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Characterization of micro-organisms isolated from dairy industry after cleaning and fogging disinfection with alkyl amine and peracetic acid

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ABSTRACT

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Aims: To characterize micro-organisms isolated from Norwegian dairy production plants after cleaning and fogging disinfection with alkyl amine/peracetic acid and to indicate reasons for survival.

Methods and Results: Microbial samples were collected from five dairy plants after cleaning and fogging disinfection. Isolates from two of these production plants, which used fogging with alkylamino acetate (plant A), and peracetic acid (plant B), were chosen for further characterization. The sequence of the 16S ribosomal DNA, fatty acid analysis and biochemical characteristics were used to identify isolates. Three isolates identified as *Rhodococcus erythropolis*, *Methylobacterium rhodesianum* and *Rhodotorula mucilaginosa* were isolated from plant A and one *Sphingomonas* sp. and two *M. extorquens* from plant B. Different patterns of resistance to seven disinfectants in a bactericidal suspension test and variable degree of attachment to stainless steel were found. The strains with higher disinfectant resistance showed lower degree of attachment than susceptible strains.

Conclusions: The study identifies and characterizes micro-organisms present after cleaning and fogging disinfection. Both surface attachment and resistance were shown as possible reasons for the presence of the isolates after cleaning and disinfection.

Significance and Impact of the Study: These results contribute to the awareness of disinfectant resistance as well as attachment as mechanisms of survival in dairy industry. It also strengthens the argument of frequent alternation of disinfectants in the food processing industry to avoid the establishment of resistant house strains.

Keywords: alkyl amine, biofilm, dairy industry, disinfection, fogging, peracetic acid.

INTRODUCTION

A main challenge in the food industry is to avoid contamination of raw materials and products by pathogens and spoilage organisms. This calls for control of micro-organisms on food contact surfaces. Different methods have been developed for decontamination, the most common being low-pressure manual application of foam or gel containing active compound(s). Automatic application such as fogging is an alternative for the food processing industry.

Fogging disinfection implies dispersal of finely disposed droplets of a disinfectant within a room. The intention of fogging is to ensure that all regions and equipment in the room receive an adequate application of the disinfectant. Fogging systems are costly, but could be cost efficient, and also result in improved hygiene if used appropriately. However, whether the systems always function as anticipated can be questioned. Fogging for disinfection of food processing factories and equipment, although not a new operation in the food industry, is not a much published

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research subject. Hedrick (1975) found that a chlorine fog reduced the air-borne count of organisms. Holah *et al.* (1995) found that fogging was less effective compared with other disinfection methods such as the use of ozone or ultraviolet radiation. Burfoot *et al.* (1999) concluded that fogging is effective in reducing the number of organisms on upward-facing surfaces but not on vertical or downward-facing surfaces. They also concluded that fogging reduced the number of viable air-borne organisms and that drop size and dispersion are important factors for the fogging procedure to be effective. Bagge-Ravn *et al.* (2003) compared the efficacy of peracetic acid-based fogging with hypochlorite-based foam in a salmon smokehouse. The results indicated that the procedure based on fogging gave similar or better reduction in micro-organisms than the foam-based method.

It has long been recognized that bacteria *in vivo* are found predominantly attached to surfaces. The term biofilm is commonly used to describe bacteria in the attached state surrounded by an extracellular matrix of polysaccharides. The biofilm state is generally believed to increase the ability of bacteria to survive antibacterial influence from its surroundings, such as heat, dehydration, foam cleaning, u.v. light, disinfectants, antibiotics, etc.

Microbial resistance to disinfectants is receiving more attention due to the frequent use of disinfectants in food processing industries. There is also incipient concern regarding disinfectant resistance contributing to resistance to antibiotics by co-selection of antibiotic resistance genes (Sidhu *et al.* 2001; Russell 2002a). The term resistance is used in different ways in the literature. McDonnell and Russell (1999) divide the term into intrinsic and acquired resistance and refer to increased survival through biofilm formation as physiological resistance. In this paper we will refer to micro-organisms surviving the given user concentration in the bactericidal test as resistant. The maximum kill in the bactericidal suspension test used here is a 5-log reduction of cell number. We will refer to <2 log kill as high resistance, 2–4 log kill as medium resistance, 4–5 log kill as low resistance and more than 5 log kill as no resistance.

The most commonly used disinfectants in the food processing industry in Norway and other European countries such as UK (Holah *et al.* 2002) are quaternary ammonium compounds (QAC), hypochlorites, amphoteric compounds and peroxides. Besides this, alcohols, aldehydes, phenolic compounds and chlorhexidine are also used but mainly in health service. In the food processing industry disinfectants may be left on the surfaces with the possibility of prolonged exposure of the micro-organism to the disinfectant used. Laboratory experiments have shown that exposure to sublethal concentrations of disinfectant may result in acquisition of resistance (Langsrud *et al.* 2003b). Russell (2002b), lists biocides to which bacterial resistance may be a problem as belonging to the groups of QAC such as benzalkonium chloride (BC), Bisbiguanides such as chlorhexidine, diamidines such as propamidine, bisphenols such as triclosan and acridines such as acriflavin. Peracetic acid-based disinfectants generally do not generate resistance. Although disinfectants based on alkyl aminoacetate are used to some extent in the food industry, they are almost neglected in the scientific literature.

Common problem-causing bacteria in the dairy industry are: *Streptococcus agalactiae* and other streptococci, coliformic bacteria, *Pseudomonas* spp. and *Corynebacterium pyogenes*. *Staphylococcus aureus*, *Bacillus cereus*, *Listeria monocytogenes*, *Yersinia enterocolitica*, *Salmonella* spp., *Escherichia coli* O157:H7 and *Campylobacter jejuni* are all bacterial pathogens of concern in raw milk and other dairy products. These micro-organisms understandably receive much attention from the scientific community. There has been less focus on the general flora surviving good cleaning and disinfection routines. In this study six strains isolated from surfaces in two Norwegian cheese factories after cleaning and fogging with alkyl amine and peracetic acid (alternating on a weekly basis) were identified, characterized and tested for resistance to different disinfectants and attachment to stainless steel.

MATERIALS AND METHODS

Media and reagents

Casitone and D/E neutralizing broth was from Difco (Detroit, MI, USA). Mueller–Hinton agar (MHA), Mueller–Hinton broth (MHB), MRSA, brain–heart infusion agar (BHIA), yeast extract (YE), tryptic soya broth (TSB), tryptic soya agar (TSA), skim milk powder and plate count agar (PCA) were from Oxoid (Hampshire, UK). An overview of the disinfectants used is given in Table 1. The disinfectant Virkon was purchased as powder in portion bags for 10-l quantity. All other disinfectants were concentrates of industrial quality. Stock solutions and further dilutions were prepared within few hours before the bactericidal tests were carried out, except the stock solution of BC, which was kept for no more than three weeks in a dark bottle at a concentration of 5000 ppm. All concentrates were kept in closed containers at room temperature. Stock solution of Virkon was prepared from 1·0 g of powder just before use.

Isolation of micro-organisms, control strains and biochemical tests

Micro-organisms were isolated by contact agar plates (CAP) with PCA from walls and equipment of five Norwegian dairy production facilities after cleaning and fogging disin-

Table 1 Disinfectants used in the bactericidal suspension tests

Trade name	Main active components	User concentration*	Supplier (Oslo, Norway)
All Des	Amphoteric tensides	0·3%	Novadan
TP99	Alkyl amine	1·0–1·3%	Ecolab
Oxonia active	Peracetic acid	0·2%	Ecolab
Titan Hypo	Hypochlorite	0·5–1·0%	Lilleborg
Benzalkonium chloride	QAC	200 μg ml	Norsk Medisinaldepot
Virkon†	Acid peroxygen	1·0%	Puls

*Recommended by manufacturer. Benzalkonium chloride is given as concentration of active component. All other concentrations given for commercial products.

†Virkon (a mixed powder product containing potassium persulphate, sodium chloride, sodium dodecylbenzene, sodium hexameta phosphate, sulphamic acid and maleic acid).

fection. From 10 to 20 control points were sampled both before and after disinfection. The samples were taken from walls, ceilings, conveyor belts, electric switches, packing machines, ventilation ducts (outside) and tanks (outside). Samples from different heights and undersides of objects were taken at different distances from the fogging nozzle. The CAPs were incubated at 20°C and inspected after 2, 5 and 7 days. *Staphylococcus aureus* ATCC 6538 and *Pseudomonas aeruginosa* ATCC 15442 were used as control strains for bactericidal suspension tests and attachment experiments. The strains were stored at −80°C in 15% glycerol and were streaked on TSA plates a few days prior to experiments. In all experiments these two control strains were treated as the other isolates. Bacterial isolates were analysed by Gram staining and oxidase and catalase tests. Oxidase activity was tested using 'Identification sticks – oxidase' (Oxoid). Catalase activity was tested by mixing a colony in a drop of MQ water and then adding one drop of 30% hydrogen peroxide. Gas production indicated a positive catalase test.

Growth conditions

The isolates were streaked on different agar media and incubated at 25 and 30°C for 10 days. Plates were inspected for visual colony appearance after 1, 3 and 10 days. Growth at different temperatures was tested on both PCA and TSA. Frozen glycerol cultures were streaked on agar plates and incubated at given temperature. The plates were inspected for visual colony appearance after 1, 3 and 10 days. A liquid medium based on the components of PCA was made and named plate count broth 1 (PCB1). The PCB1 medium contains 2·5 g l^{-1} YE, 5 g l^{-1} casein and 1·0 g l^{-1} glucose. PCB1 medium with no glucose was named PCB2.

Growth in various media (TSB, PCB1, PCB2, MHB) was studied by using 500 ml baffled shake flasks, incubating at 25°C in a rotary shaker cabinet at 200 rev min^{-1}. Precultures were prepared by inoculation from agar plate colonies to 1·5 ml of the same medium as tested for the main culture followed by incubation over night at 200 rev min^{-1} and 25°C. The main culture was made by inoculating 1 ml of preculture in 99 ml medium. Samples were taken every hour and CFU ml^{-1} and optical density at wave length 600 nm ($O.D._{600}$) was measured. CFU ml^{-1} was measured by 10-fold dilutions of sample in peptone water (500 μl + 4·5 ml) and streaking of 100 μl on agar plates (PCA and TSA).

Anaerobic growth was tested by streaking frozen glycerol culture on agar plates, sealing the plates into anaerobic cabinets, containing AnaeroGen (Oxoid), and incubating for 10 days at 25 and 30°C.

16S ribosomal DNA sequencing

DNA was purified by binding cells to monodisperse beads (Rudi *et al.* 1998). Cells from agar plate colonies were mixed with 5 μl magnetic, monodisperse beads (Dynal, Oslo, Norway) in 50 μl lysis buffer containing 4·0 mol l^{-1} GTC and 1% sarcosyl, and dispersed in this solution for 15 min at 65°C. Then 100 μl of 96% EtOH was added and the tube was placed next to a magnet for 5 min at room temperature. DNA was washed twice with 500 μl 70% EtOH on the beads and the supernatant was removed before the DNA was resuspended in 50 μl sterile MilliQ water. To evaporate EtOH the samples were incubated at 65°C for 10 min. To avoid possible leftovers of lysis buffer the samples were transferred to a new sterile Eppendorf tube. The samples were amplified by PCR, heating for 95°C for 3 min followed by 35 three-step cycles of 95°C for 15 s, 49°C for 15 s and 72°C for 90 s, and finally 72°C for 7 min. The samples were kept at −20°C until used. 16S rDNA genes were amplified by PCR. Forty microlitres of PCR product was mixed with 4 μl cold exonuclease and 4 μl cold alkaline phosphatase and the total volume of 48 μl was heated to 37°C for 15 min. The enzymes were then inactivated at 80°C for 15 min. Four microlitres of this product was then added to a mix of 8 μl termination mix, 1 μl sequencing primer and 7 μl of sterile water for 10 different primers. The primers used were 357 F, 515 F, 906 F, 1100 F, 1237 F, 338 R, 519 R, 907 R, 1100 R and 1391 R (Turner *et al.* 1999). The PCR apparatus was preheated to 96°C before inserting the sample tubes. Twenty-five three-step cycles were run using the steps: 96°C for 10 s, 50°C for 5 s and 60°C for 4 min. Finally, the product was cooled to 4°C for at least 7 min. Then each sample was transferred into 2 μl 3 M NaAc in 50 μl cold 95% EtOH. After 15 min at −80°C, the DNA was centrifuged at 4°C for 30 min and washed once with 250 μl cold 70% EtOH. After another spin at 4°C for 5 min

DNA were vacuum dried for 5 min. Twelve microlitres of template buffer was added before heating to 95°C for 2 min and cooling on ice for 5 min. The samples were sequenced in a 16S rDNA-sequencing machine (ABI Prism 310 Genetic Analyzer; Applied Biosystems, Foster City, CA, USA). The sequences were compared with the NCBI database.

Fatty acid analysis

Fatty acid analysis of the bacterial isolates was carried out as published by Sundheim *et al.* (1998) with the identification based on Similarity Index (SI). SI is a numerical value, which expresses how closely the fatty acid composition of an unknown compares with the mean fatty acid composition of the strains used to create the library entry or entries listed as its match. This index value is a computer-generated calculation of the distance, in multidimensional space, between the profile of the unknown and the mean profile of the most similar library entry. SI is not a 'probability' or percentage, but an expression of relative distance from the population mean. An exact match would result in an SI of 1·000.

Identification of yeast

The yeast (Vi4b) was identified from morphological and physiological characteristics by Centraalbureau voor Schimmelcultures (CBS, Yeast Division, Delft, the Netherlands).

Bactericidal tests

The tests were performed following the procedure described by the European Committee for Standardisation (Anon. 1997), with modifications. In brief, test tubes containing 4 ml of each disinfectant to be tested were prepared from fresh disinfectant stock solutions. A 10% skim milk protein solution was mixed with cell culture such that the concentrations in the test tube would be *ca* 10^7 cells ml^{-1} and 1% skim milk. In 20 s intervals, 1 ml of this solution was added to the disinfectants and a control of MilliQ water and whirl mixed. After 5 min reaction time 500 μl were transferred to 4·5 ml D/E neutralization broth (Difco) and whirl mixed. Neutralized samples were diluted 10-fold in peptone water and plated on TSA or PCA plates to measure CFU ml^{-1}. The plates were incubated at 25°C for 10 days and colonies were counted after 2, 5 and 10 days.

All solutions were held at 20 ± 1°C before usage. Stock solutions were prepared just prior to the experiments. For Virkon 1·0 g of powder was dissolved in 50 g MilliQ water, TP99 was diluted 1·0–10·0 g while all other disinfectants were diluted 1·0–100 g in MilliQ water. Final concentrations were prepared and the disinfectants as well as the control were transferred to test tubes and tempered to 20 ± 1°C before usage. Table 1 lists the disinfectants and concentrations used in the bactericidal suspension tests. The experiments were performed three times on different days and with all solutions freshly prepared.

Attachment to stainless steel

Steel coupons (AISI 304, 2B finish, 75 mm × 22 mm × 1 mm) were sonicated for 30 min in Decorex (Borer Chemie, Zuchwil, Switzerland), rinsed with MilliQ water and submerged in acetone for 1 h before autoclaving at 121°C. The coupons were placed vertically in 50 ml glass tubes with 45 ml TSB. The medium was then inoculated with overnight culture, the final cell concentration being 10^5–10^6 CFU ml^{-1}. The tubes were incubated in an incubation shaker at 100 rev min^{-1} and 25°C. The CFU ml^{-1} in the cell culture surrounding the coupons was determined by serial dilution of 500 μl culture in peptone water, spreading on agar plates and incubating at 25°C for 7 days. The steel coupon was then transferred to a Petri dish containing 25 ml peptone water and rinsed for 2 min to remove unattached cells. The coupon was then transferred to a new 50 ml glass tube containing 45 ml of peptone water. Attached cells were detached from the coupon by sonication in an ultra sound bath (Branson 3510; Branson Ultrasonic, Dansbury, CT, USA) at 40°C for 15 min. The CFU ml^{-1} was then measured by serial dilution in peptone water, spreading on TSA plates and incubating at 25°C for 7 days. The degree of attachment was calculated as the percentage of cells attached to the steel coupon from the total number of cells in the growth medium and on the steel coupon, that is all cells attached equals 100%. The experiments were performed three times on different days and with all solutions freshly prepared.

RESULTS

Isolation of strains

Three of the five dairy production plants where cleaning and disinfection routines were observed had obvious flaws in their technical routines. In one of the production plants the equipment failed to produce a real fog and did not cover the room. In another dairy the capacity of the fogging equipment was too little for the room it was used in, resulting in no fog in a larger part of the room. One dairy had problems with the computer program controlling the fogging process. The six isolates from the two remaining dairy production plants were chosen for further work. Both plants disinfected once a week and alternated between Oxonia aktiv (0·5–1% at dairy A and 2% at dairy B) and 2% TP99 (alkyl amino acetate) every third month. The chosen isolates from dairy A were designated Vi16, Vi4a, Vi4b and the isolates from dairy B 7a, 7b and 7c. At dairy A the three chosen isolates were

the only survivors after disinfection with TP99 and they were found on two of 16 control points. Vi16 was isolated from the down-facing surface of a steel trolley table. Vi4a and Vi4b were both isolated from a rubber hose by a sink in a cheese ripening room. At dairy B the three isolates 7a, 7b and 7c were all isolated from wall tiles next to a door in a cheese-ripening room, after disinfection with Oxonia aktiv. These three isolates were isolated from three of 20 samples. Besides these isolates, heavy growth of both bacteria and moulds was also found under conveyor belts. These samples were excluded from further study as the fog had probably not reached these locations.

Identification of isolates

The isolation on PCA initially showed that Vi16 appeared as large cream coloured and smooth colonies. Vi4a, 7b and 7c colonies were pink and small. Vi4b colonies were pink, smooth and circular. 7a colonies had a deep yellow colour and the colonies were small and with a rough surface. Light microscopy of the isolates clearly showed Vi4b as yeast buds. Vi16 appeared as thick, almost short coccoid-shaped rods. Vi4a had a rod-shaped appearance, while 7a showed as long and narrow rods. 7b appeared as thick rods and 7c could typically be seen with irregular shape and both branched and curved rods containing inclusion bodies.

Strain Vi16 was identified as *Rhodococcus erythropolis* by 16S rDNA sequencing (ID 100%), while the results from the fatty acid analysis suggested the strain to be *Nocardia globerula* or *Rhodococcus erythropolis* (SI 0·570/0·542). Strain Vi4a was identified as *Methylobacterium rhodesianum* by 16S rDNA sequencing (ID 98%), while the results from the fatty acid analysis suggested the strain to be *Methylobacterium* sp. (SI 0·917). Strain Vi4b was identified as the yeast *Rhodotorula mucilaginosa* by morphological and physiological characteristics. Strain 7a was identified as being similar to *Sphingomonas* sp. by 16S rDNA sequencing (ID 96%), while the results from the fatty acid analysis gave no ID. Strains 7b and 7c were identified as *Methylobacterium* spp. by both 16S rDNA sequencing (ID 98% and 99%) and fatty acid analysis (SI 0·956 and 0·821). Table 2 summarizes the identification including results of the catalase test, oxidase test and Gram staining. It should be mentioned that fatty acid analysis identified *Methylobacterium* sp. among isolates from all five factories.

Growth

Growth on different media was tested to decide which media to use for further work on the isolates. When frozen glycerol cultures were streaked directly on agar plates, TSA and MHA supported growth within 3–5 days at 25 and 30°C for *R. erythropolis* Vi16, *Rh. mucilaginosa* Vi4b and *Sphingomonas* sp. 7a. The isolates *M. rhodesianum* Vi4a, *M. extorquens* 7b and 7c showed no growth on MHA and only poor growth on TSA. The richer medium BHIA supported growth for *M. rhodesianum* Vi4a and *Sphingomonas* sp. 7a, but not for *M. extorquens* 7b and 7c even after 9 days of incubation. The glucose-containing medium (MRSA) showed only weak growth of *M. extorquens* 7c after 9 days at 30°C. The strains were isolated on PCA, which contains casein. PCA generally supported growth for all isolates at 25 and 30°C. In addition, skim milk agar supported growth of *M. rhodesianum* Vi4a, *Sphingomonas* sp. 7a and *M. extorquens*

Table 2 Results of 16S rDNA sequencing and fatty acid analysis, Gram staining, catalase and oxidase testing, colour of colonies on agar plates (PCA/TCA), light- and electron scanning microscopy (SEM) and growth rate for isolated strains

Factory	Isolate	Gram G+/G−	Oxidase ±	Catalase ±	Colour of colonies (TSA or PCA)	Morphology microscopy	Fatty acid analysis*	16s rDNA sequence analysis
A	7a	G−	−	+	Yellow	Long and narrow rods	No ID	*Sphingomonas* sp. (96%)
	7b	G−	+	+	Pink	Thick rods	*Methylobacterium extorquens* (SI 0·956)	*Methylobacterium* sp. (98%)
	7c	G−	+	+	Pink	Irregular rods, inclusion bodies	*Methylobacterium extorquens* (SI 0·821)	*Methylobacterium* sp. (99%)
B	Vi16	G+	−	+	Cream	Short, thick rods	*Nocardia globerula* or *Rhodococcus erythropolis* (SI 0·570/0·542)	*Rhodococcus erythropolis* (100%)
	Vi4a	G−	+	+	Pink	Rods	*Methylobacterium rhodesianum* (SI 0·917)	*Methylobacterium* sp. (98%)
	Vi4b	Yeast	NT	NT	Pink	Yeast buds	*Rhodotorula mucilaginosa*	NT

*Fatty acid analysis for the five bacteria by A. Sletten, The Agricultural University of Norway. The yeast (Vi4b) was analysed by CBS, the Netherlands.

Table 3 Log_{10} reduction of CFU after exposure to disinfectants for 5 min at 20°C given as the average of three independent experiments ± standard error of the mean. A log_{10} reduction of 5·00 is the maximum measurable reduction in CFU ml^{-1}

Disinfectant	Vi16	Vi4a	Vi4b	7a	ATCC 6538	ATCC 15442
All Des	0·05 ± 0·01	−0·05 ± 0·03	1·80 ± 0·46	3·57 ± 0·44	0·01 ± 0·02	0·17 ± 0·02
TP99	>5·00	4·48 ± 0·52	>5·00	>5·00	>5·00	>5·00
Benzalkonium chloride	≥5·00 ± 0·03	0·64 ± 0·11	>5·00	>5·00	>5·00	3·85 ± 1·07
Oxonia active	0·48 ± 0·03	>5·00	4·81 ± 0·19	>5·00	>5·00	>5·00
Titan Hypo	4·51 ± 0·25	0·01 ± 0·03	>5·00	>5·00	>5·00	>5·00
Virkon	>5·00	>5·00	4·16 ± 0·45	>5·00	>5·00	>5·00

7b, but not for *M. extorquens* 7c, even after 9 days of incubation. TSA and PCA were chosen as agar media for further work. Incubating isolates on plates under anaerobic conditions showed in no growth on plates even after 10 days of incubation.

Growth in 5 ml liquid medium (TSB, PCB1, PCB2) in 10-ml test tubes proved difficult for the strains *Sphingomonas* sp. 7a, *M. extorquens* 7b, *M. extorquens* 7c and *M. rhodesianum* Vi4a. Growth was very slow and typically resulted in a surface ring on the glass tube and clusters of pink (Vi4a, 7b and 7c) and yellow (7a) aggregates on the bottom of the tubes. Growth in baffled 500-ml shake flasks at 200 rev min^{-1} gave improved growth conditions and a more homogeneous culture. Therefore, growth in TSB in 500 ml shake flasks was tested at 30°C. Preculture grown in 10 ml tubes was used for inoculum after vigorous vortexing to dissolve the aggregates. Growth was clearly seen from turbidity for *R. erythropolis* Vi16, *M. rhodesianum* Vi4a, *Sphingomonas* sp. 7a and *Rh. mucilaginosa* Vi4b after 3 days of incubation. For *M. extorquens* 7b and *M. extorquens* 7c there was no visible growth in TSB after 10 days. The PCB1 and PCB2 media both supported visible growth (turbid culture) for *M. extorquens* 7b and 7c at 30°C after 10 days, while no visible growth was seen after 3 days. Cell numbers on agar plates was in the order of 10^5–10^6 CFU ml^{-1} as a maximum. TSB and PCB2 were chosen as liquid mediums.

At this stage we decided to perform further experiments with the four strains that showed satisfactory growth, that is *R. erythropolis* Vi16, *M. rhodesianum* Vi4a, *Rh. mucilaginosa* Vi4b and *Sphingomonas* sp. 7a. It proved difficult to raise the cell number for *M. extorquens* 7b and 7c above 10^6 CFU ml^{-1} and this would complicate the bactericidal tests. *Rhodococcus erythropolis* Vi16, *Rh. mucilaginosa* Vi4b and *Sphingomonas* sp. 7a reached stationary phase after overnight incubation while *M. rhodesianum* Vi4a did so within 48 h when grown from a fresh inoculum with vigorous shaking in baffled flasks.

Growth at different temperatures was tested on TSA and PCA agar plates incubated at temperatures from 4 to 37°C for up to 14 days. All isolates grew at 12, 15, 25, 30 and 37°C. No isolates grew at 50°C. *Rhodococcus erythropolis* Vi16 and *Rh. mucilaginosa* Vi4b grew at 4°C while the other four isolates did not.

Resistance to disinfectants

The results of the bactericidal suspension tests are shown in Table 3. The results showed different patterns of resistance for the different isolates and control strains. *Sphingomonas* sp. 7a showed a medium grade and all other strains showed a high grade of resistance to All Des (based on amphoteric tensides). *Rhodococcus erythropolis* Vi16 showed high resistance to Oxonia active (peracetic acid) and also some resistance towards Titan Hypo (hypochlorite). *Methylobacterium rhodesianum* Vi4a showed high resistance to Titan Hypo (hypochlorite) and BC (QAC) as well as some resistance to TP99 (alkyl aminoacetate). *Rhodotorula mucilaginosa* Vi4b showed relatively low resistance to Oxonia active (peracetic acid) and Virkon. In general, the isolate *Sphingomonas* sp. 7a showed little or no resistance. *Staphylococcus aureus* showed no resistance besides All Des and *P. aeruginosa* showed some resistance towards BC.

Attachment to steel coupons

The degree of attachment to stainless steel is shown in Fig. 1. A low attachment percentage expresses a low tendency to attach to the steel surface while a high percentage indicates a higher tendency to attach. The results showed that *R. erythropolis* Vi16 had a very low tendency (<0·01%) for early attachment to stainless steel under the given conditions. *Sphingomonas* sp. 7a and *P. aeruginosa* expressed a relatively high tendency to attach to the steel coupons (5–10%). The remaining isolates, as well as *S. aureus*, showed a medium level of attachment (0·1–1%) compared with the other strains.

DISCUSSION

Few studies have been published on fogging as a disinfection method. Burfoot *et al.* (1999) showed that the effect of

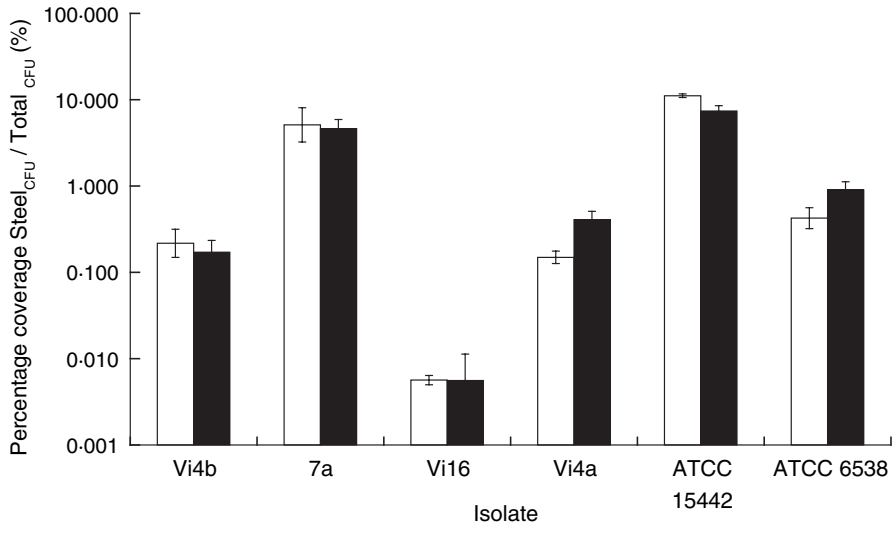

Fig. 1 Attachment of isolates to stainless steel coupons 1 h (□) and 2 h (■) after inoculation of *ca* 10^6 CFU ml^{-1}.

fogging is reduced on vertical and down-facing surfaces when compared with up-facing horizontal surfaces. Four of the six isolates in our study were isolated on walls and under the table of a steel trolley, indicating a possible reduced killing effect from the disinfectant fog.

Methylobacterium spp. and *Rhodotorula* spp. are common strains isolated in the dairy industry (Haridy 1992; Costa *et al.* 1993; Viljoen and Greyling 1995; Canganella *et al.* 1998; Westall and Filtenborg 1998; Krukowski *et al.* 2001; Petersen *et al.* 2002), while *Rhodococcus erythropolis* is less commonly reported. *Sphingomonas* has so far not been isolated in the dairy industry, but as it resembles *Pseudomonas* spp., which are quite commonly found in such locations, it is no surprise to find *Sphingomonas* sp. in the tested locations. Bagge-Ravn *et al.* (2003) compared the efficacy of peracetic acid-based fogging with hypochlorite-based foam in a salmon smokehouse. Their results indicated bacteria to be very sensitive to fogging disinfection in the studied location. They found that yeasts accounted for close to half of the surviving microflora after fogging, while Gram-negative bacteria, mainly *Pseudomonas* spp., dominated after foam sanitization.

Three of the six isolates studied and several isolates from the other three dairies all belonged to the genus *Methylobacterium*, which was first listed in the Approved list of Bergey's Manual of Systematic Bacteriology in 1980. Bergey's Manual from 1994 describes *Methylobacterium* as growing slowly or not at all on nutrient agar, occasionally occurring in rosettes or branched and pleomorphic, often containing large sudanophilic inclusions and sometimes volutin granules, growing strictly aerobic and growing as a pink surface ring or pellicle in liquid media (Holt *et al.* 1994). The isolates *M. rhodesianum* Vi4a, *M. extorquens* 7b and 7c fitted this description, although only *M. extorquens* 7c branched and typically showed asymmetrical rods and inclusion bodies. *Methylobacterium* spp. rarely cause human disease, but are known to be opportunistic pathogens and has been isolated from AIDS patients and others with reduced immune system (Truant *et al.* 1998; Sanders *et al.* 2000). These bacteria are also found in biofilm in potable water (Percival *et al.* 1998, 1999). Although the present study and other studies have demonstrated that the presence of *Methylobacterium* spp. is rather common in dairies, the role of this bacterium as regards product quality is yet an open question. Due to slow growth it sometimes escapes detection from routine hygiene controls. The fact that two of the three isolates identified as *Methylobacterium* grew on skim milk agar, and that all these three isolates grew at 12°C, might suggest that the conditions in dairy production plants promote growth of these bacteria. The *Methylobacterium* strain tested in the bactericidal test showed high resistance towards hypochlorite, which might be due to the cells producing a glutathione-utilizing hydrolytic dechlorinating enzyme (Obrien and Murphy 1993). It showed no resistance towards peracetic acid and only a low level of resistance towards alkyl amine which were used in the fogging process.

Rhodotorula mucilaginosa is a pathogenic yeast (Haridy 1992) and care should be taken to avoid it in production facilities. Growth studies show that it grows at 4°C and other literature reports growth in dairy products such as yoghurt at 8 and 4°C (Canganella *et al.* 1998). Yeast can cause spoilage of many food products and has been isolated from different food sources such as cheese (Corsetti *et al.* 2001), juice (Shearer *et al.* 2002), salami (Abunyewa *et al.* 2000) and intermediate moisture meats (Wolter *et al.* 2000).

Sphingomonas was proposed as an independent genus first in 1990 (Yabuuchi *et al.* 1990). Previously, it was classified as belonging to the genus *Pseudomonas*. The genus *Sphingo-*

monas is a homogeneous group of organisms in the α-4 subclass of *Proteobacteria* (Takeuchi *et al.* 1994; White *et al.* 1996). It is of industrial interest due to the production of gellan gum (White *et al.* 1996; Giavasis *et al.* 2000). *Sphingomonas* is an opportunistic pathogen (Miyazaki *et al.* 1995) and many members of the genus are capable of degrading refractory pollutants, for instance 4-fluoro, 4-chloro and 4-bromo derivatives (Schmidt *et al.* 1993; White *et al.* 1996; Stolz *et al.* 2000).

The actinomycetes genus *Rhodococcus* is well known for its ability to degrade hydrocarbons and *Rhodococcus erythropolis* can degrade various chloroaromatics, nitroaromatics and polychlorobiphenyls (PCB) (Warhurst and Fewson 1994). Rhodococci are also implicated in foam formation in activated sludge waste water treatment plants (Blackall 1994).

The fact that these isolates were present after thorough cleaning and fogging disinfection can be explained in several ways. Resistance towards the chosen disinfectants could be one explanation, biofilm formation another. Micro-organisms living within a biofilm may be 100–1000 times less susceptible than in a free living state (Gilbert *et al.* 2002). Resistance of this kind has been demonstrated towards antibiotics and antiseptics as well as highly reactive chemical biocides, including QAC (Evans *et al.* 1990) and halogens and halogen-releasing agents (Walker *et al.* 1994).

Our results indicate that the *Sphingomonas* sp. 7a isolate, in showing no resistance in bactericidal suspension tests to neither peracetic acid nor alkyl amine, did not survive due to intrinsic or acquired resistance disinfectant resistance. On the contrary, it showed a relatively high rate of attachment to stainless steel. This led to the assumption that the *Sphingomonas* isolate might have survived disinfection from its ability to attach to surfaces. Our work focuses on early attachment rather than on established biofilm. This is due to the fact that the well-established cleaning and disinfection routines in the dairies investigated, including alternation between different disinfectants, would probably exclude the micro-organism's opportunity to form an established biofilm. However, the ability of the micro-organism to quickly attach to surfaces is critical for it to survive the cleaning and fogging routine. Another fact is that some micro-organisms acquire resistance through controlled growth rate. Ostrowski *et al.* (2001), showed *Sphingomonas alaskensis* to express specific growth rate control of hydrogen peroxide resistance. This could explain the low level of resistance the isolate *Sphingomonas* sp. 7a shows in bactericidal suspension tests after being grown in rich medium, while still surviving the fogging disinfection in a potentially starved situation.

The *Rhodococcus* isolate showed high resistance towards peracetic acid. Good routines in the tested production plant, including frequently alternating between two different disinfectants, supposedly would have secured killing of this organism as it was killed by the alternative disinfectant alkyl amine. None of the isolates showed resistance to both peracetic acid (Oxonia active) and alkyl amine (TP99) used in the fogging procedures at the two dairies. This implies that the chosen combination of disinfectants is also effective for removing resistant micro-organisms. Choosing the right combination of disinfectants for effective alternation is a challenge. The recommended approach (Langsrud *et al.* 2003a) is to apply disinfectants that hold different killing mechanisms, for example an oxidizing agent alternating with a surfactant. In a recent study of resistance in *Pseudomonas* spp. Langsrud *et al.* (2003c) suggest a combination of BC and hypochlorite for alternation purposes. In the present work the resistance of *Methylobacterium* towards both BC and hypochlorite shows that the microflora in the facilities tested can also survive this combination. The combination of peracetic acid and hypochlorite does not secure killing of the *Rhodococcus* isolate. Rotation of biocides is a common practice in the Norwegian food industry, but less common in, for instance, the UK (Holah *et al.* 2002). Murtough *et al.* (2001) reviewed the scientific evidence for rotation of biocides in the clinical area and concluded that rotation of biocides could be advocated in 'dirty environments' containing high concentrations of micro-organisms.

Two common ATCC strains were included in the experiments for comparison. *Pseudomonas aeruginosa* and *S. aureus* were chosen due to their known ability to grow in biofilm and also because *P. aeruginosa* exhibits a high level of resistance towards BC (Langsrud and Sundheim 1997). This was also confirmed in the present study. *Sphingomonas*, although earlier classified as *Pseudomonas*, does not share this level of resistance in our study. The identification was only 96% by 16S rDNA for this isolate and it should also be noted that some *Pseudomonas* strains are less resistant than others. The identification of the isolate 7a could be verified by analysis of the outer membrane which is typical of *Sphingomonas*, containing glycosphingolipids and lacking lipopolysaccharides (White *et al.* 1996).

The high rates of survival of all the tested organisms towards the amphoteric tensides disinfectant All Des is probably due to the lowest recommended user concentration being too low. Virkon was definitively the most potent disinfectant in the bactericidal suspension tests. However, the yeast showed some resistance towards this potent disinfectant. Virkon combines several mechanisms of killing and its low toxicity has made it a common choice in hospital environments.

Many different factors contribute to the survival of micro-organisms in food production plants. The general access to water and nutrients, temperature as well as the design of the production facilities inflicts a selective pressure resulting in the basic microflora of the production plant. Cleaning and

disinfection routines intended to eliminate unwanted micro-organisms from the facilities add to the selective pressure on this microflora. The micro-organisms are known to adapt to this hostile pressure and acquire higher levels of resistance. This resistance can be of different natures including attachment to surfaces followed by biofilm formation often referred to as physiological resistance (McDonnell and Russell 1999). This type of resistance seemed to be the survival mechanism of the 7a isolate in our work. This work has shown the presence of micro-organisms in two dairy production plants after thorough cleaning and disinfection. Some factors concerning their survival of cleaning and disinfection routines have been studied. Four of the isolates belonged to the genus *Methylobacterium* and *Sphingomonas* and may potentially cause spoilage of product. *Methylobacterium* isolates seem to be common in Norwegian dairy facilities. The isolated yeast identified as *Rhodotorula mucilaginosa* is a human pathogen. It constitutes a public health hazard and steps should be taken to avoid it in dairy products. These three types of isolates have also been reported in dairy products elsewhere. The last isolate *Rhodococcus erythropolis* is well known for its ability to break down complex substrates, but it is uncertain to what extent it causes spoilage of food or whether it implies any other risk to the consumer.

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