilm than the mutants lacking chromosomal *sodC* gene (RG101 and RG105). Furthermore, we observed no significant difference between RG101 and RG105 in biofilm accumulation.

We investigated whether oxidative stress could affect the ability of *sodC* mutants to form biofilm. Cells were

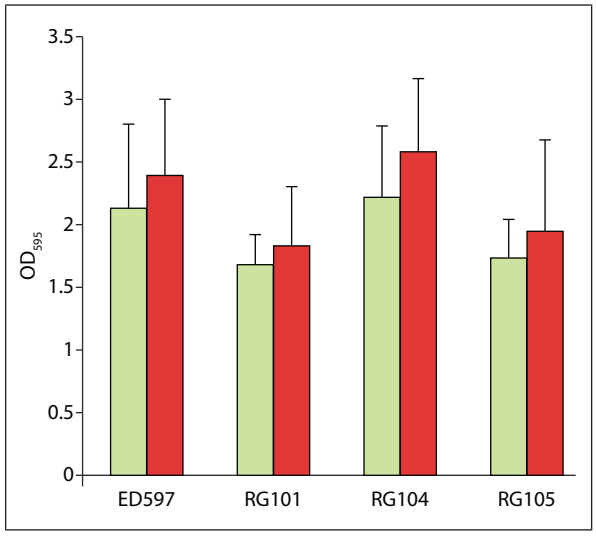

Figure 1
The strains, after an incubation at 28 °C for 6 h, were grown further 42 h in modM9 (solid bars) or modM9 in presence of 250 µg l^{-1} H_2O_2 (open bars). Differences were observed between wild type (ED597) and RG101 or RG105 both in modM9 and in presence of H_2O_2 as well as between wild type or RG104 in modM9 or in presence of H_2O_2. All differences were significant with $p < 0.01$ except for RG104 where the difference was $p < 0.05$. Error bars indicate standard deviations of at least six independent experiments.

initially grown for 6 h, to allow their adhesion to the surface, and then exposed to 250 µg l^{-1} of H_2O_2 for 42 h. Wild type and RG104 strains under stressful conditions formed a comparable amount of biofilm higher than that produced by RG101 and RG105 mutants. Furthermore, H_2O_2 stimulated biofilm formation in the wild type and RG104 strains, whereas in RG101 and RG105 mutants (*Figure 1*) we observed no significant effect.

Survival to oxidative stress induced by hydrogen peroxide

Figure 2 shows the different resistance to the oxidative stress of wild type or *sod*C deletion mutant cells organized in biofilm. The treatment of 96-well plates with 1.5 mg l^{-1} H_2O_2 for 1 h caused in all mutants the recovery of a smaller number of cells than in wild type. However, we obtained no statistical difference in survival between the mutants, in agreement with our previous results obtained in planktonic culture grown in LB medium [10].

Biofilm observation by SEM

In order to determine whether morphological changes occurred in biofilm architecture challenged with H_2O_2, the biofilm formed on coverslips was analyzed by SEM. We observed no significant difference between wild type and deletion mutants in the organization of 48 h-biofilm obtained in modM9. The SEM micrographs (*Figure 3a, 3b*) showed a well-organized biofilm consisting of cells surrounded by exopolysaccharide matrix; several cellular pillars and curli filaments attached the cells to the substratum, promoting the formation of an interconnecting mesh between cells (data not shown for RG104 and RG105). After an initial growth of 6 h in modM9, changes in biofilm organization were visible when cells were grown for further 42 h in the presence of 250 µg l^{-1} of H_2O_2 (*Figure 3c, 3d*). A slight cell elongation was the only alteration observed in the wild type (*Figure 3c*), an effect related to H_2O_2 mediated injury. The RG104 mutant showed a morphology very similar to the wild type, conserving a densely packed structure (data not shown). On the contrary, the chromosomal *sod*C deletion mutants exhibited more consistent morphological alterations with a few areas with partial fusion of cellular walls (*Figure 3d* represents RG101, data not shown for RG105). Morphological changes appeared also in the wild type when 48 h-biofilms were challenged with 250 µg l^{-1} of H_2O_2 for 1 h. In fact, the *Figure 3e* shows extensive areas where the cell-cell network was destroyed, and the cells have lost their structural integrity. However, the biofilm of all deletion mutants showed more severe damages when exposed to H_2O_2 (*Figure 3f* represents RG101, data not shown for RG104 and RG105). In this case, the entire biofilm organization was dramatically altered showing a loss of individuality of single cells and a total absence of interconnections.

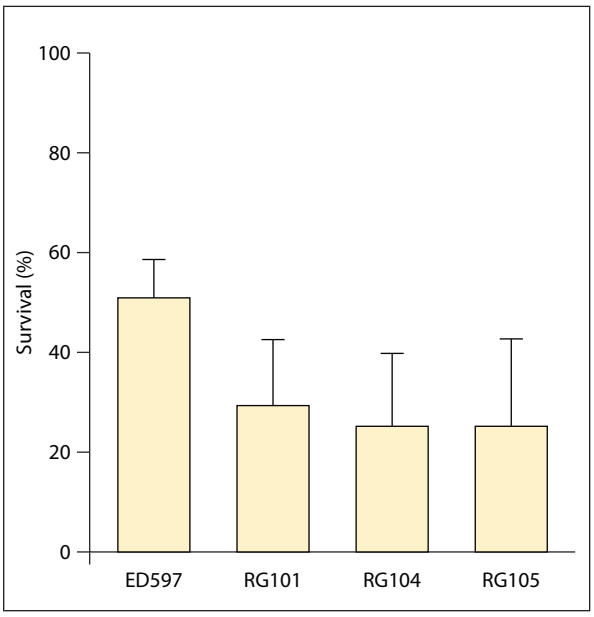

Figure 2
Susceptibility to 1.5 mg l^{-1} H_2O_2 of wild type (ED597) and *sod*C deletion mutant biofilms. The survival percentage is calculated by the ratio of the number of CFU ml^{-1} obtained from incubation in H_2O_2 and the number of CFU ml^{-1} obtained from incubation in PBS. Differences were significant with $p < 0.01$. Error bars indicate standard deviations of at least six independent experiments.

DISCUSSION

In a previous research, we compared the functional, structural and regulatory properties of the three SodC enzymes. We demonstrated that, despite the SodCs are all involved in cellular protection against H_2O_2, the *sod*C genes located in prophagic sequences are differently regulated with respect to the chromosomal *sod*C copy and encoded for proteins with distinct structural/functional features [10]. Kim *et al.* [8] demonstrated the role of chromosomal *sod*C gene product (SodC) in *E. coli* O157:H7 biofilm formation, but they did not consider the prophagic genes in their study. Therefore, in this work we investigated for the first time the possibility that the prophagic *sod*C genes products (SodC-F1/SodC-F2) may be involved in biofilm formation. Moreover, we studied the role that SodCs play in protecting bacteria, embedded in a biofilm structure, from oxidative stress. Our results indicate the involvement of the only SodC in biofilm formation, excluding any role of prophagic proteins in this process. In fact, the biofilm produced in modM9 by the wild type and the RG104 mutant was higher than that produced by the RG101 and RG105 mutants. In addition, the CV values of single mutant and triple mutant were comparable, the latter appearing to be unaffected by the further absence of prophagic copies. Unexpectedly, the SEM observations (*Figure 3a, 3b*) did not identify morphological differences between the strains grown in modM9. This result suggested that the SodC does not participate in the biofilm morphology, but it is probably involved in the process of the adhesion to the surface, resulting in higher biofilm production. On the other hand, these differences in cell adhesion ability cannot be fully appreciated by SEM because it is a qualitative and not quantitative investigation method.

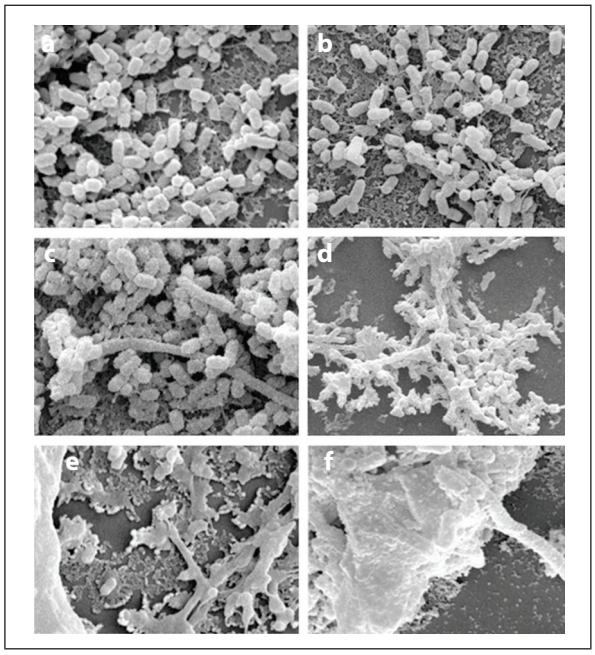

Figure 3
Letters *a, c* and e indicate wild type; letters *b, d* and f indicate RG101 mutant (RG104 and RG105 are not shown). The strains, after an incubation at 28 °C for 6 h, were allowed to grow further 42 h in modM9 (*a* and *b*) or in the presence of 250 µg l^{-1} H_2O_2 (*c* and *d*). Micrographs *e* and *f* show 48 h-biofilm of wild type (ED597) and RG101 respectively, after 1 h treatment with 250 µg l^{-1} H_2O_2.

Several reports showed that the defence mechanisms against oxidative stress might be involved in biofilm formation [9, 14]. Recent works have shown that in *E. coli* O157:H7 [8, 14] H_2O_2 induced *sod*C genes and that SodCs contributed to the resistance against exogenous reactive oxygen species. In this work we investigated whether the three SodCs are differently involved in the protection against H_2O_2 of biofilm cells. Our results confirm that *E. coli* O157:H7 biofilm cells are more resistant to H_2O_2 than cells in planktonic culture [10]. Furthermore, the wild-type strain showed a higher survival capacity towards H_2O_2, compared to deletion mutants, that have not showed significant differences, indicating the absence of any additive effect of the deletions. The lower resistance of the *sod*C deletion mutants to stressful conditions was also observed by SEM analyses, carried out on 48 h-biofilm after 1 h of H_2O_2 treatment. Although in the wild type biofilm the cellular structures appeared injured, all mutants reacted similarly to the oxidative stress, showing more severe damages. Morphological differences between the deletion mutants appeared only when the H_2O_2 exposure was continuous. In fact, after 42 h of oxidative treatment RG104 biofilm was structurally comparable to wild type and did not show the same cellular alterations observed in RG101 and RG105 biofilms, suggesting the importance of SodC in the biofilm formation in the presence as well as in the absence of H_2O_2. This suggestion is reinforced by CV results, which showed a significant increase in H_2O_2 induced biofilm only in the strains that possess chromosomal *sod*C copy.

Interestingly, the physiological state of cells at the moment of the insult resulted to be a critical factor for H_2O_2 resistance. In fact, we obtained different results by exposing to H_2O_2 for 42 h the developing biofilm (after only 6 h of growth in modM9) or by exposing to H_2O_2 for 1h the 48 h-biofilm. All strains were able to adapt to the presence of H_2O_2 for 42 h and to form a biofilm, despite damages observed by SEM (*Figure 3c, 3d*). Probably, during the initial formation of biofilm certain cells, called "persisters" [15], were in a physiological state that allowed them to resist to the oxidizing agent and to proliferate until the maturation of biofilm itself. On the other hand, after 1 h of treatment, survival results and SEM observations (*Figure 3e, 3f*) revealed that H_2O_2 damaged all structural organization of the 48 h-biofilm. Therefore, our data indicate that the dose can be lethal or sublethal depending on specific development stage of the biofilm during the application of the biocide. Indeed, we demonstrated that the dose of 250 µg l^{-1} H_2O_2 used on a developing biofilm had a sublethal effect, inducing further a biofilm increase. On the contrary, when we used the same dose on a 48 h-biofilm it had a lethal effect destroying the cellular structure. It's known that the use of sublethal doses of bactericidal agents or a prolonged exposure to them could select resistant bacteria [16] able to tolerate a further dose of the same or other bactericides, thus developing a cross-resistance to several classes of biocides [14, 17]. Therefore, treatment with a biocide needs to be immediately effective to avoid the opposite effect as a further biofilm increase and the onset of some form of resistance.

CONCLUSIONS

Taken together, our data exclude that the prophagic *sod*C genes, unlike the chromosomal *sod*C, are necessary for an efficient biofilm formation, confirming that the three *E. coli* SodCs play distinct physiological roles. However, despite their different involvement in biofilm formation, all SodCs contribute to the bacteria protection against the toxic action of exogenous H_2O_2, when they are organized in biofilm. Finally, this study underlines that the biofilm development phase is critical in order to ensure effective sanitation and to reduce the environmental persistence of pathogens.

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Conflict of interest statement

There are no potential conflicts of interest or any financial or personal relationships with other people or organizations that could inappropriately bias conduct and findings of this study.

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