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Biofilms isolated from washing machines from three continents and their tolerance to a standard detergent

Jasmin Gattlen[a], Caroline Amberg[b], Manfred Zinn[a]* and Laurie Mauclaire[a]

[a]Empa, Swiss Federal Laboratories for Materials Science and Technology, Laboratory for Biomaterials, Lerchenfeldstrasse 5, CH-9014 St. Gallen, Switzerland; [b]Empa Testmaterials AG (ETM), Mövenstrasse 12, CH-9015 St. Gallen, Switzerland

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The goal of this comparative study was to investigate biofilm forming microorganisms living in washing machines (WMs). Biofilms were sampled from 11 washing machines from four countries and three continents. Among the 94 isolated strains, 30% were potential human pathogens. Representative strains were selected and biofilm formation was evaluated with the crystal violet (CV) assay. The majority of the WM isolates formed more biofilm than their reference strains. Biofilms of P. putida WM (the largest biofilm producer) were exposed to different concentrations (0.0007–7 g l^{-1}) of the standard detergent IEC-A* at 308C for 30 min and observed with confocal laser scanning microscopy. Using quantitative CVA, P. putida WM biofilm removal required higher detergent concentrations than the type strain. However, for both strains the recommended detergent concentration (7 g l^{-1}) was insufficient to completely clean surfaces from cell debris and exopolymeric substances.

Keywords: biofilm; tolerance; detergents; household; crystal violet; cleaning

Introduction

Biofilms are not only an issue in the medical field or in the food industry, they have also been found to inhabit surfaces of sanitary installations such as toilet bowls (Pitts et al. 1998), showerheads (Feazel et al. 2009) or household devices like refrigerators (Michaels et al. 2001) and washing machines (Terpstra 1998; Weide and Heinzel 2000). In washing machines, microbes are introduced by dirty laundry (soil, debris of human skin) or by insufficiently treated water. The biofilm formed in the washing machines has not been reported to be health-threatening but it is responsible for malodour (Munk et al. 2001).

Biofilms are more tolerant to chemicals and therefore more complicated to control and to eliminate than planktonic cells (Costerton et al. 1987; Stewart 1996; Simões et al. 2006). Moreover, standard tests on planktonic cells overestimate detergent efficiency in comparison to the real conditions in a washing machine (Block and Stelter 2002). Despite this knowledge, standard tests for bactericidal activity of detergents, disinfectants and antiseptics are still conducted with planktonic cells (eg European Committee for Standardisation 1997a,b; ASTM International 2004). Another limitation of the standard tests mentioned above is the representativeness because test microorganisms are clinical strains such as P. aeruginosa ATCC 15442, Staphylococcus aureus ATCC 6538, Klebsiella pneumoniae ATCC 4352 or Candida albicans ATCC 10231, which rarely colonize household devices (McBain et al. 2003; Feazel et al. 2009). The motivation for this study was to improve knowledge on microorganisms colonizing washing machines and to define an appropriate method to determine the efficacy of washing detergents on biofilm removal. In this study microorganisms growing as biofilms in household washing machines were first identified, as well as the hot spots of biofilm formation. In a second step, the ability of washing machine isolates to form biofilms was evaluated and compared with that of their type strain. In a third step, the biofilm tolerance against detergents was determined by estimating the detergent concentrations that were necessary to remove the biofilm.

Materials and methods

Sample isolation

If not otherwise mentioned all chemicals were provided by Sigma-Aldrich/Fluka, Buchs, Switzerland. Microorganisms were isolated from 11 household washing machines and washing machine parts coming from four countries, viz. the USA (n ¼ 3), Switzerland

*Corresponding author. Email: manfred.zinn@empa.ch
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(n ¼ 7), South Korea (n ¼ 1 and washing machine parts) and Germany (washing machine parts). Three of the washing machines were top loading the others front loading. The sampled washing machines were selected by manufacturers as relevant for biofilm investigations because, for example, they had been in use for several years or had malodour problems and eventually showed technical problems due to deposition of carbonate and detergent. The washing machines were opened and key locations for biofilm formation were visualized after staining the washing machine parts with crystal violet. Fifteen locations, such as the detergent drawer, the crossbar, the pump, the filter, the rubber ring of the door, the drum inside, the drum outside, the outer drum, the hose outlet, and the hose drum-pump were sampled with a sterile medical cotton swab (Food and Agricultural Products Standards Committee 1997). Briefly, a 2 cm × 2 cm surface was first sampled with the wet swab (sterile 0.9% NaCl solution) and then scraped again with a dry swab. Both swabs were transferred into 5 ml of 0.9% NaCl solution, vortexed for 1 min and kept overnight at room temperature to reactivate slowly growing biofilm cells. The swabs were vortexed again for 1 min followed by a 10-fold dilution series in 0.9% NaCl and plating on tryptic soy agar (TSA, Difco™, Le Pont de Claix, France) or Sabouraud dextrose agar (SDA, Oxoid, Pratteln, Switzerland). The plates were incubated at 30°C for 24 h and 48 h, respectively. Pure cultures of the isolates were established and cultured on TSA or SDA. Cryogenic stocks were prepared with overnight culture frozen in 30% sterile glycerol (1:1).

Strain identification

Gram-staining was applied followed by estimation of oxidase (oxidase reagent, bioMérieux, Lyon, France) and catalase activity (Bactident® catalase, Merck, Darmstadt, Germany), lactose fermentation (Mac Conkey agar, Oxoid) and the haemolytic pattern (Blood agar, Merck, Darmstadt, Germany). The cell size, form and the presence of endospores were determined by microscopy. Biochemical identification was conducted with API® tests (API 20 NE, REF 20050 identification system for non-fastidious Gram-negative rods; API 20 E, REF 20100/ 20160 identification system for Enterobacteriaceae and other non-fastidious Gram-negative rods; API 20 C AUX, REF 20210 yeast identification system, bioMérieux). When biochemical analysis led to ambiguous identification, strains were sent to BaseClear (Leiden, Netherlands) for sequencing and identification. For the initial samples 16S rDNA was sequenced with following primers: 16SR TACCTTGTTACGACTTCGTCCCA, 16SF AGTTTGATCCTGGCTCAG, 16S AGAGTT TGATCCTGGCTCAG, 16S ACGGCTACCTTGT-TACGACTT and for further sequencing and identification the validated MicroSEQ® systems (16S rDNA (bacteria) or D2-LSU rDNA (fungi)) from Applied Biosystems (Nieuwerkerk, Netherlands) were used.

Biofilm formation and quantification

Biofilm formation was quantified for the microorganisms isolated from at least two countries that could easily be cultivated (ie able to grow in defined medium and not flocculating). In total 15 isolates were screened belonging to Gram-negative, Gram-positive bacteria and yeast. The washing machine (WM) isolates were compared with their type strains from the German strain collection (DSMZ) that served as reference (Table 1). The microorganisms were transferred from frozen stock into 5 ml of the appropriate medium and streaked on agar plates of the corresponding medium: (i) TS medium: Tryptic soy broth and agar, (ii) SD medium: Sabouraud dextrose broth and Sabouraud 4% glucose agar, (iii) Trypticase soy yeast extract medium (DSMZ medium 92): 30 g l^{-1} TS broth, 3 g l^{-1} yeast extract, 15 g l^{-1} agar, (iv) Gym Streptomyces medium (DSMZ medium 65): 4 g l^{-1} glucose, 4 g l^{-1} yeast extract, 10 g l^{-1} malt extract, 2 g l^{-1} $CaCO_3$, 12 g l^{-1} agar, (v) Universal medium for yeast (DSMZ medium 186): 3 g l^{-1} yeast extract, 3 g l^{-1} malt extract (Oxoid), 5 g l^{-1} peptone from soybeans (peptone N-Z-Soy BL 7, enzymatic hydrolysate), 10 g l^{-1} glucose, 15 g l^{-1} agar. A single colony was picked from an agar plate, inoculated into 15 ml of the appropriate medium and incubated (30°C, 150 rpm, Infors HT, Bottmingen, Switzerland) for ca 15–18 h. Five ml of the overnight culture were centrifuged (10,000 × g, 4°C, 15 min, Heraeus® Multifuge® 3 S-R, Thermo Fisher Scientific, Zurich, Switzerland). The pellet was resuspended in 10 ml of 0.9% NaCl. Aliquots were taken for cell counting by flow cytometry (CyFlow®, Partec, Münster, Germany) after staining with Syto9 (final concentration: 0.5 mM, Molecular probes®, Invitrogen, Lucerne, Switzerland). The remaining cells were stored at 4°C, centrifuged and resuspended in sterile biofilm medium to a final cell number of 10^5–10^6 cells ml^{-1}. Biofilm minimal medium (pH ¼ 7) consisted of 1 g l^{-1} 3-(N-morpholino)propanesulfonic acid (MOPS), 1.1 g l^{-1} $(NH_4)_2SO_4$, 0.15 g l^{-1} KH_2PO_4, 0.25 g l^{-1} $MgSO_4 \times 7H_2O$, 0.1 g l^{-1} $FeSO_4 \times 7H_2O$, 0.2 g l^{-1} Na_2-EDTA × $2H_2O$. Autoclaved medium was supplemented with 1 g l^{-1} heat-sterilized D(þ)-glucose as carbon source and 1 ml of filter-sterilized (0.22 mm, Millex®, Milipore™ AG, Zug, Switzerland) trace element stock solution (1.5 g l^{-1} $CaCl_2 \times 2H_2O$, 3.96 g l^{-1} $MnCl_2 \times 4H_2O$, 5.62 g l^{-1} $CoSO_4 \times 7H_2O$, 0.34 g l^{-1} $CuCl_2 \times 2H_2O$, 1 g l^{-1} $ZnSO_4 \times 7$

Table 1. Strains (WM isolates and type strains) used for biofilm screening in 96-well microtiter plates and their corresponding medium for optimal growth of the pre-cultures (Atlas 1995, 1996).

Strain isolated from washing machines	Medium	Type strains	Medium
P. putida WM	TS[a]	P. putida (DSMZ 50026)	TS
P. fluorescens WM	TS	P. fluorescens (DSMZ 6147)	TS
Citrobacter freundii WM	TS	Citrobacter freundii (DSMZ 30039)	TS
Microbacterium oxydans WM	TS	Microbacterium oxydans (DSMZ 20578)	TS
Microbacterium aurum WM	TS	Microbacterium aurum (DSMZ 20028)	TSY[c]
Microbacterium sp. WM 1	TS	Microbacterium sp. (DSMZ 8600)	TSY
Microbacterium sp. WM 2	TS		
Microbacterium sp. WM 3	TS		
Staphylococcus cohnii subsp. urealyticum WM	TS	Staphylococcus cohnii subsp. urealyticum (DSMZ 6718)	TS
Cellulosimicrobium sp. WM	TS	Cellulosimicrobium cellulans (DSMZ 43879)	GSM[d]
Rhodotorula mucilaginosa WM 1	SD[b]	Rhodotorula mucilaginosa (DSMZ 70403)	YM[e]
Rhodotorula mucilaginosa WM 2	SD		
Rhodotorula mucilaginosa WM 3	SD		
Rhodotorula minuta WM	SD	Rhodotorula minuta (DSMZ 3016)	YM
Rhodotorula slooffiae WM	SD		

[a]TS, tryptic soy medium; [b]SD, Sabouraud dextrose medium; [c]TSY, trypticase soy yeast extract medium; [d]GSM, gym Streptomyces medium; [e]YM, universal medium for yeast.

H_2O, 1 g l^{-1} $MoO_4Na_2 \cdot 2H_2O$, pH ¼ 1). The optical density was measured at 600 nm (Spectronic[1] Genesys™ 6, UV-visible spectrophotometer, Thermo Electron Schweiz AG, Allschwil, Switzerland) and the cell abundance was checked by 10-fold dilution series and plating. The suspension (0.2 ml) was loaded into sterile 96-well plates (TPP92096, flat bottom, Trasadin- gen, Switzerland). Care was taken that the same lot number of the microtiter plate was always used (Lot Nr. 20080234). The plates were sealed with adhesive tape. The cells were spun down to the bottom of the well by centrifugation (2260 g, 7 min, 48C) and then incubated (24 h, 308C, 50 rpm, Lab-Therm LT-W, Kü hner AG, Birsfelden, Switzerland). After 24 h, the suspension was discarded and the biofilm formed was washed three times (3 × 300 ml sterile-filtered tap water) and dried in a laminar sterile bench. Crystal violet (0.1% CV [w/v] in MilliQ[1] water) was used to stain the biofilm at room temperature for 30 min. The biofilm was washed five times (5 × 300 ml sterile-filtered tap water) and dried again. To destain the biofilm, 200 ml of dimethylsulfoxide (DMSO) (Huber et al. 2002) were added (308C, 50 rpm, 2 h) and 100 ml were transferred into sterile 96-well plates (Nunc, Cat. Nr 260836, Denmark) for the measurement of absorbance at 595 nm (Varian Cary 50[1] MPR microplate reader coupled to a Varian Cary 50[1] Bio UV/visible spectrophotometer, Varian AG, Steinhausen, Switzerland). The biofilm formation experiments were conducted with three colonies (clones) on triplicate 96-well plates. Each well was considered as an independent measurement (n ¼ 378). The OD values were used as measured. The average OD value and the 95% confidence level were calculated. One way ANOVA (a ¼ 0.05) was used to compare the results.

Washing detergent assay against biofilm in 96-well plates

The efficacy on biofilm removal by the standard washing detergent IEC-A* was assessed for Pseudomonas putida WM and its type strain. The washing detergent was tested on 1-day-old biofilms of P. putida produced as described above. The washing detergent IEC-A* (5.39 g l^{-1} IEC-A base [IEC/SC 59D Home laundry appliances 2010]), 1.4 g l^{-1} Na-perborate, 0.21 g l^{-1} tetra acetyl ethylene diamine (TAED, bleach activator; IEC/SC 59D Home laundry appliances 2010; provider Empa Testmaterials, St Gallen, Switzerland) was dissolved in cold tap water (water hardness 16.028 fh ¼ 98 dH) and constituted the fresh stock solution. To obtain different concentrations, the stock solution was diluted with cold tap water in 10-fold dilution steps (0.0007–7 g l^{-1}). The detergent (200 ml) was added to each well. The plates were incubated (308C, 50 rpm, 30 min). The wells were rinsed five times and air dried in a laminar flow bench. Removal of biofilm was evaluated by CV staining according to the protocol already described. The experiments were conducted with three colonies (clones) on triplicate plates. Each well was considered as an independent measurement (n ¼ 72 to 108 depending on the tested concentrations). The average values of the blanks (detergent without biofilm, n ¼ 99) were subtracted from the measured values. The value obtained was standardized using the average of the negative controls (biofilm rinsed with tap water; n ¼ 108) and the 95% confidence level was calculated.

Confocal laser scanning microscopy (CLSM)

Biofilms of P. putida isolated from the washing machines and its type strain were cultivated in 6-well

plates (Costar 3516, Corning Inc., NY, USA) and exposed to IEC-A* as described above. Exopolymeric substances (EPS) were stained with the lectin Concanavalin-Alexa633 (final concentration 0.1 mg ml^{-1}, Molecular probes; Invitrogen) and DNA was stained with Syto BC (final concentration 0.5 mM, Molecular probes, Invitrogen) for at least 30 min (Neu et al. 2001). The samples were analysed with a confocal laser scanning microscope (CLSM, Axioplan 2 Imaging LSM 510, Zeiss). Alexa633 and SytoBC were excited at 632 and 488 nm, respectively. Images were recorded and treated with the software Zeiss LSM Image Examiner (version 4.0.0.241).

Results

Biofilm formation in household washing machines

Eleven washing machines were dismantled and various parts were sampled to identify hot spots of biofilm formation. Biofilm was formed on different materials within the washing machines, in particular on metal, rubber and polypropylene (Table 2). Corrosion could be seen in some cases on the crossbar and some calcium carbonate precipitate in the outer drum. Depending on the shipping conditions of the washing machines some biofilms were already dry while the rest still remained humid. The microbial population differed from machine to machine whereas the microbial load (plate counts) was in the same range in all the washing machines. Biofilms were abundant at places permanently in contact with water (eg the evacuation tube) that were hidden and not easily accessible for maintenance cleaning. Locations with increased biofilm formation were the plastic filter, metal parts of the outer drum, and rubber tubes (Figure 1a–e). In the inner drum, where the washing cycle takes place, no biofilm formation was observed (Figure 1f). Table 2 summarizes the 94 microorganisms that were isolated and identified. They belonged to mesophilic (30°C), fast growing bacteria. Thirty percent among them were potential human pathogens (eg Pseudomonas aeruginosa, Citrobacter freundii and Serratia marcescens).

The majority of the tested WM microorganisms formed more biofilm than the type strains

Fifteen strains were chosen that cover the three groups Gram-negative, Gram-positive bacteria and yeast, belonging to risk group 1 (exception Citrobacter freundii). They were reactivated from the dried biofilm in the sampled washing machines and grown in complex and defined minimal media. In a series of experiments, the ability of WM isolates to form biofilm was quantified and compared to the one of their type strain. The initial cell loads were the same for the WM and the type strains, viz. 10^5 and 10^6 cells ml^{-1} for yeast and bacteria, respectively. After growth for 24 h in biofilm minimal medium an increase in CV staining for nine of the WM isolates was observed in comparison to their reference counterparts (Figure 2). These results indicated that these nine isolates were forming more biofilm than their type strains. Out of the 15 WM isolates, nine formed more, two formed similar amounts, and four formed less biofilm than their reference counterpart obtained from the culture collection (Figure 2). The Gram-positive Cellulosimicrobium sp. WM (p-value: 1.7×10^{-42}) and Microbacterium oxydans WM (p-value: 2.3×10^{-42}), as well as the yeast Rhodotorula mucilaginosa (WM 1 and 3) (p-values: 3.2×10^{-26} and 9.4×10^{-6}, respectively) formed significantly more biofilm than their type strains. In contrast, Rhodotorula minuta (Rhodotorula sp. WM A, p-value: 1.7168E-09) and Rhodotorula slooffiae (Rhodotorula sp. WM B, p-value: 1.7×10^{-29}) formed significantly less biofilm than their type strain. The largest difference in biofilm formation was observed for P. putida and Pseudomonas fluorescens. P. putida WM from the washing machine produced twice as much biofilm as the reference strain from the collection. P. putida WM was also the best biofilm former of all the tested strains.

To support the findings of the CV assay, the EPS and cells of the P. putida biofilm were stained and observed with CLSM. These observations confirmed that P. putida WM formed greater amounts of EPS than the type strain (Figure 3). The cells of the WM isolates often appeared yellow (overlay of red and green), indicating that they were embedded in the EPS matrix. In the biofilm of the type strain the cells were mainly found at the bottom of the well and not protected by EPS.

Tolerance of 1-day-old P. putida biofilm towards washing detergents

CLSM observations of P. putida WM and the reference biofilms revealed that the highest concentration of IEC-A* detergent (7 g l^{-1}) removed the cells but not all the EPS and cell debris (Figure 3). The remaining biological material was more abundant and dense for the WM isolate than for the type strain. The treatment with low concentrations of detergent only partially removed the cells and the EPS for both the type and WM strains. Tolerance towards detergent was evaluated in terms of biofilm removal by CV assay. After contact with 7 g l^{-1} of the IEC-A* detergent solution for 30 min, no remaining P. putida biofilms was detected by the CV assay. With lower

Table 2. Overview of isolated strains and their location within the washing machines.

Organism	Risk group[a] Endospore[b]	Identification[c]	Humidity[d]	Material[e]
Gram-negative strains				
Acinetobacter sp.	1 and 2	G	w	P
Bacteroides bacterium	1 and 2	G	d	M
Brachybacterium sp.	1	G	d	P
Brevundimonas diminuta	2	G	w	P
Brevundimonas vesicularis	1	B	d	P
Brevundimonas sp. / Caulobacter sp.	1 and 2	B	d	P
Burkholderia cepacia	2	B	d	M
Caulobacter vibrioides	1	G	w	M
Chryseobacterium indologenes	2	B	w	R/P
Chryseobacterium meningosepticum	2	B	d	P
Chryseobacterium sp.	1 and 2	G	w	R
Chryseomonas luteola	2	B	w/d	R/M
Citrobacter braakii	2	B	w	P
Citrobacter freundii	2	B	w	M
Comamonas acidovorans	n. c.	B	w	P
Ensifer sp. / Sinorhizobium sp.	n. c., 1	G	d	M
Enterobacter cloacae	2	B	d	M
Kaistia sp.	1	G	d	M
Klebsiella pneumoniae	2	B	w	P
Methylobacterium mesophilicum	1	B	d	P
Ochrobactrum anthropii	2	G/B	w	P/R
Pantoea sp.	1 and 2	B	w	R
Pantoea spp.	1 and 2	B	d	M
Pseudomonas asplenii/putida	1	G	d/w	M/P
Pseudomonas aeruginosa (ATCC 10145)	2	G	w	P
Pseudomonas aeruginosa	2	B	w	P
Pseudomonas boreopolis	1	G	w	P
Pseudomonas fluorescens	1	B	w/d	P/R
Pseudomonas fluorescens/putida	1	B	w	R
Pseudomonas putida	1	B	w	R
Pseudomonas stutzeri	1	B	d/w	M/R
Pseudomonas sp.	1 und 2	G	d	M
Ralstonia sp.	1 and 2	G	w	P
Rhizobium sp. / Agrobacterium sp.	n. c., 1	G/B	d	M/P
Rhizobium sp. / Agrobacterium sp. / Azospirillum sp.	1	B	w	R
Roseomonas genomospecies	n. c.	B	w	P
Roseomonas sp.	n. c.	G	d	P
Roseomonas massilieae	n. c., 1	G	d	M
Serratia marcescens	2	B	w	P
Sphingobacterium spiritivorum	2	B	w	P
Sphingobium cloacae	1	G	w	P
Sphingobium yanoikuyae	1	G	d	M/P
Sphingomonas paucimobilis	2	B	d/w	M/P
Sphingomonas sp. V1	1	G	d	M
Sphingopyxis chilensis	1	G	d	P
Stenotrophomonas maltophila	2	B	d/w	M/P/R
Stenotrophomonas maltophila, Stenotrophomonas sp.	2	G	d	P
Stenotrophomonas sp.	2	G	d	M
Vibrio metschnikovii	2	B	w	P
Gram-positive strains				
Bacillus pumilus	1,E	G	d	P
Bacillus sp. CNJ905 PL04	n. c., E	G	w	R
Bacillus sp.	1 and 2	B	w	M
Bacillus sp. / Lysinibacillus sp.	n. c.	B	w	M
Bacillus thuringiensis	1, E	G	w	P
Cellulosimicrobium cellulans	n. c., E	G	w	R
Cellulosimicrobium cellulans/funkei	n. c.	B	w	M/P
Cellulosimicrobium sp.	1 and 2	B	w	R R
Exiguobacterium sp. India orange	1, E	G	w	R
Exiguobacterium sp. BTAH1	1, E	G	w	M
Microbacterium aurum	1	G	d	

(continued)

Table 2. (continued)

Organism	Risk group[a] Endospore[b]	Identification[c]	Humidity[d]	Material[e]
Microbacterium liquefaciens, maritypicum, oxydans	1	G	w	P
Microbacterium oxydans	1, E	G	w/d	P/M
Microbacterium paraoxydans	1	G	w	M
Microbacterium sp.	1 and 2	B/G	w/d	P/M
Microbacterium sp. SKJH-22	1	G	d	M
Micrococcus luteus	1	G	d/w	M/R
Paenibacillus sp. (Bacillus-relative)	1 and 2	G	w	R
Rhodococcus fascians (DSM 43673)	1	G	d	M/P
Rhodococcus sp.	1 and 2	G	d	M/P
Rhodococcus sp./Nocardia sp.	1 and 2	G	d	M/P
Staphylococcus cohnii subsp. urealyticum (ATCC 49330)	1	G	d	M
Williamsia sp. KTR4	n. c.	G	d	M
Yeast and filamentous fungi				
Alternaria (sterile mycelium)	n. c.	B	d	M
Alternaria	n. c.	B	w	P
Aspergillus ochraceus	n. c.	B	w	P
Aspergillus versicolor	n. c.	B	w	P
Capronia coronata	n. c.	G	d	M
Cladosporium sphaerospermum	1	B	w	R
Cladosporium sp.	n. c.	B	d	P
Cryptococcus sp.	n. c.	G	d	M
Cryptococcus sp. HB949	1	G	d	M
Dematiaceous (sterile mycelium)	n. c.	B	d	M
Dematiacea (phoma-like)	n. c.	B	d	M
Rhodotorula minuta	1; rare 2	B	w	R
Rhodotorula mucilaginosa	1; rare 2	G/B	d	P/R/M
Rhodotorula mucilaginosa AFTOL-ID 1548	1	G	w	P
Rhodotorula mucilaginosa AFTOL-ID	1	G	d	M
Rhodotorula mucilaginosa SJ 197	1	G	w	R
Rhodotorula slooffiae	1	G	d	M
Penicillium	n. c.	B	w	P
Penicillium (conidia)	n. c.	B	w	R
Penicillium sp.	1	B	w	P
Sphaeropsidales (phoma-like)	n. c.	B	d	M
Trichosporon domesticum	2	G	w	P

[a]n. c., not classified; [b]E, endospore formation; [c]B, biochemical; G, genetic; [d]d, dry weight; w, wet weight; [e]M, metal; P, plastic; R, rubber.

concentrations of the detergent (0.0007–0.07 g l^{-1}) the CV signal was detected, indicating that the biofilms were not completely removed. The WM strain which formed more EPS was more tolerant than the type strain (Figure 4).

Discussion

Formation of biofilm in washing machines

Ninety-four species isolated from washing machines from four countries were cultured and identified. The isolates were typical environmental microorganisms inhabiting soil, water and the human body, including, among others, members of the Enterobacteriaceae and Pseudomonadaceae. The majority of the WM isolates were also found on other domestic surfaces or in freshwater. On shower heads the predominant colonizers were Mycobacterium spp., Staphylococcus spp., Escherichia spp. and Pseudomonas spp. (Feazel et al. 2009), whereas Methylobacterium spp. and Sphingomonas spp. were the main colonizers of shower curtains (Kelley et al. 2004). These microorganisms were also found on kitchen sponges or dishrags (Enriquez et al. 1997; Michaels et al. 2003).

The identified microorganisms were mesophilic and fast growing on rich medium (TS or SD). About 30% of the microorganisms isolated from the washing machines belonged to potential human pathogens such as P. aeruginosa and K. pneumoniae. The percentage of potential pathogens (risk group 2) was found to be surprisingly high compared to the 3.6% of the 56 bacterial strains isolated from toilet bowls (Egert et al. 2010). In a healthy person, an infection with an opportunistic pathogen is controlled by the immune system. However, opportunistic pathogens are the main cause for morbidity and mortality in immunocompromised individuals (Brieland et al. 2000; Wanke et al. 2000).

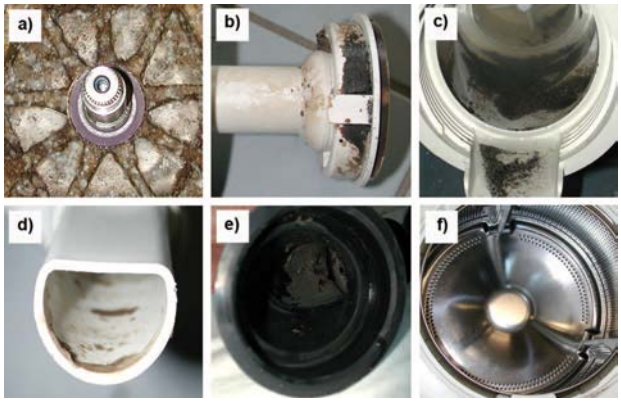

Figure 1. Parts of household washing machines prone to biofouling. Mainly hardly accessible, wetted parts of the washing machine were colonized. a ¼ crossbar (metal alloy); b ¼ filter lid (thermoplastics); c ¼ filter (thermoplastics); d ¼ air trapping (thermoplastics); e ¼ rubber tube (rubber); f ¼ inner drum (metal alloy) without biofilm formation.

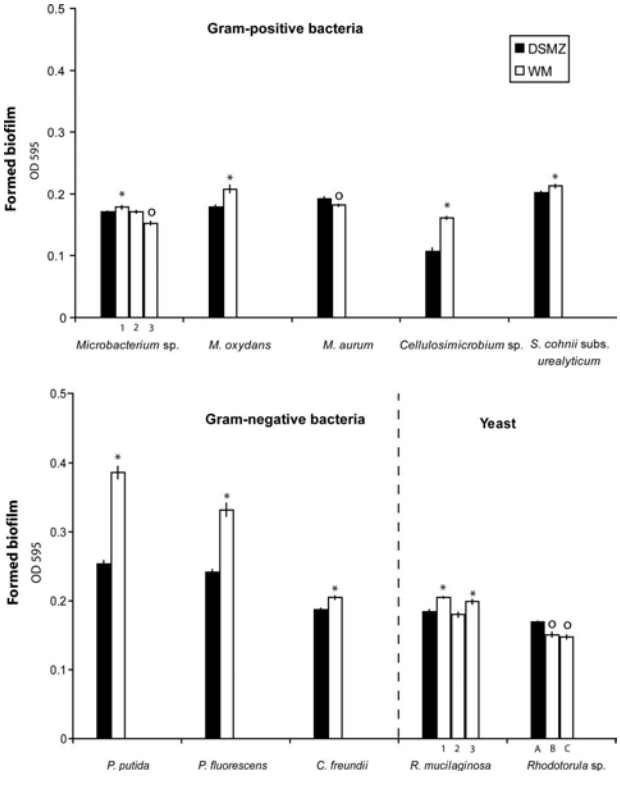

Figure 2. The biofilm formed by the washing machine isolates (¤) and their type strains (&) within 24 h (n ¼ 378 wells, + 95% confidence level). Formation of biofilm was measured by CV assay and is reported as the OD at 595 nm. (•) and (o) indicate when WM isolates formed quantitatively more or less biofilm than type strains, respectively. A ¼ Rhodotorula minuta (DSMZ 3016); B ¼ Rhodotorula minuta WM; C ¼ Rhodotorula slooffiae WM.

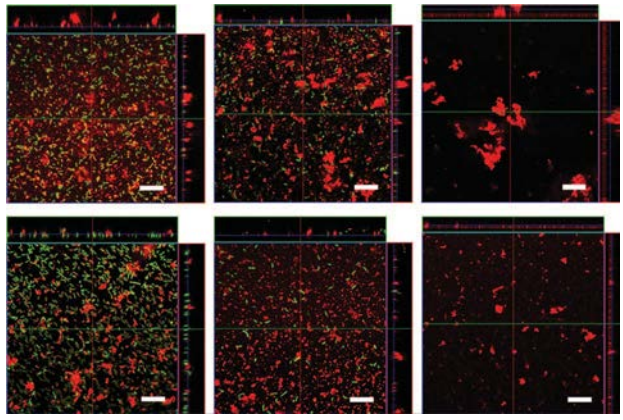

Figure 3. Overlay of confocal micrographs of 1-day-old biofilms of a P. putida strain isolated from a washing machine (upper panel) compared with its type strain (DSMZ 50026) (lower panel) after exposure to IEC-A* detergent at different concentrations: 0.0007 g l^{-1} (left), 0.07 g l^{-1} (middle) and 7 g l^{-1} (right). The DNA of the cells was stained with SytoBC (488 nm, green) and EPS was stained with Concanavalin-Alexa633 (632 nm, red). Scale bar ¼ 20 mm.

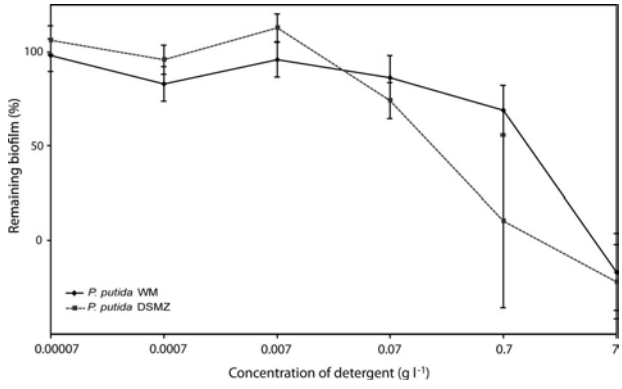

Figure 4. Effect of IEC-A* detergent concentration on P. putida biofilms. Reduction of biofilm measured as the average of OD value of CV staining and 95% confidence, n ¼ 72 to 108. A P. putida strain isolated from a washing machine (WM) was compared with the type strain (DSMZ 50026).

Moreover, it was observed that the microbial composition varied depending on the geographical origin of the washing machine. Washing machines from South Korea comprised more fungi and yeasts than washing machines from Europe or the USA. The reason(s) for this observation could be (i) a different occurrence and distribution of microorganisms in the environment, (ii) different environmental conditions such as temperature or relative humidity and/or (iii) the use of different washing detergents and washing conditions.

The surfaces inhabited by microorganisms were not limited to permanently wet environments and

comprised metal, rubber and plastics (typically polypropylene). Biofilms were never detected in the drum where the laundry is placed. However, rubber and plastic parts in direct contact with the operator were prone to biofilm formation. In general, biofilms developed to a larger extent in the inner parts of the washing machine and were hidden to the user's eyes. Considering these findings, it is recommended that precautions are taken, especially to limit the dispersion of the spores during dismantling of the washing machine. Surprisingly, the detergent drawer, into which the highly concentrated washing detergent is added, was also prone to biofilm formation.

Tolerance of biofilm towards washing detergents

Detergents are primarily formulated to remove soil from clothes under dynamic conditions. In particular, bleach containing detergents are known to reduce the microbial load in washing machines (Terpstra 1998; Wilson et al. 2007). However, clothes are not the only place where microorganisms may be found and should be removed. Potential biofilm formation on mechanical parts of washing machine exposed to gentle mixing should also be considered. Although detergents were not designed for this particular purpose, the efficacy of a non-phosphate standard powder formulation containing bleach and bleach activator were tested against 1-day-old biofilm. The recommended concentration of the washing detergent IEC-A* (7 g l^{-1}) was insufficient to remove EPS and cell debris as demonstrated by microscopic observations. Even a 1-day-old biofilm could withstand relatively high concentrations of detergent. CV staining, which is less sensitive than direct microscopic observations, showed that half of the biofilm of a WM isolate could be removed at a detergent concentration between 0.7 g l^{-1} and 7 g l^{-1}. Concentrations that were 10 times lower were sufficient to remove the biofilm formed by the type strain. This indicated how inefficient detergents are against biofilms really growing inside washing machines. Numerous theories have been proposed to explain why biofilms are more tolerant to disinfection (Fux et al. 2005; Walker and Marsh 2007). Several studies even demonstrated resistance and/or adaptation towards sanitizers such as active chlorine compounds (Yildiz and Schoolnik 1999; Russell 2004) or quaternary ammonium bases (Sundheim et al. 1998; Langsrud et al. 2003). Experiments performed under real washing conditions have shown that the bleach component has the main impact on the survival of bacteria on textiles in both detergent solution and waste water (Munk et al. 2001). Therefore, liquid detergents or powder formulations lacking bleach will most likely have a lower efficacy of biofilm removal. Beadle and Verran (1999) have already shown that liquid detergents without bleach allow the recovery and growth of microorganisms in a low nutrient environment.

P. putida WM produced more biofilm and appeared to be more tolerant towards detergent than its type strain. This observation is in agreement with other studies reporting that a larger amount of biofilm leads to better protection of the cells (Davies et al. 1998; Cochran et al. 2000). Microscopy showed that 7 g l^{-1} of IEC-A* detergent was sufficient to remove the cells but a lot of EPS was still present. However, the amount of remaining biofilm was not sufficient to be detected by CV staining. Antoniou and Frank (2005) showed that biofilms of P. putida treated with different concentrations of NaOH at 66°C for 3 min reduced the cellular coverage on surfaces more easily than the polysaccharide/EPS coverage. Deposition of organic materials such as EPS is problematic because it serves as site for attachment of other organisms including cells which are not able to produce EPS (Neu 1992; Gomez-Suarez et al. 2002). Among all the constituent of EPS, the carbohydrates are the most relevant in term of bacterial attachment (Jain and Bholse 2009). Since the EPS may help the cells to re-colonize the surface the detergent should not only remove the cells but also the EPS.

Conclusions

Microorganisms were able to form biofilms on diverse materials and locations within household washing machines. Tests under laboratory conditions demonstrated that the recommended concentration of a standard powder formulation (IEC-A*) was not sufficient to entirely remove a 1-day-old biofilm. These findings question the validity of the standard procedures based on planktonic cells to test the efficacy of washing detergents on biofilm removal. To be more representative, it is recommended, firstly that tests with biofilms are to be conducted because this is the main form of bacterial life in a household washing machine, and secondly that microorganisms isolated from washing machines are to be used because they are already adapted to chemical and mechanical stresses. Better knowledge of tolerance and adaptation to washing detergents will also help to improve the efficiency of detergents as well as washing programs towards biofilm removal. This is especially of interest because washing behaviour has changed a lot in the last few years. In particular, the user tends to wash at low-temperature and with bleach-free detergents. The influence of these changes on formation of biofilms in the washing machine is still unknown.

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References

Antoniou K, Frank JF. 2005. Removal of Pseudomonas putida biofilm and associated extracellular polymeric substances from stainless steel by alkali cleaning. J Food Prot 68:277–281.

ASTM International. November. 2004. ASTM standard test method E 2406-04: test method for evaluation of laundry sanitizers and disinfectants for use in high efficiency washing operations, West Conshocken, PA, USA.

Atlas RM. 1995. Handbook of media for environmental microbiology. London, UK: CRC Press.

Atlas RM. 1996. Handbook of microbiological media. 2nd ed. London, UK: CRC Press.

Beadle IR, Verran J. 1999. The survival and growth of an environmental Klebsiella isolate in detergent solutions. J Appl Microbiol 87:764–769.

Block C, Stelter N. 2002. Microbicidal effect of laundry detergents. SOFW J 128:48–54.

Brieland JK, Loebenberg D, Menzel F, Hare RS. 2000. Efficacy of SCH27899 in an animal model of legionnaires' disease using immunocompromised A/J mice. Antimicrob Agents Chemother 44:1333–1336.

Cochran WL, Suh SJ, McFeters GA, Stewart PS. 2000. Role of RpoS and AlgT in Pseudomonas aeruginosa biofilm resistance to hydrogen peroxide and monochloramine. J Appl Microbiol 88:546–553.

Costerton JW, Cheng KJ, Geesey GG, Ladd TI, Nickel JC, Dasgupta M, Marrie TJ. 1987. Bacterial biofilms in nature and disease. Annu Rev Microbiol 41:435–464.

Davies DG, Parsek MR, Pearson JP, Iglewski BH, Costerton JW, Greenberg EP. 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science 280:295–298.

Egert M, Schmidt I, Bussey K, Breves R. 2010. A glimpse under the rim – the composition of microbial biofilm communities in domestic toilets. J Appl Microbiol 108:1167–1174.

Enriquez C, Eniquez-Gordillo R, Kennedy DI, Gerba CP. 1997. Bacteriological survey of used cellulose sponges and cotton dishcloths from domestic kitchens. Dairy Food Environ Sanit 17:20–24.

European Committee for Standardisation. 1997a. European standard EN 1275: chemical disinfectants and antiseptics – quantitative suspension test for the evaluation of basic fungicidal or basic yeasticidal activity of chemical disinfectants and antiseptics – test method and requirements (phase 1). Brussels, Belgium: European Committee for Standardisation.

European Committee for Standardisation. 1997b. European standard EN 1276: chemical disinfectants and antiseptics – quantitative suspension test for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic, and institutional areas – test method and requirements (phase 2/step 1). Brussels, Belgium: European Committee for Standardization.

Feazel LM, Baumgartner LK, Peterson KL, Frank DN, Harris JK, Pace NR. 2009. Opportunistic pathogens enriched in showerhead biofilms. Proc Natl Acad Sci USA 106:16393–16398.

Food and Agricultural Products Standards Committee. 1997. DIN 10113-1: determination of surface colony count on fitment and utensils in foodareas – part 1: quantitative swab method. Berlin, Germany:DIN German Institute for Standardization.

Fux CA, Costerton JW, Stewart PS, Stoodley P. 2005. Survival strategies of infectious biofilms. Trends Microbiol 13:34–40.

Gomez-Suarez C, Pasma J, van der Borden AJ, Wingender J, Flemming HC, Busscher HJ, van der Mei HC. 2002. Influence of extracellular polymeric substances on deposition and redeposition of Pseudomonas aeruginosa to surfaces. Microbiol-UK 148: 1161–1169.

Huber B, Riedel K, Kothe M, Givskov M, Molin S, Eberl L. 2002. Genetic analysis of functions involved in the late stages of biofilm development in Burkholderia cepacia H111. Mol Microbiol 46:411–426.

IEC/SC 59D Home laundry appliances. 2010. IEC 60456: clothes washing machines for household use – methods for measuring the performance. Geneva, Switzerland: IEC International Electrotechnical Commission.

Jain A, Bhosle N. 2009. Biochemical composition of the marine conditioning film: implications for bacterial adhesion. Biofouling 25:13–19.

Kelley ST, Theisen U, Angenent LT, St Amand A, Pace NR. 2004. Molecular analysis of shower curtain biofilm microbes. Appl Environ Microbiol 70:4187–4192.

Langsrud S, Sundheim G, Borgmann-Strahsen R. 2003. Intrinsic and acquired resistance to quaternary ammonium compounds in food-related Pseudomonas spp. J Appl Microbiol 95:874–882.

McBain AJ, Bartolo RG, Catrenich CE, Charbonneau D, Ledder RG, Rickard AH, Symmons SA, Gilbert P. 2003. Microbial characterization of biofilms in domestic drains and the establishment of stable biofilm microcosms. Appl Environ Microbiol 69:177–185.

Michaels B, Ayers T, Celis M, Gangar V. 2001. Inactivation of refrigerator biofilm bacteria for application in the food service environment. Food Service Technol 1: 169–179.

Michaels B, Gangar V, Schultz A, Curiale MS. 2003. A microbial survey of food service can openers, food and beverage can tops and cleaning methodology effectiveness. Food Service Technol 3:123–132.

Munk S, Johansen C, Stahnke LH, Adler-Nissen J. 2001. Microbial survival and odor in laundry. J Surfact Deterg 4:385–394.

Neu TR. 1992. Microbial footprints and the general ability of microorganisms to label interfaces. Can J Microbiol 38:1005–1008.

Neu TR, Swerhone GDW, Lawrence JR. 2001. Assessment of lectin-binding analysis for in situ detection of glycoconjugates in biofilm systems. Microbiol-UK 147:299–313.

Pitts B, Stewart PS, McFeters GA, Hamilton MA, Willse A, Zelver N. 1998. Bacterial characterization of toilet bowl biofilm. Biofouling 13:19–30.

Russell AD. 2004. Bacterial adaptation and resistance to antiseptics, disinfectants and preservatives is not a new phenomenon. J Hosp Infect 57:97–104.

Simõ es M, Simõ es LC, Machado L, Pereira MO, Vieira MJ. 2006. Control of flow-generated biofilms using surfactants: evidence of resistance and recovery. Food Biopr Proc 84:338–345.

Stewart PS. 1996. Theoretical aspects of antibiotic diffusion into microbial biofilms. Antimicrob Agents Chemother 40:2517–2522.

Sundheim G, Langsrud S, Heir E, Holck AL. 1998. Bacterial resistance to disinfectants containing quaternary ammonium compounds. Int Biodeterior Biodegr 41:235–239.

Terpstra PMJ. 1998. Domestic and institutional hygiene in relation to sustainability. Historical, social and environmental implications. Int Biodeterior Biodegr 41:169–175.

Walker JT, Marsh PD. 2007. Microbial biofilm formation in DUWS and their control using disinfectants. J Dent 35:721–730.

Wanke B, dos Santos Lazera M, Nucci M. 2000. Fungal infections in the immunocompromised host. Mem Inst Oswaldo Cruz 95:153–158.

Weide MR, Heinzel M. 2000. Situation analysis of hygiene in the home. SOFW J 126:6–11.

Wilson JA, Loveday HP, Hoffman PN, Pratt RJ. 2007. Uniform: an evidence review of the microbiological significance of uniforms and uniform policy in the prevention and control of healthcare-associated infections. Report to the Department of Health (England). J Hosp Infect 66:301–307.

Yildiz FH, Schoolnik GK. 1999. Vibrio cholerae O1 El Tor: identification of a gene cluster required for the rugose colony type, exopolysaccharide production, chlorine resistance, and biofilm formation. Proc Natl Acad Sci USA 96:4028–4033.