Escherichia coli Resistance to Chlorine and Glutathione Synthesis in Response to Oxygenation and Starvation

SÉBASTIEN SABY, PIERRE LEROY, AND JEAN-CLAUDE BLOCK

LCPE-LSE, UMR Université CNRS 7564, and Centre du Médicament-UPRES EA-ER 635, Faculté de Pharmacie, 54001 Nancy Cedex, France

Received 28 May 1999/Accepted 13 September 1999

Reduced glutathione (GSH) levels and resistance to chlorine were measured for two isogenic Escherichia coli strains stressed by oxygenation and/or starvation. The E. coli mutant deficient in GSH was not more sensitive to the oxidant than its parent strain when the bacteria were cultured with a low oxygenation rate. Starvation or oxygenation increased the resistance of the parent strain to chlorine, while the resistance of the deficient strain remained unchanged.

All aerobic organisms cope with reactive oxygen species, such as superoxide anions, hydrogen peroxide, and hydroxyl radicals, which accumulate in cells as products of the incomplete reduction of molecular oxygen (10, 14, 15). Cells are equipped with several defense systems to protect them from the harmful effects of these reactive oxygen species (36). They include antioxidant systems, such as reduced glutathione (GSH; l-γ-glutamyl-l-cysteinylglycine) and enzymes (catalases and superoxide dismutases). GSH is usually the most abundant intracellular low-molecular-weight thiol in Escherichia coli. It is a major component of all the cell systems involved in protection against oxidants and free-radical-mediated cell injuries, since the redox status (Eo’ = −240 mV) is mainly maintained by thiol-disulfide equilibrium inside the bacterial cell (2, 24, 32). GSH is also indirectly implicated in the regulation of the OxyR transcription factor (38), SoxR (7), and SOS (26) systems.

Recently, the question was raised of the benefit of such a protective system for microorganisms in specific environments, such as drinking water distribution networks, in which the bacteria grow in a low-nutrient environment (17, 33) that contains variable concentrations of oxidative species, such as chlorine used for postdisinfection (4). Water distribution networks are continuously colonized by autochthonous bacteria which are impossible to eradicate with the usual disinfectants. The limited transfer of oxidants through biofilms and bacterial envelopes (6, 25, 30, 31) and genetically controlled resistance are the two major explanations for this situation. Glutathione homeostasis and a high intracellular GSH level were recently proposed as a potential resistance pathway of E. coli cells to chlorine (5).

In this study we tested the validity of the hypothesis that oxygenation (oxygenation of the growth medium from 10% to saturation) and starvation (24 h of starvation in minimal medium) of cultures of E. coli lead to significant changes in glutathione homeostasis (concentration of GSH and its precursor, the γ-glutamylcysteine GluCyS) and hence modify E. coli sensitivity to further chlorine disinfection.

Bacterial models. Experiments were carried out with an isogenic pair of E. coli strains, since GSH was not detectable in indigenous biomass recovered from drinking water treatment plants. E. coli AB1157 and the GSH-deficient strain JTG10 (derived in Bruce Demple’s laboratory, Harvard University, Cambridge, Mass., reported to be deficient in GSH synthetase (13), were provided by Danièle Touati (Institut Jacques Monod, Paris, France). E. coli AB1157 and JTG10 were grown in completely mixed 5-liter chemostats (dilution rate, 0.05 h−1; 30 ± 1°C; regulated oxygen) and continuously fed with Luria-Bertani (LB) medium (Difco), and 50 μg of kanamycin monosulfate ml−1 (final concentration) was added every 24 h to the JTG10 culture. The steady-state bacterial density in the reactor was ca. 2 × 109 ml−1 (determined by 4,6-diamidino-2-phenylindole [DAPI] staining) (31), including about 50% culturable bacteria (counted as CFU after dilution in sterile 0.9% NaCl solution and incubation for 48 h at 30 ± 2°C in LB agar [Difco] containing 50 μg of kanamycin ml−1). The E. coli parental strain (AB1157) produced GSH (ca. 6 μmol/1012 cells, which is close to previously reported values) (13). A cellular GSH level was measurable in the mutant strain (JTG10), but the values were always low (5- to 35-fold lower than that of its parent strain) (Table 1). This fact could be explained by a mutation mechanism that was easily reversible even when the strain was cultured in the presence of kanamycin. Nevertheless, the difference between the GSH levels of the two strains was considered great enough to use them as an experimental model for the examination of the role of GSH in the response to stress factors.

Determination of thiol concentrations. Suspensions of bacteria (180 ml) were centrifuged at 10,000 × g for 20 min at 20°C. The pellets were washed twice by resuspending them in 0.9% NaCl and centrifuging them at 10,000 × g for 5 min at 20°C. Thiols were extracted from the bacteria by immediately suspending each pellet in 10 ml of 3.3% HClO4 containing 2 mM disodium EDTA, with vigorous vortexing and sonication for 5 min in an ultrasonic bath (Prolabo 670/H; power, 9) at 6°C. The suspensions were incubated for 10 min in an ice bath and centrifuged at 10,000 × g for 5 min at 4°C. The resulting supernatants were immediately frozen and stored in liquid nitrogen. The frozen samples were fast thawed in a water bath at 30°C and diluted with cold 0.1 M HCl containing 2 mM disodium EDTA (4°C), and a 50-μl aliquot was injected onto the column of the high-performance liquid chromatography system optimized for thiol measurement (18). This technique included reverse-phase separation, postcolumn derivatization with ortho-phthalaldehyde, and fluorimetric detection. The technique selectively measured the GSH and GluCyS. Total GSH (GSH−; GSH plus oxidized forms [symmetrical and mixed disulfides]) was measured by the same high-perfor-
mance liquid chromatography technique, but the samples were reduced with 1,4-dithiothreitol. GSH was expressed as GSH equivalents. Thiols concentrations were expressed as micromoles (or micromolar concentrations) per \(10^{12}\) total cells.

**Chlorination assay.** The washed bacterial suspension (20 ml) to be tested was filtered through a 5-μm-pore-size filter (Millipore) to remove or break up the bacterial aggregates. The filtered suspension was aseptically placed in 500-ml brown flasks (previously cleaned of trace organic matter by heating them to \(550^\circ\text{C}\) for \(4\) h) containing 400 ml of sterile phosphate-buffered saline (PBS). Sodium hypochlorite (20 mg liter \(^{-1}\)) was added to the diluted \(E.\ coli\) suspension (approximately \(10^6\) cells ml \(^{-1}\))-1) to obtain final concentrations of from \(2 \times 10^{-3}\) to 0.1 mg of Cl \(_2\) liter \(^{-1}\) (one flask used as a control was not spiked with chlorine). Solutions of sodium hypochlorite (Sigma Chemical Co.) (20 mg liter \(^{-1}\) ) were prepared daily in sterile bacterium-free distilled water and adjusted to pH 7.0 with dilute HCl. The sodium hypochlorite concentration was determined by the \(N,N\)-diethyl-p-phenylene diamine method (1), and the results were expressed in milligrams of chlorine liter \(^{-1}\). The flasks were incubated for 30 min at \(20^\circ\text{C}\) in the dark with gentle shaking (160 rpm); then traces of residual chlorine were neutralized by adding sterile sodium thiosulfate (final concentration, 0.02%) to all flasks. The culturable bacteria were then counted, and the number of surviving bacteria was determined as CFU per milliliter. Plate counts (CFU/milliliter) were log\(_{10}\) transformed, and the means and standard deviations of the means were calculated. The calculated means for each assay corresponding to each chlorine concentration tested were compared by one-way analysis of variance (\(P = 0.05\)) (11). Differences in the chlorine sensitivities of the two strains and the influence of oxygenation rates or starvation were then analyzed. All data were evaluated by analysis of variance testing with StatView F.4.5. (Abacus Concepts, Inc., Berkeley, Calif.).

**Effect of oxygenation on \(E.\ coli\) cultures.** The \(E.\ coli\) cultures were oxygenated with pure oxygen at 10, 50, 95, and 100% oxygen saturation of the medium (\(n = 1\) for each value). One hundred milliliters of steady-state AB1157 and JTG10 cultures was washed twice by centrifugation at 10,000 \(\times\) \(g\) for 5 min at \(20^\circ\text{C}\) and resuspended in 200 ml of sterile PBS (pH 6.5) (the washing protocol did not decrease the viable CFU [data not shown]). Samples (20 ml) were used for disinfection assays, and 180 ml was used to measure thiol concentrations. Changing the oxygenation rate of the parental \(E.\ coli\) growth cultures (AB1157) from 10 to 100% (Table 1) drastically increased its GSH content (threefold). There was a marked conversion of GSH to the oxidized form, as indicated by the decrease in the GSH/GSH\(_{\text{r}}\) ratio from 99 to 80%. The concentration of the GSH precursor, Glucys, also increased when the oxygenation rate was increased from 10 to 50% and decreased when the Table 1. Glucys, GSH, and GSH\(_{\text{r}}\) concentrations in \(E.\ coli\) AB1157 and JTG10 cultured under different oxygenation conditions

<table>
<thead>
<tr>
<th>(E.\ coli) strain</th>
<th>Oxygenation rate (%)</th>
<th>Glucys (μmol/10(^{12}) cells)</th>
<th>GSH (μmol/10(^{12}) cells)</th>
<th>GSH(_{\text{r}}) (μmol/10(^{12}) cells)</th>
<th>GSH/GSH(_{\text{r}}) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB1157</td>
<td>10 (n=1)</td>
<td>0.040</td>
<td>5.92 ± 0.59</td>
<td>6.51 ± 1.04</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>50 (n=3)</td>
<td>0.20 ± 0.08</td>
<td>7.59 ± 1.90</td>
<td>8.37 ± 2.17</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>95 (n=6)</td>
<td>0.19 ± 0.04</td>
<td>13.49 ± 0.62</td>
<td>16.92 ± 0.78</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>100 (n=3)</td>
<td>0.03 ± 0.01</td>
<td>1.11 ± 0.15</td>
<td>1.27 ± 0.15</td>
<td>74</td>
</tr>
<tr>
<td>JTG10</td>
<td>10 (n=1)</td>
<td>0.04</td>
<td>0.95</td>
<td>1.29</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>50 (n=3)</td>
<td>0.06 ± 0.03</td>
<td>1.37 ± 0.72</td>
<td>1.40 ± 0.65</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>95 (n=6)</td>
<td>0.07 ± 0.03</td>
<td>0.42 ± 0.13</td>
<td>0.49 ± 0.20</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td>100 (n=3)</td>
<td>0.02 ± 0.01</td>
<td>0.13 ± 0.01</td>
<td>0.16 ± 0.01</td>
<td>52</td>
</tr>
</tbody>
</table>

* The data are reported with standard deviations.
spite of a threefold decrease in GSH (Fig. 2A). The sensitivity of the mutant strain to chlorine did not change after starvation ($P, 95\%$) (Fig. 2B).

Starvation is one of the most important factors that influence the sensitivity of bacteria to disinfectants (3, 19, 22, 23, 28, 35). However, the way in which bacteria develop resistance is poorly understood. The consensus is that the resistance of starved bacteria to chlorine is primarily due to limited chlorine transfer to intracellular target bacteria because of changes in the cell membrane permeability (19, 37), accumulation of exopolymers on the bacterial cell surface (29), and/or formation of aggregates (12, 27, 34, 35). We have demonstrated that only the wild-type GSH-containing $E. coli$ strain increased its resistance to chlorine after 24 h of starvation, while the mutant strain without GSH did not develop such a resistance. These results suggest that GSH metabolism plays a role in the general starvation response, perhaps by activation of $\sigma^5$, the major regulator of the general starvation response in $E. coli$ produced by the rpoS gene (21). This potential action of GSH in the regulation of starvation has never been described, but it was demonstrated that GSH increased significantly during the transition from exponential to stationary phase (9, 20). More work is needed to understand the importance of GSH in the defense of starving cells against chlorine.

We conclude that starvation and oxidative stress cause $E. coli$ to become resistant to chlorine in less than 24 h. GSH plays a key role in the cell defense against chlorine, acting as an oxidant scavenger and activator of defense systems. The same pathways involving GSH are probably also implicated in defense against other disinfectants, such as chloramines, $\text{H}_2\text{O}_2$, and ozone, which all deplete the intracellular GSH pool ($5, 16$). Tap water treatment processing includes the reduction of
TABLE 2. Glucys, GSH, and GSH concentrations in E. coli AB1157 and JTG10 before and after 24 h of starvation in PBSa

<table>
<thead>
<tr>
<th>E. coli strain</th>
<th>Starvation</th>
<th>Glucys (μmol/1012 cells)</th>
<th>GSH (μmol/1012 cells)</th>
<th>GSHi (μmol/1012 cells)</th>
<th>GSH/GSHi (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB1157</td>
<td>Before</td>
<td>0.014 ± 0.002</td>
<td>6.99 ± 0.38</td>
<td>7.39 ± 0.39</td>
<td>94.6 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>After</td>
<td>0.07 ± 0.01</td>
<td>0.84 ± 0.06</td>
<td>2.48 ± 0.59</td>
<td>35.1 ± 8.6</td>
</tr>
<tr>
<td>JTG10</td>
<td>Before</td>
<td>0.005 ± 0.0003</td>
<td>1.27 ± 0.09</td>
<td>1.31 ± 0.10</td>
<td>97.0 ± 19.1</td>
</tr>
<tr>
<td></td>
<td>After</td>
<td>0.013 ± 0.003</td>
<td>0.12 ± 0.08</td>
<td>0.17 ± 0.08</td>
<td>66.6 ± 19.4</td>
</tr>
</tbody>
</table>

a The data are reported with standard deviations; n = 3.

organic matter and the inactivation of bacteria by ozonation and chlorination. The resulting low nutrient level and the initial exposure of bacteria to oxidants could make the final chlorination less effective. Consequently, the ability of E. coli to remain physiologically active and develop resistance to chlorine after sublethal stresses has potential public health implications and may require further changes in water treatment practices, particularly where the disinfectant residual in the water is less than 0.1 mg of chlorine/liter.

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