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Mechanisms of biofilm formation in paper machine by *Bacillus* species: the role of *Deinococcus geothermalis*

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Mechanisms for the undesired persistence of *Bacillus* species in paper machine slimes were investigated. Biofilm formation was measured for industrial *Bacillus* isolates under paper machine wet-end-simulating conditions (white water, pH 7, agitated at 45ºC for 1–2 days). None of the 40 tested strains of seven *Bacillus* species formed biofilm on polished stainless steel or on polystyrene surfaces as a monoculture. Under the same conditions, *Deinococcus geothermalis* E50051 covered all test surfaces as a patchy thick biofilm. The paper machine bacilli, however, formed mixed biofilms with *D. geothermalis* E50051 as revealed by confocal microscopy. Biofilm interactions between the bacilli and the deinococci varied from synergism to antagonism. Synergism in biofilm formation of *D. geothermalis* E50051 was strongest with *Bacillus coagulans* D50192, and with the type strains of *B. coagulans*, *B. amyloliquefaciens* or *B. pumilus*. Two *B. licheniformis*, one *B. amyloliquefaciens*, one *B. pumilus* and four *B. cereus* strains antagonized biofilm production by *D. geothermalis*. *B. licheniformis* D50141 and the type strain of *B. licheniformis* were the strongest antagonists. These bacteria inhibited deinococcal growth by emitting heat-stable, methanol-soluble metabolite(s). We conclude that the persistence of *Bacillus* species in paper machine slimes relates to their ability to conquer biofilms formed by primary colonizers, such as *D. geothermalis*. *Journal of Industrial Microbiology & Biotechnology* (2001) **27,** 343–351.

Keywords: paper machine; biofilm; stainless steel; coadhesion; *Deinococcus geothermalis*

Introduction

Slimes impair the quality of endproducts and runability of paper machines [2,4,12,32,33]. *Bacillus* species, including *Bacillus cereus*, are frequently found in paper machine water and slimes [2,4,12,24,29,30,32,33]. Several *Bacillus* species can deteriorate raw materials of papermaking [29]. In paper products, they frequently are the dominant microbial contaminant [12,23, 27,30,33] because their heat-resistant spores survive the hot drying section of the machines. *B. cereus* is capable of food poisoning and classified to biohazard category 2; therefore, its occurrence in paper products is undesirable [24]. Paper mills use biocides to control microbial growth in the wet-end water circuits and sizing starches, but this has not eradicated bacilli from the paper products [12,23,27,30,33]. Mechanisms by which the bacilli are retained in the paper machine environment are not known. Some *Bacillus* species are resistant to several industrial biocides [22,29]. Biofilm formation protects bacteria against antimicrobial agents [5,34]. Bacilli can form biofilms on surfaces in dairy processes [9,10,18,35], which are rich in nutrients. Paper machine white water (WW) contains only 3–18 mg l^{-1} of total N and 2–6 mg l^{-1} of total P [32,33].

Understanding the mechanisms by which *Bacillus* species survive in paper machines should help in developing strategies for their elimination from paper products. We isolated *Bacillus* spp. from biofilms grown on stainless steel coupons in the water circuits of three paper machines. We describe here the biofilm-forming properties of paper-industry-contaminating *Bacillus* strains, belonging to seven species. The results show that monocultures of *Bacillus* strains formed no biofilm under paper machine wet-end-simulating conditions on any of the tested surfaces. The paper industry bacilli coadhered with the paper machine strain, *Deinococcus geothermalis* E50051, a primary colonizer of stainless steel [33].

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Materials and methods

Isolation and characterization of biofilm bacteria from paper machines

Coupons of stainless steel AISI 316 (15 mm×75 mm), cleaned with 94% (v/v) ethanol, were immersed in the wet-end areas of three paper machines. Pure cultures were isolated from biofilms formed on the coupons, using half-strength Tryptic Soy Agar (TSA; BBL, Becton Dickinson, Cockeysville, MD, USA) and nutrient agar (Lab M, Bury, England) at 45°C as described by Väisänen *et al* [33]. Gram reaction, cell morphology and the presence of intracellular inclusions were recorded from cells grown on plates of (BBL, Becton Dickinson) or half-strength TSA for 1–2 days at 45°C.

Whole cell fatty acids were analyzed from cultures grown on Tryptic Soy Broth Agar (TSBA) plates for 24 or 48 h at 45°C or 24 h at 28°C. Fatty acids were extracted, methylated and analyzed as described [32,33]. Gram-positive or Gram-variable rods (1 μm×2–3 μm or larger) were considered presumptive members of the genus *Bacillus* (Table 1), when over 80% of the whole cell fatty acids were branched chain fatty acids [15], 13-methyltetradecanoic acid being the most abundant (37–66%), followed by 14-methyltetradecanoic or 15-methylhexadecanoic acids (*ca.* 20%). Isolates were identified as *B. cereus* based on the presence of signature fatty acids 11-methyldodecanoic acid and *trans*-9-

Table 1 Bacteria isolated at 45°C from biofilms grown on stainless steel coupons during immersion of 2–24 h in the water circuits of the wet-end areas of three paper machines

Isolates[a]	Numbers of isolates from paper mill		
	A	B	C
Gram-positive isolates			
B. cereus	6	4	
Bacillus sp. (*B. licheniformis*, *B. pumilus*+other)	4	9	2
Other Gram-positive rods	7	1	3
Deinococcus sp.		5	
Other Gram-positive cocci	2	1	
Gram-negative isolates			
Thermomonas-like strains	8		3
Ochrobactrum sp.			2
Other Gram-negative rods			4
Gram-variable cocci			3
Total number of isolates (64)	27	20	17

[a]Naming and grouping of the isolates based on Gram reaction, morphology and whole cell fatty acid composition.

hexadecenoic acid [23]. Isolates with whole cell fatty acid compositions resembling that of *Thermomonas haemolytica* sp. nov., isolated from a paper machine [3], were named as *Thermomonas*-like. Other bacterial strains were named as received (Table 2).

Polystyrene plate assay for biofilm formation

To prepare WW medium, authentic wire water of a publication paper machine operated at neutral pH was collected, amended with 100 mg l^{-1} of yeast extract, filtered through a metal sieve with pore size of 74 μm (to remove fibres but not bacteria, process fines or fillers), sterilized (121°C, 15 min) and adjusted to pH 7.0 with sterile 2 M HCl. The potential of WW medium to support growth of the bacterial cultures was verified by spreading 100 μl of the inoculum on a WW agar plate (WW medium solidified with 15 g l^{-1} agar) because opacity of the medium prevented turbidometric measurements. Plates were read after 3 days at 45°C.

The biofilm formation was analyzed by a 96-well microtiter plate assay, modified from Cowan and Fletcher [6] using plates of regular polystyrene (MicroWell 269787) or of Tissue Culture Treated Polystyrene (Nunclon 167008; Nalge Nunc International, Roskilde, Denmark). Each well with 240 μl of sterile WW medium was inoculated with 2.5 μl of a culture grown in Tryptic Soy Broth (TSB; Oxoid Unipath, Hampshire, UK) for 1 day at 37°C. The microtiter plates were placed on a rotary shaker (160 rpm, 45°C) for 22 h. The wells were emptied, stained with 300 μl of aqueous acridine orange (0.1 g l^{-1}) or crystal violet (4 g l^{-1} in 20% (v/v) methanol) for 3 min, washed three times under a running water tap to remove nonadhering biomass and allowed to air-dry. Stain retained by the biofilm was dissolved in ethanol (300 μl per well, 1 h) and A_{500} (acridine orange) or A_{595} (crystal violet) was measured with a plate reader (iEMS Reader MF; Labsystems, Helsinki, Finland).

To correlate the absorbance values with biomass quantity, a TSB culture of *D. geothermalis* E50051 with a known density of cells was stained with crystal violet (4 g l^{-1}) for 3 min, spun down (2600×*g*, 5 min) and washed with water three times. The retained stain was dissolved in ethanol, aliquots were pipetted into microtiter plate wells, filled to 300 μl with ethanol and scored for absorbance with a plate reader. Lower detection limit of the polystyrene plate assay was 1×10^4 cfu per well (*ca.* 5×10^3 cfu cm^{-2}). A_{595} values between 0 and 4.0 correlated linearly with cfu.

Assay of biofilm formation on stainless steel

Discs (39 mm^2) of stainless steel AISI 316 were polished to 320 grit with a water sanding paper, degreased with acetone and

Table 2 Bacterial strains used in this study

Strain	Origin of the strain	Reference/source
Originating from paper industry environment		
B. coagulans E50L1	paper machine, slime from steel in wire area	[33]
B. coagulans E50L3	paper machine, slime from steel in wire area	[33]
B. coagulans D50192	paper machine, WW	[33]
B. cereus JN11002	slime on steel coupon exposed to paper machine WW	this study
B. cereus Cal1[a]	board machine, calander water	[24]
B. cereus BM-VIII P1[a]	board machine, slime	[31]
B. amyloliquefaciens TSP55	food packaging board	[25]
B. pumilus TSP66	food packaging board	[25]
B. licheniformis D50141	bentonite	[33]
B. sphaericus D28143	bentonite	[33]
B. circulans C28141	bentonite slurry	[33]
D. geothermalis E50051	paper machine, slime from wire area. A known primary colonizer of stainless steel	[33]
Reference strains		
B. amyloliquefaciens DSM7^T	culture collection strain	MSS[b]
B. cereus ATCC14579^T	culture collection strain	MSS
B. coagulans DSM1^T	culture collection strain	MSS
B. licheniformis DSM13^T	culture collection strain	MSS
B. pumilus DSM27^T	culture collection strain	MSS

[a]Enterotoxin-producing strain [24].
[b]MSS=strain collection of Mirja Salkinoja-Salonen.

mounted into a hole drilled in the vertical wall of a 100-ml HDPE flask, flushed with the inner surface of the flask to avoid edge effects. The interior of the flask was cleaned for 1 h with 94% (v/v) ethanol. Each flask with 65 ml of sterile WW medium was inoculated with 150 μl of the test bacteria pregrown in TSB (24 h, 45°C, 160 rpm). For air supply, the cap of each flask was pierced with an injection needle protected by a 0.45-μm sterile filter. The discs were collected after incubation for 1 or 2 days (45°C, 160 rpm), rinsed twice under running tap water, stained for 10 min with aqueous acridine orange (100 mg l^{-1}; Molecular Probes Europe, Leiden, the Netherlands) and rinsed twice with tap water. The heights of cell clusters grown on the discs were measured under an epifluorescence microscope (Nikon Eclipse E800, Tokyo, Japan) using a 100× oil immersion objective (Nikon Plan Fluor, NA1.3) and a scale in the z-focus screw. Cells were counted from 30 randomly selected fields of view per disc. The cumulative fluorescence of the acridine-orange-stained biofilm on steel discs was measured using a scanning fluorometer (Fluoroskan Ascent; Labsystems) with the filter pair 485/538 nm and an integration time of 100 ms for each of the 40 measurement points covering the whole surface area of the disc.

Confocal laser scanning microscopy (CLSM) of biofouled steels

One-day-old biofilms on the stainless steel discs were stained with acridine orange. If not inspected immediately, the stained biofilms were fixed with 2% v/v glutaraldehyde (TAAB, Reading, UK) in sterile phosphate buffer (0.1 M, pH 7.5, 2 h), rinsed three times with the buffer and stored in the dark at 4°C. The biofilms were examined using a BioRad MRC-1024 confocal laser scanning system with an inverted Zeiss Axiovert 135M light microscope. The 488-nm excitation line of a KrAr laser (3% of the maximum power of 15 mW), emission filters BP 506–538 nm and EFLP 585 nm (based on the fluorescence emission maxima of acridine orange bound to DNA or to RNA; 526 and 650 nm [11]) and pinholes of 2.0 mm were used. The discs were mounted in water on sample holders as described in Ref. [16]. Pieces of coverglass were used as spacers to prevent compression of the biofilm when it was examined with a 63× oil immersion objective (Zeiss Plan-Apochromat, NA1.4) of the inverted microscope. Image collection and processing were performed with the software described in Ref. [16].

Assays of growth inhibition

Plate assay: The test strains were grown for 3 days in 4 ml of TSB (37°C, 160 rpm), 4 ml of methanol was added and the culture was shaken for 2 h. The extract was sterile-filtered (0.2 μm), and its aliquot boiled in a sterile screw-capped glass vial for 10 min. An overnight culture (100 μl) of *D. geothermalis* E50051 was spread on TSA agar plates and air-dried in a laminar flow hood for 30 min. Aliquots (10 μl) of the bacterial filtrates (50% v/v methanol, boiled and nonboiled) were pipetted on the agar surface. Inhibition zones around the pipetted spots were read after 1 day at 45°C from four to eight parallel spots. Filtrates (50% v/v methanol) of sterile TSB and of *D. geothermalis* E50051 TSB cultures were used as negative controls.

Microtiter plate assay: Extracts were prepared of the test strains, grown on TSA plates (37°C, 10 days), in 100% methanol as described by Andersson *et al* [1]. Antagonism towards biofilm formation by *D. geothermalis* was tested by the polystyrene plate method with 5–40 μl of the methanol extract (or methanol as negative control) and 230 μl of R2 broth per well, inoculated with 5 μl of *D. geothermalis* E50051 in overnight culture in TSB.

Results

Bacteria colonizing paper machine surfaces

Adherence to surfaces and growth as biofilms are the mechanisms by which biofouling persists in industrial processes. Bacteria were isolated from biofilms grown on stainless steel coupons immersed in wet-end areas of three paper machines. Table 1 shows that 39% of the 64 isolates were presumptive members of the genus *Bacillus* and 16% were identified as *B. cereus*. The biofilms may thus represent a reservoir shedding vegetative cells and spores of *Bacillus* sp. into the paper machine water circuits.

The biofilm-forming abilities of the 25 *Bacillus* sp. isolates (Table 1) and *D. geothermalis* E50051, a known steel-adhering paper machine strain, were examined. This was done by the polystyrene plate assay at 45°C in sterilized paper machine WW-based medium with an agitation of 160 rpm equivalent to a peripheral flow of 60 mm s^{-1} in the wells. These parameters were chosen to simulate paper machine wet-end conditions. Biofilms formed on surfaces of the wells within 22 h were stained with acridine orange and quantified by scoring the absorbance at 500 nm. Absorbance yields of the 25 *Bacillus* sp. cultures were less than twice those obtained for control, noninoculated WW medium (A_{500} 0.1). Biofilm production was similarly poor on regular and on tissue-culture-treated (more hydrophilic) polystyrene. Under the same conditions, *D. geothermalis* E50051 produced biofilms of 0.7–1.0 absorbance units. The inefficiency in generating biofilms in paper machine water shared by the 25 *Bacillus* isolates from paper machine biofilms was unexpected considering their high number in the paper machine slimes (39% of all isolates).

Mono- and coculture biofilms on polystyrene

Biofilm production by paper industry *Bacillus* species was investigated further with well-characterized *Bacillus* strains, isolated from the machines, raw materials and endproducts of paper and board mills (Table 2). The *Bacillus* strains were grown in wells of the polystyrene plates as monocultures or as cocultures with *D. geothermalis* E50051, as the above-described results suggested that the presence of *Bacillus* spp. in the biofilms might depend on interactions with other species. The results in Figure 1 show that the 16 live *Bacillus* monocultures adhered no more than killed indigenous bacteria from autoclaved WW medium, whereas *D. geothermalis* E50051 formed thick biofilms both on the regular and the tissue-culture-treated polystyrene surfaces (A_{595} 2.20±0.31 and 2.16±0.38, equivalent to $>3\times10^6$ cfu cm^{-2}). Failure of the 16 *Bacillus* strains to produce biofilm was not caused by their inability to propagate in WW medium: they grew similarly to *D. geothermalis* E50051, except *B. sphaericus* D28143 and *B. circulans* C28141, which failed to grow on WW agar plates at 45°C.

Figure 1 shows that when the *Bacillus* strains were grown in coculture with *D. geothermalis* E50051, six of the cocultures produced clearly more biofilm than the deinococci did alone. This synergy was not limited to the paper machine isolates, e.g., *B. coagulans* D50192, but also occurred with type strains of

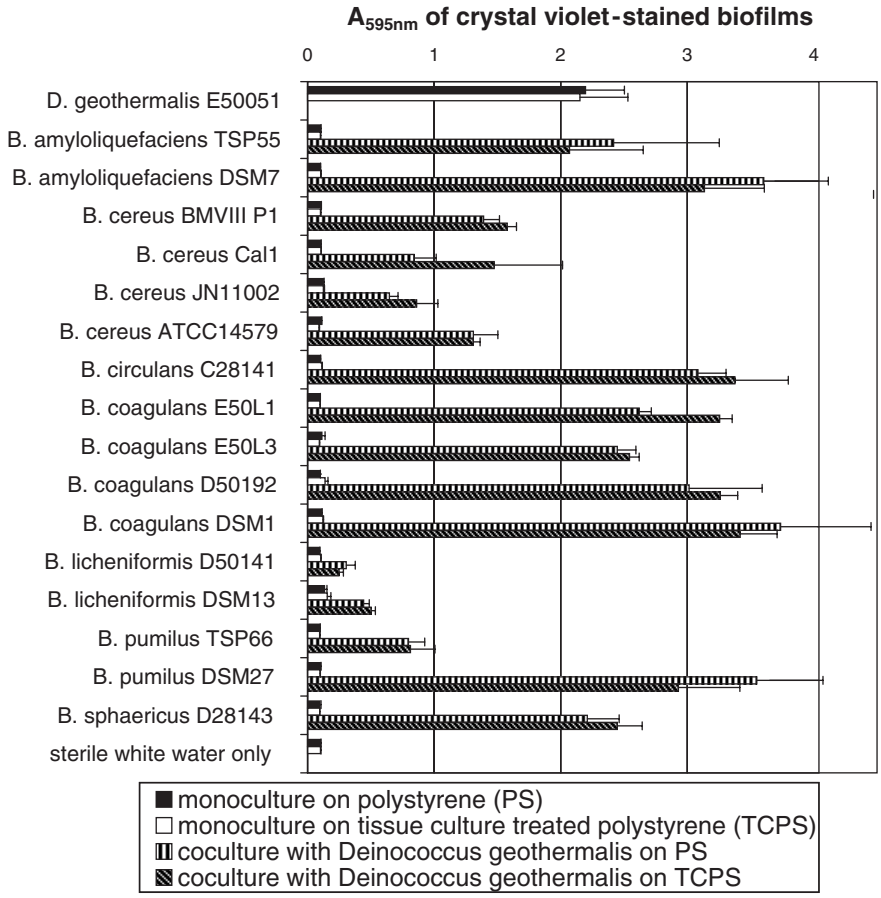

Figure 1 Biofilm formation of 11 *Bacillus* strains of paper industry origin and five *Bacillus* type strains in monoculture and in coculture with *D. geothermalis* E50051. Biofilm formation was measured in WW medium (22 h, 45°C, 160 rpm) in wells of the 96-well plates of polystyrene and tissue-culture-treated polystyrene. The bars show the average A_{595} ± SD of triplicate wells of the crystal-violet-stained biofilms after dissolution of the stain in ethanol. The highest A_{595} values were equivalent to $> 7\times10^6$ cfu cm^{-2}.

B. coagulans, *B. amyloliquefaciens* and *B. pumilus* (Figure 1). Similar results were obtained on the regular and tissue-culture-treated polystyrene surfaces (Figure 1). Seven *Bacillus* strains (*B. pumilus* TSP66 and the strains of *B. cereus* and *B. licheniformis*) antagonized biofilm formation of *D. geothermalis* E50051 (Figure 1). The two *B. licheniformis* strains were the strongest *Deinococcus* antagonists.

Biofilm formation on stainless steel

Biofilm-forming properties of the paper industry *Bacillus* strains (topmost nine strains listed in Table 2) were inspected on stainless steel. Panels A–F in Figure 2 show stereomicroscope images of acridine-orange-stained monoculture biofilms formed on discs of stainless steel within 2 days in WW medium. None of the nine *Bacillus* monocultures formed a visible biofilm on the steel (Panels B–F), whereas *D. geothermalis* E50051 (Panel A) formed a patchy biofilm up to 45±17 μm in height (Table 3). The cumulative fluorescence yields (index of biofilm biomass) of the stainless steel discs incubated with *Bacillus* monocultures were less than 2% of that emitted by the biofilm of a *D. geothermalis* monoculture on a similar steel disc (Table 3). Packing of deinococci in the cell clusters was dense; therefore, the epifluorescence microscopic estimate of 1×10^8 cells cm^{-2} is approximate. Epifluorescence microscopic cell counts of *Bacillus* monocultures on the steel surface ranged from 400 cells cm^{-2} for *B. pumilus* TSP66 to 2×10^5 cells cm^{-2} for *B. cereus* Cal1 (Table 3), i.e., 0.0004–0.16% of those of *D. geothermalis* E50051. The *Bacillus* cells and spores developed no multilayered cell clusters on steel, but scattered on the steel surface in an arbitrary manner, also inferred from the high coefficient of variation in the cell counts (Table 3).

The microscopic count of 1×10^8 deinococcal cells cm^{-2} on steel is higher than the 3×10^6 cfu cm^{-2} estimated for the well-grown *D. geothermalis* E50051 biofilms on polystyrene surfaces. In the polystyrene plate assay, the crystal violet may have penetrated only the topmost layers of the dense cell clusters, leaving the core of the biofilm unstained. This would explain the underestimation of the cell numbers in the microtiter plate assay, where suspension-stained cells were used to calibrate absorbance values.

Panels G–M in Figure 2 show stainless steel coupon surfaces exposed to *Bacillus* sp. with *D. geothermalis* E50051. All cocultures produced more biofilm than any of the tested *Bacillus* strains did in monoculture (Panels B–F in Figure 2; Table 3). *D. geothermalis* E50051 produced more biofilm with *B. coagulans* D50192 and *B. cereus* Cal1 than it did in monoculture (Figure 2, Table 3), but less in the presence of *B. amyloliquefaciens* TSP55, *B. cereus* JN11002, *B. coagulans* E50L3 or *B. licheniformis* D50141 (Table 3; Panels G and I in Figure 2). This is similar to the strong antagonism by *B. cereus* JN11002 and *B. licheniformis*

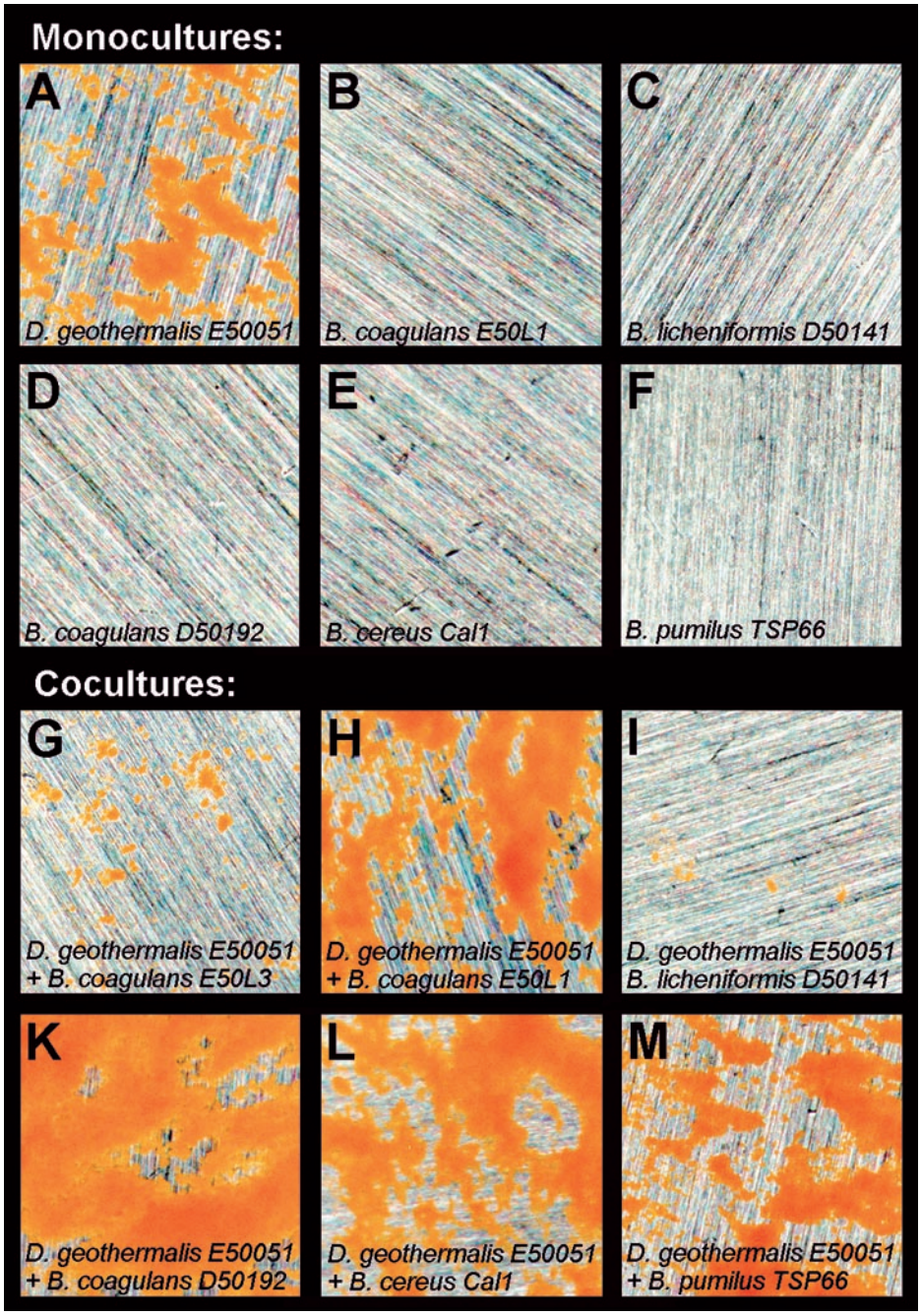

Figure 2 Stereomicroscope images of acridine-orange-stained biofilms grown on discs of polished (320 grit) stainless steel. Sterilized WW medium was inoculated with one *Bacillus* culture at a time, alone or in coculture with *D. geothermalis* E50051. Each panel displays 1 mm^2 of the steel surface after growth of 2 days at 45°C.

D50141 observed on polystyrene surfaces (Figure 1), except the strong antagonism of *B. amyloliquefaciens* TSP55 on stainless steel, which was not visible on the polystyrenes (Figure 1). *B. licheniformis* D50141 was the strongest *Deinococcus* antagonist on the all three types of surfaces (Figures 1 and 2, Table 3).

Confocal microscopy of coculture biofilms

Species interactions in the coculture biofilms formed by the nine *Bacillus* strains with *D. geothermalis* E50051 were inspected with CLSM. Monocultures of *D. geothermalis* E50051 formed compact colonies on stainless steel (Figure 3A). Microfibrils visible within the colony amidst the coccoidal cells are fibrous fines of pulp origin from the WW. CLSM images from the biofilms formed by strains of *B. cereus*, *B. coagulans* or *B. licheniformis* in coculture with *D. geothermalis* E50051 show rod-shaped *Bacillus* sp. abundant inside (Panels B–F in Figure 3) or anchored on the surface of the deinococcal colonies (Panel D). The CLSM images show that true mixed biofilms were formed on the steel surfaces exposed to the cultures of two species.

Most of the acridine-orange-stained deinococci displayed a strong red fluorescence, indicating high RNA content of the cells

Table 3 Dimensions of biofilms generated on discs of stainless steel by strains of *Bacillus* species isolated from the paper industry in monoculture and in coculture with *D. geothermalis* E50051[a]

Strain	Biofilm formation by *Bacillus* strains in				
	Monoculture			Coculture with *D. geothermalis*	
	Epifluorescence microscopic count of the acridine-orange-stained cells		RFU[b] (cumulative and range)	Height of cell clusters μm (mean±SD)[c]	RFU[b] (cumulative and range)
	Cells cm^{-2}	CV[d]			
B. amyloliquefaciens TSP55	500	2.04	6 (0.2)	8±2	18 (1.6)
B. cereus JN11002	7×10^4	1.10	6 (0.1)	9±4	37 (2.2)
B. cereus Cal1	2×10^5	0.88	4 (0.1)	58±14	512 (34.5)
B. coagulans E50L1	7×10^3	1.93	5 (0.2)	37±7	346 (30.3)
B. coagulans E50L3				17±5	78 (4.5)
B. coagulans D50192	4×10^4	0.66	5 (0.1)	55±19	799 (74.8)
B. licheniformis D50141	9×10^3	1.97	6 (0.2)	16±5	6 (0.1)
B. pumilus TSP66	400	2.44	5 (0.1)	41±20	403 (35.3)
D. geothermalis E50051	1×10^8		403 (28.2)	45±17	403 (28.2)

[a]The biofilms were grown in WW medium (2 days, 45°C, 160 rpm).
[b]RFU=relative fluorescence units. Fluorescence of the stained biofilm was measured from 40 fields covering the surface of the whole stainless steel disc (39 mm^2). From sterile control disc, the cumulative rfu was 3 and the range between highest and lowest scores was 0.1 rfu.
[c]Measured with an epifluorescence microscope from cell clusters (n=20).
[d]Coefficient of variation in 30 microscopic fields.

and thus high metabolic activity, in monoculture (Figure 3A) or in cocultures, e.g., with *B. coagulans* D50192 (Figure 3E). Panels B, C and F in Figure 3 show coculture biofilms, in which deinococci emitted green or slightly yellow fluorescence, suggesting that the deinococcal cells had less RNA and a lower metabolic activity in the vicinity of cells of *B. cereus* BM-VIII P1 or JN11002 (Panels B and C), *B. licheniformis* D50141 (Panel F) or *B. amyloliquefaciens* TSP55 (not shown in Figure 3). When cumulative fluorescence yields (index of biofilm biomass) were measured from the microscoped biofilms, the cocultures of *D. geothermalis* E50051 with *B. licheniformis* D50141, *B. amyloliquefaciens* TSP55 or *B. cereus* JN11002 yielded 1%, 4% or 32%, respectively, of that produced by *Deinococcus* monocultures. These three *Bacillus* species thus suppressed metabolic activity and slowed down biofilm formation by deinococci on stainless steel in paper machine water.

The CLSM results indicate that *D. geothermalis* E50051 served as a platform for the paper industry bacilli to adhere to steel, also for those species which subsequently antagonized the metabolic activity and biofilm production of their host (*D. geothermalis* E50051).

Inhibition of D. geothermalis by bacilli

To explore the nature of the observed antagonisms, extracts from broth and plate cultures of the 16 *Bacillus* strains (Table 2) were investigated for their effects on *D. geothermalis* E50051.

Filtrates in 50% v/v methanol from TSB broth cultures of *B. pumilus* TSP66, *B. licheniformis* D50141, *B. amyloliquefaciens* TSP55 and *B. licheniformis* DSM13^T produced inhibition zones of 12±1, 11±1, 10±1 and 9±1 mm (n=4) to *D. geothermalis* E50051 grown on TSA plates, i.e., significantly more than the ≤2 mm produced by negative controls (filtrates in 50% v/v methanol from sterile TSB or from *D. geothermalis* E50051 itself). Antagonism of these four *Bacillus* strains towards the biofilm formation of *D. geothermalis* (Figures 1–3, Table 3) was thus mediated by growth-inhibiting diffusible cell-free substances. Heating (100°C, 10 min) did not reduce the inhibitory effect of the extracts, nor affect the negative controls, indicating that no proteins were involved. Extracts from the type strains of *B. pumilus* and *B. amyloliquefaciens* also inhibited growth of *D. geothermalis* (inhibition zones of 8±1 and 8±0 mm, n=4) on TSA plates, although these strains did not reduce the biofilm mode of growth of *D. geothermalis* in WW medium (Figure 1). Methanol filtrates from TSB broth cultures of 10 further *Bacillus* strains (Table 2) were noninhibitory towards *D. geothermalis* (inhibition zone ≤2 mm), including the four strains of *B. cereus* that moderately antagonized growth of *D. geothermalis* biofilms on polystyrene in WW medium (Figure 1). The relationship between growth on TSA plates and biofilm antagonism is thus of a complex nature.

The four *Bacillus* strains that showed the strongest biofilm antagonism on the stainless steel (Table 3) were grown for 10 days on TSA plates, extracts were prepared in 100% methanol and tested for their effects on biofilm formation of *D. geothermalis* E50051 on polystyrene plates. Extracts (0.2 mg ml^{-1} dry weight) from *B. licheniformis* D50141, *B. amyloliquefaciens* TSP55 and *B. cereus* JN11002 reduced biofilm production by *D. geothermalis* E50051 by 88±2%, 84±3% and 70±2% (reduction in the A_{595} of the crystal-violet-stained biofilm). A three-times-higher concentration of the extract from *B. pumilus* TSP66 was required for a similar decline. Relatively high concentrations were needed; thus, the metabolites were only moderately toxic. Effective inhibition may require the presence of bacilli in close proximity to *D. geothermalis* cells in the biofilms. The results show that the microtiter plate assay served better than the agar plate assay for the inhibition study. The strains of four *Bacillus* species produced methanol-soluble metabolite(s) capable of partially repressing biofilm formation of *D. geothermalis*,

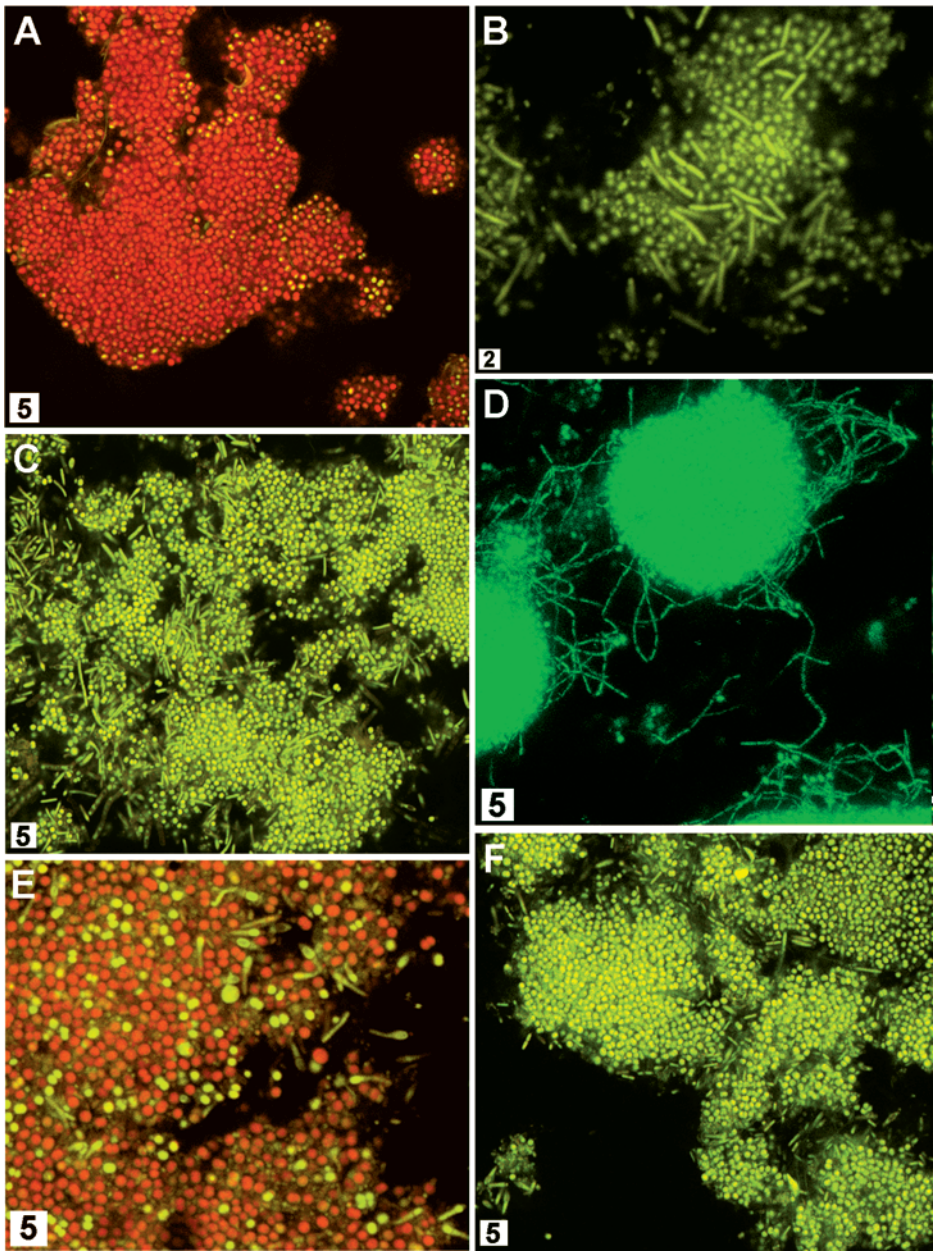

Figure 3 Confocal laser scanning microscope images of acridine-orange-stained biofilms of *Bacillus* species from paper machines in coculture with *D. geothermalis* E50051 on discs of polished (320 grit) stainless steel. WW medium was inoculated with *D. geothermalis* E50051 and with none (A), *B. cereus* BM-VIII P1 (B), *B. cereus* JN11002 (C), *B. coagulans* E50L1 (D), *B. coagulans* D50192 (E) or *B. licheniformis* D50141 (F), and grown for 1 day at 45°C. Panels A, B, C, E and F show single optical sections lateral to the steel surface (dual channel detection: 506–538 nm and > 585 nm). Panel D is a sum projection built using maximum intensity method from 10 optical sections lateral to the steel surface with z-steps of 1.0 μm (single channel detection only: 506–538 nm). The scalebox is 2 or 5 μm as shown in each panel.

explaining the antagonism observed in the coculture biofilms (Table 3, Figures 2 and 3).

Discussion

Several species of *Bacillus* are ubiquitous in paper machine biofilms, but were incapable of independent biofilm formation on stainless steel or on polystyrene surfaces in paper machine water in the laboratory. In the presence of *D. geothermalis*, these bacilli were incorporated into biofilms. Our results indicate that persistence of *Bacillus* species in paper machines depends on primary colonization of the clean surfaces by other, adhesion-promoting species. The species *D. geothermalis* is heat-tolerant, first isolated from hot springs [8]. Deinococci resist desiccation [21]. These two properties may explain its propagation in the splash zones of paper machines, where slimes often form.

To our knowledge, this is the first detailed study on adhesive bacterial interactions related to biofouling of paper machines. In drinking water distribution systems, adhesion of pathogenic bacteria into biofilms formed by indigenous water bacteria has been shown [28]. Interactions of pathogenic *Listeria monocyto-*

genes with other bacteria in biofilms of food processing plants have been observed [9]. Elvers *et al* [7] isolated bacteria and fungi from the photoprocessing industry and showed differences in colonization rates between single species biofilms and a seven-species bacterial–fungal biofilm. Skillman *et al* [26] showed cooperation between industrial isolates of *Klebsiella pneumoniae* and *Enterobacter agglomerans* resulting in enhanced biofilm formation. Most intensively, the coadhesion has been investigated in oral biofilms [17,20]. Specific lectin–sugar interactions and also lipoprotein interactions have been proposed to play a role in the coaggregation between Gram-positive bacteria [20]. Our CLSM examinations revealed the integration of *Bacillus* cells within the deinococcal colonies irrespective of the strain or species tested; thus, a specific coadhesion mechanism is improbable.

Bacillus spores adhere as monolayers on many kinds of surfaces, hydrophobic spores of *B. cereus* being the most adhesive [13]. Spores of *B. cereus*, *B. licheniformis*, *B. subtilis*, *B. stearothermophilus* and *Paenibacillus polymyxa* adhered more than vegetative cells of these species [10,13]. Slimy biofilms were reported on stainless steel by *B. subtilis* in a nutrient-rich medium with 50 g l^{-1} of sucrose [35] and by dairy strains of *B. cereus*, *B. licheniformis*, *B. pumilus*, *B. subtilis* and *B. stearothermophilus* in milk-containing medium [10,18]. Problems caused by single species biofilms of *B. subtilis* or *B. stearothermophilus* have been reported in dairy manufacturing plants [9]. In a mini review of slime problems in the paper industry, Blanco *et al* [2] listed *Bacillus* species as one of the primary organisms responsible for slimy deposits subsequently accumulating secondary bacteria. Direct evidence, however, for the role of bacilli as primary biofilm formers on paper machine surfaces has not been presented. This assumption is merely based on their frequent presence when bacteria are isolated from mature slimes. We showed that the *Bacillus* strains (n=40) of paper industry origin generated no biofilm in paper machine water. Väisänen *et al* [33] also showed that although *B. coagulans* E50L1 was isolated from paper machine slime, only individual cells and spores adhered to steel and no cell cluster or biofilm was formed under conditions, where *D. geothermalis* E50051 grew as a thick biofilm.

Many *Bacillus* species produce bacteriocins [36]. Bacilli grow fast on nutrient-rich cultivation media like TSA, plate count agar or nutrient agar at 37°C — conditions used for bacterial isolation in many studies [2,4,12,32]. These conditions may mask the eventual presence of slower-growing bacteria in the machine slimes. It is therefore possible that the role of *Bacillus* spp. in paper machine biofilms has been overestimated by biased isolation procedures. This study demonstrates that *D. geothermalis* E50051 formed biofilms similarly on stainless steel and different polystyrene surfaces; thus, the polystyrene plate assay is a convenient tool for evaluation of biofilm formation potential of paper machine isolates.

Intercellular communication, including lethal signals, is believed to be a critical factor in the formation of mixed-species biofilms in nature [34]. Oral streptococci produce hydrogen peroxide in biofilms [17]. This paper shows that the antagonistic *Bacillus* strains adhered in the primary biofilm formed by *D. geothermalis*, and subsequently partially antagonized the growth of the host by producing what were probably nonproteinaceous inhibitory metabolite(s). The moderate toxicity of these substances indicates efficacy only against bacteria in close proximity, analogously as it has been suggested for oral biofilms that most metabolic signals may communicate only microdistances [17]. The interactions of the bacilli with deinococci in the growing biofilms ranged from synergism to antagonism even within a single *Bacillus* species. The strongest antagonists were the paper industry isolate *B. licheniformis* D50141 and the type strain of *B. licheniformis*. Food poisoning-related strains of *B. licheniformis* produce lipopeptides that can inhibit bacterial growth [19].

Jayaraman *et al* [14] showed that antimicrobial peptides produced by the gramicidin-S-overproducing *Brevibacillus brevis* 18 strain inhibited the formation of a corrosive biofilm on steel by sulfate-reducing *Desulfovibrio vulgaris* in modified Baar's medium. Jayaraman *et al* suggested the production of a protective biofilm by an antimicrobial-producing primary colonizer (*B. brevis* 18) as an attractive alternative for conventional biocides in corrosion control. We found several antagonistic *Bacillus* strains, but they were incapable of primary colonization. The persistence of paper machine bacilli depended on coadhesion, suggesting that the target of *Bacillus* prevention in paper machine slimes should be the primary colonizers, e.g., deinococci. Our results open new viewpoints in development phases of biofilms and in prevention of biofouling in paper machines. Our observations of coadhesion also explain why *Bacillus* strains are always found when paper machine slimes are analyzed [2,4,12,24,29,30,32,33], even though they proved to be inefficient biofilm formers as monocultures in the paper machine WW.

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