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Lucilia sericata Chymotrypsin Disrupts Protein Adhesin-Mediated Staphylococcal Biofilm Formation

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***Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms cause chronic infections due to their ability to form biofilms. The excretions/secretions of *Lucilia sericata* larvae (maggots) have effective activity for debridement and disruption of bacterial biofilms. In this paper, we demonstrate how chymotrypsin derived from maggot excretions/secretions disrupts protein-dependent bacterial biofilm formation mechanisms.**

Chronic infections are commonly associated with biofilms formed by staphylococci such as *Staphylococcus aureus* and *Staphylococcus epidermidis* (1). Staphylococcal biofilm formation involves a number of steps: first, the attachment of bacteria to a biomaterial surface via cell wall-associated adhesins (2), followed by their accumulation to a multibacterial layer. *S. aureus* and *S. epidermidis* use several different intercellular adhesive mechanisms, such as the polysaccharide intercellular adhesin (PIA), also termed polymeric *N*-acetylglucosamine (PNAG), which is synthesized by the *icaADBC* locus, to accumulate and form biofilms (3–6); proteinaceous factors independent of *icaADBC* and PIA have emerged as alternatives and include surface protein G (SasG) (7) and biofilm-associated protein (Bap) (8) in *S. aureus* and the accumulation-associated protein (Aap; homolog to SasG) (9) and extracellular matrix binding protein (Embp) (10) in *S. epidermidis*.

Lucilia sericata larvae (maggots) have been applied to chronic wounds for centuries, and sterile maggots have been shown to effectively debride necrotic tissue (11) and disinfect wounds (12) and are also reputed to influence healing (13, 14). Components of maggot secretions that aid debridement, such as metalloproteases, serine-proteases, and aspartyl compounds (15), that have antibacterial activities (16–18), and which may assist healing (19, 20) have been identified. Specifically of interest to this present study is the isolation from excretions/secretions (ES) of a chymotrypsin-like proteinase (15), which, as a recombinant enzyme, effectively degrades wound eschar *ex vivo* (21, 22). Thus, we studied the potential of this recombinant chymotrypsin (rChymotrypsin) to interfere with staphylococcal biofilms. This was facilitated by the availability of clinically important biofilm-forming *S. aureus* and *S. epidermidis* strains that employ either PIA (3, 23) or proteinaceous adhesins such as Aap/SasG (7, 9) for biofilm formation.

The previously described semiquantitative adherence assay using 96-well tissue culture plates (Nunc, United Kingdom) was used to measure attachment and accumulation of *S. epidermidis* 1457 (*icaADBC* and PIA positive) (23) and 5179-R1 (Aap positive and *icaADBC* negative) (9) and *S. aureus* SA113 (ATCC 35556; *icaADBC*, PIA, and SasG positive) (3, 7, 24) biofilms on the plastic surface in the presence of 0.1, 1, or 10 μg/ml rChymotrypsin (12, 18, 23). No rChymotrypsin was added to the control wells. rChymotrypsin was prepared and tested as previously described (21, 22) and had a specific activity of 10.1 pmol/min/mg (22). Data were analyzed using a one-way analysis of variance (ANOVA) with Tukey's test and with the level of significance set at a P value of <0.05. All experiments were performed three times, each time in triplicate ($n = 9$).

The greatest effect of rChymotrypsin on both nascent and preformed biofilms was seen on *S. epidermidis* 5179-R1, with less of an effect on *S. epidermidis* 1457 and *S. aureus* SA113 (Fig. 1). Nascent *S. epidermidis* 1457 biofilm formation was inhibited by 20 to 33% by 0.1 to 10 μg/ml rChymotrypsin compared to that of the control (Fig. 1a), while a disruption of 11 to 51% was observed on the preformed biofilms (Fig. 1b). The effect of rChymotrypsin was not significant on either the nascent or preformed *S. epidermidis* 1457 biofilms. In the case of *S. epidermidis* 5179-R1, a significant decrease of 69 to 72% in nascent biofilm formation was observed (Fig. 1a), while rChymotrypsin disrupted preformed biofilms by 6 to 77%, with a significant difference between 10 μg/ml and the control and 0.1 μg/ml (Fig. 1b). The results for *S. aureus* SA113 were more variable. A significant decrease of 32 to 61% was seen when nascent biofilms were exposed to 1 and 10 μg/ml rChymotrypsin (Fig. 1a), while no effect was seen with 0.1 μg/ml of rChymotrypsin. On *S. aureus* SA113 preformed biofilms, an 11 to 51% disruption in biofilm, which was not a significant change from the biofilm formation of the control, was observed (Fig. 1b).

To visualize the effect of rChymotrypsin on the staphylococcal biofilms, light microscopy was used (Fig. 2) (18). rChymotrypsin clearly disrupted *S. epidermidis* 5179-R1 and *S. aureus* SA113 biofilms (Fig. 2f and 2j); the effect was not so apparent on *S. epidermidis* 1457 (Fig. 2b). To support the hypothesis that the cell-cell adhesion disruptions observed were due to the proteolytic activity of rChymotrypsin, the respective

Received 28 November 2012 **Accepted** 30 November 2012
Published ahead of print 7 December 2012
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doi:10.1128/AEM.03689-12

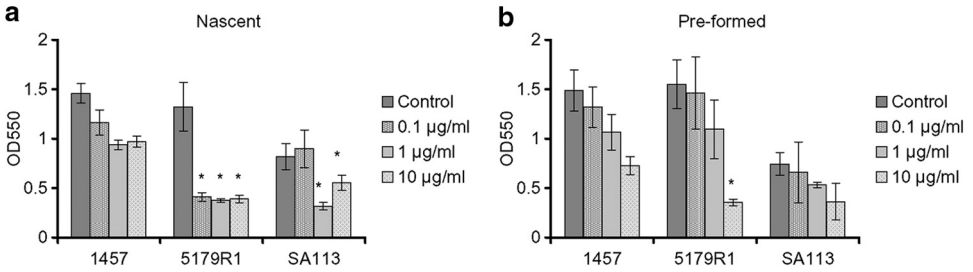

FIG 1 Effect of rChymotrypsin on nascent *S. epidermidis* 1457 and 5179-R1 and *S. aureus* SA113 biofilms (a) and preformed *S. epidermidis* 1457 and 5179-R1 and *S. aureus* SA113 biofilms (b). Significant effects were seen on nascent *S. epidermidis* 5179-R1 and *S. aureus* SA113 biofilm formation (*, $P < 0.05$), but on the preformed biofilms, rChymotrypsin had a significant effect only on *S. epidermidis* 5179-R1 (*, $P < 0.05$). OD550, optical density at 550 nm. Error bars indicate the standard errors of the means.

biofilms were exposed to sodium *meta*-periodate and proteinase K, chemicals known to disintegrate the intercellular adhesins PIA/PNAG and Aap/SasG employed by *S. epidermidis* and *S. aureus* (3, 7, 9, 23). Results showed similar disruption of cell aggregates to rChymotrypsin (Fig. 2), specifically on *S. epidermidis* 5179-R1, which is Aap dependent.

Aap is a cell wall-associated protein comprising an A domain and a repetitive B domain. The intercellular adhesive properties of Aap are located in the N-terminal domain B, which becomes active only after the A domain has been proteolytically cleaved by an endogenous staphylococcal protease or an exogenous host protease (9). Rohde et al. showed that different proteases can either encourage or inhibit Aap-mediated biofilm formation by *S. epidermidis* 5179-R1 in a dose-dependent manner (9). Thus, rChymotrypsin may affect the proteolytic processing mechanism of Aap in nascent *S. epidermidis* 5179-R1, which is essential for the activation and mediation of intercellular adhesion and biofilm formation. Alternatively, with preformed biofilms, rChymotrypsin may affect Aap activity by cleaving the Aap peptide bonds as observed with proteinase K (9). The exact influence of rChymotrypsin on Aap is under investigation, as is the reversible nature of its effect. The fact that rChymotrypsin works only on the proteinaceous-adhesin-dependent strains and the fact that different clinical staphylococci, in particular, PIA-dependent *S. epidermidis* strains and an *S. aureus* strain, use a polysaccharide and/or proteinaceous biofilm-forming mechanism suggest that chymotrypsin is unlikely to represent a standalone agent. Work is also under way to study the effect of rChymotrypsin on a range of clinical *S. epidermidis* and *S. aureus* isolates, including methicillin-resistant *S. aureus* and other clinically relevant staphylococci.

In conclusion, our study has clearly demonstrated that maggot rChymotrypsin can interfere with bacterial adhesion, adding further to our understanding of the way maggots exert their antibacterial effects. Clearly, protein adhesins are not the only mechanism used by bacteria to adhere to wound tissue, and we believe that maggots attack bacterial adhesins *in vivo* by secreting a repertoire of bioactive antibiofilm agents, of which chymotrypsin is one key component.

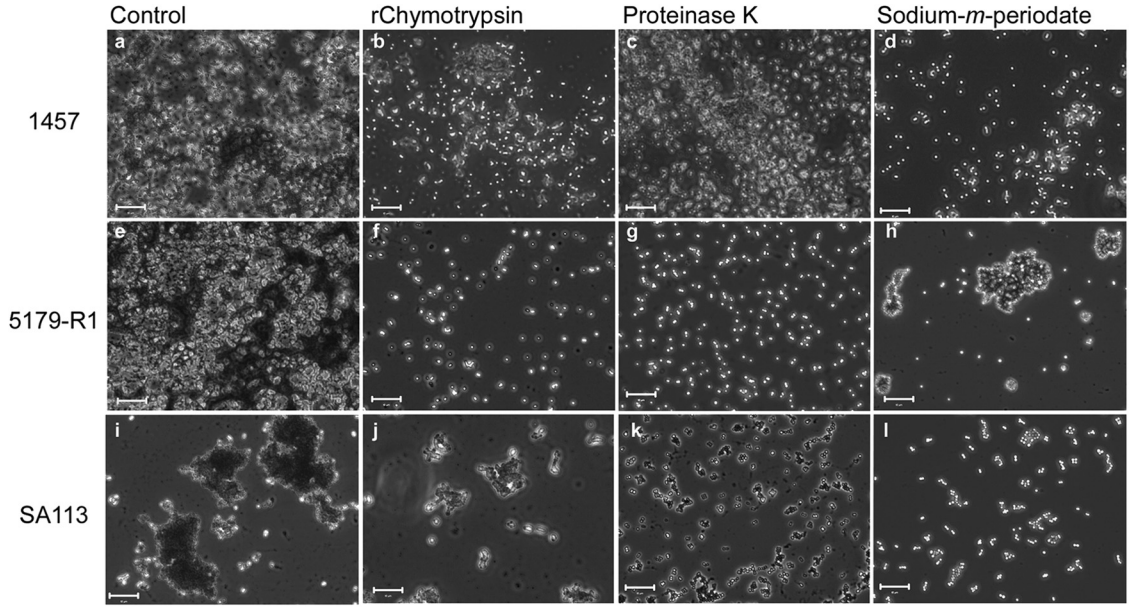

FIG 2 Light-microscopy images showing the effect of rChymotrypsin, proteinase K, and sodium *meta*-periodate on preformed *S. epidermidis* 1457 (a to d), *S. epidermidis* 5179-R1 (e to h), and *S. aureus* SA113 (i to l) biofilms. (a, e, and i) Untreated bacteria, controls; (b, f, and j) 10 μg/ml rChymotrypsin; (c, g, and k) proteinase K; (d, h, and l) sodium *meta*-periodate. Bar = 10 μm.

ACKNOWLEDGMENTS

We thank the Technology Strategy Board and MRC DPFS for funding Alan Brown and Gary Telford (Immune Modulation Research Group, School of Pharmacy, University of Nottingham) to produce maggot recombinant chymotrypsin.

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