Lack of intracellular trehalose affects formation of *Escherichia coli* persister cells

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Persisters are dormant antibiotic-tolerant cells that usually compose a small fraction of bacterial populations. In this work, we focused on the role of trehalose in persister formation. We found that the Δ*otsA* mutant, which is unable to synthesize trehalose, produced increased levels of persisters in the early stationary phase and under heat stress conditions. The lack of trehalose in the Δ*otsA* mutant resulted in oxidative stress, manifested by increased membrane lipid peroxidation after heat shock. Stationary Δ*otsA* cells additionally exhibited increased levels of oxidized proteins and apurinic/apyrimidinic sites in DNA as compared to WT cells. Oxidative stress caused by the lack of trehalose was accompanied by the overproduction of extracellular indole, a signal molecule that has been shown to stimulate persister formation. Our major conclusion is that intracellular trehalose protects *E. coli* cells against oxidative stress and limits indole synthesis, which in turn inhibits the formation of persisters.

INTRODUCTION

Persisters are dormant or slow-growing cells that are highly antibiotic-tolerant and usually compose a small fraction of bacterial populations. In contrast to drug-resistant bacteria, persisters are phenotypic variants of WT cells and become susceptible to antibiotics upon regrowth. It was suggested that persisters can escape the effect of antibiotics because their cellular activities (translation, cell wall synthesis, topoisomeraze activity), which are targets of antibiotics, are strongly reduced (Lewis, 2010). Therefore, persister cells can often be the main cause of the recalcitrance of bacterial infections. Current studies indicate that multiple and parallel mechanisms are involved in cell persistence. It has been proposed that so called type I persisters are formed in response to external conditions, including starvation, whereas the formation of type II persisters is a stochastic, continuous process, resulting from fluctuations in gene expression in individual cells (Balaban et al., 2004, 2011; Wood et al., 2013). Apart from starvation conditions, other stresses can promote persistence (Poole, 2012). For instance, oxidative stress, acid treatment and heat shock induced persister formation in *Streptococcus mutans* (Leung & Lévesque, 2012). High levels of persisters appeared in *E. coli* cultures exposed to acid or oxidative stress (Hong et al., 2012; Vega et al., 2012; Wu et al., 2012). Recent studies indicate that antibiotics can also be implicated in persister formation (Dörr et al., 2010; Johnson & Levin, 2013; Kwan et al., 2013). Kwan et al. (2013) demonstrated that antibiotics that halt transcription or translation caused the generation of high levels of antibiotic-tolerant *Escherichia coli* cells. Johnson & Levin (2013) found that treatment of *Staphylococcus aureus* with subMIC concentrations of antibiotics induced persisters tolerant to drugs other than those with which the cells were treated. Recent studies also indicate that some of these conditions may lead to persister formation through the activation of the toxin–antitoxin (TA) modules. Usually, the TA systems consist of a stable toxin which perturbs essential cellular functions, and an unstable antitoxin which controls and inactivates the toxin. The best studied toxin involved in persister is HipA kinase, which phosphorylates the glutamyl-tRNA synthetase, leading to the inhibition of aminoacylation and protein synthesis (Germain et al., 2013). Other examples are the mRNase MqsR, degrading mRNA, (Kim & Wood, 2010) and a membrane-acting peptide, TisB, which inhibits cell growth by decreasing the proton motive force and ATP level (Dörr et al., 2010). It was found that overproduction of toxins led to enhanced persister frequencies, whereas deletion of single or multiple genes coding for toxins resulted in the reduction of persistence (Kim & Wood, 2010; Maisonneuve et al., 2011). Maisonneuve et al. (2011) revealed that another crucial factor required for persistence and linked to the TA systems is ATP-dependent protease Lon, which degrades antitoxins and thereby causes activation of toxins. The alarmone guanosine tetraphosphate (ppGpp), synthesized in response to various stresses, is also required for persister formation (Korch et al., 2003; Nguyen et al., 2011; Amato et al., 2013). It was proposed that ppGpp...
controls the cellular level of polyphosphate, which in turn activates Lon (Gerdes & Maisonneuve, 2012). Recently, Vega et al. (2012) demonstrated that persister formation can be controlled by indole, an extracellular signalling molecule. Indole is produced by E. coli under nutrient-limited conditions and has been demonstrated to affect biofilm growth (Lee et al., 2008; Kuczyńska-Wiśniki et al., 2010) and increase antibiotic resistance via drug efflux pumps and oxidative stress protective mechanisms (Hirakawa et al., 2005; Lee et al., 2010). It was found that indole-dependent persistency is mediated by the oxidative stress response (oxyR) and phage shock response (psp) (Vega et al., 2012). Interestingly, it was also reported that in a mixed culture indole produced by E. coli induced antibiotic tolerance of Salmonella typhimurium, a pathogen which is not able to synthesize indole (Vega et al., 2013).

Previously, we reported that persister formation in a stationary phase E. coli culture was inhibited in a medium supplemented with trehalose or other osmolytes (Leszczyńska et al., 2013). We also found that trehalose caused reversion of persisters to antibiotic-sensitive cells. It has been reported that trehalose protects prokaryotic and eukaryotic cells from various stresses including freezing and dessication. Hence, trehalose is widely used as a desiccation and cryoprotectant which stabilizes proteins and lipid bilayers (Elbein et al., 2003; Jain & Roy, 2009). It is known that trehalose can act as a chemical chaperone preventing denaturation of proteins and facilitating the formation of native oligomers (Diamant et al., 2001). It has also been demonstrated that trehalose acts as a chemical chaperone and inhibits the aggregation of proteins involved in Alzheimer’s (Liu et al., 2005), Huntington’s (Tanaka et al., 2004), Parkinson’s (Yu et al., 2012) and prion diseases (Béranger et al., 2008). There are various theories explaining the protective properties of trehalose. According to the prevalent exclusion theory, trehalose sequesters water molecules from proteins, decreasing their hydrated radius. In consequence, proteins become more compact and stable (Jain & Roy, 2009).

In E. coli, trehalose synthesis is catalysed by two enzymes: trehalose-6-phosphate synthase, encoded by otsA, and trehalose-6-phosphate phosphatase, encoded by otsB (Ruhal et al., 2013). E. coli produces trehalose in response to the cold (Kandror et al., 2002) and osmotic stresses (Giaever et al., 1988), during the entry into stationary phase (Hengge-Aronis et al., 1991) and in response to desiccation (Zhang & Yan, 2012). Inability to synthesize trehalose impairs E. coli growth during cold and osmotic stress but has no effect at high and low pH or at elevated temperatures (Kandror et al., 2002; Purvis et al., 2005). It was found, however, that expression of the otsBA operon is required for stationary phase thermo-tolerance (Hengge-Aronis et al., 1991). Overproduction of trehalose increases thermal tolerance (Purvis et al., 2005) whereas osmotically induced accumulation of trehalose protects E. coli against desiccation (García De Castro et al., 2000) In summary, there is accumulating evidence pointing to a connection between trehalose, oxidative stress, indole signalling, antibiotic tolerance and formation of persisters; however, the nature of this connection is not clear.

In this work, we aimed to clarify the role of trehalose in persister formation. Using the ΔotsA mutant, which is unable to synthesize trehalose, we demonstrated that the lack of intracellular trehalose resulted in an increase of oxidative stress, overproduction of extracellular indole and increased antibiotic tolerance of the cells.

**METHODS**

**Bacterial strains, growth conditions and plasmids.** E. coli MC4100 [araD139Δ(lacProA285) argF1 U169 flaRelA rpsL] and its derivatives were used in this study. MC4100 ΔotsA::kan and MC4100 ΔgfpA::kan were constructed by P1 transduction (Sambrook et al., 1989) using E. coli JW5312-3 and JW3392-1 (E. coli Genetic Stock Center, Yale University), respectively, as donors. To obtain MC4100 ΔotsAAgfpA::kan, the resistance cassette was removed from the MC4100 ΔotsA::kan using pCP20 plasmid expressing the Flp recombinase (Datsenko & Wanner, 2000). In the next step, the resulting strain MC4100 ΔotsA::A and JW3392-1 strain were used as the recipient and donor, respectively. To construct the double mutant MC4100 ΔotsAΔtnaA::kan, the ΔtnaA::kan mutation was transferred from JC3686 (E. coli Genetic Stock Center, Yale University) to MC4100 ΔotsA by P1 transduction. The presence of mutations was verified by PCR using the appropriate primers, and the absence of trehalose and indole (see below) in the ΔotsA and ΔtnaA mutants was confirmed.

The strains were grown in Luria–Bertani (LB) medium in Ehrenmeyer flasks with agitation (200 r.p.m., aerobic cultures). The strains transformed with the pGB2 vector and its derivative pGB-otsBA were grown in LB medium supplemented with 30 µg spectinomycin ml⁻¹. To obtain reproducible results, the medium was sterilized by filtering. To prepare glycerol stocks, a culture grown to an OD₆₀₀ of 1.0 was supplemented with glycerol at a final concentration of 10%, divided into 25 µl aliquots and frozen at −80 °C. The 25 µl stock was used for inoculating an overnight culture (1:1000). After 18 h, the overnight culture was diluted 1:100 into a fresh LB medium and incubated at 37 °C for 24 h. The cultures submitted to heat stress were first incubated at 30 °C to an OD₆₀₀ of 0.3, and then transferred to 50 °C for 15 min. Before desiccation and cold stress, bacteria were cultured in LB at 37 °C to an OD₆₀₀ of 0.5. Cold stressed bacteria were transferred to 16 °C for 3 h. In desiccation experiments, cells from 1 ml cultures were pelleted and resuspended in 100 µl water (Nocker et al., 2012). Fifty microlitre droplets of the cell suspensions were placed on glass microscope slides and incubated at 30 °C. After drying (which took approximately 40 min as judged by eye) the samples were resuspended in 1.5 ml 0.85% NaCl.

Plasmid pGB-otsBA was constructed by cloning between the PstI and Smal sites of plasmid pGB2 (Churchward et al., 1984) a PCR fragment containing the otsBA operon with its own promoter. The PCR fragment was generated with primers 5’ATCTCGAGTAGCT-GGTTCGGGCTTAACTCGTG3’ and 5’GACCGGGTAGAC-GTAAATCTGGAATGATGTG3’ using E. coli chromosomal DNA as the template and digested with PstI and Smal.

**Determination of the number of persister and dead cells.** To determine the number of persisters, the cultures were diluted 1:100 in fresh LB medium, supplemented with 5 µg ofloxacin (Ofx) ml⁻¹ or 200 µg ampicillin (Amp) ml⁻¹ and incubated at 37 °C for at least 4 or 6 h, respectively. The surviving persisters were plated on LB agar for colony counts. To rule out the possibility that antibiotic-tolerant cells were resistant mutants, persister colonies were plated on LB agar plates with Ofx or Amp. The frequency of persisters was estimated in relation to the total number of c.f.u. or total number of cells before
The thiobarbituric acid-reactive sub-mutant, E. coli cells (50 ± was measured was estimated using epifluorescence microscopy (Zeiss Axio Scope.A1) after staining with a LIVE/DEAD BacLight viability kit (Molecular Probes) according to the manufacturer’s protocol.

Extracellular indole assay. To measure extracellular indole concentrations, 0.4 ml Kovac’s reagent (10 g p-dimethylaminobenzaldehyde, 50 ml HCl and 150 ml amyl alcohol) was mixed for 2 min with 1 ml of medium collected after sedimentation of the bacteria. The reaction mixture was diluted 1:10 in HCl/amyl alcohol solution (50 ml HCl and 150 ml amyl alcohol) and the Abs was measured (Domka et al., 2006). The concentration of indole was calculated based on the calibration curve.

Trehalose and glycogen assay. Cells (1.5 ml) were pelleted, washed, resuspended in 300 ml 10 mM Tris/HCl pH 7.4 and disrupted by sonication (Vibra-Cell sonicator). To determine trehalose activity, the extracts were incubated with trehalase (20 mU sample−1; Sigma), which hydrolyses trehalose to glucose, for 18 h at 37 °C. To quantify the amounts of glycogen, cell extracts were heated at 100 °C for 15 min. After the addition of 95 % ethanol crude glycog en was precipitated, dissolved in the reaction mixture containing glucoamylase (Abcam) and incubated for 30 min at room temperature. The amounts of glucose generated from either trehalose or glycogen were measured at 360 nm using a glucose (hexokinase) assay kit (Sigma). In control samples without trehalase and glucoamylase, amounts of pre-existing glucose were determined and subtracted from the total glucose level.

Immunodetection of oxidized proteins. Carbonyl groups, the major products of protein oxidation, were immunodetected after reaction with 2,4-dinitrophenylhydrazine (DNPH) (Levine et al., 1990). The bacteria were collected, washed in 10 mM Tris/HCl pH 8.0, resuspended in buffer A (0.5 M Tris/HCl pH 6.8, 6 % SDS, 10 mM EDTA) and lysed at 95 °C for 5 min. Aliquots of extracts or protein aggregates were incubated with 10 mM DNPH in 2 M HCl for 30 min at room temperature. After neutralization with 2 M NaOH, the proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Sambrook et al., 1989). Protein-bound 2,4-dinitrophenylhydrazones were detected using anti-2,4-dinitrophenylhydrazino-d-biotin) reacted specifically with AP sites in the genomic DNA. To investigate the influence of intracellular trehalose on persister formation during the stationary phase, we compared the frequency of persisters in the WT E. coli culture and the ΔotsA mutant, which lacks active trehalose-6-phosphate synthase. The intracellular concentration of trehalose in WT cells reached

antibiotic treatment (Table 1). Total cell counts were determined using a Neubauer chamber at a 1000-fold magnification. The level of dead cells was estimated using an epifluorescence microscope (Zeiss Axio Scope.A1) after staining with a LIVE/DEAD BacLight viability kit (Molecular Probes) according to the manufacturer’s protocol.

Detection of AP sites in the genomic DNA. Apurinic/apyrimidinic (AP) sites in DNA are one of the main types of damage generated by oxidative stress. AP sites are formed when oxidized bases are removed during the process of DNA repair (Kurisu et al., 2001). A QIAamp DNA Mini kit (Qiagen) was used for isolation of DNA (0.5 μg for the reaction). The AP sites were detected using a DNA Damage-AP sites– Assay kit (Abcam). In this assay a biotinylated aldehydereactive probe (ARP) reagent (N’-aminooxymethylcarbonylhydrazino-biotin) reacted specifically with AP sites, followed by colorimetric detection. Standard ARP-DNA was used for the calibration curve.

Isolation of protein aggregates. Cells were pelleted, converted into spheroplasts and lysed by sonication as described previously (Leszczynska et al., 2013). The cell extracts were incubated with 2 % Triton X-100 for 15 min at room temperature and subsequently loaded on a two-step sucrose gradient (1 ml 55 %, w/w, sucrose; 5 ml 17 %, w/w, sucrose). After 1.5 h ultracentrifugation (200000 g), a 1 ml sample containing insoluble proteins was collected from the bottom of the gradient.

RESULTS AND DISCUSSION

Intracellular trehalose inhibits persister formation in the early stationary phase culture of E. coli

All experiments were performed using LB medium that contained trehalose derived from yeast extract. Trehalose concentration in LB medium can be variable depending on the supplier and the batch of the yeast extract. It has been reported that trehalose content can range from 2 to 17 % in various batches of the extract (Zhang et al., 2003). Therefore, to obtain reproducible results, all the experiments were performed using one batch of yeast extract that ensured a relatively low concentration (0.014 %) of trehalose in the medium. To estimate the level of persisters in stationary cultures, we used Ofx, a DNA gyrase inhibitor that is able to kill non-growing cells. To investigate the influence of intracellular trehalose on persister formation during the stationary phase, we compared the frequency of persisters in the WT E. coli culture and the ΔotsA mutant, which lacks active trehalose-6-phosphate synthase. The intracellular concentration of trehalose in WT cells reached

Table 1. The levels of persister, nonculturable and dead cells in stationary and heat-shocked cultures. The frequency of persisters was estimated in relation to the total number of cells before Ofx treatment. The total number of cells ml−1 (100 %) was calculated using a haemocytometer. One hundred percent corresponded to 3.4×10⁸ cells (12 and 24 h cultures) and 2.4×10⁸ cells (50 °C).

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<th>Persister (%)</th>
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<td>37 °C (12 h)</td>
<td>0.04 ± 0.008</td>
<td>0.22 ± 0.03</td>
<td>2.5 ± 0.5</td>
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<td>37 °C (24 h)</td>
<td>0.69 ± 0.08</td>
<td>0.67 ± 0.1</td>
<td>8 ± 1</td>
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<td>30 °C → 50 °C</td>
<td>0.02 ± 0.005</td>
<td>0.23 ± 0.03</td>
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The MDA-TBA adducts were quantified colorimetrically at 532 nm. Standard MDA solutions were used for the calibration curve.

The levels of live and dead cells were estimated by epifluorescence microscopy (Zeiss Axio Scope.A1) after staining with a Live/Dead BacLight viability kit (Molecular Probes) according to the manufacturer’s protocol.

Light viability assay. The number of nonculturable cells was calculated by subtracting c.f.u. counts from the number of live cells; c.f.u. counts were estimated by plating serial dilutions on LA.
Trehalose affects formation of E. coli persisters

Intracellular trehalose inhibits the formation of E. coli persisters. E. coli WT and ΔotsA were grown for 24 h at 37 °C in LB medium. (a) The trehalose concentration was estimated using a trehalase assay. (b) At the times indicated, the cultures were diluted 1:100 in fresh LB medium and exposed to Ofx (5 μg ml⁻¹) for 4 h at 37 °C. Antibiotic-tolerant bacteria were plated for colony counting. One hundred percent corresponds to the number of colonies before antibiotic treatment. (c) Representative growth curves of WT and ΔotsA cells. (d, e) Stationary cultures (12 h) were diluted 1:100 and incubated with Ofx (5 μg ml⁻¹). Trehalose (0.2 %) was added to the ΔotsA strain 1 h before the culture was exposed to the antibiotic. (e) Complementation of the otsA mutant using the low-copy-number plasmid pGB-otsBA and empty plasmid pGB2 as a control. The bacteria were plated for colony counting at the times indicated on the graphs (d, e). The error bars indicate SD of three independent experiments. For (b), (d) and (e) a paired two-tailed t-test was used to determine statistical significance (*P<0.05; **P<0.02). In (d) and (e) the asterisks indicate significant differences between the mutant and the respective complementation strain.

Fig. 1

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In the course of the experiment. To rule out the possibility that the antibiotic-tolerant cells were resistant mutants, persister colonies were retested by replica plating on LB plates with Ofx. No Ofx-resistant colonies of either of the tested strains were detected. The most pronounced difference in the persister levels between the WT and ΔotsA cultures was observed by 12 h (Fig. 1b). At the 12 h time point, the frequency of persisters produced by the mutant strain was 10-fold higher than in the control WT culture. The Δots mutation did not affect the growth rate (Fig. 1c) but led to a significant rise in the level of nonculturable cells, whereas the number of dead bacteria in the mutant culture was only slightly increased at the end of the experiment (24 h) (Table 1). The frequency of persisters shown in Fig. 1(b) was estimated in relation to the number of c.f.u. before antibiotic treatment. Taking into account the presence of nonculturable and dead cells, the level of persisters was recalculated in relation to the total number of cells (Table 1). After 12 h, the frequency of persisters estimated in relation to the total cell number was higher in the mutant strain than in the WT culture, but it was comparable in both strains after 24 h.

In the WT culture (Fig. 1a), the level of persisters was maximal (Fig. 1b). We supposed that the lack of trehalose in ΔotsA cells would accelerate the appearance of persisters or would increase the frequency of persisters. It should be noted that low amounts of trehalose, 0.02–0.09 μmol (mg protein)⁻¹, were detected in ΔotsA cells (Fig. 1a). This might result from the uptake of trehalose that was present in the LB medium, as well as from an alternative pathway of trehalose synthesis from glycogen, which does not require the otsA gene product (Pan et al., 2008). The MICs of Ofx for both WT and ΔotsA were similar, indicating that the mutation did not affect intrinsic antibiotic resistance. In the mutant culture, persisters were already detected after 4 h (persisters constituted approximately 0.01 % of the ΔotsA culture), and their level was higher than in the WT culture during almost the entire course of the experiment. To rule out the possibility that the antibiotic-tolerant cells were resistant mutants, persister colonies were retested by replica plating on LB plates with Ofx. No Ofx-resistant colonies of either of the tested strains were detected. The most pronounced difference in the persister levels between the WT and ΔotsA cultures was observed by 12 h (Fig. 1b). At the 12 h time point, the frequency of persisters produced by the mutant strain was 10-fold higher than in the control WT culture. The Δots mutation did not affect the growth rate (Fig. 1c) but led to a significant rise in the level of nonculturable cells, whereas the number of dead bacteria in the mutant culture was only slightly increased at the end of the experiment (24 h) (Table 1). The frequency of persisters shown in Fig. 1(b) was estimated in relation to the number of c.f.u. before antibiotic treatment. Taking into account the presence of nonculturable and dead cells, the level of persisters was recalculated in relation to the total number of cells (Table 1). After 12 h, the frequency of persisters estimated in relation to the total cell number was higher in the mutant strain than in the WT culture, but it was comparable in both strains after 24 h.

In (d) and (e) the asterisks indicate significant differences between the mutant and the respective complementation strain.

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cells was only approximated. The bacteria of E. coli restored the ability of Amp (200 μg ml\(^{-1}\)). The bacteria were plated for colony counting at the times indicated on the graphs. (c,d) E. coli WT/pGB2, ΔotsA/pGB2 and ΔotsA/pGB2-otsBA were grown at 30 °C to an OD\(_{900}\) of 0.3 and then submitted to heat stress (50 °C, 15 min). (c) The frequency of persisters formed after heat shock was estimated as described in the legend to Fig. 1(b). (d) The intracellular trehalose concentration was estimated as described in Methods. The error bars indicate the standard deviations of three independent experiments. Statistical differences between samples were determined as described for Fig. 1 (*P<0.05; **P<0.01). Cultures of ΔotsA and WT were exposed to 50 °C were compared in (a) and (b).

**ΔotsA mutation influences persister frequency under heat stress**

We next asked the question whether the effect of the ΔotsA mutation on antibiotic tolerance can be observed not only in the stationary phase but also under other conditions that are known to induce trehalose synthesis, including desiccation, cold and heat stress (Elbein et al., 2003; Kandror et al., 2002; Zhang & Yan, 2012). In these experiments, exponentially growing cultures were tested. Therefore, Ofx and Amp were used to estimate persister levels. We found that the ΔotsA strain produced a considerably higher number of persisters than the WT strain after heat shock (Fig. 2a,b). To confirm that heat shock-induced persisters were not resistant mutants, the antibiotic-tolerant cells were replica plated on LB plates with Ofx or Amp. We did not detect any mutants. After cold stress and desiccation the differences between the strains were less pronounced or statistically insignificant (P>0.05, data not shown). After heat shock, the level of Ofx-tolerant cells in the ΔotsA culture was approximately 15-fold higher than in the WT strain (Fig. 2a), whereas the frequency of Amp-tolerant ΔotsA cells was only approximately threefold higher than in the WT culture (Fig. 2b). It is worth noting that the ΔotsA mutation caused a moderate increase in the number of nonculturable and dead cells after heat shock (Table 1). Complementation of the ΔotsA mutant with the pGB-otsBA plasmid fully restored the WT level of antibiotic susceptibility (Fig. 2c). The intracellular
concentration of trehalose in the WT/pGB2 cells significantly increased after heat shock from 0.01 to 0.9 µmol trehalose (mg protein)$^{-1}$ (Fig. 2d). Similar results were observed in the WT strain without vector (data not shown). It seems that a signal that stimulated the production of WT persisters at 50 °C was stronger than the inhibitory effect of intracellular trehalose observed when the WT culture entered the stationary phase (Fig. 1b,c). The $\Delta$otsA/pGB-otsBA culture accumulated up to 1.7 µmol trehalose (mg protein)$^{-1}$ (Fig. 2d) and generated only 0.08 and 0.09% Ofx- and Amp-tolerant cells, respectively. These results confirmed that the presence of intracellular trehalose inhibited the formation of persisters after heat shock.

**Low persister frequencies in $\Delta$glgA cells correlates with increased levels of intracellular trehalose**

UDP-glucose utilized for trehalose synthesis is also a substrate for glycogen production; therefore we suspected that a $\Delta$glgA mutant, which lacks glycogen synthase, would accumulate trehalose and in consequence produce fewer persisters. Indeed, we found that $\Delta$glgA cells overproduced trehalose [0.6 µmol (mg protein)$^{-1}$ as compared to 0.1 µmol mg$^{-1}$ in WT cells] and were unable to form persisters after heat shock and during the stationary phase (Fig. 3). On the other hand, $\Delta$otsA cells accumulated almost twofold more glycogen [70 µg (mg protein)$^{-1}$] than the WT bacteria [40 µg (mg protein)$^{-1}$]. Glycogen has been found to enhance the survival of cells (Montero et al., 2009; Chandra et al., 2011); hence, it was plausible that the $\Delta$otsA culture produced more persisters due to overproduction of glycogen. However, the double mutation $\Delta$glgA$\Delta$otsA, which abolished the synthesis of both trehalose and glycogen, restored the ability to produce persisters at 50 °C as well as during the stationary phase (Fig. 3). This result indicated that the increased frequency of persisters in the $\Delta$otsA culture was not related to the enhanced accumulation of glycogen.

**Lack of intracellular trehalose results in oxidative damage in E. coli cells**

Since it has been demonstrated that trehalose protects cells against oxidative damage (Elbein et al., 2003) and oxidative stress is implicated in persister formation (Hong et al., 2012; Leung & Lévesque, 2012; Vega et al., 2012; Wu et al., 2012), we supposed that the signal, which enhanced persister frequencies in the $\Delta$otsA cultures, was generated by oxidative stress. To verify this hypothesis, we first tested whether main macromolecules – proteins, DNA or lipids, were susceptible to oxidative damage in the absence of intracellular trehalose. We found that the main target for oxidative stress in the $\Delta$otsA strain was the unsaturated fatty acids in cell membranes (Fig. 4a). After heat shock and at the stationary phase, the level of lipid peroxidation in $\Delta$otsA cells was increased by approximately 50%, as compared to the WT strain. The levels of AP sites that are formed in the DNA after the removal of the oxidized bases (Kurisu et al., 2001) and of the oxidized proteins were significantly higher in the $\Delta$otsA strain than in the WT at the stationary phase (P<0.05), whereas at 50 °C no differences were observed (Fig. 4b,c). Our results are in agreement with previous studies showing that trehalose inhibited lipid peroxidation in Saccharomyces cerevisiae cells exposed to oxidative stress induced by menadione (da Costa Morato Nery et al., 2008; Herdeiro et al., 2006). The protection of membranes by trehalose can be based on the water replacement mechanism. Oku et al. (2003) demonstrated that in vitro, one trehalose molecule interacts specifically with one cis-double bond of unsaturated fatty acid and some hydroxyl groups. Trehalose can also act directly as a scavenger of hydrogen peroxide and superoxide anions (Elbein et al., 2003; Luo et al., 2008).

Previously, we found that the frequency of persisters correlated with the level of protein aggregates accumulated in E. coli stationary phase cultures (Leszczynska et al., 2013). It was also demonstrated that trehalose prevented protein aggregation in vivo (Leszczynska et al., 2013; Schultz et al., 2007; Singer & Lindquist, 1998). Therefore, we expected that the $\Delta$otsA mutant would accumulate more insoluble proteins, particularly under conditions that enhance persister frequency. However, the levels of proteins aggregated after heat shock or during the
stationary phase were comparable in both the WT and ΔotsA strains (Fig. 4d). Since it has been suggested that oxidized proteins are prone to denaturation and aggregation (Cecarini et al., 2007), it was possible that the ΔotsA mutation might promote aggregation of some oxidized proteins without influencing the total level of protein aggregates. However, we found that the aggregates contained only trace amounts of carbonylated proteins (compare Fig. 4c and d), indicating that the vast majority of oxidized proteins remained soluble in both strains. These results therefore revealed that the lack of intracellular trehalose in ΔotsA did not affect protein aggregation either after heat shock or during the stationary phase. The discrepancies between these and the previous results (Leszczynska et al., 2013; Schultz et al., 2007; Singer & Lindquist, 1998) could be explained by the fact that different conditions were used in the studies. For example, aggregation of recombinant proteins was investigated (Schultz et al., 2007; Singer & Lindquist, 1998) and trehalose was overproduced or externally added instead of being depleted (Leszczynska et al., 2013; Schultz et al., 2007). It is also possible that small soluble aggregates, which could not be isolated in a sucrose gradient, were formed in the mutant cells. Various studies suggest that protein misfolding and aggregation can lead to oxidative stress, lipid peroxidation and membrane lipid rearrangements (Bednarska et al., 2013). Ami et al. (2009) demonstrated that recombinant protein misfolding and aggregation caused a change in the lipid content and reduced E. coli membrane permeability. Interestingly, these changes were induced by small soluble protein complexes but not by large insoluble aggregates. Therefore, it is not
excluded that the increased lipid peroxidation in the ΔotsA mutant (Fig. 4a) was associated with the presence of soluble toxic protein aggregates.

ΔotsA culture overproduces extracellular indole which stimulates persisters formation

It was previously reported that persister formation can be stimulated by the signalling molecule indole via induction of the oxidative response (OxyR) and Psp pathway (Vega et al., 2012). On the other hand, it was demonstrated that tryptophanase TnaA, the enzyme which catalyses synthesis of indole, was overproduced under oxidative stress (Kuczynska-Wiśn, Pomposiello et al., 2001; Zheng et al., 2001). Hence, we supposed that oxidative stress in the ΔotsA strain activated the OxyR pathway, and additionally led to the overproduction of extracellular indole which in turn enhanced the oxidative response and antibiotic tolerance (Hirakawa et al., 2005; Vega et al., 2012). Indeed, we observed increased levels of extracellular indole in the stationary and heat-shocked ΔotsA/pGB cultures (Fig. 5). Moreover, in the ΔotsA/pGB-otsBA culture, in which trehalose synthesis was restored, the level of extracellular indole was significantly reduced (Fig. 5). Similar differences were observed between the ΔotsA and WT strains without plasmids (data not shown). The concentrations of extracellular indole in the stationary and heat-shocked ΔotsA/pGB cultures were higher by 0.13 and 0.2 mM, respectively, as compared to the WT/pGB strain (Fig. 5). To confirm that this excess of extracellular indole was sufficient to enhance persister frequencies in the ΔotsA culture, we estimated the level of persisters in WT cultures supplemented with adequate concentrations of indole (0.05–0.2 mM). We found that indole added exogenously to LB medium did not affect growth rate (data not shown) but significantly stimulated persister formation (Fig. 6a). We also found that the production of extracellular indole by WT cells was gradually reduced in the presence of increased amounts of trehalose added to the culture (Fig. 6b). To elucidate further the role of indole in the formation of ΔotsA persisters, we used the ΔotsAΔtnaA mutant, which was unable to synthesize both trehalose and indole. We supposed that if indole was required to enhance persister frequencies in the trehalose-deficient culture, the WT and ΔotsAΔtnaA strains would generate comparable levels of persisters. Indeed, the lack of indole in the double mutant culture reduced antibiotic tolerance (Fig. 6c), which was in agreement with earlier studies (Vega et al., 2013). However, the level of persisters was still higher in the ΔotsAΔtnaA culture submitted to heat stress than in the control WT strain (Fig. 6c).

In conclusion, we demonstrated that the intracellular trehalose protected E. coli cells against oxidative stress and thereby inhibited the formation of persisters at the stationary phase and after heat shock. In the absence of trehalose, ΔotsA cells were exposed to endogenous
oxidative stress associated with the overproduction of extracellular indole, which stimulated antibiotic tolerance. However, our results clearly indicated that other mechanisms, apart from indole signalling, could also be responsible for the enhanced formation of persisters when the trehalose level is low. Since the level of extracellular indole was strongly affected by the concentration of trehalose in a medium, we suppose that trehalose can influence not only antibiotic tolerance, but also other processes that are regulated by indole, including biofilm formation. Previously, we found that externally added trehalose caused reversion of persisters to β-lactam-sensitive cells (Leszczyńska et al., 2013). Allison et al. (2011) demonstrated that specific metabolites, including glucose and other sugars, induce proton motive force in dormant cells, thereby facilitating aminoglycoside uptake and eradication of persisters. Neither β-lactams nor quinolones kill persisters exposed to these metabolic stimuli. Therefore, trehalose, which increases Amp- and Ofx-tolerance, affects persisters most probably by a different mechanism. There is a growing interest in factors that sensitize persister bacteria to antibiotics and/or facilitate eradication of biofilms (Allison et al., 2011; Barraud et al., 2013; Conlon et al., 2013; Lebeaux et al., 2014; Kim et al., 2011; Leszczyńska et al., 2013). We believe that the results presented here may help to find new strategies for more efficient antibacterial therapies.

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