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Sugar fatty acid esters inhibit biofilm formation by food-borne pathogenic bacteria

Soichi Furukawa^{1,†,*}, Yuko Akiyoshi¹, George A. O'Toole², Hirokazu Ogihara¹, and Yasushi Morinaga^{1,†,*}

¹Department of Food Science and Technology, College of Bioresource Sciences, Nihon University, 1866, Kameino, Fujisawa-shi, 252-8510, Japan

²Department of Microbiology and Immunology, Dartmouth Medical School, Rm 505 Vail Bldg, Hanover, NH 03755

Abstract

Effects of food additives on biofilm formation by food-borne pathogenic bacteria were investigated. Thirty-three potential food additives and 3 related compounds were added to the culture medium at concentrations from 0.001 to 0.1% (w/w), followed by inoculation and cultivation of five biofilm-forming bacterial strains for the evaluation of biofilm formation. Among the tested food additives, 21 showed inhibitory effects of biofilm formation by *Staphylococcus aureus* and *Escherichia coli*, and in particular, sugar fatty acid esters showed significant anti-biofilm activity. Sugar fatty acid esters with long chain fatty acid residues (C14-16) exerted their inhibitory effect at the concentration of 0.001% (w/w), but bacterial growth was not affected at this low concentration. Activities of the sugar fatty acid esters positively correlated with the increase of the chain length of the fatty acid residues. Sugar fatty acid esters inhibited the initial attachment of the *Staphylococcus aureus* cells to the abiotic surface. Sugar fatty acid esters with long chain fatty acid residues (C14-16) also inhibited biofilm formation by *Streptococcus mutans* and *Listeria monocytogenes* at 0.01% (w/w), while the inhibition of biofilm formation by *Pseudomonas aeruginosa* required the addition of a far higher concentration (0.1% (w/w)) of the sugar fatty acid esters.

Keywords

Sugar fatty acid esters; biofilm; food-borne pathogenic bacteria

INTRODUCTION

Biofilms are defined as adherent, matrix-enclosed bacterial populations (Costerton, Lewandowski, Caldwell, Korber, Lappin-Scott, 1995; Costerton, Stewart, Greenberg, 1999; Davey, O'Toole G, 2000; Greenberg, 2003; Kolter, Greenberg, 2006; O'Toole, Kaplan, Kolter, 2000). In nature, microorganisms usually attach to solid surfaces, especially at the liquid-solid

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*Corresponding author: Yasushi Morinaga and Soichi Furukawa, Department of Food Science and Technology, College of Bioresource Sciences, Nihon University, 1866, Kameino, Fujisawa-shi, 252-8510, Japan. TEL: +81-0466-84-3971, FAX: +81-0466-84-3971, morinaga@brs.nihon-u.ac.jp, furukawa@brs.nihon-u.ac.jp.

[†]S.F. and Y.M. contributed equally to this study.

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interface. In general, biofilms are very resistant to antibiotics and some physical treatments (Costerton et al., 1999; Davey et al., 2000; Mah et al., 2003; O'Toole, Stewart, 2005), and are also considered to be a serious problem for infectious disease (Costerton et al., 1999; Davey et al., 2000; Furukawa, Kuchma, O'Toole, 2006). In food hygiene, biofilms are also a very important potential hazard and a source of bacterial contamination of foods (Kumar, Anand, 1998; Wong, 1998; Zottola, Sasahara, 1994).

There are a few studies on the chemicals with strong biofilm-specific inhibitor (Chmielewski, Ftank, 2007), and, in the food processing environment, some of these anti-biofilm compounds are difficult to be used due to safety concerns. Food additives are safe and if some of these additives had biofilm inhibitory activity, they would be desirable for use in controlling biofilms in the food environment, particularly from the viewpoint of eliminating potential safety concerns. Thus, we investigated the effects of thirty-three potential food additives and 3 related compounds on biofilm formation by food-borne pathogenic bacteria.

MATERIALS AND METHODS

Microorganisms and culture conditions

Escherichia coli K-12 MG 1655 was obtained from the National Institute of Genetics (Shizuoka, Japan). *Pseudomonas aeruginosa* ATCC27853, *P. aeruginosa* PA14 (Rahme et al., 1995) and *Listeria monocytogenes* ATCC19114 were obtained from American Type Culture Collection (Manassas, USA). *Staphylococcus aureus* A7510 was obtained from Tokyo Metropolitan Institute of Hygiene (Tokyo, Japan). *Streptococcus mutans* MT 8148 was obtained from the National Institute of Infectious Diseases (Tokyo, Japan). Bacteria were cultivated in Trypticase Soy Broth (TSB; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) overnight at 37°C.

Methods for evaluation of biofilm formation

Biofilm formation was quantified according to a previously described method (Jackson et al., 2002; Narisawa, Furukawa, Ogihara, Yamasaki, 2005; O'Toole, Kolter, 1998). To assess mixed culture biofilm formation, overnight cultures of each strain were diluted 1:100 into fresh TSB (total 100 µl) and grown in 96-well microtiter plates at 30°C for 24 h.

Food additives

Food additives used in this study are described in Table 1. Sugar fatty acid esters with fatty acid residues of different chain length and with similar hydrophilic-lipophilic balance (HLB; C8:HLB16, C10:HLB16, C12:HLB16, C14:HLB16, C16:HLB16, C16:HLB18) were obtained from Mitsubishi-Kagaku Foods Co. (Tokyo, Japan). Mono- and Poly-glycerine fatty acid esters (C8:1G, C12:1G, C12:10G, C14:10G) were obtained from Taiyo Kagaku Co. (Mie, Japan). Poly-γ-glutamic acid was supplied from Ajinomoto Co., Inc. (Tokyo, Japan), and enzymes were obtained from Amano Enzyme Co. (Nagoya, Japan). Other food additives tested are listed in Table 1. Food additives were dissolved in water (0.03 to 1% (w/v)) and were sterilized by filtration.

Statistical analysis

The data presented in all studies are the means of three replicate experiments. Significant differences were determined with 5% level of significance ($P < 0.05$) by Student's *t*-test.

RESULTS

Effect of food additives on biofilm formation

The effects of food additives on biofilm formation by food-borne pathogenic bacteria were investigated. Thirty-three potential food additives and 3 related compounds (Table 1), were screened for their ability to inhibit biofilm formation. Of the food additives tested, 21 showed inhibitory effects against some or all of the bacterial species tested (Table 2). Sugar fatty acid esters containing long chain fatty acid residues (>C12) showed significant inhibition of biofilm formation by *S. aureus* and *E. coli*, and showed moderate inhibition of biofilm formation of *L. monocytogenes*. The growth of these three strains was largely unaffected at the minimum inhibitory concentration for biofilm formation (0.001% (w/w)) in the case of *S. aureus* and *E. coli* and 0.01% (w/w) in the case of *L. monocytogenes*. This finding suggested the specific ability of these compounds to inhibit biofilm formation. Biofilm formation by *S. mutans* was also inhibited moderately by sugar esters at 0.01 to 0.1% (w/w). At this concentration, the growth of *S. mutans* was inhibited, thus growth inhibition is likely the basis of the observed biofilm inhibition. On the other hand, sugar fatty acid esters showed only a weak inhibition of biofilm formation by *P. aeruginosa*. Activities of the sugar fatty acid esters positively correlated to the increase of the fatty acid chain length (Figure 1 to 4).

Glycerine fatty acid esters also showed inhibition of biofilm formation by *S. aureus*, *E. coli* and *S. mutans*, however the activities were lower compared with the sugar fatty acid esters with long chain fatty acid residues.

ϵ -Polylysine and chitosan inhibited biofilm formation by *S. aureus* at low concentrations (0.001%), however a higher concentration was needed to inhibit the biofilm formation by *E. coli* (0.01%).

Among the enzymes tested, PROTIN^R (a powerful protease derived from *Bacillus* used for bating and dehairing in the leather industry) altered the biofilm formed by *E. coli*. The *E. coli* biofilm formed in the presence of PROTIN^R was fragile and easily peeled off from the solid surface of the wells.

Effect of sucrose mono-palmitate (C16:HLB16) on the initial attachment of bacterial cells

Effects of the timing of the addition of one sugar fatty acid ester on the inhibition of biofilm formation were investigated (Fig.5). The sugar fatty acid ester C16:HLB16 (sucrose mono-palmitate) was added to the culture of *S. aureus* at 0 to 8 hrs cultivation to the final concentration of 0.001% (w/w). Addition of sucrose mono-palmitate at the initiation of bacterial growth (0 to 2.5 hrs cultivation) inhibited biofilm formation more than 80%, while when added to the actively growing culture (after 4 hr cultivation) the inhibition ratio was less than 40%. This result suggested that sugar fatty acid esters inhibited the initial attachment of the *S. aureus* cells to the abiotic surface.

DISCUSSION

Sugar fatty acid esters containing long chain fatty acid residues (>C12) showed inhibitory effects on biofilm formation by *S. aureus* and *E. coli*. In particular, sucrose mono-myristate (C14:HLB16) and sucrose mono-palmitate (C16:HLB16) significantly inhibited biofilm formation by *S. aureus* and *E. coli* at a low concentration (0.001% w/w)(Table 2, Fig. 1 and Fig. 2). The addition of sucrose mono palmitate at the early growth stage of *S. aureus* exhibited a strong inhibitory effect (Fig 5), suggesting that the ester inhibited the initial attachment of the *S. aureus* cells to the abiotic surface. In a previous study, it was reported that the sugar fatty acid esters at 0.05% also inhibit the adhesion of *Salmonella* Enteritidis (Miyamoto, Kawagichi, Shimotsu, Kawagishi, Honjoh, 2009).

In contrast, biofilm formation by *P. aeruginosa* was not inhibited with low concentrations of sugar fatty acid esters. *P. aeruginosa* is known to have esterase on its cell surface (Ohkawa I., Shiga, Kageyama, 1979), therefore the added sugar fatty acid esters may be decomposed by this esterase. However, *P. aeruginosa* PA14 is a primary clinical isolate and is a multihost pathogen that is virulent in a variety of mammalian and nonvertebrate hosts (Jander, Rahme, Ausubel, 2000; Lau et al., 2003; Liberati et al., 2005; Mahajan-Miklos, Tan, Rahme, Ausubel, 1999; Rahme et al., 1995; Tan, Mahajan-Miklos, Ausubel, 1999), therefore the inhibitory effects of sugar fatty acid esters on the *P. aeruginosa* PA14 biofilm formation at even 0.1% would be noteworthy.

The minimum inhibitory concentrations of sugar fatty acid esters for the inhibition of biofilm formation were similar for Gram-positive *S. aureus* and Gram-negative *E. coli*. This result suggested that differences in cell surface chemical structure were not important for this inhibition. We hypothesize the physical factors such as nature of electric charge of the cell surface or physical interaction between cell and solid surface might be important.

Polycations such as ϵ -Polylysine and chitosan inhibited biofilm formation by *S. aureus* at low concentrations, while their inhibitory effect was relatively weak in the case of *E. coli* biofilm formation. The reason for this difference is not clear at present. Sugar fatty acid esters (surfactants) and polycations (positive charge carriers) may inhibit biofilm formation by different mechanisms. Further studies will clarify the difference of the inhibitory mechanisms of these two different compound groups.

Biofilms formed on the pipelines or other surfaces in the food processing environment are recognized as a potential source for food poisoning. Biofilms formed on the pipelines are typically removed by cleaning in place (CIP) washing or by hand washing after breaking down the machinery, and these procedures required a large amount of labor. Therefore the inhibition of biofilm formation may be the best way for controlling biofilms in food processing equipment. Food additives are safe. Thus, for controlling biofilm formation in the food environment food additives are desirable. In this study, we show that sugar fatty acid esters, which are registered food additives and used as emulsifiers in processed foods, are promising agents for inhibiting biofilm formation by food poisoning bacteria. The inhibitory mechanisms and the practicality of using sugar fatty acid esters in the food-processing environment should be studied further.

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Fig. 1. Effect of sugar fatty acid esters on the biofilm formation of *S. aureus*

Shown are the results of biofilm assays testing the ability of sugar fatty acid esters to inhibit biofilm formation by *S. aureus* (black bars, left-hand Y-axis). Growth of the strains in the indicated sugar fatty acid esters is presented as CFU/ml (diamonds, right-hand Y-axis). Assays were performed as described in the Materials and Methods.

^a: Minimum inhibitory concentrations for the biofilm formation.

^b: Fatty acid chain number of sugar fatty acid esters.

Fig. 2. Effect of sugar fatty acid esters on the biofilm formation of *E. coli*

Shown are the results of biofilm assays testing the ability of sugar fatty acid esters to inhibit biofilm formation by *E. coli* (black bars, left-hand Y-axis). Growth of the strains in the indicated sugar fatty acid esters is presented as CFU/ml (diamonds, right-hand Y-axis). Assays were performed as described in the Materials and Methods.

^a: Minimum inhibitory concentrations for the biofilm formation.

^b: Fatty acid chain number of sugar fatty acid esters.

Fig. 3. Effect of sugar fatty acid esters on the biofilm formation of *S. mutans*

Shown are the results of biofilm assays testing the ability of sugar fatty acid esters to inhibit biofilm formation by *S. mutans* (black bars, left-hand Y-axis). Growth of the strains in the indicated sugar fatty acid esters is presented as CFU/ml (diamonds, right-hand Y-axis). Assays were performed as described in the Materials and Methods.

^a: Minimum inhibitory concentrations for the biofilm formation.

^b: Fatty acid chain number of sugar fatty acid esters.

Fig. 4. Effect of sugar fatty acid esters on the biofilm formation of *L. monocytogenes*

Shown are the results of biofilm assays testing the ability of sugar fatty acid esters to inhibit biofilm formation by *L. monocytogenes* (black bars, left-hand Y-axis). Growth of the strains in the indicated sugar fatty acid esters is presented as CFU/ml (diamonds, right-hand Y-axis). Assays were performed as described in the Materials and Methods.

^a: Minimum inhibitory concentrations for the biofilm formation.

^b: Fatty acid chain number of sugar fatty acid esters.

Fig. 5. Effect of time-of-addition of sugar fatty acid esters (C16) (0.001%) on the inhibition of biofilm formation by *S. aureus*

Sugar fatty acid esters were added at the time indicated on the X-axis, then the biofilm allowed to form for a total of 24 hrs. The biofilm inhibition ratio (%) indicates the extent to which biofilm formation was blocked compared to untreated control biofilm assays.

Table 1**Additives Used for Screening.**

<u>Emulsifiers</u>	<u>Preservatives</u>	<u>Enzymes</u>
☐ Sugar fatty acid esters	☐ Potassium Sorbate	☐ Protease
☐ C8:HLB16, sucrose caprylate	☐ Sodium Benzoate	☐ PROTIN ^R
☐ C10:HLB16, sucrose caprate	☐ Sodium Propionate	☐ Proleather ^R
☐ C12:HLB16, sucrose laurate	☐ Potassium Metabisulfite	☐ Hemicellulase
☐ C14:HLB16, sucrose myristate	☐ Sodium Metabisulfite	☐ β -glucanase
☐ C16:HLB16, sucrose palmitate		☐ Polyphenol oxidase
☐ C16:HLB18, sucrose palmitate	<u>Gelling agents</u>	☐ Nuclease
	☐ Pectin	
☐ Glycerine fatty acid esters	☐ Poly- γ -glutamic acid	<u>Non food additive</u>
☐ C8 :1G, glycerine caprylate	☐ Sodium alginate	☐ Surfactin
☐ C12:1G, glycerine laurate		☐ Sodium Surfactin
☐ C12:10G, glycerine laurate	<u>Non-nutritive sweetner</u>	
☐ C14:10G, glycerine myristate	☐ Xylitol ☐	<u>Food component</u>
		☐ PARATINIT ^R
		☐ reduced isomaltulose)
<u>Miscellaneous</u>		
☐ Calcium Monohydrogen		
Phosphate		
☐ Calcium dihydrogen		
Pyrophosphate		
☐ Chitosan		
☐ Trehalose ☐		

Table 2

Effect of food additives on the biofilm formation and growth.

Category	Name	Structure	Minimum inhibitory concentrations (%)											
			<i>S. aureus</i> A7510		<i>E. coli</i> K-12 MG 1655		<i>S. mutans</i> MT 8148		<i>L. monocytogenes</i> ATCC19114		<i>P. aeruginosa</i> ATCC27853		<i>P. aeruginosa</i> PA14	
			Biofilm	Growth	Biofilm	Growth	Biofilm	Growth	Biofilm	Growth	Biofilm	Growth	Biofilm	Growth
Emulsifiers	Sugar fatty acid esters	C8:HLB16	>0.1	>0.1	>0.1	>0.1	>0.1	>0.1	>0.1	>0.1	>0.1	>0.1	>0.1	>0.1
		C10:HLB16	0.1	>0.1	0.1	>0.1	0.1	0.1	>0.1	>0.1	>0.1	>0.1	>0.1	>0.1
		C12:HLB16	0.01	>0.1	0.01	>0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	>0.1
		C14:HLB16	0.001	>0.1	0.001	>0.1	0.01	0.01	0.01	>0.1	0.1	>0.1	0.1	>0.1
		C16:HLB18	0.001	0.01	0.001	<0.1	0.01	0.01	0.01	<0.1	<0.1	<0.1	0.1	>0.1
	Glycerine fatt acid esters	C8 :1G	>0.1	>0.1	>0.1	>0.1	0.1	0.1	>0.1	>0.1	>0.1	>0.1	>0.1	>0.1
		C12:1G	0.01	0.1	<0.1	<0.1	0.1	0.1	>0.1	>0.1	>0.1	>0.1	>0.1	>0.1
		C12:10G	0.01	<0.1	0.1	<0.1	0.001	0.1	0.1	0.1	0.1	0.1	0.1	>0.1
		C14:10G	0.01	<0.1	0.001	<0.1	0.01	0.01	>0.1	>0.1	>0.1	>0.1	>0.1	>0.1
Preservatives	Na-Benzozate		0.1	>0.1										
	Na-Propionate		0.1	>0.1										
	K-Pyrosulfite				0.1	>0.1								
	Na-Pyrosulfite				0.1	>0.1	0.1	>0.1						
	ε-Polylysine		0.001	>0.1	0.1	>0.1								
Miscellaneous	Chitosan		0.001	>0.1	0.01	>0.1								
Enzymes	PROTIN ^R			>0.1	0.001	>0.1	0.1	0.1	>0.1	>0.1	>0.1	>0.1	>0.1	>0.1
	Proleathe ^R				0.1	>0.1	0.1	0.1	>0.1	>0.1	>0.1	>0.1	>0.1	>0.1
	Protease		0.1	>0.1	0.1	>0.1	0.1	0.1	>0.1	>0.1	>0.1	>0.1	>0.1	>0.1
	Hemicellulase		0.1	>0.1										
	Laccase		0.1	>0.1										
	Tunicase				0.1	>0.1								

* Blank boxes show not-tested.