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Isolation and Characterization of Biofouling Bacteria in Ultra-High Purity Water Used in the Semiconductor Manufacturing Process

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Abstract Bacteria were isolated and identified from an advanced high-purity water system that supplies ultra-high purity water (UHPW) for 16-megabyte DRAM semiconductor manufacturing. Scanning electron microscopic and microbiological observations revealed that the primary source of the bacteria isolated from the UHPW was detached cells from biofilms developed on the pipe wall through which the UHPW, a man-made and extremely nutrient poor environment, was passing. About 63–65% of the bacteria isolated from the UHPW and the pipe wall were Gram-positive, whereas only 10% of the bacteria isolated from the feed water were Gram-positive. The UHPW bacteria were a diverse group, including nine genera of Gram-positive bacteria and seven genera of Gram-negative bacteria. Strains of the UHPW bacteria effectively adhered to and formed a biofilm on the surface of polyvinyl chloride (PVC) pipe.

Key words: Biofouling, Biofilm bacteria, semiconductor, ultra-high purity water

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 [5, 7]. Over the past 10 years, control of the bacterial contamination in ultra-high purity water (UHPW) has become a major concern for integrated circuit wafer processing in the semiconductor industry, since bacteria, as either a live or dead forms of microorganisms or their fragments are particle sources as well as a source of low level metal contamination. Therefore, it is essential to monitor and control the number of bacteria per unit volume of UHPW.

The UHPW used by semiconductor manufacturing companies is made by an 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 [5, 12]. 254- and 185-nm UV and ozone are common sanitants used for microbial control in high-purity water systems [9]. The concentrations of total organic carbon and metal ions in the final products are strictly controlled to less than 0.1 PPB and 0.05 PPB, respectively, for manufacturing high quality semiconductors. Even though the UHPW used by the semiconductor industry is an extreme oligotrophic environment, microorganisms can still survive, grow, and multiply under these conditions [6, 9, 10, 14, 15]. Most of the heterotrophic bacteria isolated from UHPW are facultative oligotrophic bacteria which can grow in the presence of both low and high concentrations of organic substances [12].

Although there have been an increasing number of reports about methodologies for the prevention of bacteria contamination in UHPW [2, 3, 10], little has been understood for microbiological characteristics of UHPW bacteria. In order to control bacterial contamination, it is necessary to elucidate the microbial composition and physiological characteristics of UHPW bacteria. Accordingly, in the present study, we isolated and identified biofouling bacteria from an advanced high purity water system that supplies water for 16-megabyte DRAM semiconductor manufacturing at the Samsung Electronic Co., LTD, Kiheung-Eup, Yongin-Goon, Kyunggi-Do, Korea. The ability of the bacteria to adhere to the polyvinyl chloride (PVC) pipe, which is a substratum for biofilm development, was also characterized.

Scanning Electron Microscopic Observation of UHPW Bacteria

Formaldehyde was added to the UHPW samples to make a final concentration of 2% immediately after the UHPW was aseptically sampled through a sampling valve from the main piping of the high-purity water system. The samples were kept for 24 h at 4°C. The fixed bacteria were then filtered through 0.2 μm-porosity polycarbonate filters (Millipore),

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loaded on specimen stubs coated with silver-paste, and dried under a vacuum for 10 min. The dried samples were gold-coated using a Polaron SC502 Coater (Fisons, U.K.) at 15 mA for 1 min under a vacuum, and finally examined using a Stereoscan 260 scan electron microscope (Cambridge Ltd., U.K.).

The scanning electron microscopic observation of the filters revealed not only planktonic (free-floating single cell) bacteria but also bacterial clumps. Representative photographs revealing single cell bacteria and clumps of bacteria are presented in Fig. 1. All the single cell bacteria were coccoid-shaped and were less than 0.3 μm in diameter (Fig. 1A). This small coccoid-shaped morphology may be an adaptive response of bacteria for their survival in the transition from feast to famine, because this morphology increases the surface-to-volume ratio and a high surface-to-volume ratio is advantageous for nutrient uptake in oligotrophic conditions [16]. The bacterial clumps consisted of bacteria surrounded by extracellular polymeric substances, indicating that the clumps were detached biofilm (Fig. 1B). Biofilm, a thin layer of bacteria and organic matter, occurs under the viscous boundary layer, at the interface between a bulk water phase and solid system component such as piping [11]. There

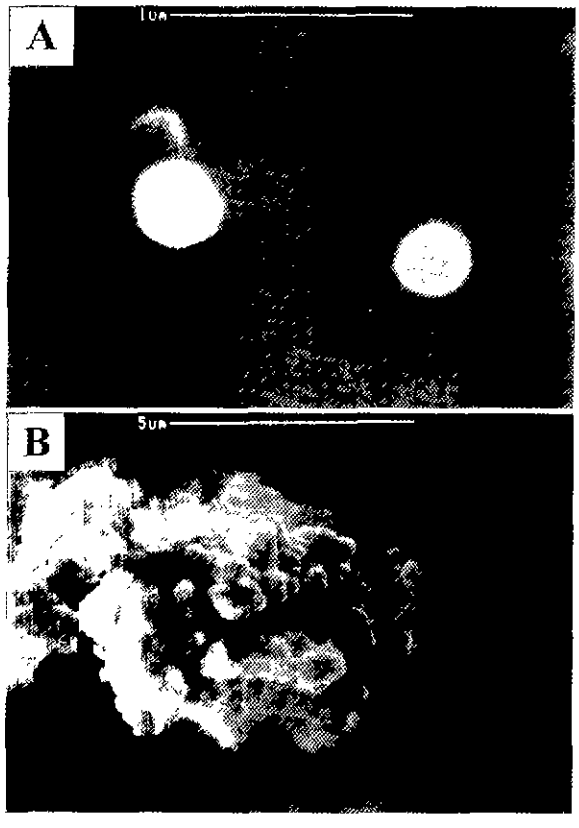

Fig. 1. Scanning electron microscopic observation of UHPW bacteria.
(A) shows the single cell bacteria and (B) shows a bacterial clump consisting of bacteria surrounded by extracellular polymeric substances.

have been several papers that have demonstrated that the primary source of planktonic bacteria is detachment of cells from interfacial biofilms, although experimental data have not yet been clearly presented [13, 14]. Husted *et al.* [14] demonstrated that a rough correlation exists between the bacterial concentration and the total oxidizable carbon (TOC) in UHPW and suggested that a slight increase in carbon or other limiting nutrients stimulates the release of cells from biofilms. A correlation between the bacterial concentration and the TOC in UHPW was also observed in the high purity water system at the Samsung Electronic Co. (data not shown).

Optimization of Culture Conditions for Isolation of Bacteria

In order to optimize the recovery of bacteria from UHPW, three media were evaluated. The bacteria present in the UHPW were collected aseptically by filtration (0.2 μm-porosity GS filter, Millipore). The filters were then aseptically transferred onto: 1) a nutrient broth agar medium (NB; Difco), 2) a one-hundred-fold diluted nutrient broth agar medium (DNB), and 3) an R_2A agar medium, and then incubated for 7 days at 28°C. During the incubation, the colonies that developed on the media were counted periodically.

The average number of bacteria grown on the DNB agar was larger than those grown on the R2A and NB agars (Fig. 2). The number of colonies on the NB and R2A agars became maximal within 120 h and 150 h, respectively. Although the initiation time of colony development on the DNB agar was retarded compared to those on the R2A and NB agars, the final counts on the DNB agar were 1.5-fold and 2-fold

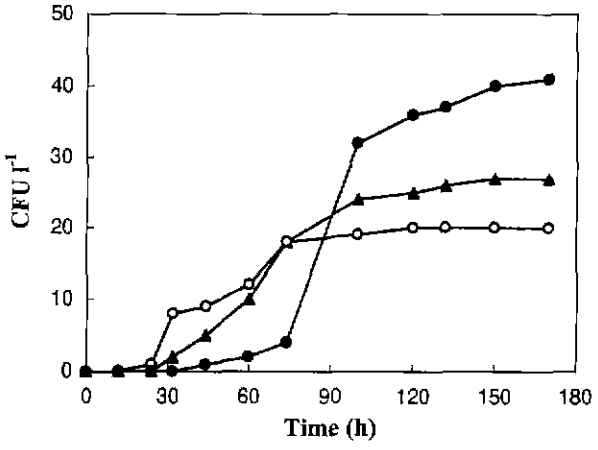

Fig. 2. Comparison of numbers of colonies on R_2A, NB, and DNB agar plates at incubation time.
The bacteria present in 1 l of UHPW were collected aseptically by filtration (0.2 μm-porosity GS filter, Millipore). The filters were aseptically transferred onto an R_2A agar medium (▲), NB agar medium (○), and DNB agar medium (●), and then incubated for 7 days at 28°C. During the incubation, colonies that developed on the media were counted periodically. These results are the mean values of six independent experiments. CFU l^{-1} means colony forming unit per 1 l of UHPW.

Table 1. Distribution of gram-positive and gram-negative bacteria in feed water, ultra-high purity water, and PVC pipe.

Bacterial source	Number of bacteria tested	% of gram-positive bacteria	% of gram-negative bacteria
Feed water	400	10.2	89.8
Ultra-high purity water	516	63.4	36.6
PVC pipe	350	65.1	34.9

higher than those on the R2A and NB agars, respectively. The same observations have been previously reported for the isolation of bacteria from an oligotrophic environment. The use of a 100-fold dilution of the full-strength nutrient broth to isolate soil bacteria resulted in the isolation of more bacteria which were unable to grow on the full strength nutrient broth [8, 17].

Isolation and Identification of the Bacteria

Five-hundred-and-sixteen bacteria were isolated from 12 l of UHPW. 63.4% of the bacteria were gram-positive and 36.6% of them were gram-negative, although only 10.2% of bacteria isolated from the feed water (which was city water used for manufacturing high purity water) were Gram-positive (Table 1). This dramatic change in the composition of the gram-positive bacteria during water purification showed that gram-positive bacteria were abundant in the biofilms which had developed on the pipe wall of the high-purity water system.

In order to clearly elucidate the cause of the high frequency of gram-positive bacteria in the UHPW, several parts of the pipe were removed from the high-purity water system, and then the bacteria on the pipe wall were isolated. The bacteria on the pipe wall were recovered in 0.1 M-phosphate buffered saline by scraping the surfaces of the pipe with a sterile spatula under aseptic conditions [4]. The recovered bacteria were completely homogenized and then serially diluted with 0.1 M-phosphate buffered saline. One-tenth milliliter of each dilution was spread on a DNB agar medium plate. All the agar plates were incubated for 7 days at 28°C. The colonies developed on the DNB agar plates were randomly selected and subcultured on the same medium until pure culture isolates were obtained. About 5×10^3 heterotophic bacteria were recovered from 1 cm^2 of pipe wall and 65.1% of the bacteria from the 350 isolates were gram-positive. This result suggested that the primary source of bacteria isolated from the UHPW was cells detached from biofilms developed on the pipe wall through which the UHPW, a man-made and extremely nutrient-poor environment, was passing. It became obvious from the above result that the pipe lines serve as a substratum for biofilm growth in high-purity water systems; once a pioneer biofilm develops on the pipe wall, this biofilm then serves as a very effective medium for the capture of scarce nutrients, thereafter, the biofilm grows and releases planktonic pioneer cells that establish the biofilm in another section of the pipe.

Table 2. Identification of gram-positive bacteria isolated from ultra-high purity water.

Bacterial species identified	Number of isolates identified (%)
Micrococcus luteus	17 (21.3)
Kocuria roseus	12 (15.0)
Kocuria kristinae	6 (7.5)
Nocardia globerula	9 (11.3)
Nocardia asteroides	5 (6.3)
Arthrobacter protophormiae	5 (6.3)
Bacillus cereus	3 (3.8)
Brevibacillus brevis	3 (3.8)
Methylobacterium rhodesianum	2 (2.5)
Staphylococcus hominis	1 (1.3)
Microbacterium arborescens	1 (1.3)
Not identified	16 (20.0)

Bacteria were identified by Biolog (BIOLOG, Inc. Hayward, CA, U.S.A.) and MIDI (MIDI, Newark, U.S.A.) identification systems.

Eighty strains, randomly selected from the 327 gram-positive bacteria isolated from the UHPW, were identified by the Biolog identification system (BIOLOG, Inc. Hayward, CA). Some of the strains were further identified by a cellular fatty methyl ester analysis using a MIDI microbial identification system (MIDI, Newark, U.S.A.) with gas chromatography (Hewlett Packard 5890) and a flame ionization detector. The majority of the bacteria were identified as *Micrococcus luteus* (21.3%), *Kocuria roseus* (15.0%), *K. kristinae* (7.5%), and *Nocardia globerula* (11.3%). *N. asteroides* (6.3%), *Arthrobacter protophormiae* (6.3%), *Bacillus cereus* (3.8%), *Brevibacillus brevis* (3.8%), *Staphylococcus hominis* (1.3%), *Methylobacterium rhodesiaum* (1.3%), and *Microbacterium arborescens* (1.3%) were also identified (Table 2).

One-hundred-and-twenty-four strains, randomly selected from the 189 gram-negative bacteria isolated from the UHPW, were also identified by Biolog (BIOLOG, Inc. Hayward, CA, U.S.A.) and MIDI (MIDI, Newark, U.S.A.) identification systems. The majority of the bacteria were identified as *Stenotrophomonas maltophilia* (27.4%), *Chryseobacterium meningosepticum* (16.1%), *Comamonas acidovorans* (11.3%), and *Ralstonia pickettii* (8.1%). *Xanthomonas oryzae* pv *oryzae* E (3.2%), *X. campestris* pv *translucens* (1.6%), *X. campestris* pv *aglanomena* (1.6%), *Pseudomonas fluorescens* type F (1.6%), and *Acinetobacter* sp. (1.6%) were also identified (Table 3).

Table 3. Identification of gram-negative bacteria isolated from ultra-high purity water.

Bacterial species identified	Number of isolates identified (%)
Stenotrophomonas maltophilia	34 (27.4)
Xanthomonas oryzae pv. *oryzae* E	4 (3.2)
Xanthomonas campestris pv. *translucens*	2 (1.6)
Xanthomonas campestris pv. *aglaonema*	2 (1.6)
Chryseobacterium meningosepticum	20 (16.1)
Comomonas acidovorans	14 (11.3)
Ralstonia pickettii	10 (8.1)
Acinetobacter sp.	2 (1.6)
Pseudomonas fluorescens type F	2 (1.6)
Not identified	34 (27.4)

Bacteria were identified by Biolog (BIOLOG, Inc. Hayward, CA, U.S.A.) and MIDI (MIDI, Newark, U.S.A.) identification systems.

These UHPW bacteria were clearly a diverse group, including nine genera of Gram-positive bacteria and seven genera of Gram-negative bacteria. It is interesting to note that the highly frequent genera for the Gram-positive bacteria were *Micrococcus* and *Kocuria*, and that for the Gram-negative bacteria was *Stenotrophomonas*. Most strains of *Stenotrophomonas* produced slimy or mucoid colonies, indicating that these bacteria produce extracellular polymeric substances, which may be responsible for the biofilm formation on the surface of the pipe lines. The scanning electron microscopic observation of the *Micrococcus* strains revealed that the cells of the *Micrococcus* strains were tetrads or clusters which were connected with bridging polymers (data not shown), which may play a role in the anchorage of the biofilms developed on the substratum surfaces.

Adhesion of Bacteria to PVC Pipe

The adhesion to the surface of the PVC pipe of 8 strains of *Stenotrophomonas*, 3 strains of *Micrococcus*, and 3 strains of *Kocuria* isolated from the UHPW was investigated. The isolates were grown in a ten-fold diluted nutrient broth medium for 24 h at 28°C. The cells were then washed three times and suspended in a 0.01 M phosphate buffer (pH 7.0). The cell suspension (30 ml) (approximately 10^8 cells per ml) was added to a 50 ml conical tube containing PVC pipe (1.5 cm×1.5 cm). After incubation for 2 h at 28°C, the PVC pipes were gently washed three times with 30 ml of a 0.01 M phosphate buffer (pH 7.0) to remove any loosely attached bacteria. The remaining attached bacteria were fixed with 2% glutaraldehyde in a sodium cacodylate solution. Thereafter, the specimens with bacteria were dried in a desiccator and placed in OsO_4 vapor for 2 h. After the specimens were coated with a thin gold layer, the number of bacteria was counted using a Stereoscan 260 scanning electron microscope (Cambridge Ltd., U.K.). A total of 25 sites were selected to count the bacterial number. Each site covered an area of 2,500 μm^2, from which the number of cells per square centimeter was then determined [1]. All the bacteria tested effectively adhered to the surface of the PVC pipe. The number of adhered bacteria ranged from 10^5 to 10^6 per cm^2 of PVC pipe.

In summary, the results described herein reveal that the primary source of the bacteria isolated from the UHPW was cells detached from biofilms developed on the pipe wall through which the UHPW was passing. Although UHPW is an extreme oligotrophic environment, bacteria can still survive, grow, and multiply as biofilm. Due to this biofilm fouling, the periodic cleaning of a high-purity water system is necessary. This is the first detailed microbiological characterization of biofouling bacteria in UHPW used in a semiconductor manufacturing process.

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