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Biofilm formation of *Staphylococcus aureus* dairy isolates representing different genotypes

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ABSTRACT

The objective of this study was to compare the biofilm-forming capabilities of different genotypes of *Staphylococcus aureus* dairy isolates from Switzerland and northern Italy, including *Staph. aureus* genotype B (GTB) and methicillin-resistant *Staph. aureus* (MRSA). We hypothesized that biofilm formation might be more pronounced in the contagious GTB isolates compared with other genotypes affecting individual animals. Twenty-four dairy isolates, including 9 MRSA, were further characterized by genotyping by using ribosomal spacer PCR, *spa* typing, biofilm formation under static and dynamic conditions, and scanning electron microscopy. The GTB isolates (n = 6) were more able to form biofilms than other genotypes at 37°C and at 20°C after 48 and 72 h of incubation in the static assay using polystyrene microtiter plates. This result was supported by scanning electron micrographs showing a GTB isolate producing strong biofilm with extracellular matrix in contrast to a genotype C isolate. Furthermore, none of the MRSA isolates formed strong biofilms in the static assay. However, some MRSA produced low or moderate amounts of biofilm depending on the applied conditions. Under dynamic conditions, a much more diverse situation was observed. The ability of GTB isolates to be strong biofilm formers was not observed in all cases, emphasizing the importance of growth conditions for the expression of biofilm-related genes. No specific genotype, *spa* type, or MRSA isolate could be categorized significantly into one level of biofilm formation. Nineteen percent of isolates behaved similarly under static and dynamic conditions. The results of this study expand our knowledge of different dairy-related *Staph. aureus* subtypes and indicate the benefit of genotyping when biofilms are studied.

Key words: *Staphylococcus aureus*, biofilm, genotype, *spa* type

INTRODUCTION

Staphylococcus aureus is a foodborne pathogen considered the third most important causative bacterial agent of foodborne illnesses worldwide (Hennekinne et al., 2012); it is of great concern to the dairy industry (De Buyser et al., 2001; Oliver et al., 2009). In particular, dairy cow mastitis is the most important disease in the global dairy industry and *Staph. aureus* is one of the most important etiological agents of contagious mastitis (Silva et al., 2013; Voelk et al., 2014). Another major concern is that *Staph. aureus* can form biofilms (Santos et al., 2014). Biofilms are aggregates of microbial cells surrounded by a matrix of exopolymers (Costerton et al., 1999). Besides the production of exotoxins and surface proteins, the formation of these highly organized multicellular complexes is increasingly recognized as an important virulence factor in *Staph. aureus* (Tang et al., 2012; Lee et al., 2014). Biofilm formation can lead to persistent contamination or infection because the cells within the biofilm are very resistant to sanitation procedures and to the action of the host immune system and antimicrobial agents (Song et al., 2016). Different sources of *Staph. aureus* in the dairy cow environment have been described (Zadoks et al., 2002). Infected animals (cow-to-cow transmission), workers, and equipment and utensils used for milking are the main sources of the microorganism (Lee et al., 2014). Although some researchers have studied the ability of members of the *Staphylococcus* genus to adhere to surfaces and form biofilm, most studies have addressed the clinical aspects related to biofilm formation by *Staphylococcus intermedius* on medical implants and materials (Donlan and Costerton, 2002; de Souza et al., 2014). Moreover, few studies have reported biofilm formation by *Staph. aureus* isolated from ready-to-eat-foods (Oniciuc et al., 2016). Additionally, recent studies have identified several genotypes of *Staph. aureus* that differ in their contagiosity and pathogenicity (Fournier

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et al., 2008; Voelk et al., 2014; Cosandey et al., 2016). Graber et al. (2009) further demonstrated that genotype was highly associated with virulence gene pattern. Among different genotypes, *Staph. aureus* genotype B (**GTB**) is associated with high within-herd prevalence, indicating an increased contagious and virulence potential compared with other genotypes (Graber et al., 2009; Voelk et al., 2014). In particular, *Staph. aureus* GTB, a major contaminant in Swiss raw milk cheese (Hummerjohann, et al., 2014), was characterized by the presence of the enterotoxin genes *sea*, *sed*, and *sej*, and a SNP *lukE* gene (lukEB; Cosandey et al., 2016). Genotype B has been found not only in Switzerland, but also in other countries of central Europe, including Italy, indicating that it is a relevant international problem in cow milk production (Cosandey et al., 2016). Regarding these aspects, the current study was carried out to compare the biofilm-forming capabilities of different genotypes of *Staph. aureus* dairy isolates, including *Staph. aureus* GTB and methicillin-resistant *Staph. aureus* (**MRSA**), because MRSA are a severe problem in the human population and have been isolated from milk, cheese, and other foodstuffs in different countries (Normanno et al., 2007; De Boer et al., 2009; Kav et al., 2011). We evaluated the ability of *Staph. aureus* dairy isolates to form biofilm under static and dynamic conditions and by using scanning electron microscopy. We hypothesized that biofilm formation might be more prevalent in the more contagious GTB strains compared with other genotypes (**OGT**).

MATERIALS AND METHODS

Bacterial Isolates

The experiment was conducted on 24 isolates (including 6 GTB strains) from milk and milk products. One isolate from poultry meat (PR 281), previously described as strong biofilm producer (Di Ciccio et al., 2015), and 3 strains from a culture collection (ATCC3556, ATCC12600, ATCC12228; American Type Culture Collection, Manassas, VA) were included as reference strains (Table 1). Stock cultures were stored at −80°C, and strains were incubated for 24 h at 37°C in tryptic soy broth (**TSB**, BBL Becton Dickinson, Le Pont de Claix, France) before experiments.

Extraction of Nucleic Acids

A single colony of *Staph. aureus* was resuspended in 100 μL of Tris-EDTA buffer (10 m*M* Tris/HCl, 1 m*M* EDTA, pH 8.0), incubated at 95°C for 10 min, and immediately placed into ice. For PCR analysis, the lysate was diluted 1:100 in H_2O and directly used for amplification.

Genotyping

Genotyping of the strains was based on PCR amplification of the 16S-23S rRNA intergenic spacer region (**RS-PCR**) and was performed as described by Fournier et al. (2008). Briefly, the PCR reaction mix (total volume of 25 μL) contained 1× HotStarTaq Master Mix (Qiagen AG, Hombrechtikon, Switzerland), 800 nmol of each primer G1 and L1 (Jensen et al., 1993), and 30 μg of the lysate nucleic acids. The PCR conditions were as follows: denaturation at 95°C for 15 min followed by 27 cycles of 94°C for 1 min, 2-min ramp time, annealing at 55°C for 7 min, 2-min ramp time, and extension for at 72°C for 2 min on a T-Professional thermal cycler (Biometra, Göttingen, Germany). The PCR products were analyzed by the miniaturized electrophoresis system DNA 7500 LabChip (Agilent Technologies, Basel, Switzerland). The resulting amplification patterns were interpreted according to Fournier et al. (2008), using a computer program developed in-house (Syring et al., 2012).

spa Typing

The *spa* typing was based on the amplification of the spacer region of the *spa* gene of *Staph. aureus* which

Table 1. Optical density (OD) at 550 nm and biofilm production index (BPI) of reference strains on polystyrene

Reference strain	OD[1]	BPI
Staphylococcus aureus ATCC 35556 (positive control, strong biofilm producer)	0.756 ± 0.15	0.758
Staph. aureus ATCC 12600 (moderate biofilm producer)	0.450 ± 0.07	0.405
Staphylococcus epidermidis ATCC 12228 (negative control)	0.343 ± 0.05	0.294
Staph. aureus PR 281 (poultry isolate, very strong biofilm producer)	0.979 ± 0.255	1.09

[1]Values are expressed as OD mean ± SD.