Research Article

Assessment of Damage to Nucleic Acids and Repair Machinery in Salmonella typhimurium Exposed to Chlorine

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Water disinfection is usually evaluated using mandatory methods based on cell culturability. However, such methods do not consider the potential of cells to recover, which should also be kept as low as possible. In this paper, we hypothesized that a successful disinfection is achieved only when the applied chlorine leads to both intracellular nucleic acid damage and strong alterations of the DNA repair machinery. Monitoring the SOS system responsiveness with a umuC-’lacZ reporter fusion, we found that the expression of this important cellular machinery was altered after the beginning of membrane permeabilization but prior to the total decline of both the cell culturability and the nucleic acid integrity as revealed by Sybr-II staining. Rapid measurement of such nucleic acid alterations by fluorochrome-based staining could be used as an alternative method for assessing the effectiveness of disinfection with chlorine.

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1. Introduction

Chlorine (a mixture of HClO and ClO⁻) is the most widely used bactericidal agent for disinfection of drinking waters. Chlorine reacts with various biological molecules: proteins [1–3], lipids [4, 5], and nucleic acids [6–9]. By way of consequence, this strong oxidant affects structures and several metabolic processes such as membrane permeability [10–12], ATPase activity [13, 14], respiration [11], and the proton motive force of the cell [15]. All these deleterious effects were previously shown to occur very rapidly [16, 17].

One of the problems related to water disinfection with chlorine is linked to the control of the effectiveness of disinfection, which requires carrying out mandatory methods such as culturing bacteria on standard nutritive agar media. These mandatory methods give delayed results and, additionally, do underestimate the real number of viable bacteria in drinking water, especially when oxidative stress has been applied [11, 18, 19]. Then, the question of an optimal and effective dose of disinfectant (the dose which should prevent the repair of injured cells and their regrowth) has been left unanswered both (i) because the key functions or structures to be irreversibly targeted by the disinfection process have not been defined yet, and (ii) because there is no accurate and rapid method currently available for detecting irreversible injuries to be used as an indicator of treatment effectiveness.

Reactivity of HClO at lethal concentrations with nucleic acids is governed by chlorine diffusion into the cells and its direct action on cell polymers as well as by reactive oxygen species generated upon exposure to the oxidant [17, 20, 21]. Moreover, chlorine attacks preferentially exocyclic-NH₂ groups of cytidine and adenosine at specific sites [8] and may also lead to DNA backbone cleavage [20, 22]. Saby et al. [23] first showed that chlorine-induced damage to nucleic acids could be revealed by the inability of fluorochromes, such as DAPI, to stain chlorinated bacteria. Other studies have corroborated this result and showed that chlorine reacting with nucleic acids in vitro and in vivo caused damage, thus resulting in a reduced fluorescence of the complex (nucleic acid + fluorochromes) stained with SYBR-II or propidium iodide (PI) [12, 24, 25].

A rapid analytical method which could confirm the irreversible and growth inhibitory nature of the damage suffered by chlorinated cells would clearly help practitioners...
to take the appropriate corrective actions to address various urgent needs (e.g., water disinfection, network cleaning, etc.). Therefore, there is a need for an alternative disinfection assessment method to be explored that would lie midway between usual methods such as culture, the limits of which are listed above, and methods measuring the complete ravages of cellular internal structure through observation of a fluorochrome staining drop.

In this paper, we hypothesized that a successful disinfection is achieved only when the applied chlorine concentration leads to both intracellular nucleic acid damage and strong alterations of the DNA repair machinery. Indeed, the inefficient fluorochrome staining of chlorinated bacteria could be used as a new criterion for a rapid water disinfection control. However, it does not give any indication on either the extent of the damage or its reversibility knowing that bacterial cells are equipped with a repair system. Therefore we investigated the effect of chlorine on the SOS system expression of Salmonella typhimurium (used as a laboratory model) and compared it to the loss of membrane permeability to propidium iodide (PI), DNA integrity assessed by Sybr-II staining, and bacterial culturability on nutritive agar medium. Finally, the pleiotropic effects of chlorine on various cell components are discussed, and we propose to rank these criteria for assessing disinfection efficiency and to define a threshold chlorine dose for a safe disinfection.

2. Materials and Methods

2.1. Bacterial Strain and Chlorine Treatment. All experiments were carried out using the strain TA1535/pSK1002 of Salmonella typhimurium [26], where the umuC‘-lacZ fusion of plasmid pSK1002 can be used as a reporter to monitor the induction of the SOS system by genotoxic agents. S. typhimurium was grown in stirred batch culture at 37°C in trypt-case soy agar (TSA) medium supplemented with 25 μg mL⁻¹ ampicillin, until OD₆₀₀ reached 0.4. Bacterial cells were washed twice in PBS medium and adjusted to 2.8 × 10⁸ bacteria mL⁻¹ in reverse osmosis water. Aliquots of the cell suspension were spiked with various concentrations of chlorine (commercial solution of bleach—Javel Jarrie water, Oxalis), ranging from 0.1 to 3 mg L⁻¹ (measured as Cl₂ by DPD method; Rodier [27]), and incubated for 90 minutes at 22 ± 1°C. A nonchlorinated control prepared in the same conditions was included in all assays. The pH of the assays ranged from 6.6 (nonchlorinated suspension) to 7.0 (chlorinated suspension with 3.5 mg Cl₂ L⁻¹). Although chlorine was very rapidly consumed in all assays (<2 minutes), all analyses were performed after any residual chlorine was neutralized by systematic addition of sodium thiosulfate.

2.2. Bacterial Counts. Bacterial cell culturability (Colony Forming Units, CFU) was estimated on TSA medium using plate count methods (incubation at 37°C for 72 hours), while total cell counts and membrane-altered cell counts were obtained by flow cytometry after staining, respectively, with Sybr-II and PI according to Phe et al. [12]. Additionally, the use of PI and Sybr-II fluorescent dyes allowed the assessment of nucleic acid integrity as previously shown by Phe et al. [12].

2.3. SOS/umu Chromotest Procedure. The bacterial response to DNA damage was assessed using the SOS reporter system of the strain by means of an “SOS umu-test” [26]. A 2 mL aliquot of the treated cells was buffered with 2 mL pH 7 PBS and was incubated at 37°C for 2 hours with 0.5 mL TSA medium to give the cells a chance to express the umuC‘-‘lacZ fusion. The induction level of the SOS system was then evaluated by assaying β-galactosidase specific activity according to Miller [28]. The reactivity of the SOS system was controlled in duplicate experiments by adding a known genotoxic agent, the 4-nitroquinoline-1-oxide (4-NQO, final concentration 50 ng mL⁻¹).

3. Results

3.1. Effect of Chlorine on Bacterial Counts and Fluorescence. The total number of fluorescent cells counted by flow cytometry after cell staining with SYBR-II decreased by 12% for low chlorine exposure (0.3 mg Cl₂ L⁻¹) compared to the nonchlorinated control (Figure 1). This initial drop, which had been previously reported with chlorinated water samples [12, 25], could result from an alteration of a subset of fragile cells. For higher chlorine concentrations, the number of fluorescent cells remained steady but the fluorescence of the bacteria stained with SYBR-II decreased significantly after application of 1.5 mg Cl₂ L⁻¹ (Figure 2).

Some cells were already detectable by PI staining before any chlorine treatment was applied (4% of the total cell
counts), indicating the occurrence of chlorine-independent membrane alterations in this laboratory-grown suspension (Figure 1). The increase in PI-positive cells in the suspension was found to be chlorine concentration dependent. At $3 \times 10^6$ bacteria mL$^{-1}$, virtually 100% of the cells were stained by PI (around $2.6 \times 10^6$ PI$^+$ cells mL$^{-1}$) indicating membrane permeation of the major part of the bacterial population (Figure 1). However the increase in the number of PI$^+$ cells was not proportionally correlated with the PI fluorescence increase (Figures 1 and 2), which could be explained by a partial alteration of the complex (PI + nucleic acid) formation in the Salmonella cells in agreement with the previous observations obtained with chlorinated Escherichia coli [25].

Without chlorine exposure, the culturable fraction of the Salmonella suspension represented no more than 35% of the total cell counts (Figure 1). The decrease in the number of colony forming units on TSA fits a simple inactivation kinetic model [29]. At $3 \times 10^6$ bacteria mL$^{-1}$, $3.6 \times 10^5$ bacteria mL$^{-1}$ were still able to form colonies on plates even though these culturable bacteria may have been initially permeabilized by chlorine.

3.2. Effect of Chlorine on the Expression of the SOS System of S. typhimurium. As previously mentioned, the effect of chlorine on the SOS system was monitored using an umuC’-lacZ fusion. When bacteria were solely exposed to chlorine, the β-galactosidase specific activity increased only slightly (1.6-fold) between 0 and $0.5 \text{ mg Cl}_2 \text{ L}^{-1}$ and went back to background level after treatment with $1.5 \text{ mg Cl}_2 \text{ L}^{-1}$ and over (Figure 3).

When nonchlorinated bacterial suspensions were treated with 4-NQO, a genotoxic agent, the β-galactosidase specific activity was 6- and 7-fold higher compared to the control without 4-NQO. For bacterial suspensions subjected to chlorination followed by a 4-NQO treatment, the β-galactosidase specific activity increased from 100 to 200 Miller units, until $[\text{Cl}_2] = 0.5 \text{ mg L}^{-1}$. This significant increase, compared to the assays without 4-NQO, showed that (i) the SOS system could still react after low chlorination (i.e., $<0.5 \text{ mg L}^{-1}$), and (ii) chlorine by itself had only a slight genotoxic effect. The synergistic effect occurring between chlorine and 4-NQO could be due to increased membrane permeability as demonstrated by PI fluorescence staining, leading in turn to better diffusion of the 4-NQO into the bacterial cells, and higher damage to the DNA.

From 0.5 to $1.5 \text{ mg Cl}_2 \text{ L}^{-1}$, the decrease in β-galactosidase specific activity, from 200 to 15 Miller units, could result from general chlorine cytotoxicity against cell machineries including the SOS system. For higher chlorine concentrations, the SOS specific activity remained at background level indicating that most of the cells were not able to respond anymore.

### 4. Discussion

The pleiotropic effect of chlorine results from its reactivity with numerous biological molecules (on the cell surface and inside the cell after rapid diffusion), causing alterations of cell functions and inhibiting the bacterial culturability. In our assays carried out with laboratory-grown bacteria, we identified two subgroups in the initial cell population, one being more sensitive to the chlorine treatment than the other. This “sensitive” subpopulation was “bleached” (undetectable by Sybr-II) with $0.5 \text{ mg L}^{-1}$ chlorine (Figure 1) while the remaining “resistant” subpopulation, representing about 88% of the initial cell population, persisted physically at chlorine concentrations as high as $3 \text{ mg L}^{-1}$. The culturable counts started to decrease at chlorine concentrations for which only the resistant subpopulation persisted, thus
suggesting that the sensitive subpopulation was already non-
culturable prior to chlorine treatment. PI staining showed
that before any chlorine treatment was applied, about 4% of
the initial cell population displayed altered membrane
properties. At this point, it is tempting to speculate that these
4% of membrane-damaged cells were part of cells forming
the sensitive subpopulation.

The decrease observed in the mean fluorescence of
Sybr-II-stained bacteria results from chlorine-damaged
nucleic acids (especially at 3 mg Cl₂ L⁻¹) as expected from
assays carried out with nucleic acid solutions [24], tap
water bacteria, and E. coli suspensions [12, 25]. The
increase in mean fluorescence after PI staining showed that
chlorine affected membrane permeability, as reported by
others [15], and allowed better diffusion of PI fluorochrome
into the bacterial cells. It should be noted that, as evidenced
by previous observations [25], the PI fluorescence plotted
in Figure 2 probably should have been higher as chlorinated
nucleic acids are not stained efficiently with propidium
iodide.

As revealed by the specific activity of β-galactosidase, low
chlorine exposure of cells had only a slight effect on the
SOS system expression which is in agreement with reports
from Le Curieux et al. [30], Thomas et al. [31], and Włod-
kowski and Rosenkranz [32]. The SOS system's functionality
was not altered for chlorine concentrations <0.5 mg L⁻¹
as shown by its significant increase after addition of [4-
NQO] = 50 ng mL⁻¹ or 100 ng mL⁻¹. The synergistic effect
of chlorine combined with 4-NQO could be due to higher
membrane permeability to 4-NQO as a result of chlorine
treatment. However, for concentrations >0.5 mg Cl₂ L⁻¹,
chlorine cytotoxicity overrides rapidly the responses of the
cells prohibiting any mutagenic effect measurement. Then
chlorine potential mutagenic activity cannot be compared
directly with that of less toxic or reactive agents [31]. The
decrease in β-galactosidase specific activity for [Cl₂]
>0.5 mg L⁻¹ can be the consequence of chlorine reacting with
various cellular targets. Then, this loss in specific activity
rather reflects the collapse of various cellular machineries,
including the SOS system itself.

Interestingly, beyond the shift point of 0.5 mg L⁻¹ of
chlorine a substantial decrease both in culturability and
SOS response was observed. This unexpected result sparks
a renew interest in the culture method as chlorine-stressed
nonculturable bacteria appear to be quite unable to repair
damage caused by chlorine. However, at a chlorine con-
centration of 3 mg L⁻¹, 3.6 × 10² bacteria mL⁻¹ were still
culturable and SOS expression was not measurable anymore.
Besides, only a partial reduction in the fluorescence of the
bacterial population stained with SYBR-II was recorded
for the same treatment (3 mg Cl₂ L⁻¹). This ranking of the
responsiveness of the different methods may be caused by
a relatively low sensitivity of the umu-test, especially with
chlorinated bacteria, compared to that of the plate count
method and by the partial alteration of nucleic acids by
chlorine in the cells, which may be stained even for higher
chlorine exposure. Nevertheless, whatever the mechanisms
of chlorine action, it appears that this oxidant causes an
immediate permeabilization of the cell envelopes combined
with a loss in culturability and, at the very least for higher
chlorine concentrations, a significant loss in fluorescence for
the fluorochromes that stain nucleic acids.

5. Conclusions

This study has shown that shock chlorination on a relatively
dense laboratory-grown bacterial population has pleiotropic
effects on bacterial cells at the different levels of cellular
organization. On the one hand, chlorine reacts at the
bacterial cell surface increasing membrane permeability as
revealed by a rise in the number of PI+ cells and in the mean
fluorescence of PI-stained cells. On the other hand, chlorine
diffuses into the cell and damages polymers, such as nucleic
acids, as shown by a decrease in the mean fluorescence of
Sybr-II-stained cells. Additionally, chlorine spoils the cellular
machinery expression and de facto the SOS system expres-
sion. These new results support our initial hypothesis that
efficient and safe disinfection (i.e., low risk of bacterial repair
and regrowth) is definitively achieved only when a dramatic
reduction in the fluorescence of DNA/RNA fluorochromes
that stain bacterial cells is observed. Rapid measurement of
such nucleic acid alterations by fluorochrome-based staining
(results obtained within 1 hour) can be proposed as a
new alternative method for assessing the effectiveness
of disinfection.

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References

[1] R. W. R. Baker, “Studies on the reaction between sodium
hypochlorite and proteins,” Biochemical Journal, vol. 41, pp.
337–342, 1947.
[2] C. L. Hawkins, D. I. Pattison, and M. J. Davies, “Hypochlorite-
induced oxidation of amino acids, peptides and proteins,”
Amino Acids, vol. 25, no. 3-4, pp. 259–274, 2003.
[3] E. L. Thomas, “Myeloperoxidase, hydrogen peroxide, chloride
antimicrobial system: nitrogen-chlorine derivatives of bacte-
rial components in bactericidal action against Escherichia coli,”
Infection and Immunity, vol. 23, no. 2, pp. 522–531, 1979.
[4] J. J. M. van den Berg, C. C. Winterbourn, and F.
A. Kuypers, “Hypochlorous acid-mediated modification of
cholesterol and phospholipid: analysis of reaction products
by gas chromatography–mass spectrometry,” Journal of Lipid
Research, vol. 34, no. 11, pp. 2005–2012, 1993.
[5] C. C. Winterbourn, J. J. M. van den Berg, E. Roitman, and F. A. Kuypers, “Chlorohydrin formation from unsaturated fatty acids reacted with hypochlorous acid,” *Archives of Biochemistry and Biophysics*, vol. 296, no. 2, pp. 547–555, 1992.

[6] C. Bernofsky, “Nucleotide chloramines and neutrophil-mediated cytotoxicity,” *FASEB Journal*, vol. 5, no. 3, pp. 295–300, 1991.

[7] J. J. Burrows and G. J. Muller, “Oxidative nucleobase modifications leading to strand scission,” *Chemical Reviews*, vol. 98, no. 3, pp. 1109–1151, 1998.

[8] C. L. Hawkins and J. M. Davies, “Hypochlorite-induced damage to nucleosides: formation of chloramines and nitrogen-centered radicals,” *Chemical Research in Toxicology*, vol. 14, no. 8, pp. 1071–1081, 2001.

[9] W. A. Prütz, “Interactions of hypochlorous acid with pyrimidine nucleotides, and secondary reactions of chlorinated pyrimidines with GSH, NADH, and other substrates,” *Archives of Biochemistry and Biophysics*, vol. 349, no. 1, pp. 183–191, 1998.

[10] C. Venkobachar, L. Iyengar, and A. V. S. Prabhakara Rao, “Mechanism of disinfection: effect of chlorine on cell membrane functions,” *Water Research*, vol. 11, no. 8, pp. 727–729, 1977.

[11] A. K. Camper and G. A. McFeters, “Chlorine injury and the enumeration of waterborne coliform bacteria,” *Applied and Environmental Microbiology*, vol. 37, no. 3, pp. 633–641, 1979.

[12] M.-H. Phe, M. Dossot, H. Guilloteau, and J.-C. Block, “Nucleic acid fluorochromes and flow cytometry prove useful in assessing the effect of chlorination on drinking water bacteria,” *Water Research*, vol. 39, no. 15, pp. 3618–3628, 2005.

[13] W. C. Barrette Jr., D. M. Hannum, W. D. Wheeler, and J. K. Hurst, “General mechanism for the bacterial toxicity of hypochlorous acid: abolition of ATP production,” *Biochemistry*, vol. 28, no. 23, pp. 9172–9178, 1989.

[14] D. M. Hannum, W. C. Barrette Jr., and J. K. Hurst, “Subunit sites of oxidative inactivation of *Escherichia coli* F1-ATPase by HOCl,” *Biochemical and Biophysical Research Communications*, vol. 212, no. 3, pp. 868–874, 1995.

[15] W. C. Barrette Jr., J. M. Albrich, and J. K. Hurst, “Hypochlorous acid-promoted loss of metabolic energy in *Escherichia coli*,” *Infection and Immunity*, vol. 55, no. 10, pp. 2518–2525, 1987.

[16] J. M. Albrich and J. K. Hurst, “Oxidative inactivation of *Escherichia coli* by hypochlorous acid. Rates and differentiation of respiratory from other reaction sites,” *FEBS Letters*, vol. 144, no. 1, pp. 157–161, 1982.

[17] S. Dukan, S. Dadon, D. R. Smulski, and S. Belkin, “Hypochlorous acid activates the heat shock and soxRS systems of *Escherichia coli*,” *Applied and Environmental Microbiology*, vol. 62, no. 11, pp. 4003–4008, 1996.

[18] M. R. Barer and C. R. Harwood, “Bacterial viability and culturability,” *Advances in Microbial Physiology*, vol. 41, pp. 93–137, 1999.

[19] R. R. Colwell and D. J. Grimes, Eds., *Non-Culturable Microorganisms in the Environment*, ASM Press, Washington, DC, USA, 2000.

[20] S. Dukan and D. Touati, “Hypochlorous acid stress in *Escherichia coli*: resistance, DNA damage, and comparison with hydrogen peroxide stress,” *Journal of Bacteriology*, vol. 178, no. 21, pp. 6145–6150, 1996.

[21] S. Dukan, S. Belkin, and D. Touati, “Reactive oxygen species are partially involved in the bacteriocidal action of hypochlorous acid,” *Archives of Biochemistry and Biophysics*, vol. 367, no. 2, pp. 311–316, 1999.