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PEER REVIEW SNAPSHOT

TITLE: The Biocide Chlorine Dioxide Stimulates Biofilm Formation in *Bacillus subtilis* by Activation of the Histidine Kinase KinC

Moshe Shemesh, Roberto Kolter, and Richard Losick – Dec 2010

INTRODUCTION

This study, originally published in the *Journal of Bacteriology*, was conducted in order to investigate the effects of ClO2, a known biocide, on biofilm growth. Biofilm is a collection of different types of bacteria, fungi, and protists in a colony enveloped in secreted slime that allows them to adhere to moist surfaces ideal for growth. Biofilm is one of the most common techniques for growth and protection for these microorganisms, and is not only a nuisance, but a potential hazard.

PURPOSE

The purpose of this research was to evaluate the effect of chlorine dioxide on biofilm growth across multiple bacterial species and environments.

METHOD

- The researchers isolated multiple strains of biofilm-producing bacteria and allowed to proliferate to an observable culture in two different mediums – the biofilm-inducing medium MSgg, and typically biofilm inhibiting medium, TSS.
- The cultures were treated with chlorine dioxide and allowed to incubate for 3 days.
- The cultures were then tested for growth of biofilms.

RESULTS

- The research confirms that sublethal doses of chlorine dioxide accelerates the formation of biofilm across multiple species of bacteria.
- The researchers found that biofilm grew in both the typically biofilm inducing medium as well as the biofilm-inhibiting medium at an exponential rate.
- It was further inspected that chlorine dioxide causes the activation of KinC in the cells of the bacteria, triggering and inducing biofilm formation.
- Biofilms protect microorganisms like bacteria from biocides.

NOTEWORTHY

- In application, if a chlorine dioxide dosing system were to experience mechanical or human error, the plumbing system associated would experience amplification of both biofilm and bacterial growth.
- The researchers also tested another biocidal oxidant, hydrogen peroxide, and interestingly found that it did not accelerate biofilm formation, suggesting that this issue is unique to chlorine dioxide.

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The Biocide Chlorine Dioxide Stimulates Biofilm Formation in *Bacillus subtilis* by Activation of the Histidine Kinase KinC[▿] [†]

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ABSTRACT

Bacillus subtilis forms biofilms in response to signals that remain poorly defined. We report that biofilm formation is stimulated by sublethal doses of chlorine dioxide (ClO_2), an extremely effective and fast-acting biocide. ClO_2 accelerated biofilm formation in *B. subtilis* as well as in other bacteria, suggesting that biofilm formation is a widely conserved response to sublethal doses of the agent. Biofilm formation depends on the synthesis of an extracellular matrix that holds the constituent cells together. We show that the transcription of the major operons responsible for the matrix production in *B. subtilis*, *epsA-epsO* and *yqxM-sipW-tasA*, was enhanced by ClO_2, in a manner that depended on the membrane-bound kinase KinC. Activation of KinC appeared to be due to the ability of ClO_2 to collapse the membrane potential. Importantly, strains unable to make a matrix were hypersensitive to ClO_2, indicating that biofilm formation is a defensive response that helps protect cells from the toxic effects of the biocide.

The spore-forming bacterium *Bacillus subtilis* can form structurally complex, multicellular communities at air/liquid interfaces (3, 10). These floating biofilms, known as pellicles, consist of long chains of cells that are held together by an extracellular matrix (3). Production of the matrix is governed by an intricate regulatory network, at the heart of which is the transcriptional repressor SinR, which directly binds to the promoters of the *epsA-epsO* and *yqxM-sipW-tasA* matrix operons and an additional regulatory gene, *slrR* (6, 7, 12). At the initiation of biofilm formation, SinR is sequestered by its antagonist SinI, resulting in the derepression of the matrix and the *slrR* gene (7, 12). SlrR, in turn, sets in motion a self-reinforcing, double-negative feedback loop that augments matrix production and promotes cell chaining (4). Whereas SinR is produced constitutively, SinI is produced under the positive control of the phosphorylated form of the transcription factor Spo0A (18). Spo0A is phosphorylated via a multiple-component phosphorelay by four principal histidine kinases, KinA, KinB, KinC, and KinD (11, 13).

Current thinking in the field is that the kinases respond to different environmental signals, but the nature of these signals and how the kinases respond to them are not known in most cases. Some progress has been made in the case of the membrane-bound kinase KinC, which is indirectly activated by the cyclic lipopeptide surfactin (14). Surfactin is both a surfactant and a quorum-sensing signaling molecule that apparently exerts its indirect effect through its ability to cause potassium leakage (14). Just how potassium leakage leads to KinC activation is not known, but other unrelated natural products that cause potassium leakage also activate KinC and trigger biofilm formation. Here we report that chlorine dioxide (ClO_2), an extremely effective and fast-acting biocide, is a potent stimulator of biofilm formation at sublethal doses. We further report that ClO_2 works by activating KinC in a manner that is associated with a reduction in membrane potential. Finally, we show that biofilm formation is a defensive response that helps protect cells from the toxic effects of the biocide.

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MATERIALS AND METHODS

Strains and growth media.

Strains used in the study are listed in Table S1 in the supplemental material and were isogenic other than as indicated. For routine growth all the strains were propagated in Luria-Bertani broth (LB; 10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter) or on solid medium containing LB supplemented with 1.5% agar. The *B. subtilis* wild-type (WT) strain NCIB3610 and its derivatives were regularly cultured in LB medium. The biofilms were generated in either MSgg minimal medium (5 mM potassium phosphate, pH 7, 100 mM MOPS [morpholinepropanesulfonic acid], pH 7, 2 mM $MgCl_2$, 700 μM $CaCl_2$, 50 μM $MnCl_2$, 50 μM $FeCl_3$, 1 μM $ZnCl_2$, 2 μM thiamine, 0.5% glycerol, 0.5% glutamate) or TSS glucose minimal medium (50 mM Tris [pH 7.5], 37 mM NH_4Cl, 0.035% $K_2HPO_4·3H_2O$, 0.004% $FeCl_3$, 0.004% trisodium citrate dihydrate, 1 mM $MgSO_4·7H_2O$, 0.1% glutamine, 0.5% glucose). For assaying pellicle formation, the cells were grown to exponential growth phase, washed in phosphate-buffered saline (PBS; pH 7) (the buffer was autoclaved and filtered through a 0.22-μm Corning filter system prior to use), and inoculated into either MSgg broth or TSS glucose minimal medium. The cells were grown to early log phase (optical density at 600 nm [OD_{600}] of ~0.1), treated with freshly made ClO_2 at indicated concentrations, and incubated at 22°C for 3 days in Falcon Multiwell plates or for 2 days in glass tubes. (Because the cells were not collected by centrifugation and washed, the ClO_2 was expected to remain active in the cell suspensions for a prolonged period.)

The broth microdilution method (20) was used to determine the MIC, the lowest concentration of ClO_2 inhibiting visible growth of bacteria after overnight incubation. For a coculture experiment, the *lacZ*-bearing wild-type (WT) and unlabeled mutant cells were grown as separate cultures, centrifuged, and washed in PBS. Equal volumes of the washed suspensions of the cells were mixed to create the coculture, which was grown to exponential phase. One portion of the coculture was treated with ClO_2 (16 μg/ml), and the other portion was left untreated. The cell mixtures were then plated out on LB solid medium containing 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-Gal; Sigma) at a final concentration of 40 μg/ml. The cell ratio in ClO_2-

treated as well as untreated cocultures was determined from the numbers of WT and mutant CFU.

Preparation of ClO_2 solution.

The stabilized ClO_2 S-TAB10 tablets (BASF, Florham Park, NJ) were dissolved in 500 ml deionized water for preparing stock solutions of approximately 450 μg/ml. The concentration of stock solution was measured using the colorimetric method with the Pocket Colorimeter II analysis system (Hach Company, Loveland, CO). To ensure the accuracy of ClO_2 concentrations, we routinely used freshly prepared stock solutions of ClO_2 and the concentrations were determined before each experiment.

Flow cytometry.

The membrane potential of the cells was assayed by flow cytometry using the Bac*Light* bacterial membrane potential kit (Molecular Probes) according to the manufacturer's instructions. The metabolically active bacteria generate a membrane potential of approximately −100 mV; the diethyloxacarbocyanine dye $DiOC_2$ (3,3′-diethyloxacarbocyanine iodide), which allows the variation in cell size to be normalized by analysis of the ratio of red fluorescence to green fluorescence, was used to report changes across the range of −30 to −130 mV. Cells that had been grown to late exponential phase were diluted to an OD_{600} of 0.1 in PBS and treated with 1, 2, and 4 μg/ml of freshly prepared ClO_2. As a positive control for depolarization, we used 10 μl of 500 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), and as a negative control, the cells were untreated. The samples were analyzed using a BD LSR II flow cytometer (BD Biosciences) with a 488-nm excitation and emission filter, which was suitable for fluorescein and Texas Red dye. For each sample approximately 20,000 events were collected at the low flow rate, and the signal was acquired with logarithmic amplification. Data were captured using FACS Diva software (BD Biosciences) and further analyzed using FlowJo 8.5.2 software.

Microscopy analysis.

For fluorescence microscopy analysis, the cells were grown in MSgg broth to early exponential phase (OD_{600} of ~0.1), treated with 4 μg/ml ClO_2, and then further incubated to late exponential phase. Afterwards 1 ml of the treated and untreated cultures was harvested and centrifuged. Cells were washed with cold PBS buffer and resuspended in 50 μl cold PBS buffer. Three microliters of resuspended cells was placed on the center of an agar-coated microscopy slide (VWR; catalogue number 48311-702) and covered by an 0.15-mm microscopy cover slide (VWR; catalogue number 48366-045). Cover slides were pretreated with poly-l-lysine as previously described (9). Samples were examined using an Olympus workstation BX61 microscope. Images were taken and analyzed using an automated software program, SimplePCI. For assaying cell chaining during pellicle development, cells were collected from pellicle-forming wells after 1 day of incubation and were washed with cold PBS buffer. Cells were suspended in 50 μl of cold PBS buffer and were analyzed using phase-contrast microscopy.

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RESULTS

Chlorine dioxide accelerates biofilm formation.

As a starting point, we determined that ClO_2 was lethal at concentrations above 32 µg/ml in the biofilm-inducing medium MSgg, as judged by measuring growth rate and MIC. Next, we investigated the effect of a sublethal dose (4 µg/ml) of ClO_2 on biofilm formation. Figure Figure11 shows that ClO_2 treatment stimulated the formation of a thick, floating biofilm (pellicle) (Fig. 1A and D) composed of bundled chains of cells (Fig. (Fig.1B).1B). Sublethal doses of ClO_2 also stimulated biofilm formation in the glucose minimal medium TSS, which ordinarily does not induce biofilm formation effectively (Fig. (Fig.1C).1C). Interestingly, these effects of ClO_2 were not restricted to *B. subtilis*, as a similar effect was seen with *Pseudomonas aeruginosa* (see Fig. S1 in the supplemental material). We conclude that bacterial biofilm formation is a widely conserved response to sublethal doses of ClO_2.

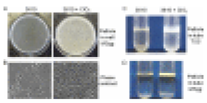

FIG. 1.

The effect of sublethal doses of ClO_2 on *B. subtilis* 3610. (A) ClO_2 (4 µg/ml) accelerates pellicle formation during the static growth in MSgg medium in polystyrene multiwell plates. (B) Phase-contrast images of cells, collected from a pellicle **...**

Chlorine dioxide stimulates matrix gene transcription.

A defining feature of biofilm formation is the synthesis of an extracellular matrix that binds the constituent cells together. In *B. subtilis* biofilms the matrix consists of an exopolysaccharide (EPS) and an amyloid-like fiber composed of the protein TasA (2, 3, 17). The operon responsible for the production of the exopolysaccharide is *epsA-epsO*, and the operon encoding TasA and responsible for the production of the fibers is *yqxM-sipW-tasA* (5). Under conditions that promote biofilm formation, a subpopulation of cells expresses these two operons to high levels (5, 14, 19). We hypothesized that the ability of ClO_2 to augment biofilm formation was due to upregulation of the genes involved in matrix synthesis. To test this hypothesis, we analyzed the effect of ClO_2 on matrix gene expression by using transcriptional fusions of the promoters for *epsA-epsO* and *yqxM-sipW-tasA* to genes encoding fluorescent proteins. Fluorescence microscopy showed that ClO_2 treatment markedly increased the number of cells expressing P_{epsA}-*gfp* and P_{yqxM}-*cfp* and their fluorescence intensity (Fig. (Fig.22).

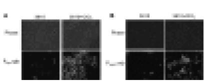

FIG. 2.

ClO_2 stimulates transcription of the *eps* and *yqxM* operons. Fluorescence microscopy of wild-type cells demonstrating the induction in expression of P_{eps}-*gfp* (A) and P_{yqxM}-*cfp* (B) in the presence of 4 µg/ml ClO_2. Samples were examined using an Olympus **...**

Chlorine dioxide is sensed by KinC.

Next, we investigated the step in the biofilm regulatory circuit at which ClO_2 acts. A potential candidate was the histidine kinase KinC, as we explain. Part of the signaling circuitry that regulates biofilm formation involves two sequentially acting signaling molecules, ComX and surfactin (15, 16). The prenylated peptide ComX activates the membrane histidine kinase ComP. ComP, in turn, phosphorylates the transcriptional factor ComA, resulting in the activation of a regulon that includes the *srf* operon, which is responsible for the synthesis of the cyclic lipopeptide surfactin (8, 16). Surfactin is a quorum-sensing molecule that activates KinC by causing the leakage of K^+ ions from across the cytoplasmic membrane (14). KinC, in turn, phosphorylates the response regulator Spo0A via a multicomponent phosphorelay. Finally, phosphorylated Spo0A (Spo0A~P) turns on the synthesis of SinI, an antirepressor for SinR, a repressor of the *epsA-epsO* and *yqxM-sipW-tasA* operons (12, 14). In sum, this biofilm-inducing pathway involves a linear sequence in the order ComX, ComP, surfactin, KinC, Spo0A, SinI, SinR, and the matrix operons. Given that the nonspecific oxidative activity of ClO_2 is known to cause membrane damage (1, 21) and given that surfactin acts at the membrane to cause K^+ leakage (13), we reasoned that ClO_2 might be accelerating biofilm formation by causing ion leakage and thereby stimulating the activity of KinC.

As a first test of this hypothesis and to pinpoint the step in the pathway at which ClO_2 might be acting, we examined the effect of the biocide on mutants of *comP*, *srfAA* (one of the genes involved in surfactin synthesis), *kinC*, *spo0A*, *sinI*, and *epsH* and on an *epsH tasA* double mutant. The results showed that sublethal doses of ClO_2 accelerated biofilm formation by the *comP* and *srfAA* mutants but not by the *kinC*, *spo0A*, *sinI*, *epsH*, and *epsH tasA* mutants (Fig. (Fig.3).3). We conclude that ClO_2 acts just upstream of KinC, presumably by stimulating KinC activity in a surfactin-independent manner. The dependence on KinC was specific in that KinA, KinB, and KinD mutants were unaffected in their response to ClO_2 (data not shown).

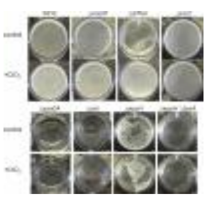
FIG. 3.
ClO_2 acts upstream of KinC in biofilm regulatory circuitry. ClO_2 (4 μg/ml) induces pellicle formation by *srfAA* and *comP* mutants but not by *kinC*, *spo0A*, *sinI*, or *epsH* mutants or an *epsH tasA* double mutant.

Next, we tested the sensitivity of the *kinC* mutant to ClO_2. Growth curve analyses (Fig. (Fig.4)4) as well as MIC experiments (data not shown) revealed that the *kinC* mutant strain was particularly sensitive to ClO_2, consistent with the idea that KinC is responsible for stimulating biofilm formation as a protective response to ClO_2.

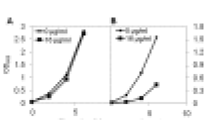
FIG. 4.

A *kinC* mutant is sensitive to ClO_2 stress. Growth curves of *B. subtilis* 3610 (A) and *ΔkinC* (B) strains grown in MSgg medium at 37°C in shaking culture.

Chlorine dioxide disrupts membrane potential.

As a further test of the idea that sublethal doses of ClO_2 activate KinC by causing ion leakage across the membrane, we asked whether the biocide impairs membrane potential. To investigate changes in membrane potential as a consequence of ClO_2 treatment, we carried out flow cytometry analyses using the carbocyanine dye $DiOC_2$ (3,3′-diethyloxacarbocyanine iodide). Cells with a normal membrane potential fluoresce red, and those with impaired potential fluoresce green. The results show that there was a significant decrease in the red/green fluorescence ratio in cells treated with ClO_2, a finding consistent with the idea that KinC is activated by the decrease in membrane potential caused by ClO_2 (Fig. (Fig.5).5). In sum, we propose that alterations in membrane potential, caused by ClO_2 treatment, are sensed by KinC as a stress signal that induces biofilm formation (Fig. (Fig.66).

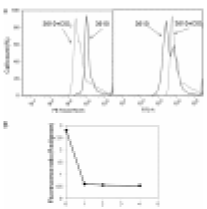

FIG. 5.
Flow cytometry analysis of *B. subtilis* 3610 cells. (A) The cells were stained with the carbocyanine dye $DiOC_2$ and analyzed with a BD LSR II flow cytometer using a 488-nm excitation and emission filter suitable for fluorescein and Texas Red dye. The ClO **...**

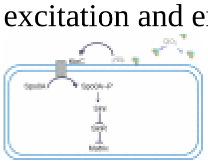

FIG. 6.
Model for the induction of biofilm formation by ClO_2. A decrease in membrane potential caused by sublethal doses of ClO_2 is sensed as an emergency signal by KinC, which induces the phosphorylation of Spo0A, which, in turn, stimulates the expression of **...**

Mutants blocked in matrix production are sensitive to chlorine dioxide.

Given that the matrix operons are induced by ClO_2 treatment, we asked whether mutants unable to make a matrix are more sensitive to ClO_2 than are wild-type cells. We addressed this question by applying an agar diffusion test, which demonstrated that the *epsH* mutation significantly enhanced sensitivity to ClO_2 (Fig. (Fig.7).7). In addition, we carried out a coculture survival experiment in which we treated a mixture of wild-type (3610) cells and cells with mutations of *epsH* or *spo0A* (Table (Table1)1) that had been grown in shaking culture in biofilm-inducing medium (MSgg). Assuming that there is no matrix sharing in shaking culture, the EPS produced by wild-type cells would not be expected to provide protection to mutant cells in *trans*. We distinguished the two kinds of cells by using a *lacZ* reporter. The results show that cells unable to make matrix were approximately 10-fold more sensitive to ClO_2 than were wild-type cells (Table

(Table1).1). Staining with crystal violet followed by treatment with 20% copper sulfate solution revealed a halo of exopolysaccharide surrounding the wild-type cells (Fig. (Fig.8).8). We propose that the exopolysaccharide helps to prevent ClO_2 from reaching the cytoplasmic membrane.

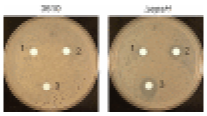

FIG. 7.
Killing of matrix production mutant cells by ClO_2. Antibiogram showing the susceptibility of strains to discs impregnated with ClO_2 (disc 1, 50 μg/ml; disc 2, 100 μg/ml; disc 3, 200 μg/ml) on MSgg agar plates.

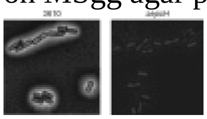

FIG. 8.
A halo of exopolysaccharide surrounds 3610 cells. Transmitted light images of the cells grown to late log phase in shaking culture and stained with crystal violet followed with treatment with 20% copper sulfate solution. Samples were visualized **...**

TABLE 1.

ClO_2 preferentially kills cells mutant for matrix production[a]

Coculture[b]	Cell ratio[c]	
	In untreated culture	After treatment
3610/ *epsH* mutant	0.72	6.4
3610/ *spo0A* mutant	0.90	9.9

[a]The experiment assumes that, in shaking culture, the EPS produced by wild-type cells did not provide protection to mutant cells in trans.
[b]*lacZ*-bearing 3610 cells were mixed with unlabeled mutant cells, and the mixture was grown to exponential phase and then split in two, with one portion being treated with ClO_2 (16 g/ml) and the other portion being left untreated.
[c]Determined by measuring the numbers of wild-type and mutant CFU.

TABLE 1.
ClO_2 preferentially kills cells mutant for matrix production[a]
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DISCUSSION

The principal finding of this study is that sublethal doses of ClO_2 accelerate biofilm formation, not only in *B. subtilis* but in other bacteria as well. We showed that ClO_2 acted via KinC to induce expression of the genes involved in matrix production. These results thus indicate that biofilm formation is a response to the stress caused by ClO_2. The response was selective in that another oxidant, hydrogen peroxide, did not accelerate biofilm formation at sublethal doses. It is curious that both surfactin and ClO_2 exert their effects via KinC. Both are membrane active but in different ways. Surfactin causes selective potassium ion leakage whereas ClO_2 causes a collapse in membrane potential. An important challenge for the future will be to elucidate how KinC senses membrane perturbations.

In keeping with the idea that biofilm formation is a stress response, a coculture experiment demonstrated that matrix production confers partial protection against ClO_2. Staining with copper sulfate revealed a halo of exopolysaccharide, leading us to propose that this matrix component provided a protective barrier against oxidative damage by the biocide. Once again, the effect was selective in that hydrogen peroxide did not discriminate between a matrix production mutant and the wild type in a coculture survival experiment as well as in an agar diffusion test (data not shown). *In toto*, these findings are consistent with the idea that ClO_2 acts primarily at the membrane whereas at sublethal doses hydrogen peroxide acts on targets inside the cell. Our findings are also in keeping with the work of Young and Setlow (21), who concluded that ClO_2 kills spores mainly by causing damage to the membrane.

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SUPPLEMENTARY MATERIAL

[Supplemental material]
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ACKNOWLEDGMENTS

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FOOTNOTES

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[†]Supplemental material for this article may be found at http://jb.asm.org/.

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