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MINIREVIEW

Secretory Proteases of the Human Skin Microbiome

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ABSTRACT The human skin is our outermost layer and serves as a protective barrier against external insults. Advances in next-generation sequencing have enabled the discoveries of a rich and diverse community of microbes—bacteria, fungi, and viruses that are residents of this surface. The genomes of these microbes also revealed the presence of many secretory enzymes. In particular, proteases which are hydrolytic enzymes capable of protein cleavage and degradation are of special interest in the skin environment, which is enriched in proteins and lipids. In this minireview, we will focus on the roles of these skin-relevant microbial secreted proteases, in terms of both their widely studied roles as pathogenic agents in tissue invasion and host immune inactivation and their recently discovered roles in intermicrobial interactions and modulation of virulence factors. From these studies, it has become apparent that while microbial proteases are capable of a wide range of functions, their expression is tightly regulated and highly responsive to the environments the microbes are in. With the introduction of new biochemical and bioinformatics tools to study protease functions, it will be important to understand the roles played by skin microbial secretory proteases in cutaneous health, especially the less studied commensal microbes with an emphasis on contextual relevance.

KEYWORDS *Candida*, *Cutibacterium*, dermatophytes, *Malassezia*, proteases, skin microbiology, *Staphylococcus*, *Streptococcus*

SKIN MICROBIOME

The skin is our outermost layer that interfaces with the external environment (1) and is also the site of residence of a rich and diverse microbial community composed of bacteria, viruses, and fungi (2). The major advance in understanding the community composition of skin microbes came in the early 2000s with the adoption of culture-independent techniques (3) together with next-generation sequencing which allows direct ecological profiling of the skin microbiome (4–6). This is a tremendous step forward in studying human skin microbes because culture-independent profiling of microbes greatly reduces the growth bias associated with laboratory culture conditions, providing significantly more accurate microbial composition analysis.

The human skin is divided into 3 main subtypes: oily/sebaceous, moist, and dry (7). Each type of skin site is associated with a particular microbial composition signature (6). The adult skin microbiome is very stable over a long period, as shown by the minimal changes to the composition especially at the sebaceous sites (8). This is fairly surprising, given the skin is constantly exposed to environmental perturbations and in contact with opportunistic pathogens. This stability underlines how skin microbes are masters of their environment—the microbial genes and associated products enable these microbes to thrive in this nutrient-deprived environment (9).

The skin environment. The human skin is a stratified epithelium consisting of dividing basal layers of keratinocytes which differentiates into corneocytes in the uppermost layer of the skin (1). This layer, known as the stratum corneum, was thought to be the major residence site of microbes. Recent studies have shown the presence of

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a rich microbial community in skin invaginations and hair follicles, especially microbes that thrive in a more anaerobic environment (10, 11). In healthy individuals, the skin forms a formidable barrier against external insults through tight junctions in the stratum granulosum and the stratum corneum, which consists of highly keratinized cells in a lipid matrix (12). Compared to the gut environment that is enriched in carbohydrates, the main components of the skin surface are proteins and lipids (9). It is perhaps not surprising that skin microbes, the superior survivors in this dry, acidic environment, correspondingly harbor a repertoire of hydrolytic enzymes involved in protein and lipid metabolism. In this review, we will focus on how microbes use enzymes of protein metabolism to facilitate their growth, survival, and/or invasion on the skin surface.

Proteases and protein metabolism. Proteins are one of the two major components of the stratum corneum (13). In order to utilize this resource, proteins need to be broken down into peptides and amino acids for uptake by the microbes. This catabolic process is performed by a class of hydrolytic enzymes called proteases (or proteinases) (14, 15). Proteases are divided into 7 main classes based on the catalytic mechanism: serine, metallo-, cysteine, aspartate, threonine, asparagine, and glutamate proteases (16). Serine, metallo- and cysteine proteases account for the majority of the proteases in bacteria and fungi, while aspartyl proteases are prevalent in many fungal genomes (17).

Secretory proteases are especially intriguing because microbial cells need to expend energy in synthesizing and secreting proteins, and this has to be carefully regulated under nutrient-deprived conditions (18). The conventional understanding is that secretory proteases, such as the *Cutibacterium acnes* proteases that release arginine from skin proteins (19), mainly function in nutrient acquisition (20). However, the roles of proteases in mediating processes beyond general catabolism have been increasingly evident, especially in mammalian systems (21). Through catalyzing irreversible peptide bond hydrolysis, proteases can precisely mediate biological events crucial for intermicrobial and host-microbial interactions. Most importantly, the repertoire of secretory enzymes is optimized for the environment where they reside (22); expression and activities of these enzymes are dynamic and change as the skin environment is altered.

The roles of secretory skin microbial proteases have been extensively studied, though the focus in early studies is mostly on cutaneous infection and invasion. With our renewed understanding of the human skin microbiome, many recent studies have revealed novel roles that microbial proteases play in regulating key biological processes beyond tissue invasion. In this minireview, we will outline the functional roles of these secretory proteases with relevance to the skin environment, with an emphasis on recent discoveries on both the pathogenic and potentially beneficial roles of these microbial proteases (Fig. 1).

SKIN FUNGAL SECRETORY PROTEASES

Historically, fungal secretory proteases are regarded as key virulence agents in skin and systemic infections. The most prominent members associated with skin infections are the dermatophytes, *Candida* and *Aspergillus* spp. However, next-generation sequencing studies of skin samples from healthy individuals in recent years revealed that aside from the feet, the skin is overwhelmingly populated by a single genus—*Malassezia* (23). This has led to a renewed understanding of fungal growth on human skin—colonization of commensals (growth without host tissue degradation) and infection by pathogens (involving host tissue destruction and penetration) (24). Recent studies on secretory proteases are increasing our understanding of how these proteases can be essential elements that facilitate colonization and infection (Table 1).

Candida. *Candida* is a genus of yeasts in which many species are commensals or symbionts of the human skin and gut. *Candida* spp. are often opportunistic pathogens, causing disease when the host skin or mucosal barriers are disrupted or when the host is immunocompromised (25). *Candida albicans* is the main species of *Candida* found on human skin and is linked to many common superficial infections (26). *Candida* spp. are only a minor component of the healthy skin microbiome. Despite this, *Candida* secreted aspartyl proteases (Saps) are perhaps the most well-studied among the

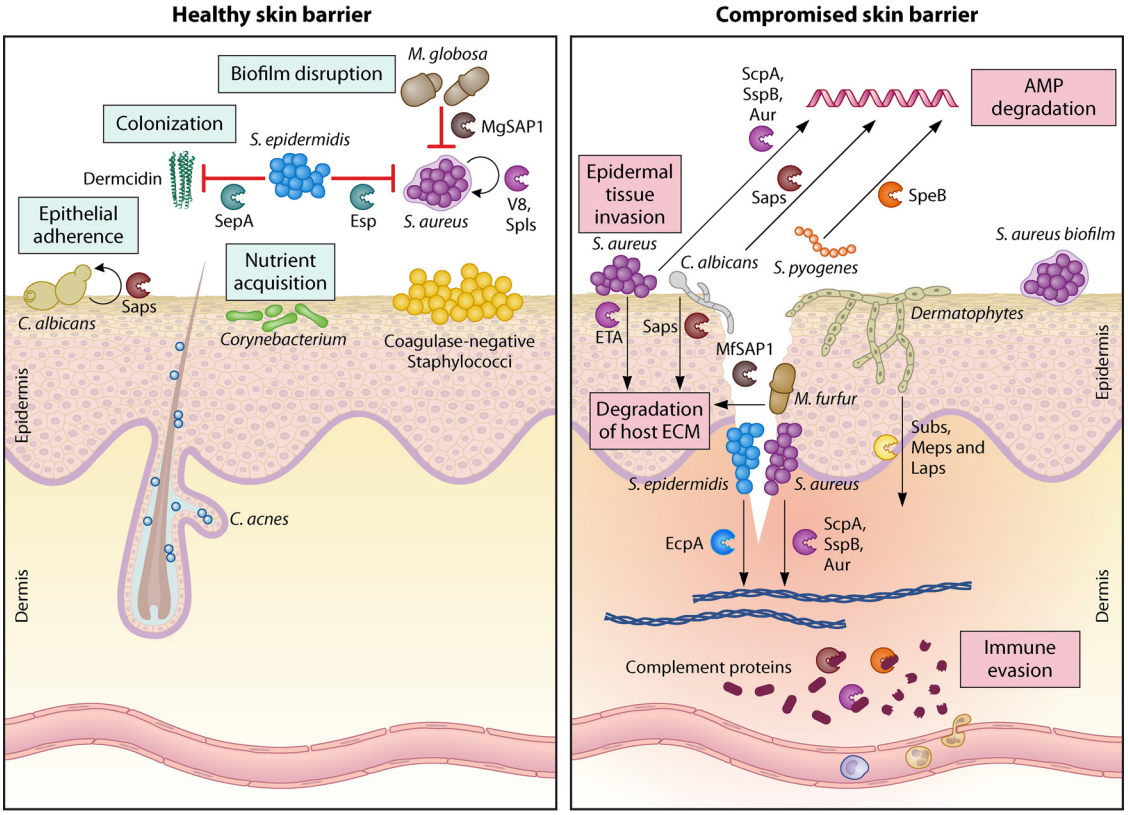

FIG 1 The role of skin-resident microbial secretory proteases in healthy and diseased skin. When the epidermal barrier is intact, the microbes and their secreted proteases are localized to the stratum corneum, the topmost layer of the epidermis, and epidermal invaginations. When the epidermal barrier is breached or compromised, these secretory proteases can reach the deeper layers of the epidermis and dermis, resulting in skin tissue damage and inflammation. The functions of the proteases depend on the skin environment—the same protease can have different roles depending on whether the skin barrier is intact. Only key secreted proteases from each species are shown. The color of the protease icons corresponds to the color of the microbe. Figure not drawn to scale. AMP, antimicrobial peptide; ECM, extracellular matrix.

human fungal skin microbiome (see reference 27 for a more extensive review). *Candida albicans* secretes 10 Saps belonging to 2 main families of aspartyl proteases—the candidapepsins (Sap1 to -8) and the yapsins which are bound to the fungal cell wall and membrane via a glycosylphosphatidylinositol anchor (Sap9 and -10) (28–30). The large number of protease-encoding genes is likely due to the need for specialized proteases during the various stages of host invasion as well as the specific location of infection, as these Saps have various pH optima and substrate specificities (30–32). Expression of Sap1 to -3 is often associated with *C. albicans* yeasts, while Sap4 to -6 are associated with hyphal growth (33); the morphological association of Sap7 to -10 has not been confirmed (30). In both the commensal and infection states, Sap9 is the most highly expressed protease (29, 34).

Saps are involved in both colonization and infection of epithelial surfaces; these enzymes are considered to be significant virulence factors in *C. albicans* invasion of host tissues (31, 35). First, Saps are involved in colonization by processing adhesion proteins, and increased Sap activity was correlated with stronger adherence of *C. albicans* to host cells (36). Saps1 to -4 and -9 are further able to degrade the human α1-protease inhibitor, which results in an increase in human neutrophil elastase activity, leading to further tissue damage and colonization by *C. albicans* (37). Second, Saps can degrade a wide range of human structural proteins *in vitro*, including cytokeratin, collagen, and vimentin (31), which enable epithelial cavitation and penetration to establish deeper infection. Third, Saps can modulate host immune responses in multiple ways. Saps contribute to *Candida* evasion of the host's immune response by degrading complement system proteins C3b, C4b, and C5 (38). Sap2 degrades

TABLE 1 Fungal proteases and their associated targets and functions

Organism	Protease		Target(s)	Process(es) involved	Key reference(s)
Candida albicans	Secreted aspartyl proteases (Saps)	Sap1 to -3	Complement C3b, C4b, C5; α1-protease inhibitor; His5; LL-37 (Sap2 only): α2-macroglobulin; cystatin A; cytokeratin; collagen; factor H; FHR-1 receptor; kininogens; macrophage factor-H receptors CR3, CR4; mucin; vimentin	Colonization; epidermal barrier invasion; immune modulation and evasion	31, 33, 38, 40, 42, 46
		Sap4	α1-Protease inhibitor; His5; LL-37	Immune modulation	33, 42
		Sap5	E-cadherin; His5	Degradation of connective tissue; biofilm formation	48, 49
		Sap6	Unknown	Biofilm formation	48
		Sap7	His5	Colonization; (Sap9 only) biofilm formation	43
		Sap8	His5; LL-37		
		Sap9	α1-Protease inhibitor; His5; LL-37		29, 50
		Sap10	His5		
Candida parapsilosis	Secreted aspartyl proteases (SAPPs)	SAPP1	IgA; complement 3b, 4b	Immune modulation and evasion	54, 56
		SAPP2	Complement 3b, 4b; factor H-related protein 5		
Dermatophytes	Metalloprotease carboxypeptidases (Mcps), leucine aminopeptidases (Laps), deuterolysins (Npls)	McpA and -B; Lap1 and -2; NpIIA and -B	Keratin fragments	Epidermal barrier invasion	75, 76
	Metalloprotease fungalysins (Meps)	Mep1, -3, -4	Keratin	Epidermal barrier invasion	71, 74, 75
	Serine carboxypeptidases	CpyA, ScpA and -B	Keratin fragments	Epidermal barrier invasion	75, 76
	Serine dipeptidyl-peptidases (Dpps)	DppIV and -V (Tri 4)	Keratin fragments	Epidermal barrier invasion	74, 75
	Serine protease subtilisins (Subs)	Sub1 to -7	Keratin; pro-Mcps	Epidermal barrier invasion; (Sub3 and -4) activation of pro-Mcps	70, 76, 79, 85
Malassezia globosa	Aspartyl protease	MgSAP1	*S. aureus* SpA	Reduce *S. aureus* biofilm	61
Malassezia furfur	Aspartyl protease	MfSAP1	Cytokeratins; denatured collagen I/IV; fibronectin; thrombospondin-1; vitronectin	Epidermal barrier invasion	66

TABLE 2 Predicted secreted proteases in common *Malassezia* species

Protease class	No. of proteases in strain:				
	Malassezia furfur CBS14141	*Malassezia globosa* CBS7966	*Malassezia pachydermatis* CBS1879	*Malassezia restricta* CBS7877	*Malassezia sympodialis* ATCC 42132
Aspartate	5	14	6	12	6
Metallo-	2	1	0	1	3
Serine	7	2	9	4	6
Total	**14**	**17**	**15**	**17**	**15**

factor H, a complement regulator which promotes host immune cell recognition and binding to *C. albicans* (39). This degradation results in a lower host fungicidal response, facilitating fungal cell survival during infection. However, Sap2 or Sap5 overexpression in a hypofilamentous strain is insufficient to cause immunopathology and likely works in combination with various hypha-associated factors (40). Saps are further able to efficiently degrade some host antimicrobial peptides (AMPs), which promotes fungal survival in the host. AMPs are usually short, cationic, and amphiphilic peptides secreted by various cell types in the skin (41). Saps can inactivate human LL-37 (active fragment of cathelicidin), a key epidermal innate immunity AMP (42); the salivary AMP histatin5 (His5) (43); and AMPs liberated from kininogens by host proteases (44). Finally, these aspartyl proteases also elicit proinflammatory responses independent of their proteolytic activity—Sap1 to -3 stimulate macrophages, increasing interleukin-1β (IL-1β) and tumor necrosis factor alpha (TNF-α) production, while Sap1 and Sap3 also increase IL-6 production (45, 46).

Beyond host protein degradation, Saps are also involved in biofilm formation—a process that can reduce *C. albicans* susceptibility to current therapeutic agents. Global protease substrate profiling utilizing synthetic pooled peptides (47) revealed that Sap5 and Sap6 are upregulated and highly specific to *C. albicans* biofilms. *C. albicans* mutants harboring *sap5* and/or *sap6* deletions exhibit reduced biofilm formation both *in vitro* and in a rat catheter biofilm model (48). However, the precise substrates of Sap5 and Sap6 are undefined. The cell wall-associated Sap9 is also involved in this process; *sap9* mRNA expression is upregulated in *C. albicans* biofilms (49), and a knockout mutant of *sap9* displays flatter biofilm structure (50). This is likely attributed to Sap9's role in the proteolytic processing of cell wall proteins, such as Eap1, involved in surface adhesion and biofilm formation (50). This role of Saps in biofilm formation has been targeted as a potential enhancer of antifungals. Several aspartyl protease inhibitors when used in combination with commonly prescribed antifungals, which alone have little to no inhibitory effect on biofilms, resulted in reduced formation of *C. albicans* biofilms. Lopinavir, the HIV aspartyl protease inhibitor, was able to both inhibit biofilm formation and disrupt mature biofilm when used in combination with the antifungal caspofungin (51). Overall, these studies demonstrate that aspartyl proteases are important for the formation and maintenance of the *C. albicans* biofilm structures.

Other skin-resident *Candida* species are also known to possess and express genes coding for Sap homologues (31). *Candida parapsilosis*, *Candida tropicalis*, and *Candida dubliniensis* possess SapP1 to -3, SapT1 to -4 (52, 53), and SapCD1 to -4, respectively. Of the 3 species, *C. parapsilosis* is the most studied, with SapP1 to -3 observed to degrade host proteins and activate proteolytic cascades in a similar fashion as *C. albicans* Saps (54–56).

Malassezia. *Malassezia* (formerly *Pityrosporum*) is a genus of commensal fungi commonly found on human and animal skin. Currently, there are 17 known species of *Malassezia* (57). *Malassezia* species dominate the skin mycobiome in healthy individuals but are also associated with several skin conditions, such as pityriasis versicolor, seborrheic dermatitis, and atopic dermatitis (AD) (58). Common anthropophilic *Malassezia* species include *Malassezia globosa*, *Malassezia restricta*, *Malassezia sympodialis*, and *Malassezia furfur* (59). Functional genomic analysis of these recently sequenced *Malassezia* species has revealed that these *Malassezia* species possess many aspartyl proteases (Table 2) (60).

Malassezia globosa. The *M. globosa* genome possesses 17 predicted secretory proteases, of which 14 are aspartyl proteases. In *M. globosa* culture, MgSAP1 is the

secretory protease that dominates the extracellular proteolytic landscape of this yeast (61). MgSAP1 is ubiquitously expressed by *M. globosa* on healthy skin at the RNA level. Using an unbiased, mass spectrometry-based technique (47), we determined that MgSAP1 has a strong preference for positively charged residues at the P1 position. Interestingly, this cleavage preference is also observed in various fungal aspartyl proteases such as *C. albicans* Saps (48) and is likely due to the presence of an aspartate residue in the catalytic flap that confers S1 (binding pocket next to the cleavage site) specificity (62). MgSAP1 coincubation with *Staphylococcus aureus* inhibits biofilm formation without affecting planktonic growth, and this effect is likely attributable to the *M. globosa* protease cleaving *Staphylococcus aureus* protein A (SpA), an extracellular protein involved in *S. aureus* biofilm formation (63). Since *Malassezia* spp. and *S. aureus* share several common skin niches such as the anterior nares (6), this finding suggests that *Malassezia* could play a potentially beneficial role by preventing the formation of *S. aureus* biofilms which can be reservoirs for pathogenic dissemination (Fig. 1) (64).

Malassezia furfur. *M. furfur* is the *Malassezia* species most often associated with pityriasis versicolor and systemic infections (65). *M. furfur* harbors 14 secretory proteases, in which 5 are aspartyl proteases. MfSAP1 is the homologue of MgSAP1 and is also the main extracellular aspartyl protease secreted by *M. furfur* (66). MfSAP1 cleaves many key extracellular matrix (ECM) proteins associated with the human skin, such as collagens I and IV, fibronectin, cytokeratins, thrombospondin-1, and vitronectin. Compared to MgSAP1 (61), this protease has similar preferences for substrate cleavage as determined by a synthetic fluorogenic substrate (66). However, MfSAP1 is a more catalytically efficient enzyme than MgSAP1 (66). This highlights that even though *M. furfur* is much less abundant than *M. globosa* on human skin, the high enzymatic activity of MfSAP1 makes this species functionally relevant. This is important considering that a high concentration of MfSAP1 can interfere with wound healing, as assessed in a three-dimensional (3D) skin wound model (66).

Dermatophytes. The dermatophytes are a broad group of fungi comprising 3 genera—*Microsporum*, *Trichophyton*, and *Epidermophyton* (67). These fungi are largely pathogenic in nature, unlike *Malassezia* and *Candida*, which are mostly commensals. Dermatophyte infections are typically superficial, but in certain immunocompromised patients, deeper dermal infections can occur (68). *Trichophyton rubrum* is the most common anthropophilic dermatophyte.

Genes coding for the various secreted proteases are highly conserved across all dermatophyte species studied (69–71). Unlike *Malassezia* and *Candida*, where the secretory proteases are dominated by aspartyl proteases, dermatophytes' secretory protease repertoire consists of serine and metalloproteases. The dermatophyte extracellular serine protease families include the subtilisins (Subs) (72), dipeptidyl peptidases (Dpps) (73, 74), carboxypeptidases (75, 76), and sedolysins (77). The secretory metalloprotease families consist of the metalloprotease M36 (MEPs) family (72), leucine aminopeptidases (Laps) (73), and deuterolysins (75). The expression of these proteases is regulated by the pH of the environment (78). At alkaline pH, expression is raised for the serine protease Sub3 and metalloprotease Lap1, whereas at acidic pH, secretion of aspartic protease Pep1 increases greatly, which may be relevant in the context of skin and nail infections (75).

The dermatophyte secretory proteases play important roles in degrading skin barrier and structural proteins for efficient epidermal colonization and invasion. These proteases are essential to dermatophyte colonization of host skin and nail tissues, where keratin is the major nutrient source, thereby playing a role in dermatophyte virulence and invasion of host tissues (79). It is, however, important to note that the keratinase activities of dermatophytes are likely relevant only in the presence of sulfite secretion by these fungi (80). The secretion of sulfites results in reduction of the keratin disulfide cross-linkages, allowing the proteases to access the compact keratin for proteolytic degradation (81). Degradation of keratin, which is a hallmark of dermatophytes, proceeds first by endopeptidic cleavage of reduced keratin by the Subs and fungalysins, followed by the degradation of the resulting fragments by the exoproteases Laps, Dpps, and carboxypeptidases to amino acids and small peptides for assimilation and utilization (71). Importantly, expression and secretion

of these enzymes are highly regulated and influenced by the external environment (79, 82, 83). A recent study has shown that the protease expression profile of dermatophytes when grown *in vitro* and during infection varies considerably (84, 85). Many proteases secreted by *T. rubrum* when grown on keratin-based media *in vitro* are not found in infection samples and vice versa (86, 87). This emphasizes that potential virulence factors identified *in vitro* should be verified for expression in physiologically relevant human environments.

SKIN BACTERIUM SECRETORY PROTEASES

The skin bacterial community consists of 4 main phyla—*Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* (6), where the Gram-positive *Corynebacterium*, *Cutibacterium*, and *Staphylococcus* account for over half of the skin bacteria (88). Most of the commensal bacterial strains possess few extracellular protease genes compared to the opportunistic pathogens (89). The study of skin bacterial proteases was one of the earliest investigations into the roles of microbial proteases on skin, and most studies have focused on the pathogenic bacteria utilizing proteases for tissue invasion and immune dysregulation (90). While many of these bacterial secretory proteases can serve as facile agents of invasion, recent investigations have shown that the expression of these proteases is tightly controlled and plays a complex role in modulating other virulence factors during bacterial pathogenesis (Table 3). In this minireview, we will focus on the secreted proteases produced by Gram-positive bacteria, which account for the majority of the skin bacterial community.

***Cutibacterium* (formerly *Propionibacterium*).** *Cutibacterium* species are Gram-positive, aerotolerant anaerobic bacteria that include the skin-dwelling *Cutibacterium acnes*, *Cutibacterium avidum*, and *Cutibacterium granulosum* (91, 92). They are prevalent across all skin sites and are the most abundant genus of bacteria in sebaceous skin sites due to their affinity for sebum as a nutrient source (23). As anaerobes, they are well adapted for survival in the oxygen-depleted skin invaginations, such as hair follicles and the pilosebaceous unit (93). The most well-studied species is *Cutibacterium acnes*, which is often associated with acne pathogenesis through biofilm formation which promotes bacterial adhesion to corneocytes, resulting in formation of comedones (94–96).

Early studies demonstrated that several *Cutibacterium* species have extracellular proteolytic activity when grown in synthetic media (97). Recent proteomic studies of *C. acnes* secretome (98) and biofilm (99) confirmed the presence and expression of secretory proteases including a putative subtilisin-like protease. While the exact substrates of these proteases are yet to be identified, studies have shown that *C. acnes* interacts with the host by activating host keratinocyte protease activated receptor 2 (PAR2), suggesting that *C. acnes* secretes one or more exogenous proteases (100). This activity is raised in acne lesions, which stimulate host expression of cytokines including gamma interferon (IFN-γ), IL-1α, IL-8, IL-17, and TNF-α and antimicrobial peptides human β-defensin 2 and LL-37 (101, 102). Metagenomic analysis of *C. acnes* in follicular microbiomes of acne patients and healthy individuals (103) revealed the presence of a CAAX amino protease, a membrane-bound metalloprotease involved in bacteriocin self-immunity in acne patients (104). These studies demonstrate that *C. acnes* can produce secretory proteases that have important effects on the host, but the exact proteases, their associated substrates, and molecular functions of the enzymes need further definition.

***Staphylococcus*.** *Staphylococcus* is arguably the most extensively studied genus of skin microbes. Traditionally, staphylococci are classified as coagulase positive or negative depending on the presence of coagulase that clots blood plasma (105). The skin harbors a wide range of the coagulase-negative *Staphylococcus* (CoNS) species, including *Staphylococcus epidermidis*, *Staphylococcus capitis*, *Staphylococcus hominis*, and *Staphylococcus warneri* (7). The coagulase-positive *Staphylococcus aureus* is widely regarded as an opportunistic pathogen capable of producing an arsenal of virulence factors such as extracellular enzymes involved in host epidermal invasion (Fig. 1).

TABLE 3 Bacterial proteases and their associated targets and functions

Organism	Protease		Target(s)	Process(es) involved	Key reference(s)
Cutibacterium acnes	Several unidentified proteases	Unknown	Keratinocyte protease activated receptor 2	Immune modulation	98, 100
Staphylococcus aureus	Cysteine protease (staphopains)	ScpA	Collagen; CXCR2; complement C3, C5; fibrinogen; host cystatins; LL-37	Epidermal barrier invasion; immune modulation and evasion; biofilm formation	122, 127, 135, 162
		SspB	Collagen; CD31; complement C5; chemerin; fibrinogen; fibronectin; galectin; host cystatins		
	Metalloprotease	Aureolysin	Collagen; complement C3, C5; LL-37; pro-MMP-9	Epidermal barrier invasion; immune modulation and evasion; pro-V8 activation	121, 124, 128
	Serine proteases	Exfoliative toxin A and B	Desmoglein-1	Epidermal barrier invasion	116
		V8 (SspA)	Complement C3, C5; IgG; kininogen; *S. aureus* FnBP	Immune modulation; colonization; biofilm formation; pro-SspB activation	129, 131, 137, 139
	Serine protease-like family Spl	SplA to -F	IgG4; human mucin	Biofilm formation	142
Staphylococcus epidermidis	Cysteine protease	EcpA	Collagen; elastin; fibronectin; fibrinogen; α1 protease inhibitor; IgA, IgM	Epidermal barrier invasion; immune modulation	162–165
	Metalloprotease	SepA	Dermcidin; *S. epidermidis* Aap	Colonization; biofilm formation	161, 170
	Serine protease	Esp	Various *S. aureus* extracellular proteins; fibronectin; fibrinogen; vitronectin	Regulates *S. aureus* colonization and biofilm formation; epidermal barrier invasion	166, 168, 169
Streptococcus pyogenes	Cysteine proteases	IdeS	IgG	Evasion of host phagocytes	194, 195
		Streptopain (SpeB)	Chemokines; complement C3b desmogleins; fibronectin; vitronectin; various immunoglobulins; staphylococcal SdrC	Epidermal barrier invasion; immune modulation and evasion; biofilm formation	180, 184, 186, 190, 196, 198
	Serine protease	ScpC	IL-8	Evasion of host neutrophils	187, 188
Streptococcal commensals	Metalloprotease	IgA1	Human IgA1	Immune modulation	174

***Staphylococcus aureus*.** In healthy individuals, *S. aureus* is harbored mostly in the anterior nares of about one-third of the adult population, while skin colonization is very low (106). *S. aureus* in the anterior nares can form biofilm that serves as a reservoir for the dissemination of this opportunistic pathogen (107). This coagulase-positive bacterium is historically associated with many skin disorders, ranging from inflammatory dermatological conditions such as atopic dermatitis (AD) to potentially life-threatening skin and soft tissue infections (108–111). A hallmark of *S. aureus* is its repertoire of secretory virulence factors that facilitate its survival and host invasion (112). These secretory pathogenic factors include allergens, toxins, adhesion factors, and extracellular enzymes including proteases that can facilitate superficial and invasive infections (113). The most well-characterized proteases include cysteine proteases staphopain A (ScpA) and staphopain B (SspB), metalloprotease aureolysin (Aur), serine protease V8 (SspA), and the serine protease-like family (SplA to -F) (114). Aureolysin, which is activated in an autocatalytic manner, cleaves the proenzyme of V8, which then activates SspB (115). The exfoliative toxins A and B (ETA and ETB, respectively) are serine proteases that degrade desmoglein, a key desmosome protein that maintains the barrier integrity but are present only in a few *S. aureus* strains (116–118). The functions and involvement in infection of these proteases have been reviewed extensively (90, 119, 120), and we will highlight here the recent findings on the involvement of these proteases in host-microbial interactions.

(i) Degradation of host epidermis-associated proteins. Host structural proteins such as the extracellular matrix (ECM) proteins and tight and gap junction proteins are involved in maintaining epidermal integrity. Many of these are targets of *S. aureus* extracellular proteases (90). Collagen, a major structural protein of the human dermal ECM, is a common substrate for *S. aureus* ScpA, SspB, and Aur (121, 122). A recent study using *S. aureus aur* gene knockout demonstrated that beyond its own ability to cleave collagen, Aur is able to further promote collagen degradation through its activation of host metalloprotease MMP-9 (121). While collagen is much more abundant in the dermal layer, the effect of these proteases becomes especially significant when the dermis is accessible to the bacteria, such as in wounds or skin abscesses. Interestingly, ScpA was also shown to be involved in inducing cell death when *S. aureus* propagates intracellularly, and this is likely due to its role in epithelial tissue destruction, which could facilitate bacterial exit from the host cell (123).

S. aureus ScpA, SspB, and Aur can degrade the cationic AMP LL-37 (124, 125), protecting *S. aureus* from the antimicrobial effect of cathelicidin. In a study done by Sonesson et al. (125), LL-37 fragments generated from staphopain degradation were shown to have immunomodulating effects on the host. Furthermore, the expression of ScpA and SspB was detected directly in skin biopsy specimens, demonstrating that these cysteine proteases are present in physiologically relevant environments (125).

(ii) Interference with host immune pathways. *S. aureus* secreted proteases interfere with host immune pathways in multiple ways. SspB degrades multiple immune cell surface receptors such as CD11b, CD16, and CD31 crucial for induction of phagocytosis, enabling *S. aureus* to evade phagocytosis (126, 127). ScpA, SspB, V8, and Aur are effective inhibitors of the host complement pathway (128, 129), where the proteases act upon various complement components including C3 and C5. *S. aureus* isogenic mutants lacking SspB, V8, or Aur showed decreased survival in human blood (129). Galectin, an immunomodulating lectin produced by epithelial cells, is a newly discovered substrate of SspB. In a murine subcutaneous infection model, galectin knockout mice infected with *S. aureus* showed smaller lesions than wild-type mice. As galectin activates neutrophils, SspB enhances *S. aureus* virulence through cleavage of galectin that abolishes its ability to activate neutrophils (130). A recent study by Frey et al. utilized N-terminal degradomics to unravel new human serum protein substrates of V8 protease (131). This work highlights the role of V8 in interfering with host inflammatory signaling pathways through degradation of components of the complement pathway and host protease inhibitors such as SERPINs.

While most studies have focused on the proteolytic activity of *S. aureus* proteases in affecting the host immune response, a study by Stentzel et al. (132) observed increased IgE binding to the Spl serine proteases (A to F) in asthmatic patients. This suggests that the Spl proteases, which are encoded on a single operon, can be potential allergens (132).

(iii) Self-modulating effects. *S. aureus* can form biofilms which have been associated with poor healing outcome in wounds (133) and potentially contribute to inflammation in AD (134). The staphopains ScpA and SspB were found to have an inhibitory effect on biofilm formation and maintenance (135). V8 and Aur were also reported to inhibit *S. aureus* biofilm formation (136), where V8 is known to cleave *S. aureus* adhesin FnBP (fibronectin-binding protein), a protein mediating cellular adhesion to host ECM substrates (137).

While *S. aureus* extracellular proteases are generally regarded as virulence factors, recent studies have shown that these enzymes can modulate virulence indirectly. A mutant lacking all 10 of the extracellular enzymes had dramatically reduced penetration into the deeper skin tissues (138) and decreased skin abscess formation but was found to be hypervirulent (139), likely due to the roles of these proteases in controlling the stability of secreted toxins (139–141) and surface-associated adhesion proteins (142, 143). Using a combination of protease deletion mutants, Gimza et al. identified Aur and ScpA as the two keystone proteases contributing to virulence through controlling abundance of *S. aureus* virulence factors (144). These studies highlight that the functions of *S. aureus* secreted proteases go beyond direct invasion and include modulation of other extracellular factors important to establish infection.

(iv) Importance of strain variation in pathogenicity of S. aureus. In studying *S. aureus* secretory proteases, it has become clear in recent studies that it is important to consider the pathogenicity differences among *S. aureus* strains. This is relevant for both the presence of protease genes and the complex of regulatory elements that control protease gene expression (145), of which the *agr* operon (146, 147) and *sarA* are two key regulators (148–150). In a study involving 6-month-old infants, mutations in the Agr-quorum sensing system were more frequently observed in subjects who did not develop AD (151). Comparative genomics of diabetic foot ulcer *S. aureus* strains associated with different healing outcomes further revealed disparities in the presence of virulence-associated genes between the strains (152). Overall, it is crucial to consider the strain-associated heterogenicity leading to varied expression of *S. aureus* extracellular proteases as this could contribute to differences in disease severity.

***Staphylococcus epidermidis*.** The CoNS species represent one of the most abundant bacterial communities on the human skin and are especially prevalent on moist and sebaceous skin sites (7). Species of CoNS are generally considered to be commensal or even beneficial members of the skin microbiome as they utilize several mechanisms to limit growth of pathogens on the skin (23, 153). CoNS also possess fewer extracellular toxins capable of direct tissue invasion (154, 155). Functional annotations of the CoNS reveal that these species do possess secretory proteases, but the functions and molecular substrates of these enzymes are poorly defined. The most prominent member of the skin CoNS is *S. epidermidis* (156). Several studies have demonstrated *S. epidermidis*'s role as a beneficial microbe through increasing innate skin barrier immunity by tuning T cells (157, 158). However, *S. epidermidis* can cause opportunistic infections, such as when introduced into the body through medical implants and devices (159, 160), but such occurrences are rare compared to *S. aureus*. Overall, *S. epidermidis* favors persistence on the skin rather than host invasion and tissue destruction (160).

The secreted proteases of *S. epidermidis* include the serine protease Esp, metalloprotease SepA, and cysteine protease EcpA (89). While Esp has low homology to other staphylococcal proteases, SepA is a homologue of the *S. aureus* metalloprotease Aur (161) and EcpA shares substantial homology to the *S. aureus* staphopains ScpA and SspB (162).

(i) Degradation of host epidermis-associated proteins. EcpA is expressed as part of the *ecpAB* operon upstream of its endogenous inhibitor EcpB. The cysteine protease EcpA can be found attached to the cell surface of *S. epidermidis* or secreted into the

environment, and the proportions of these two forms differ between strains (163). Williams et al. reported that several cultured *S. epidermidis* strains isolated from a patient suffering from the rare Netherton syndrome (monogenic dermatological disease from loss of the human protease inhibitor LEKTI-1) had EcpA activity (162). In a recent study on AD patients, Cau et al. (164) observed that when *S. epidermidis* is present at high density, such as on AD lesional sites, this can induce expression of *ecpA*. This protease expression is under quorum sensing control, and high density of *S. epidermidis* is correlated with increased *ecpA* expression (164). Furthermore, this cysteine protease can degrade skin structural proteins such as collagen, elastin, fibronectin, and desmoglein-1 (164), the AMP LL-37, and various blood plasma-associated endogenous protease inhibitors (165) *in vitro*. In a mouse epicutaneous exposure model, only *S. epidermidis* wild type, but not the *ecpA* knockout strain, was able to elicit skin barrier damage and inflammation (164). In addition, *S. epidermidis* serine protease Esp can also degrade fibronectin, fibrinogen, and vitronectin *in vitro* (166), while the metalloprotease SepA is simultaneously upregulated by and degrades the sweat-associated anionic AMP dermicidin (161). Overall, the degradation of these host proteins indicates that *S. epidermis* has pathogenic potential, but further studies are needed to decipher what are the factors controlling expression and activities of these proteases as *S. epidermis* is prevalent and abundant on healthy human skin (2). One emerging theory, similar to *S. aureus*, is the expansion of particular *S. epidermidis agr* types that control and increase expression of the proteases at high density in skin diseases (167).

(ii) Interactions with other microbial communities. The *S. epidermidis* serine protease Esp inhibits *S. aureus* colonization by blocking *S. aureus* biofilm formation and destroying preformed *S. aureus* biofilms (168, 169). Examination of *S. aureus* biofilm disassembly revealed the ability of Esp to degrade several *S. aureus* proteins involved in colonization and biofilm formation (166). Autolysin (Atl), extracellular matrix protein (Emp), FnBP, and SpA are some examples of *S. aureus* proteins targeted by Esp (166). The metalloprotease SepA also plays a role in biofilm formation by processing the *S. epidermidis* cell-wall-anchored Aap protein to form an adhesin that facilitates biofilm accumulation (170). Other than *S. aureus* biofilm inhibition, Esp was found to augment the bactericidal effect of human keratinocyte AMP β-defensin 2 against *S. aureus* (169). However, a follow-up study that assessed the expression of *esp* in *S. epidermidis* isolates from the nose of healthy adolescents using semiquantitative PCR did not find any correlation between *esp* expression and biofilm inhibition (171). As the gene expression analysis was done for only 9 strains, follow-up studies with a larger number of *S. epidermidis* strains are needed to decipher the relationship between *esp* expression and biofilm formation.

***Streptococcus*.** The *Streptococcus* genus is a group of Gram-positive bacteria that are commonly found in the oral and nasopharyngeal microflora of healthy individuals (172). Several *Streptococcus* species including *Streptococcus mitis*, *Streptococcus oralis*, *Streptococcus pseudopneumoniae*, and *Streptococcus sanguinis* are prevalent at the dry skin sites in healthy individuals (2). The most well-characterized *Streptococcus* is the pathogen group A *Streptococcus* (GAS) *Streptococcus pyogenes*. Though relatively uncommon in the healthy skin microbiome, *S. pyogenes* is implicated in a wide range of skin and soft tissue infections such as impetigo, ecthyma, cellulitis, and necrotizing fasciitis (111, 173).

Streptococcal commensals. The skin commensals *S. mitis*, *S. oralis*, and *S. sanguinis* produce the extracellular metalloprotease IgA1 protease, which cleaves the human IgA1 at the hinge region of the heavy chain which connects the constant fragment (Fc) region to the antigen binding region (Fab) (174). The IgA1 proteases are large proteins (around 130 to 200 kDa) that possess a Gram-positive cell wall anchoring motif and can be secreted or cell wall associated. In particular, the *iga* genes in *S. sanguinis* and *S. oralis* share high homology while *S. mitis* and *Streptococcus pneumoniae iga* genes have moderate similarities (175). The precise functions of these *Streptococcus* IgA1 proteases are unclear, and most studies have focused on their roles as a virulence factor in *S. pneumoniae*, a pathogen that causes bacterial pneumonia and meningitis (172). The cleavage of the IgA1 hinge region leaves the Fab region intact, and these Fab fragments can in turn mask the bacterial surface epitopes without eliciting a downstream

immune response as the Fc region is disconnected. Furthermore, IgA protease can inhibit phagocytic killing of *S. pneumoniae* by targeting the capsule-specific human IgA1 monoclonal antibody (176). However, the expression and functions of these IgA proteases, especially those secreted by the common streptococcal skin commensals, have yet to be investigated in detail.

***Streptococcus pyogenes*.** In contrast to the other *Streptococcus* skin commensals, *S. pyogenes* has high pathogenicity and causes infection on skin and mucosal surfaces. Similarly to *S. aureus*, *S. pyogenes* expresses a variety of extracellular virulence factors (173) including the secretory cysteine protease streptococcal pyrogenic exotoxin B (SpeB, also called streptopain), cysteine protease IdeS (IgG-degrading enzyme of *S. pyogenes*), and serine protease ScpC (also called SpyCEP).

(i) Degradation of host epidermis-associated proteins. The cutaneous barrier is composed of layers of differentiated keratinocytes joined together by intercellular junctions such as the desmosomes and tight junctions (177, 178). SpeB was found to cleave these intercellular junction proteins including desmoglein-1, desmoglein-3, E-cadherin, and occludin, facilitating the dissemination of and invasion by *S. pyogenes* in skin infections (179, 180). Host ECM proteins fibronectin and vitronectin are also targets of SpeB (181). An *S. pyogenes* insertional mutant of SpeB which lacks expression of this protease (182) results in a smaller skin abscess and lesions in murine subcutaneous infection (183). Furthermore, a mutant strain that constitutively expressed SpeB in a murine subcutaneous infection model resulted in increased lesion size compared to the wild-type control (184). However, SpeB expression and protease activity were higher in clinical isolates from nonsevere infections compared to those isolated from severe infections (185). These studies demonstrate once again that it is important to consider contextual expression of these bacterial proteases, and similar to *S. aureus*, these extracellular *S. pyogenes* proteases can play a regulatory role in controlling other virulence factors important for infection to establish.

(ii) Interference with host immune pathways. One of the best-characterized roles of *S. pyogenes* secretory proteases is their ability to degrade host immune factors ranging from cytokines (186–189) to complement proteins (190–193) and immunoglobulins (Igs). One crucial pathway that this pathogen has to overcome is the adaptive immune response facilitated by Igs. As antibodies can activate phagocytic cells and complement pathways, *S. pyogenes* gains resistance to antibody-mediated opsonophagocytosis by degrading these proteins (194, 195). Early studies uncovered the role of SpeB in degrading multiple Ig classes (196); IdeS, on the other hand, has specificity for cleavage of both circulating and Fab-bound IgG (194, 195) and was demonstrated to be a more efficient enzyme than SpeB in degrading IgG (197). Recent work by Persson et al. brought into question whether the degradation of multiple classes of Igs by SpeB is physiologically relevant as this cysteine protease can cleave the heavy chain of the Ig only when these substrates are in the reduced, semimonomeric form (197).

(iii) Interaction with other microbial communities. Most of the attention on *S. pyogenes* secreted proteases has been on their effect on cleavage of host proteins, but less is understood about their role in interaction with other microbes, especially in the context of a mixed microbial community. In a recent study, Carothers et al. (198) demonstrated by using an isogenic SpeB mutant of a skin-tropic *S. pyogenes* strain and recombinant SpeB that this protease is involved in attenuation of *S. aureus* biofilm formation. SdrC, a cell-wall-anchored *S. aureus* adhesin, was shown to be a substrate of SpeB, and its degradation leads to disruption of the biofilm (198). This study highlights the importance of understanding the roles of these secretory proteases in the context of the mixed microbial communities present at different sites.

CONCLUSION/PERSPECTIVE

The roles of skin microbial secretory proteases have long intrigued many, and historically these proteases are regarded as virulent agents in microbial pathogenesis. Recent studies have revealed that many factors need to be taken into consideration when studying microbial protease functions. First, secreted proteases from microbes involved in infection can both serve as direct agents of invasion and indirectly

modulate the abundance and stability of other virulence factors. Second, expression of the same proteases can differ dramatically within specific strains of the same species due to differences in the regulatory elements. Finally, it cannot be emphasized enough that skin host phenotype and environmental context are critical factors resulting in dynamic regulation of these secretory enzymes. The presence of potentially virulent genes does not automatically translate to constitutive expression and emphasizes the importance to go beyond metagenomic studies to validate expression *in situ*. Furthermore, the same protease could have beneficial roles when the skin barrier is intact but become an agent of pathogenesis when the skin barrier is compromised due to wounds or skin diseases (Fig. 1). It is therefore essential to consider the context and environment where the protease is expressed when elucidating the functional roles of the secreted proteases.

Many advanced analytical tools in studying microbial phenotype and protease activities have been introduced in recent years. These include large-scale culturomics studies (199, 200), mass spectrometry-based degradomics (201), and chemical probes to monitor protease activities (202, 203). Degradomics, the comprehensive functional analysis of proteases and their associated substrates (21), is a particularly powerful tool to identify novel protease substrates and quantify the extent of substrate degradation (204). The application of these techniques, together with the available database of microbe sequence databases, will advance our understanding of the molecular functions of skin microbial proteases in skin health, especially the understudied skin commensals that account for the vast majority of the skin microbiome.

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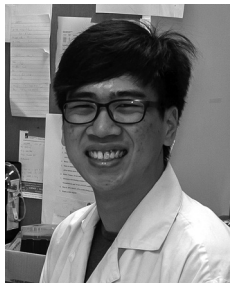

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