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Biofilm capability of staphylococcus strains isolated from food and the anti-biofilm activity of a chemically synthesized pyrrolomycin

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Abstract:
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Keywords: Biofilm; *Staphylococcus* spp; *ica; bap*; S*as* C; food isolates; anti-biofilm; pyrrolomycin; antibiofilm

Introduction

Food transmitted diseases are a big issues for public health worldwide and food-borne outbreaks from several sources are continuously reported caused by different infectious agents, as reviewed in Vitale and Schillaci (2016). Bacterial infection and contamination can be more complex and difficult to treat when pathogenic bacteria are organized in structural community named biofilms. Biofilm bacteria are more resistant to drugs and other treatments and their important role for infectious diseases in animals, has been explored in the last decades (Clutterbuck *et al.* 2007).

However biofilms are also a big concern for food safety for the contamination of surfaces and equipment in food processing industries (Srey, *et al.* 2013). *Staphylococcus aureus* is a relevant pathogen for mastitis in dairy farms and *S. aureus* biofilms are considered important in recurrent mastitis (Melchior *et al.*, 2006).

The intercellular adhesion (*ica*) locus (Cramton *et al.*, 1999*), bap* gene (Cucarella *et al.*, 2004) and *sas C* gene (Schroeder *et al.*, 2009) are involved in the organization of staphylococcal biofilm. The infected animals can contaminate milk, dairy products, meat with a consequent major risk for food-borne transmission to humans. The discovery of new drugs against sessile bacteria is considered a good strategy to control the infection and the diffusion of food transmitted pathogens (Simo˜es *et al.*, 2010).With the aim of discovering new antibacterial drugs active against planktonic bacteria and /or biofilms, we have focused on the pyrrolomycins (Schillaci *et al.*, 2010).

The evaluation of biofilm formation in food isolates of *S. aureus*, in relation to the presence of *ica, bap* and *sas C* was performed together with the assays for the antimicrobial activity of a new

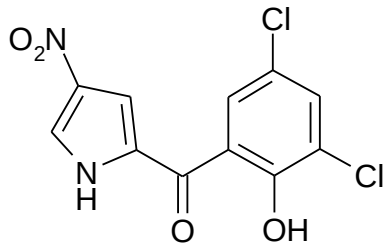
chemically synthesized nitro-derivative pyrrolomycin, the (3,5-dichloro-2-hydroxyphenyl)(4-nitro-1*H*-pyrrol-2-yl)methanone (**IV**).

Materials and Methods

Synthesis of compound IV

Synthesis of (3,5-dichloro-2-hydroxyphenyl)(4-nitro-1*H*-pyrrol-2-yl)methanone (**IV**). The compound was synthesized and characterised as already described (Raimondi *et al.,* 2006).

Figure 1: Chemical structure of (3,5-dichloro-2-hydroxyphenyl)(4-nitro-1*H*-pyrrol-2-yl)methanone **IV**



IV

Bacterial strains

Two reference strains: *S. aureus* ATCC 25923, *S. epidermidis* RP62A, and a group of 20 food strains of *S. aureus* were analysed. The isolates were subjected to Gram staining and biochemical analysis such as catalase and Voges-Proskauer (VP) test (BioMérieux), oxidase test (Oxoid), glucose and mannitol acidification in red phenol broth (Difco). Further identification was performed using the API Staph system (BioMérieux, Marcy l'Etoile, 20500, France).

MICs determination.

Minimum inhibitory concentrations (MICs) against planktonic strains were determined as previously described in Schillaci *et al.* (2010).

Molecular characterization

DNA was extracted using QIAmp DNA mini kit spin columns according to the manufacturer's instructions (Quiagen SpA, Milan, Italy). *Staphylococcus* spp. isolates were investigated by PCR for the presence of the intercellular adhesion (*ica)* locus, biofilm-associated protein (*bap*) as described in Cucarella et al. (2004) and Sas (Srinivasan *et al.* 2006).

Biofilm capability evaluation

All the staphylococcal strains were tested for their ability to form biofilms by safranin staining method (Schillaci et al., 2010). The same method was also used to evaluate a preventive activity on the three strongest strains by directly adding to the diluted suspension, sub-MIC concentrations of pyrrolomycin IV.

Biofilm susceptibility testing, methylthiazotetrazolium (MTT) method were performed as described in Schillaci *et al.* (2010)

All biofilm assays were performed in triplicate.

Results

The results on genes showed that *bap* was present in 1/20, i*ca* was detected in 16/20 strains and *sasC* in 8/20 but biofilm capability was not related to the presence of genes. (Table 1)

Table 1: Biofilm capability (Optical Density, O.D.) and genes

***Staphylococcus* strains**	**D.O**	***ica***	***bap***	***sas C***
***S.epidermidis* RP 62A**	**3.010**	+	-	-
***S.aureus* ATCC 25923**	**1.956**	+	-	+
S.aureus 708	**1.896**	+	-	+
S.aureus 637	**1.668**	-	-	-
S.aureus 1553	**1,337**	+	-	+
S.aureus 353	**1.104**	+	-	-
S. aureus 357	**1.044**	+	-	+
S. aureus 723	**1.016**	+	-	-
S.aureus 100	**0.989**	+	-	-
S.aureus 722	**0.959**	+	-	+
S.aureus 712	**0.932**	+	-	+
S. aureus 635	**0.855**	-	-	-
S.aureus 714	**0.851**	+	-	-
S.aureus 242	**0.786**	+	-	-
S.aureus 713*	**0.737**	+	+	+
S.aureus 725	**0.713**	+	-	-
S.aureus 191	**0.648**	+	-	-
S.aureus 1559	**0.641**	-	-	-
S.aureus 165	**0.585**	+	-	-
S.aureus 636	**0.541**	-	-	-
S.aureus 711	**0.528**	+	-	+
S.aureus 1704	**0.391**	+	-	+

Biofilm formation strength, was high in the two references strains followed by *S. aureus* isolate N708 (*ica*$^{+}$, *bap*$^{-}$ and *sas C*$^{+}$); similar OD values were obtained with the *S aureus* isolate (N 635.) in which all three loci are absent. The N 713 strain, presenting all three genetic loci showed a 50% biofilm capability compared to N 708. The analysis of antibacterial activity of the compound IV showed that it was effective against all planktonic forms of the tested strains with MICs values ranging from 0.4 to 0.2 µg/mL. The strongest biofilm producers N708, together with the reference strains *S.aureus* ATCC 25923, and *S. epidermidis* RP62A, were used to evaluate the anti-biofilm properties of pyrrolomycin IV on 24 hours established biofilms and before biofilm formation. The anti-biofilm activity against 24 hours pre-formed biofilm was observed at concentrations of 1.6 or 0.8 µg/mL, which were higher compared to the MIC values obtained on planktonic forms (Tab.2).

Table 2: Anti-microbial activity of pyrrolomycin **IV** against the stronger biofilm producer strains

	MIC (µg/mL) (no growth in planctonic)	**MIC (µg/mL) in preformed biofilm**			
Strains		**1.6**	**0.8**	**0.4**	**0.2**
***S. epidermidis* RP62A**	0·4	92·4	89·.5	33·6	NS
***S. aureus* ATCC 25923**	0·2	54	41	30·6	NS
***S. aureus* 353**	0·2	89	66	20	NS
***S.aureus* 708**	0·4	80·6	62·5	27	NS
S.aureus 723	0·2	88	79	19	NS

Values are the average of at least three independent determinations.

Variation coefficient was less than 15%. NS = not significant because below the 15% of inhibition.

The anti-planktonic activity showed MIC values between 0.2 and 0.4 for all isolates.

A higher MIC values (0,8-1,6) were necessary to inhibit at least 62% in the preformed biofilm of the field isolates.

An ability of the compound to prevent biofilm on two reference staphylococcal strains and *S.aureus* strain N 708, was shown at concentrations of 0.18, 0.09 and 0.045 µg/mL , lower than the MIC values established in planktonic forms. Indeed, almost 50% of biofilm was inhibited at concentration of 0.09 µg/mL (Table 3).

Table 3: Inhibition of biofim formation at sub-MIC concentrations(µg/mL)

The activity is expressed as percentage of inhibition.

Strains	0.18	0.09	0.045
***S. epidermidis* RP62A**	46·4	43.1	34.7
***S. aureus* ATCC 25923**	56·5	44.5	29·1
S. aureus 708	55	52	42·5

An average of 52% and 46% inhibitor percentage was present at sub MIC concentration of 0.18 and 0.09 µg/mL respectively. These results look very promising for the prevention of Biofilm formation, considering the very low level of the active concentration of compound IV.

Discussion

The results showed that the capability *Staphylococcus* spp. strains in biofilm formation is not related to the presence or absence of the genetic loci analysed in this study. This might be due to the fact that *in vitro* biofilm organization often depends on several factors such as the composition of the culture media (Ferreira *et al.* 2012). In contrast to human isolates, the lack of a role for the *ica* operon on the formation of biofilm by *S.aureus* isolates from bovine mastitis, has already been described (O'Gara 2007). The existence of alternative mechanisms to induce biofilm development implies that each *S. aureus* isolate might develop a particular type of biofilm matrix to better suit the environmental conditions (Schroeder *et al.*, 2009).

Bacterial infections in livestock animals represent a big concern for food safety since the pathogenic bacteria can contaminate food products and transmit infection and disease to humans. Animal with clinical or subclinical mastitis caused by *S.aureus* can contaminate the milk with strains that often harbour several virulent factors like enterotoxins and toxic shock syndrome genes (Srinivasan *et al.* 2006). More than 280 episodes of food poisoning episodes due to staphylococcal enterotoxins have been reported each year in Europe (EFSA report, *2010).* Staphylococcal enterotoxins poisoning cases and the circulation of virulent *S. aureus* strains in milk and food samples have been reported already in Sicily, Italy where mastitis problems in cattle and sheep are frequently reported (Vitale et al 2015, 2018)

Conclusion

The new synthetic pyrrolomycin **IV** showed good antimicrobial property against planktonic form of *S. aureus* and in preventing staphylococcal biofilm. A more efficacious control of bacterial biofilm can result in a lower number of antimicrobial treatments, a reduction in the spread of antibiotic resistance and a control of pathogenic bacteria in livestock farms. The community and sessile forms of pathogenic bacteria are in fact involved in the recurrence of infection in animals but also in persistent contamination of food production premises.

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Author's Contributions

MGC and MVR contributed to compound synthesis and biofilm assays. P.N performed genetic analysis, MV, D.S and VDLP supervised and planned the work, MV and DS contributed to manuscript writing, all authors approved the manuscript.

Ethics

The work did not involve any experiments on animals. All authors declare that no conflict of interest is present. The work is an original paper and is not under consideration in other journals.

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