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Monitoring and Characterization of Bacterial Contamination in a High-Purity Water System Used for Semiconductor Manufacturing

In Seop Kim*, Geon-Hyoung Lee[1], and Kye Joon Lee

Laboratory of Fermentation and Biotechnology, School of Biological Sciences, Seoul National University, Seoul 151-742, Korea
[1]Major in Biology, School of Science & Technology, College of Natural Sciences, Kunsan National University, Kunsan 573-701, Korea

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Hydrogen peroxide has been used in cleaning the piping of an advanced high-purity water system that supplies ultra-high purity water (UHPW) for 16 megabyte DRAM semiconductor manufacturing. The level of hydrogen peroxide-resistant bacteria in UHPW water was monitored prior to and after disinfecting the piping with hydrogen peroxide. Most of the bacteria isolated after hydrogen peroxide disinfection were highly resistant to hydrogen peroxide. However, the percentage of resistant bacteria decreased with time. The hydrogen peroxide-resistant bacteria were identified as *Micrococcus luteus*, *Bacillus cereus*, *Alcaligenes latus*, *Xanthomonas* sp. and *Flavobacterium indologenes*. The susceptibility of the bacteria to hydrogen peroxide was tested as either planktonic cells or attached cells on glass. Attached bacteria as the biofilm on glass exhibited increased hydrogen peroxide resistance, with the resistance increasing with respect to the age of the biofilm. These results indicate that bacteria resistant to hydrogen peroxide play an important role in biofilm regrowth on piping after hydrogen peroxide treatment. In order to optimize the cleaning strategy for piping of the high-purity water system, the disinfecting effect of hydrogen peroxide and peracetic acid on the bacteria was evaluated. The combined use of hydrogen peroxide and peracetic acid was very effective in killing attached bacteria as well as planktonic bacteria.

Key words: Hydrogen peroxide, high-purity water system, bacterial contamination, resistance, biofilm

Semiconductor device manufacturing lines require stringent water quality control because contaminated organic and inorganic materials have been shown to be a major cause of manufacturing problems and process failure. In particular, bacteria, as living organisms, contain metals and are detrimental to integrated circuit manufacturing (7, 9). Ultra-high purity water (UHPW) in semiconductor manufacturing companies is made by the advanced high-purity water system consisting of sand filters, active carbon filters, reverse osmosis units, ion exchange resins, 254- and 185 nm UV, ozone generators and finally ultrafilters (7). 254- and 185 nm UV and ozone are common sanitants continuously used for microbial control in high-purity water systems. The concentration of total organic carbon and metal ions in the final products are strictly controlled to less than 0.1 parts per billion (ppb) and less than 0.05 ppb, respectively, for manufacturing high quality semiconductors.

* To whom correspondence should be addressed. Present address: Korea Green Cross Corp. 227-3 Kugal-Ri, Kiheung-Eup, Yongin 449-900, Kyunggi-Do, Korea.
(Tel) 82-331-280-6127; (Fax) 82-331-280-6019
(E-mail) inskim@greencross.com

Although UHPW in the semiconductor industry is an extreme oligotrophic environment, microorganisms can survive, grow and multiply in those conditions. The nutrient levels in UHPW are unequivocally sufficient to permit microbial growth and reproduction to a troublesome level. Recent studies have demonstrated that nearly all high-purity water piping systems will form biofilms with time and the primary source of planktonic bacteria is detachment of cells from interfacial biofilms (6, 8, 11, 13, 15, 17-20).

Due to biofilm fouling, periodic cleaning of high-purity water system piping is necessary. In the semiconductor manufacturing industry, the common disinfectants are ozonation, quaternary ammonium solution, and hydrogen peroxide. Samsung Electronics Co., Ltd, has used hydrogen peroxide for sanitizing the piping of high-purity water system that supplies UHPW for DRAM semiconductor manufacturing, because hydrogen peroxide has the apparent ability to decompose completely to oxygen and water leaving no residuals behind that might affect product yield (3, 11).

In this study, we show that the pioneer bacteria for biofilm regrowth after hydrogen peroxide cleaning of the pip-

ing are hydrogen peroxide-resistant bacteria which may survive during hydrogen peroxide treatment. The resistant bacteria were isolated and the susceptibility to hydrogen peroxide was tested as either planktonic cells or attached cells on glass. The disinfecting effect of hydrogen peroxide and peracetic acid on the bacteria was also evaluated. This result will be useful for optimizing cleaning strategies for piping in the high-purity water industry.

Materials and Methods

Description of the ultra-high purity water

Studies were carried out with UHPW samples for manufacturing 16 megabyte DRAM in Samsung Electronic Co., LTD at Kiheung-Eup, Yongin-Goon, Kyungki-Do, Korea. The high-purity water system consists of sand filters, active carbon filters, reverse osmosis units, ion exchange resins, 254- and 185 nm UV, and finally ultrafilters. The concentration of total organic carbon and metal ions in the final products are less than 0.1 ppb and 0.05 ppb, respectively. The temperature, pH, and resistance are 26°C, 6.8-7.0, and 18 MΩ, respectively.

Hydrogen peroxide cleaning of the piping of high-purity water system

The piping of the high-purity water system was cleaned by circulating 1% hydrogen peroxide solution for 3 h. After rinsing the piping completely with UHPW until no hydrogen peroxide residues were left, the high purity water manufacturing system was operated. The final UHPW manufactured by the system was sampled periodically before and after the hydrogen peroxide treatment.

Enumeration of the total number of bacteria

The total number of bacteria in UHPW was measured using epifluorescence microscopy with fluorochrome acridine orange (4, 10, 12). Filter-sterilized formaldehyde was added to UHPW samples to make a final concentration of 2% immediately after the UHPW was aseptically sampled. The UHPW samples were filtered through 0.2 μm-porosity polycarbonate filter (Millipore) previously stained with Sudan Black B (Merck). Then, bacteria were stained with 1 ml of filter-sterilized acridine orange dye (0.05%) for 7 min. The polycarbonate filters were air dried and mounted on glass slides under coverslips with a non-fluorescing immersion oil. The number of bacteria was counted with a Zeiss epifluorescence microscope (Axioskop). The optical filter combination included a BP 450 to 490-nm excitation filter, a FT 510 chromatic beam splitter, and an LP 520 barrier filter. A total of 25 fields per sample were taken to count the bacterial number. The acridine orange solution was freshly made for each experiment.

Isolation of bacteria

Bacteria present in the UHPW were isolated by the membrane filtration viable culture method. One liter of UHPW were filtered through 0.2 μm-porosity GS filter (47 mm-diameter, Millipore) at the same velocity (0.5 $lmin^{-1}$) as the flowing stream through a sampling valve on the main piping. Filters were aseptically transferred onto one hundred-fold diluted nutrient broth (Difco) agar medium and then incubated for 7 days at 28°C (15). Fifty colonies per each sampling time were randomly isolated from the filters and then subcultured three times to pure cultures. Bacteria present in feed water used for manufacturing UHPW were also isolated by the membrane filtration viable culture method and fifty colonies were randomly isolated from the filters.

Identification of the bacteria

Bacteria isolated were first examined by light microscopy and Gram-staining. Then, the bacterial strains were identified by a BIOLOG automated microbial identification system (Biolog Inc.). Pure-culture inocula were grown on BUGM (Biolog Universal Growth Medium) agar medium for 18 h and swabbed from the agar surface and resuspended in physiological saline. Biolog GN or GP microplates wells, which contain redox indicators in all wells and a distinct carbon source in each of 95 wells, were inoculated with 150 μl of the cell suspensions that had been adjusted to the appropriate density by comparison with the turbidity standards supplied by the manufacturer. Microplates were incubated at 28°C and were read at 590 nm in the Biolog microplate reader with Microlog I computer program pertaining to the percent similarity, distance, and goodness of identification for the unknowns. When similarities with species in the database are equal to or larger than 0.5, good identification will be designated by the program. Some of the strains were further identified by cellular fatty methyl ester analysis with a MIDI microbial identification system (MIDI, Newark, Del.), using gas chromatography (Hewlett Packard 5890) and flame ionization detection. The fatty acid profiles of unknown strain were automatically compared to the stored database using covariance matrix, principal component analysis and pattern recognition software.

Disinfection of planktonic bacteria

Isolated bacteria were grown in a ten-fold diluted nutrient broth medium (Difco) at 28°C for 24 h. The cells were washed three times and suspended in sterile 0.01 M phosphate-buffered saline (pH 7.0). Thirty milliliter of cell suspension (approximately 10^8 cells per ml) was added to 50-ml conical tubes, then treated with hydrogen peroxide (Aldrich) and/or peracetic acid (Aldrich) at different concentrations at 28°C. One milliliter of the mixture was withdrawn at intervals and serially diluted in sterile phosphate-buffered saline. The diluted samples were spread on

a ten-fold diluted nutrient agar medium and incubated at 28°C for 7 days. After incubation, the number of colonies was counted.

Disinfection of attached bacteria

Isolated bacteria were grown in ten-fold diluted nutrient broth medium (Difco) at 28°C for 24 h. The cells were washed three times and suspended in sterile 0.01 M phosphate-buffered saline (pH 7.0). Fifty milliliter of cell suspension (approximately 10^8 cells per ml) was added to 500-ml beakers containing cover glasses (1.8 cm × 1.8 cm). After incubation with gentle stirring by a magnetic stirring rod at 28°C, each cover glass was taken out after 4, 8, 12, 24, and 48 h and gently washed with 30 ml of phosphate-buffered saline to remove loosely attached bacteria. The washed cover glasses were transferred to 50-ml conical tubes containing 30 ml of phosphate-buffered saline supplemented with a different concentration of hydrogen peroxide in the presence or absence of peracetic acid and then incubated at 28°C. The cover glasses were taken out at intervals and then washed with 50 ml of 0.01 M phosphate buffer (pH 7.0). The number of total bacteria and viable bacteria were measured by a direct viable count method (5). The cover glasses with attached cells were transferred into a Petri dish containing 0.3% casamino acids (Difco), 0.03% yeast extract (Difco) and 12 μg/ml nalidixic acid (Sigma). After incubation at 28°C for 4 h, cover glasses were withdrawn and fixed with formaline followed by immersing in 0.02% acridine orange solution for 2 min. The cover glasses were then allowed to air-dry, placed on glass slides and examined using a Zeiss epifluorescence microscope (Axioskop). Both total and elongated cells in 25 microscopic fields were counted.

Analysis of inactivation kinetic parameters

The inactivation constant, k, was analyzed in the equation $N/N_0 = e^{-kt}$ which describes the survivor curve, where N is the number of viable organisms surviving at time t and N_0 is the initial number of viable organisms. The D-value, i.e. the time taken to reduce the number of viable organism by 1 log cycle, was calculated (23).

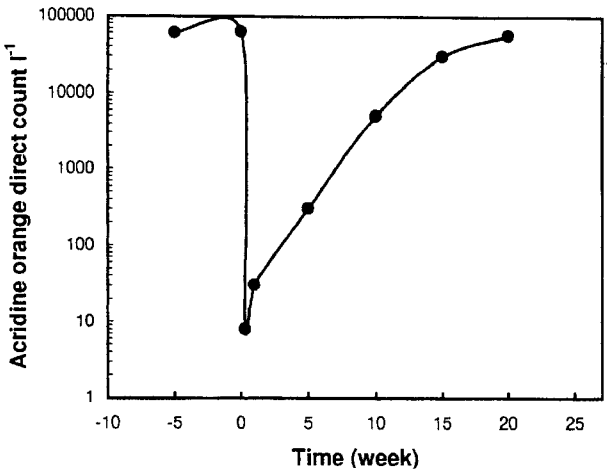

Fig. 1. The changes in total bacterial numbers in ultra-high purity water before and after hydrogen peroxide disinfection of the high-purity water system. Total numbers of bacteria were measured by acridine orange direct count. Hydrogen peroxide disinfection was carried out at week 0.

Results

High incidence of hydrogen peroxide-resistant bacteria from UHPW

The changes in total number of bacteria in UHPW before and after hydrogen peroxide disinfection of the high purity water system were measured (Fig. 1). The total bacterial number counted by the acridine-orange direct count (AODC) was significantly lowered with the hydrogen peroxide treatment. However, the total bacterial number increased following disinfection.

Bacteria present in UHPW were isolated by the membrane filtration viable culture method. Fifty strains were randomly chosen from the isolates and their inactivation constants to 1% hydrogen peroxide were evaluated. The bacteria were divided into four groups on the basis of their inactivation constant (Table 1). Group 1 bacteria (inactivation constant higher than 100) are very sensitive to hydrogen peroxide. Group 2 bacteria (inactivation constant between 50 and 100) are moderately sensitive to hydrogen peroxide. Group 3 bacteria (inactivation constant between 20 and 50) are resistant to hydrogen per-

Table 1. Classification of bacterial isolates based on their resistance to 1% hydrogen peroxide

Bacterial source	Group 1 bacteria (%) (k≥100)	Group 2 bacteria (%) (50≤k<100)	Group 3 bacteria (%) (20≤k<50)	Group 4 bacteria (%) (k<20)
Feed water	88	12	0	0
UHPW before H_2O_2 disinfection	20	38	26	16
1 week after H_2O_2 disinfection	0	0	14	86
5 weeks after H_2O_2 disinfection	0	14	38	48
10 weeks after H_2O_2 disinfection	8	26	30	36
15 weeks after H_2O_2 disinfection	18	36	28	18

k is the inactivation constant calculated from the survival curve of bacteria after 1% hydrogen peroxide was treated at 28°C.

oxide. Group 4 bacteria (inactivation constant below 20) are very resistant to hydrogen peroxide. The inactivation constants of *Escherichia coli* HB101, which is sensitive to hydrogen peroxide, and *Bacillus subtilis* ATCC9943, which is resistant to hydrogen peroxide, were 200 and 47, respectively. Most of the bacteria isolated from feed water, which was city water used for manufacturing high purity water, were sensitive to hydrogen peroxide, but bacteria isolated from UHPW exhibited increased resistance to hydrogen peroxide. Most of the bacteria isolated at one week after hydrogen peroxide treatment were very resistant to hydrogen peroxide, but the percentage of resistant bacteria decreased with time.

Identification of hydrogen peroxide-resistant bacteria

From the hydrogen peroxide-resistant Group 4 bacteria, 50 strains were randomly selected. Gram-staining showed that 31 isolates were Gram-positive and 19 isolates were Gram-negative. Most of the Gram-positive bacteria resistant to hydrogen peroxide were identified as *Micrococcus luteus* and *Bacillus cereus*. Hydrogen peroxide-resistant Gram-negative bacteria were identified as *Alcaligenes latus*, *Xanthomonas* sp. and *Flavobacterium indologenes* (Table 2).

Kinetic analysis of hydrogen peroxide disinfection on planktonic and attached bacteria

In order to elucidate the effect of bacterial attachment to substratum on 1% hydrogen peroxide disinfection, the inactivation constant and *D*-value of hydrogen peroxide disinfection on four strains of hydrogen peroxide-resistant bacteria were studied with either planktonic cells or attached cells on glass (Table 3). Each bacterial strain tested was the most hydrogen peroxide-resistant bacterium from each taxonomic group of Table 2. Attached cells on the cover glass exhibited increased hydrogen peroxide resistance compared with planktonic cells in all strains tested. *M. luteus* HR01 showed the highest resistance to hydrogen peroxide in both planktonic and attached conditions.

In order to elucidate the effect of biofilm age on hydrogen peroxide disinfection, hydrogen peroxide was treated to cells of *M. luteus* HR01 after they had been in contact with cover glasses for a predetermined period of time. Cells in the old biofilm were more resistant than cells in the young biofilm (Table 4).

Effect of combined use of hydrogen peroxide and peracetic acid on the disinfection

The effect of the combined use of hydrogen peroxide and peracetic acid on the disinfection of *M. luteus* HR01 was measured (Table 5). The combined use of hydrogen peroxide and peracetic acid was very effective for disinfecting both planktonic and attached cells compared with the single use of hydrogen peroxide or peracetic acid.

Table 2. Identification of hydrogen peroxide-resistant bacteria

Bacterial species identified	Number of isolates identified (%)
Micrococcus luteus	16 (32)
Bacillus cereus	8 (16)
Alcaligenes latus	6 (12)
Xanthomonas sp.	3 (6)
Flavobacterium indologenes	2 (4)
Not identified	15 (30)

Fifty strains of hydrogen peroxide-resistant bacteria (Group 4) were identified by the BIOLOG identification system. Some of them were further identified by the MIDI system.

Table 3. Comparison of inactivation constant (k) and decimal reduction value (*D*-value) of planktonic and attached cells of hydrogen peroxide-resistant bacteria to 1% hydrogen peroxide treatment

Bacterial strain	Planktonic cells		Attached cells[a]	
	k (h^{-1})	*D*-value (min)	k (h^{-1})	*D*-value (min)
Micrococcus luteus HR01	3.8	36.4	2.2	62.8
Bacillus cereus HR08	10.4	13.3	7.4	18.7
Alcaligenes latus HR20	8.2	16.8	4.2	32.9
Xanthomonas sp. HR38	13.3	10.4	5.7	24.2

Each bacterial strain tested was the bacteria most resistant to hydrogen peroxide among its taxonomic groups of Table 2.
[a]The attachment time of each bacteria on the cover glass was 24 h.

Discussion

Bacterial contamination control in UHPW has become a major concern for integrated circuit wafer processing dur-

Table 4. The effect of biofim age of *Micrococcus luteus* HR01 on hydrogen peroxide disinfection

Biofilm age (h)	Inactivation constant (k, h^{-1})	*D*-value (min)
4	3.3	41.9
8	2.8	49.3
12	2.5	55.3
24	2.2	62.8
48	2.0	69.1

1% hydrogen peroxide was treated to attached cells of *M. luteus* HR01 after they had been in contact with cove glasses for a predetermined period of time

Table 5. The effect of the combined use of hydrogen peroxide (HP) and peracetic acid (PAA) on the disinfection of *Micrococcus luteus* HR01

Disinfectant	Planktonic cells		Attached cells[a]	
	k (h^{-1})	*D*-value (min)	k (h^{-1})	*D*-value (min)
0.005% PAA	1.2	115.1	0.93	148.2
0.01% PAA	2.1	65.8	1.43	96.6
0.5% HP	3.2	43.2	1.8	76.8
1.0% HP	3.8	36.4	2.2	62.8
0.5% HP + 0.005% PAA	5.2	26.6	2.5	55.3
0.5% HP + 0.01% PAA	16.6	8.3	6.6	20.9
1.0% HP + 0.005% PAA	12.8	10.8	6.1	22.6
1.0% HP + 0.01% PAA	40.6	3.4	17.7	7.8

[a]The attachment time was 24 h

ing the past 10 years in semiconductor industry, because bacteria as living or dead microorganisms or as fragments are sources of particles and low level metals (7, 9). Therefore it is essential to monitor and control the number of bacteria per unit volume of UHPW.

Microscopic observation revealed that all the bacteria in UHPW, an extreme oligotrophic environment, were coccoid in shape and were less than 0.3 μm in diameter. This small coccoid morphology is an adaptive response of bacteria for their survival in the transition from feast to famine, because this morphology can increase the surface-to-volume ratio when a high surface-to-volume ratio is advantageous for nutrient uptake in oligotrophic conditions (21). The total number of bacteria present in final UHPW was time-dependent (Fig. 1). The total bacterial number was significantly lower as soon as hydrogen peroxide was treated for cleaning the piping of an advanced high-purity water system. However, it increased with time post disinfection. This result reveals that one of the primary sources of bacterial contamination in UHPW is detachment of cells from biofilms developed on the pipe wall through which UHPW is passing.

Most of the bacteria isolated from the advanced high-purity water system which had been cleaned using hydrogen peroxide were more resistant to hydrogen peroxide than the bacteria isolated from feed water (Table 1). The bacteria isolated before cleaning showed more diverse susceptibility to hydrogen peroxide than the bacteria isolated one week after hydrogen peroxide disinfection. Most of the bacteria isolated at one week after hydrogen peroxide treatment were very resistant to hydrogen peroxide, but the percentage of resistant bacteria decreased with time. These results indicate that biofilm formation on the piping starts by hydrogen peroxide-resistant bacteria which survive hydrogen peroxide disinfection. Poor disinfection will leave behind spores or biofilm that speed the bacterial recolonization of the piping system.

Attachment of organism to surfaces has been shown to alter their physiology (2, 16, 22). Attached organisms are generally found to be more active in taking up nutrients. Furthermore, attached organisms have been shown to be more resistant to such environmental stresses as starvation, heavy metals, chlorine, and antibiotics. The presence of polymeric substances in biofilm may be responsible for the resistance exhibited by the attached bacteria. The comparative study between planktonic cells and attached cells on glass showed that attached organisms are more likely to survive during hydrogen peroxide disinfection (Table 3). Interestingly cells in the old biofilm were more resistant than cells in the young biofilm (Table 4). It is therefore imperative that dosage and contact time of hydrogen peroxide must be higher than those optimized with planktonic cells when biofilm bacteria are disinfected with hydrogen peroxide. Optimization of disinfection must also be carried out with attached cells.

The antimicrobial properties of peracetic acid have been recognized for many years. Peracetic acid is in an equilibrium mixture of acetic acid, water, and hydrogen peroxide and combines the active oxygen characteristics of a peroxide within an acetic acid molecule. Peracetic acid probably disrupts sulfhydryl and sulfur bonds within enzymes; hence, important membrane components are broken by oxidative disruption. It is also likely that peracetic acid dislocates the chemiosmotic function of membrane transport through rupture or dislocation of the cell walls, seriously impeding cellular activity. Intracellular peracetic acid may also oxidize essential enzymes; thus vital biochemical pathways, active transport across membranes, and intracellular solute levels are impaired (14). The combined use of hydrogen peroxide and peracetic acid was very effective for disinfecting attached cells as well as planktonic cells (Table 5). The combination of hydrogen peroxide and peracetic acid was synergistic against *M. luteus* HR01. The synergistic interaction of the combination of hydrogen peroxide and peracetic acid against *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* was already reported (1). The combined use of 1% hydrogen peroxide and 0.01% peracetic acid was nearly eight times as effective as 1% hydrogen peroxide. This combined use of hydrogen peroxide and peracetic acid may greatly increase biofilm disinfection and reduces cost by saving hundreds of hours of production down-time in the cleaning process.

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