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Biofilm formation, *icaABCD* genes and *agr* genotyping of *Staphylococcus aureus* from fish and ground beef

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ABSTRACT

A total of 46 *Staphylococcus aureus* isolates from fish and ground beef were tested for the *agr* types, *icaABCD* genes, and biofilm formation at 12, 25 and 37 °C by the microtiter plate and the MTT assays. All isolates were positive for the *icaABD* genes, while 97.8% were positive for the *icaC*. All isolates produced biofilms at 37 and 25 °C, but 93.5% of them were also biofilm producers at 12 °C. There was no significant difference in biofilm formation between 25 and 37 °C using the crystal violet assay ($P > 0.05$). However, statistically significant differences were detected between 12 and 25 °C as well as 12 and 37 °C ($P < 0.05$). All isolates were significantly different in biofilm production by the MTT assay at all tested temperatures. Furthermore, a relationship between the presence of the *icaABCD* genes and biofilm formation was observed. The *agr* type I was the most prevalent (54.4%) among the isolates, followed by *agr* type II (41.3%) and *agr* type III (9.6%). In this study, the *S. aureus* isolates exhibited biofilm formation ability responsible for persistence of bacteria in foods, which may lead to food spoilage and human health problems.

KEYWORDS

agr typing, biofilm, *icaABCD*, meat, *Staphylococcus aureus*

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1. INTRODUCTION

Staphylococcus aureus causes wide spectrum of infections, from mild skin infections to serious invasive diseases (Götz et al., 2006). It can grow over a wide range of temperatures, pHs, salt concentrations and oxygen levels as well as under adverse environmental conditions (Götz et al., 2006; Seo and Bohach, 2011). This extreme adaptability facilitates contamination of various food products and surfaces in food processing plants (Seo and Bohach, 2011). *S. aureus* produces surface proteins, which have various functions, including adhesion, invasion of host cells, evasion of immune responses, and biofilm formation (Götz et al., 2006).

Biofilm formation includes attachment of microorganisms to surfaces and production of extracellular matrix consisting of one or more extracellular polysaccharides, DNA and proteins (Götz et al., 2006). Cells in biofilm are more resistant to heat, chemicals, and various sanitisers and antimicrobial agents than planktonic cells, making it difficult to completely remove established biofilms. Biofilms may form on surfaces such as food or food industry equipment, medical devices, and human tissues (Zhao et al., 2017). They contain spoilage and pathogenic bacteria that cause human health and economic issues (Götz et al., 2006; Vazquez-Sanchez et al., 2013).

The biofilm forming capacity of *S. aureus* has been affected by several factors such as the nature of the surface, the growth medium, and other environmental conditions (Jeronimo et al., 2012; Zhao et al., 2017). In a study, most *S. aureus* strains produced more biofilm at 37 °C than at 25 °C, and showed better biofilm production with addition of glucose (Vazquez-Sanchez et al., 2013). The optimal conditions for biofilm formation of *S. aureus* were 37 °C, pH 7, 2% glucose and 10% NaCl concentrations (Miao et al., 2019). The ability of *S. aureus* to develop biofilms on surfaces such as stainless steel, glass, polystyrene and polypropylene has been shown (Rode et al., 2007; Vazquez-Sanchez et al., 2013; Avila-Novoa et al., 2018).

Polysaccharide intercellular adhesin (PIA) is the main component of staphylococcal biofilm formation (Götz et al., 2006). The biosynthesis of PIA and its transcriptional control are encoded by the ica operon, which consists of the *icaABCD* genes and the regulatory gene icaR. The production of PIA has been eliminated with deletion of the ica locus in *S. aureus*, resulting in a loss of the ability to form biofilm (Götz et al., 2006). In *S. aureus*, the accessory gene regulator (*agr*) encoded by the *agr* locus is a well-defined quorum-sensing system. The suppression and activation of the *agr* system also affect biofilm proliferation and detachment (Tan et al., 2018).

Biofilm producing *S. aureus* in food and food processing environments creates risk for contamination of food, causing foodborne diseases and economic losses (Zhao et al., 2017). Therefore, the present study aimed to detect the presence of *icaABCD* genes associated with biofilm formation, the ability of biofilm formation under different temperatures, and the *agr* types of *S. aureus* isolated from fish and ground beef samples.

2. MATERIALS AND METHODS

2.1. Bacterial isolates

A total of 46 *S. aureus* isolates, which were identified using biochemical tests and specific Sa442 DNA fragment, including 27 ground beef, 11 seawater (*Sparus aurata*) and 8 freshwater (*Oncorhynchus mykiss*) fish were used in this study. All isolates were transferred from the stock

cultures (−20 °C) into Brain Heart Infusion broth (BHI) (Merck, Germany) and incubated overnight at 37 °C.

2.2. Detection of biofilm-associated *icaABCD* genes

Genomic DNA was isolated using the cetyl trimethyl ammonium bromide (CTAB) method (Ausubel et al., 1991). The *icaA* and *icaD* genes according to Rohde et al., (2001) and the *icaB* and *icaC* genes according to Kiem et al., (2004) were tested. The 50 µL PCR mixture contained 0.3 µM of each primer (Biomers, Germany), 5 µL DNA template (50 ng μL^{-1}), 5 µL 10× PCR buffer (Vivantis, USA), 200 µM dNTP (Thermo Fisher Scientific, USA), 4 mM $MgCl_2$ (Vivantis), 1.5 U Taq DNA polymerase (Vivantis), and 31.7 µL of PCR-grade water (AppliChem, Germany). Cycling conditions: initial denaturation (94 °C/5 min); 30 cycles of denaturation (94 °C/1 min), annealing (52 °C/30 s), extension (72 °C/1 min), and a final extension (72 °C/10 min). All reactions were carried out in a thermal cycler (Bio-Rad Lab Inc., USA). The PCR products were visualised with UV transilluminator (DNR Minilumi Bio-imaging Systems, Israel). *S. aureus* ATCC 25923 was the positive control.

2.3. Detection of biofilm formation by the microtiter plate assay

Biofilm forming ability of *S. aureus* isolates was tested as previously described with slight modification (Stepanovic et al., 2000, 2007). The isolates were cultured in Tryptic Soy Broth (TSB) (Merck) (37 °C/24 h). Cell suspensions (200 µL/each well) transferred into a plate were incubated at 12, 25 and 37 °C for 24 h. Wells were then washed three times with sterile phosphate-buffered saline (PBS) and fixed with 200 µL of 99% methanol (Merck) for 15 min. Plates were stained with 0.1% crystal violet for 15 min. The stained biofilm was solubilised with 160 µL of 33% (v/v) glacial acetic acid (Merck). The optical density was measured at 570 nm (Thermo Electron Corporation, Finland). The assays were carried out in triplicate. Negative control wells included only sterile TSB. The isolates were categorised as non-biofilm, weak, moderate, and strong biofilm producers (Stepanovic et al., 2000).

2.4. Determination of viable biofilm cells by the MTT assay

The metabolic activity of biofilm was determined by the MTT assay according to previous studies (Walencka et al., 2008; Wu et al., 2010). The isolates were incubated in TSB for 24 h. Cell suspensions were adjusted to 0.5 McFarland standard. Then, 200 µL of suspension was added to each well of a sterile plate and incubated (37 °C/24 h). The culture medium was removed and the microtiter plate was washed three times with PBS. Tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma-Aldrich, USA) was prepared in sterile PBS (pH 7.2) at a concentration of 5 mg mL^{-1} and filtered. After washing, 20 µL of MTT solution and 180 µL of TSB were added to each well and incubated (37 °C/3 h). The wells were removed and filled with 150 µL of dimethylsulphoxide (Sigma-Aldrich) to solubilise the insoluble formazan crystals. Absorbance was measured at 570 nm using the microplate reader.

2.5. *agr* genotyping

The *agr* genotyping was performed by multiplex PCR to determine the presence of four *agr* alleles using group specific primers according to Gilot et al., (2002). The amplification

products of 441, 575, 323 and 659 bp fragments represent the *agr* types I, II, III and IV, respectively. The reference strains were *S. aureus* ATCC 29213, *S. aureus* NCTC 10652 and SA07 (*mecA*-positive *S. aureus* from our collection).

2.6. Statistical analysis

The results of biofilm formation at different temperatures were compared using one-way analysis of variance (ANOVA) with Tukey's multiple comparison test. The chi-squared test was used to test the differences in the presence of *icaABCD* genes and biofilm formation at different temperatures among the *agr* types. All analyses were performed using the SigmaPlot 12.3 (Systat Software Inc., USA). $P \leq 0.05$ was accepted to be significant.

3. RESULTS AND DISCUSSION

The intercellular adhesion genes of *S. aureus* involved in biosynthesis of biofilm are located at the intracellular adhesion (*ica*) locus, which is composed of four genes, *icaABCD* (Götz et al., 2006). In this study, the 46 *S. aureus* isolates from fish and ground beef were tested for the presence of *icaABCD* genes. All isolates were positive for the *icaA*, *icaB* and *icaD* genes, but 45 (97.8%) of them were positive for the *icaC* gene (Table 1). The only isolate that did not carry the *icaC* gene was from freshwater fish. PCR amplicons of the *icaABCD* of the *S. aureus* isolates are shown in Fig. 1. Chen et al., (2020) found all *S. aureus* isolates from different foods positive for the *icaADBC*, similar to our results. In contrast, the *icaADBC* genes in *S. aureus* isolates from food contact surfaces was 52.3% (Avila-Novoa et al., 2018). Some studies reported low prevalence; 76.5% for *icaA* and 58.8% for *icaD* genes in cheese (Falaki and Mahdavi, 2017), 25% for both *icaA* and *icaD* genes in milk (Suvajdzic et al., 2017).

The results of biofilm formation of the *S. aureus* isolates detected by the crystal violet assay at 12, 25 and 37 °C are shown in Fig. 2. All isolates were positive for biofilm formation at 25 and 37 °C, while three isolates were negative at 12 °C (Tables 1 and 2). Regarding the impact of temperature, a recent study reported that the favourable temperature for *S. aureus* biofilm formation in food processing environment was 37 °C (Miao et al., 2019). In another study, biofilm production was higher at 35 °C than at 25 and 4 °C (Zeraik and Nitschke, 2012). Higher attachment capacity for *S. aureus* on polystyrene at 20, 25 and 30 °C was reported (Rode et al., 2007). In this study, there was a statistically significant difference in quantification of biofilm formation by crystal violet staining at 12 and 25 °C ($P < 0.05$) as well as at 12 and 37 °C ($P < 0.05$). However, no difference was observed between 25 and 37 °C ($P > 0.05$). Similar results were obtained by Jeronimo et al., (2012), who found no difference between 28 and 37 °C on the adherence capability of *S. aureus*. We detected cell viability of the isolates in biofilm using the MTT assay (Fig. 3). The results at 12, 25 and 37 °C were statistically significant ($P < 0.05$). In several studies, cell viability of *S. aureus* using the MTT assay was reported (Wu et al., 2010; Miao et al., 2019).

All *S. aureus* isolates were successfully genotyped using *agr* typing (Table 1). Representative results for *agr* alleles are shown in Fig. 4. The *agr* type I was the most prevalent (54.4%) among the isolates, followed by *agr* II (41.3%) and *agr* III (4.4%), which was consistent in accordance with the results of previous studies (Bardiau et al., 2014; Chen et al., 2020; Salgueiro et al., 2020). On the contrary, in a previous study, *agr* II (37.5%) and *agr* III (37.5%)

Table 1. The *icaABCD* genes, biofilm formation, and *agr* genotypes of *S. aureus* isolates from fish and ground beef

Origin	Isolate[a]	Biofilm formation			Biofilm related genes				Genotyping *agr* type
		37 °C	25 °C	12 °C	*icaA*	*icaB*	*icaC*	*icaD*	
Freshwater fish (n = 8)	A6/1	Strong	Moderate	Strong	+	+	+	+	I
	A9/7	Strong	Strong	Weak	+	+	−	+	I
	A11/2	Moderate	Moderate	Weak	+	+	+	+	I
	A13/3	Moderate	Weak	Weak	+	+	+	+	II
	A27/7	Moderate	Weak	Weak	+	+	+	+	II
	A32/1	Strong	Strong	Moderate	+	+	+	+	I
	A34/6	Strong	Weak	Weak	+	+	+	+	III
	A36/1	Moderate	Weak	Weak	+	+	+	+	II
Seawater fish (n = 11)	Ç7/6	Moderate	Strong	Weak	+	+	+	+	I
	Ç8/2	Weak	Weak	Weak	+	+	+	+	I
	Ç11/3	Weak	Weak	Weak	+	+	+	+	I
	Ç12/2	Weak	Moderate	Weak	+	+	+	+	I
	Ç14/8	Weak	Weak	Weak	+	+	+	+	I
	Ç15/1	Weak	Weak	No biofilm	+	+	+	+	II
	Ç22/5	Strong	Strong	Weak	+	+	+	+	I
	Ç29/1	Strong	Strong	Strong	+	+	+	+	I
	Ç31/5	Weak	Weak	Weak	+	+	+	+	III
	Ç32/3	Weak	Strong	Weak	+	+	+	+	I
	Ç33/4	Weak	Moderate	Moderate	+	+	+	+	I
Ground beef (n = 27)	K4/7	Weak	Moderate	No biofilm	+	+	+	+	I
	K5/5	Weak	Weak	Weak	+	+	+	+	II
	K6/2	Weak	Strong	Moderate	+	+	+	+	I
	K8/10	Weak	Moderate	Moderate	+	+	+	+	I
	K11/3	Weak	Moderate	Weak	+	+	+	+	I
	K12/1	Weak	Weak	Moderate	+	+	+	+	I
	K13/1	Strong	Strong	Weak	+	+	+	+	I
	K14/1	Strong	Strong	Weak	+	+	+	+	I
	K15/1	Moderate	Strong	Moderate	+	+	+	+	I
	K16/1	Moderate	Weak	Weak	+	+	+	+	I
	K17/1	Strong	Strong	Weak	+	+	+	+	I
	K19/2	Moderate	Strong	Moderate	+	+	+	+	I
	K22/8	Moderate	Moderate	Weak	+	+	+	+	II
	K25/3	Moderate	Weak	Weak	+	+	+	+	II
	K28/3	Moderate	Moderate	No biofilm	+	+	+	+	II
	K37/15	Weak	Moderate	Strong	+	+	+	+	I
	K40/10	Moderate	Weak	Strong	+	+	+	+	II
	K45/1	Strong	Strong	Strong	+	+	+	+	II
	K47/1	Moderate	Strong	Moderate	+	+	+	+	II
	K48/1	Strong	Strong	Strong	+	+	+	+	II
	K51/1	Strong	Strong	Strong	+	+	+	+	II
	K56/3	Strong	Strong	Strong	+	+	+	+	II
	K57/1	Moderate	Moderate	Moderate	+	+	+	+	II
	K58/1	Strong	Strong	Weak	+	+	+	+	II
	K60/2	Strong	Weak	Strong	+	+	+	+	II
	K67/4	Strong	Strong	Moderate	+	+	+	+	II
	K69/1	Strong	Weak	Weak	+	+	+	+	II

[a] Capital letters indicate the source of the isolates (A, freshwater fish; Ç, seawater fish; K, ground beef).

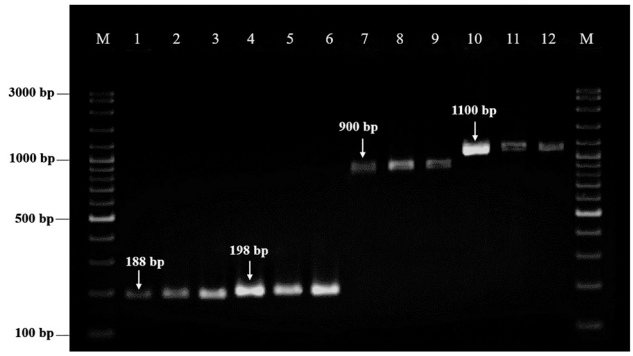

Fig. 1. Agarose gel electrophoresis of the representative *icaABCD* genes. Lane M: Marker; Lanes 1, 4, 7 and 10: positive control (*S. aureus* ATCC 25923); Lanes 2 and 3: *icaA* (188 bp) positive ground beef and fish isolates, respectively; Lanes 5 and 6: *icaD* (198 bp) positive ground beef and fish isolates, respectively; Lanes 8 and 9: *icaB* (900 bp) positive ground beef and fish isolates, respectively; Lanes 11 and 12: *icaC* (1,100 bp) positive ground beef and fish isolates, respectively

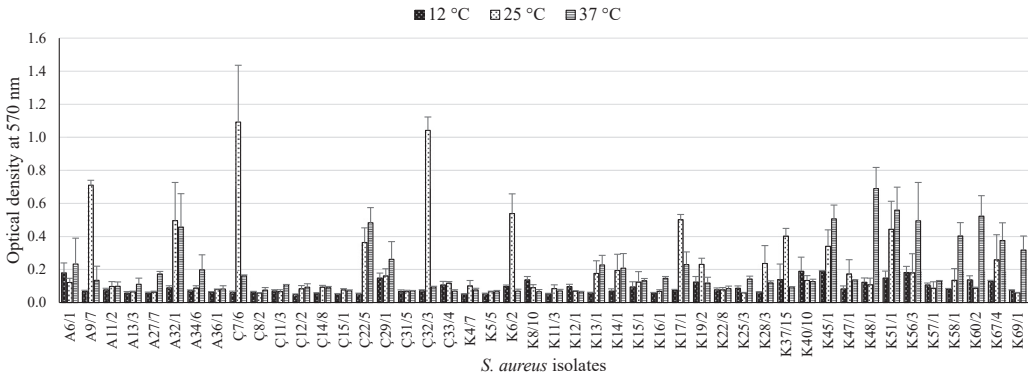

Fig. 2. Biofilm formation of *S. aureus* from fish and ground beef at 12, 25 and 37 °C by the crystal violet staining assay. Values are expressed as mean and standard deviation

were more predominant than *agr* I (25%) in *S. aureus* food strains (Achek et al., 2020). Similar to other studies, the *agr* type IV was not detected in this study (Bardiau et al., 2014; Achek et al., 2020). Our fish isolates were typed as *agr* I (68.4%), *agr* II (21.1%) and *agr* III (10.5%). However, Salgueiro et al., (2020) reported that the *S. aureus* strains from gilthead seabream were *agr* non-typable.

We found that all isolates carrying the *icaABCD* genes except a negative one for the *icaC* were biofilm producers at 25 and 37 °C (Table 2). Three isolates were negative at 12 °C. Thus, a relationship between biofilm formation and the presence of the *icaABCD* was observed. This result disagrees with the findings of Chen et al., (2020). Biofilm formation and expression of the *ica* genes may be associated with various factors such as temperature, nutrient content, osmolarity, growth under anaerobic conditions and genetics (Götz et al., 2006).

Table 2. Biofilm formation of *S. aureus* isolates at 12, 25 and 37 °C

Temperature	Origin	No. of isolates	No biofilm		Weak		Moderate		Strong	
			No (%)	OD[a]	No (%)	OD	No (%)	OD	No (%)	OD
12 °C	Freshwater fish	8	0 (0)	–	6 (75)	0.065 ± 0.007	1 (12.5)	0.089 ± 0.013	1 (12.5)	0.179 ± 0.061
	Seawater fish	11	1 (9.1)	0.053 ± 0.001	8 (72.7)	0.061 ± 0.006	1 (9.1)	0.106 ± 0.022	1 (9.1)	0.148 ± 0.031
	Ground beef	27	2 (7.4)	0.057 ± 0.002	10 (37)	0.068 ± 0.007	8 (29.6)	0.108 ± 0.017	7 (25.9)	0.158 ± 0.045
25 °C	Freshwater fish	8	0 (0)	–	4 (50)	0.074 ± 0.007	2 (25)	0.109 ± 0.028	2 (25)	0.603 ± 0.130
	Seawater fish	11	0 (0)	–	5 (45.5)	0.072 ± 0.008	2 (18.2)	0.100 ± 0.015	4 (36.4)	0.664 ± 0.140
	Ground beef	27	0 (0)	–	7 (25.9)	0.077 ± 0.008	7 (25.9)	0.154 ± 0.041	13 (48.2)	0.262 ± 0.089
37 °C	Freshwater fish	8	0 (0)	–	0 (0)	–	4 (50)	0.116 ± 0.024	4 (50)	0.256 ± 0.134
	Seawater fish	11	0 (0)	–	8 (72.7)	0.084 ± 0.008	1 (9.1)	0.163 ± 0.005	2 (18.2)	0.373 ± 0.100
	Ground beef	27	0 (0)	–	7 (25.9)	0.072 ± 0.009	9 (33.3)	0.126 ± 0.014	11 (40.7)	0.412 ± 0.109

[a] OD: Optical density; values are expressed as mean ± standard deviation.

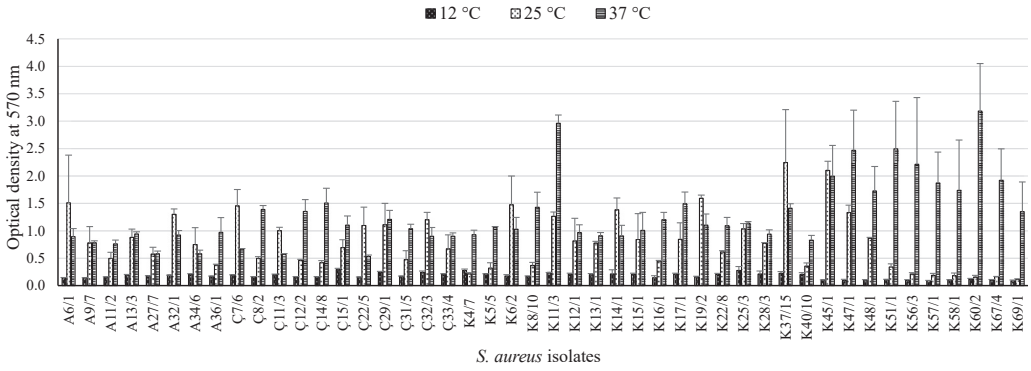

Fig. 3. The viability of *S. aureus* isolates from fish and ground beef at 12, 25 and 37 °C by the MTT assay. Values are expressed as mean and standard deviation

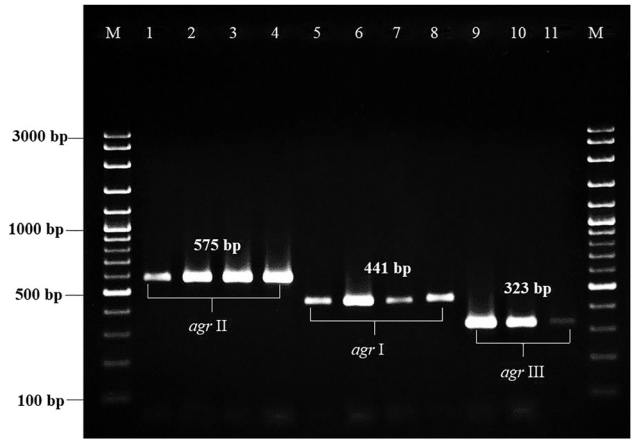

Fig. 4. Agarose gel electrophoresis of PCR products for the three *agr* types identified. Lane M: Marker; Lane 1: positive control (*S. aureus* ATCC 29213); Lanes 2, 3 and 4: positive isolates (seawater fish, freshwater fish, and ground beef, respectively); Lane 5: positive control (NCTC 10652); Lanes 6, 7 and 8: positive isolates (seawater fish, freshwater fish, and ground beef, respectively); Lane 9: Positive control (SA07); Lanes 10 and 11: positive isolates (seawater and freshwater fish, respectively)

No significant difference ($P > 0.05$) in presence of the *icaABCD* genes and biofilm formation capacity at 12, 25 and 37 °C between the *agr* types was observed.

4. CONCLUSIONS

This study demonstrated the presence of *icaABCD* genes and biofilm formation at various temperatures in almost all *S. aureus* isolates originating from fish and ground beef. Furthermore, all isolates were genotyped using *agr* typing as the *agr* types I, II and III. Incubation

temperatures of 25 and 37 °C were more effective than 12 °C in biofilm formation of *S. aureus*. There was a relationship between the presence of the *icaABCD* genes and biofilm formation among the isolates. Biofilms act as a source of food contamination and may raise human health and economic concerns. Preventative measures are needed to be taken from production to consumption to minimise the risk of food spoilage and infections caused by biofilm producing *S. aureus*.

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