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RESEARCH ARTICLE

Staphylococcus pseudintermedius isolated from atopic dogs with pyoderma induces mast cell degranulation

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ABSTRACT

Aims: First, to determine via whole genome sequencing the sequence of the *hld* gene that encodes δ-toxin and elements of the accessory gene regulator (*agr*) locus that encode quorum sensing in four *Staphylococcus pseudintermedius* isolates from atopic dogs; second, to assess degranulation of mast cells by synthetic δ-toxin *in vitro,* and by culture filtrate containing δ-toxin from the *S. pseudintermedius* isolates in canine skin *in vivo*; and third, to determine whether the genetic region (*RNAIII*) encoding the δ-toxin gene is upregulated in response to increasing bacterial density (quorum sensing) in the isolates.

Methods: Four isolates of *S. pseudintermedius* were obtained from four dogs with pyoderma and canine atopic dermatitis (cAD). All four isolates were sequenced to compare their genomes and the sequences of the *agr* and *hld* elements. Synthetic *S. pseudintermedius* δ-toxin was applied to a mast cell culture from murine fetal liver cells *in vitro*. Degranulation was assessed using a β-hexosaminidase assay. Filtered supernatants from cultures of the four *S. pseudintermedius* isolates were tested by mass spectrometry to detect δ-toxin. These filtrates were then injected into the skin of five normal dogs. The injection sites were biopsied 15 minutes later. Degranulation of canine mast cells was assessed and quantified histologically. To assess up-regulation of the genetic region encoding the δ-toxin gene in response to increasing bacterial density in the four *S. pseudintermedius* isolates, relative expression of *RNAIII* was assayed using quantitative PCR after 1, 2, 4, 7 and 8 hours of culture.

Results: Synthetic *S. pseudintermedius* δ-toxin caused comparable degranulation of MC/9 cells to δ-toxin of *Staphylococcus aureus*. Mast cell degranulation was demonstrated in the skin of all five normal dogs following intradermal injection of a purified supernatant that contained *S. pseudintermedius* δ-toxin. The genetic elements of the δ-toxins were described. As the cell density of cultures of the *S. pseudintermedius* isolates from atopic dogs increased, *RNAIII* expression increased relative to the reference gene (*gyrB*), suggesting that *RNAIII* expression may be controlled by a quorum-sensing mechanism.

Conclusions and clinical relevance: *S. pseudintermedius* isolates from atopic dogs carry genes encoding δ-toxin, a staphylococcal exotoxin that can degranulate murine mast cells *in vitro*. An agent in filtered *S. pseudintermedius* culture known to contain δ-toxin causes degranulation of dermal mast cells *in vivo* and may play a role in the initiation and/or exacerbation of cAD.

Abbreviations: AD: Atopic dermatitis; Agr: Accessory gene regulation system; cAD: Canine atopic dermatitis; LC-IDA-MS/MS: Liquid chromatography with information-dependent acquisition-mediated tandem mass spectrometry; PBS: Phosphate-buffered saline; PSM: Phenol-soluble modulins; SIG: *Staphylococcus intermedius* group; TSB: Tryptic soy broth; UPLC: Ultra-performance liquid chromatography

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Introduction

Atopic dermatitis (AD) in humans and canine atopic dermatitis (cAD) are chronic inflammatory skin diseases with a multifactorial genetic basis. They have a number of pathogenic factors in common, including ineffective skin barrier function, IgE immune responses to innocuous environmental antigens and dysbiosis of cutaneous microorganisms (Abeck *et al*. 1998; Pin *et al*. 2006; Kong *et al*. 2012; Santoro *et al*. 2015). Polyvalent antigens/allergens cross-link IgE molecules on mast cells, causing degranulation. Preformed inflammatory molecules such as histamine are released, and the activated mast cell then commences synthesis of others, including proteases, inflammatory cytokines and chemokines (Hill 2002). These inflammatory molecules are implicated in the pathogenesis of AD (Yamanaka and Mizutani 2011). Mast cell degranulation has been shown to impair skin barrier function (Gschwandtner *et al*. 2013) and facilitate the penetration of the canine epidermis by staphylococcal proteins (Mason and Lloyd 1990). A multimodal immune pathogenesis for AD has been proposed that does not imply

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causality for any one trigger or group of triggers and avoids linear interpretation of the condition (Eyerich *et al*. 2015).

Approximately 90% of humans with AD and dogs with cAD have dysbiosis of cutaneous microflora with loss of microbial diversity but increased staphylococcal colonisation. *Staphylococcus aureus* colonises human skin, and *S. pseudintermedius* that of dogs. cAD is a recognised predisposing factor for skin infection by *S. pseudintermedius* in the dog (Bannoehr and Guardabassi 2012). However, the overall relationship between staphylococcal infection and cAD is incompletely understood. No mechanism has been described to effect causation and perpetuation of cAD by staphylococci, although clinical observation would support such a relationship (DeBoer and Marsella 2001). The role of anti-staphylococcal IgE in pyoderma and cAD is also unclear but elevated levels of this antibody have been found in atopic dogs (Bexley *et al*. 2013), suggesting an allergic patho-mechanism for bacterial hypersensitivity (Miller *et al*. 2013). A putative role for staphylococcal superantigens in AD of both dogs and humans has been proposed (Nakamura *et al*. 2013; Santoro *et al*. 2015). These are antigens that cause non-specific activation of large sectors of the T-cell repertoire leading to polyclonal T-cell proliferation and massive cytokine release.

S. pseudintermedius is a member of the *Staphylococcus intermedius* group (SIG). It has been proposed (Devriese *et al*. 2009) and accepted (Bannoehr and Guardabassi 2012) that isolates obtained from dogs and identified as SIG be termed *S. pseudintermedius,* as epidemiological evidence suggests that this is the only member of the SIG that has adapted to Canidae, and the other members of the SIG are virtually absent in dogs. *S. pseudintermedius* is used for all references in this manuscript where canine isolates were described as SIG or *S. intermedius*.

Staphylococci produce virulence factors including enzymes, surface proteins and toxins, all of which are represented in various forms in the different species of the genus (Sung *et al*. 2006). They also have in common an accessory gene regulation (Agr) system which, when activated in response to changes in population density, regulates the expression of factors that facilitate colonisation and virulence (Sung *et al*. 2006). This quorum sensing (Novick and Geisinger 2008) in a population of staphylococci facilitates a concerted response when a critical cell density is reached. The *agr* locus consists of dual-directional transcription units, *RNAII* and *RNAIII*. The *RNAII* locus contains four genes, *agrB, agrD, agrC* and *agrA* (Kornblum *et al*. 1990). *agrD* encodes a peptide of the extracellular quorum signal of Agr, called autoinducing peptide (Ji *et al*. 1995), which, when secreted, binds to the AgrC transmembrane receptor and positively regulates the transcription of both *RNAII* and *RNAIII* units (Otto *et al*. 1998) (Figure 1).

RNAIII is the apparent auto-induced effector of the Agr system in *S. aureus,* inhibiting surface molecules such as Protein A, coagulase and other cell wall-anchored proteins associated with adhesion to host

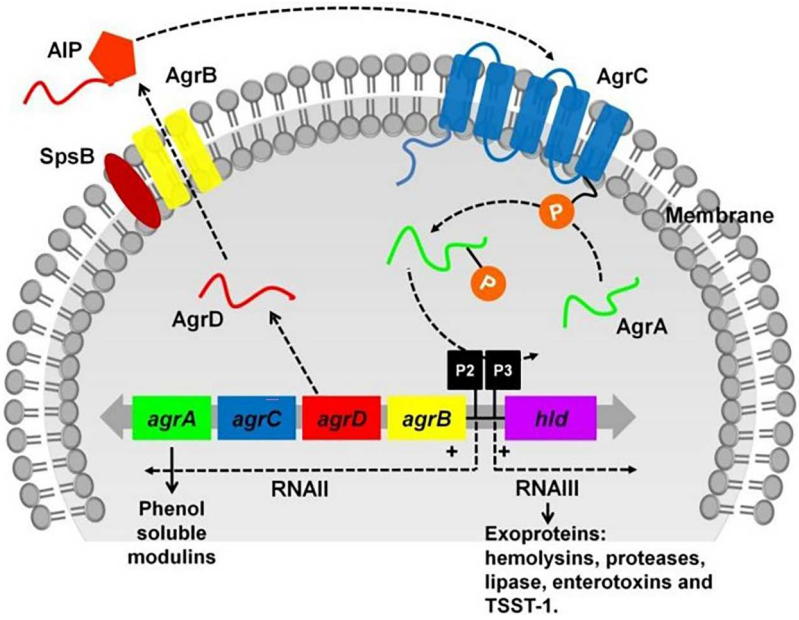

Figure 1. Schematic diagram of the *Staphylococcus aureus* Agr regulatory system. The *agr* operon consists of two transcriptional units, *RNAII* and *RNAIII*, driven by the promoters P2 and P3, respectively. *RNAII* is an operon of four genes *agrB, agrC, agrD* and *agrA*. AgrB is responsible for processing and exporting AgrD, the precursor for the auto-inducing peptide (AIP). At threshold levels of AIP, AgrC is autophosphorylated, leading to the phosphorylation of AgrA. AgrA activates RNAIII expression, thereby increasing the secretion of *S. aureus* toxins and enzymes. Reproduced from Kong *et al*. (2016) under a CC BY 4.0 license http://creativecommons.org/licenses/by/4.0 (courtesy of S Nathan).

corneocytes and extracellular matrix, while concurrently initiating a process of toxin upregulation (Novick 2003; Le and Otto 2015). Given the similarity of the genetic elements in the *agr* locus of *S. pseudintermedius,* a similar quorum-sensed production of exotoxins is likely to occur (Sung *et al*. 2006). The *agr* locus contains promoters, one of which directs the synthesis of RNAIII (Novick *et al*. 1993). Three different specificity groups of the *agr* locus from 20 isolates *of S. pseudintermedius* have been described (Sung *et al*. 2006).

A number of exotoxins from *S. aureus* and *S. pseudintermedius* have been described and are comparable (Sung *et al*. 2006; Maali *et al*. 2018). The α, β and δ phenol-soluble modulins (PSM; also called haemolysins) are highly conserved peptide toxins in staphylococci (Tsompanidou *et al*. 2010; Maali *et al*. 2018). The coding region of the gene for δ-toxin, called *hld*, is embedded in the region encoding RNAIII (Janzon *et al*. 1989; Novick 2003). Thus, concentrations of RNAIII and δ-toxin in culture supernatants correlate positively (Gagnaire *et al*. 2012) and δ-toxin can be used as a surrogate for RNAIII expression and Agr function (Chen *et al*. 2012; Gomes-Fernandes *et al*. 2017). Two analogues of *S. pseudintermedius* δ-toxin have been described, although only one is expressed in any isolate (Cheung *et al*. 2014; Maali *et al*. 2018).

A study of δ-toxin from *S. aureus,* which has approximately 64% similarity to that of *S. pseudintermedius*, demonstrated an exclusive range of effects with the potential to exacerbate and cause AD in humans (Nakamura *et al*. 2013). δ-toxin deregulated murine mast cells in the absence of antigen, creating synergy with IgE-initiated mast cell degranulation, and promoting both IgE and interleukin-4 production in mice (no other PSM initiated mast cell degranulation). The last effect was abrogated in mast cell-deficient mice. Mast cell degranulation was triggered equally by formylated and unformylated δ-toxin. Production of δ-toxin from *S. aureus* was greater from isolates obtained from lesions than from non-lesional sites on atopic humans (Nakamura *et al*. 2013).

The amount of δ-toxin produced by strains of *S. aureus* varies widely and differs depending on whether the bacterium is growing *in vivo* or *in vitro* (Hodille *et al*. 2016; Maali *et al*. 2018; Su *et al*. 2020). Further, δ-toxin from *S. pseudintermedius* causes *in vitro* cytolysis of human neutrophils and non-professional phagocytes using a human osteoblast cell line (Maali *et al*. 2018). Most forms of canine pyoderma, however, are mild to moderate superficial *S. pseudintermedius* infections with the notable exception of the scarring furunculosis of deep pyoderma (Gross *et al*. 2005). PSM cause cytolysis at micromolar concentrations, but at much lower nanomolar concentrations, they elicit inflammatory responses (Otto 2014).

Reports of dermal mast cell degranulation associated with superantigens have not been found. The relationship between cAD and *S. pseudintermedius* is incompletely understood (Santoro and Hoffman 2016), but a correlation has been established between cutaneous microbial dysbiosis and reduced skin barrier function during flares in atopic dogs (Bradley *et al*. 2016). Those authors then showed that clinical signs and barrier function improved when the dysbiosis and dominance of *S. pseudintermedius* were resolved with appropriate antibiotic treatment. Nevertheless, cause and effect roles for the two observations have not been established.

While δ-toxin from *S. aureus* is established as a cause and exacerbation of AD in humans and mice, it is unclear whether δ-toxin from *S. pseudintermedius* has similar abilities. Therefore, the overall goal of this study was to provide evidence that *S. pseudintermedius* can degranulate mast cells and thus be a mechanistic factor in the initiation and/or exacerbation of cAD. Towards this goal, four isolates of *S. pseudintermedius* were obtained from four dogs with pyoderma and cAD. We then aimed first to determine the sequence of the *hld* gene; second, to assess degranulation of mast cells by synthetic *S. pseudintermedius* δ-toxin *in vitro* and by filtered culture supernatant containing δ-toxin from the four isolates *in vivo*; and third, to determine whether the genetic region encoding the δ-toxin gene is upregulated in response to increasing bacterial density in cultures of the four isolates.

Materials and methods

Bacterial strains and culture conditions

All *S. pseudintermedius* isolates were obtained from cultures of intact pustules of infected New Zealand dogs previously diagnosed with cAD by author AB. Culture of swabs on blood agar (Fort Richard Laboratories, Auckland, NZ) resulted in colonies typical of staphylococci that were DNase-positive and hyaluronidase-negative. Each culture was stored in tryptic soy broth (TSB) (Fort Richard) with 40% glycerol at −80°C. *S. pseudintermedius* isolates were grown in TSB overnight at 37°C with shaking and then used for the following experiments.

Bacterial DNA isolation, whole genome sequencing and analysis

Bacterial DNA was isolated from the four isolates from atopic dogs using the NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany). The KAPA HyperPlus Kit (Kapa Biosystems, Wilmington, MA, USA) was used to prepare the multiplexed shotgun libraries of DNA samples. The quality of all libraries was determined by an Agilent

2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and Qubit fluorometer (Life Technologies, Carlsbad, CA, USA). DNA sequencing (300-bp read length, paired-end sequencing) was performed with MiSeq (Illumina, San Diego, CA, USA) platforms according to the manufacturer's instructions. All Illumina data sets were cleaned using Trimmomatic v.0.33 (Bolger *et al*. 2014). Trimmed reads were used to generate *de novo* assemblies of the draft genomes using SPAdes v3.11.1 (Prjibelski *et al*. 2020) with default parameters, " – cov-cutoff auto" and " – careful." Annotations of all predicted open reading frames of the draft genomes were performed using PROKKA v.1.12 (Seemann 2014).

For genome-wide phylogenetic analysis, the four *S. pseudintermedius* isolates in the current study and one reference isolate of the HKU10-03 variant (biosample SAMN02603958 downloaded from GenBank) were used to assemble the core genome. The PROKKA-annotated genome assemblies were further analysed by the software package ROARY (Seemann 2014) to provide the pangenome, core and accessory gene distribution and a gene presence/absence plot.

Degranulation of murine MC by synthetic S. pseudintermedius *δ-toxin*

Cells from a murine mast cell line (MC/9; Yamada *et al*. 2003) were re-suspended in Tyrode's buffer (Sigma, Burlington, MA, USA) at 1×10^5 cells per 100 µL, aliquoted in triplicate into a 96-well U-bottom plate and stimulated either with ionomycin (1 µM; Sigma) which causes degranulation of mast cells by mobilising stored intracellular calcium (Morgan and Jacob 1994) or with synthetic δ-toxins of *S. pseudintermedius* (from ED99 strain; amino acid sequence fMAADIISTIV EFVKLIAETV AKFIK) or *S. aureus* (amino acid sequence: fMAQDIISTIG DLVWIIDTV NKFTKK) for 1 minute. Both synthetic toxins were purchased from Peptide Institute Inc. (Osaka, Japan). The range of concentrations (1, 10, 30 and 100 µg/mL) of δ-toxins tested was selected based on the ability of *S. aureus* to produce 30 µg/mL δ-toxin in a TSB culture (Nakamura *et al*. 2013). Results are given as a relative percentage compared to freeze/thaw of the total cell culture.

Degranulation of canine mast cells in vivo *by* S. pseudintermedius *filtrate*

Culture filtrate

The four isolates of *S. pseudintermedius* from dogs with cAD described above were cultured overnight in TSB with shaking, followed by centrifugation at 5000 *g* for 5 minutes. A 600-µL aliquot of supernatant was added to 900 µL of phosphate-buffered saline (PBS; pH 7.2, 0.01 mmol/L phosphate, 0.15 mol/L NaCl), then sterilised through a 0.22 mm filter (Merck Millipore, Cork, Ireland). The filtrate was stored at −80°C and then thawed 24 hours before use; first at 4–8°C overnight and then at room temperature for 3 hours. The negative control was sterile PBS and the positive control was the selective mast cell degranulator (Mason and Lloyd 1996) compound 48/80 (Optimus Health Care, Auckland, NZ) at 1 mg/mL. A second negative control consisting of TSB was discarded when it appeared discoloured after thawing.

Identification of δ-toxin in culture supernatants

To identify the presence of δ-toxin in bacterial supernatants, these were prepared and frozen as described above. The four supernatants were then thawed, bath-sonicated for 1 minute, then briefly mixed with a vortex mixer. A 10 µL aliquot was mixed with 50 µL of methanol with a vortex mixer. Samples were incubated at 4°C for 10 minutes, then centrifuged at 16,000 *g* for 5 minutes at 5°C. The supernatant was diluted with 1:5 H_2O: methanol just prior to injection.

Liquid chromatography with information-dependent acquisition-mediated tandem mass spectrometry (LC-IDA-MS/MS) was employed to detect δ-toxin. Samples were injected onto a 0.3×10 mm trap column packed with 3 µm 300A C4 media (Dr Maisch, Ammerbuch-Entringen, Germany) and desalted for 2 minutes at 20 µL/minute, before being separated with the following gradient at 6 µL/minute using a Micro M5 Ultra-performance liquid chromatography (UPLC) system (AB SCIEX, Framingham, MA, USA): 0 minutes 15% B; 5.9 minutes 97% B; 7.9 minutes 97% B; 8 minutes 15% B; 10 minutes 15% B, where A was 0.1% formic acid in water and B was 0.1% formic acid in acetonitrile.

The liquid chromatography effluent was directed into a ZenoTOF 7600 quadrupole time-of-flight mass spectrometer (AB SCIEX) for information-dependent acquisition analysis, comprising a time-of-flight mass spectrometry scan from 350 to 1,500 m/z for 100 milliseconds, followed by tandem mass spectrometry scans (m/z 100–1,500) on the 10 most abundant multiply-charged features for 40 milliseconds each, giving a total cycle time of 0.57 seconds. The mass spectrometer and UPLC system were under the control of the Sciex OS software package (AB SCIEX).

The resulting data was searched using ProteinPilot v.5 (AB SCIEX) against a protein sequence database containing *S. pseudintermedius* entries (Uniprot.org, 2,940 entries), along with a custom sequence for δ-toxin having an amino acid substitution (MAADIISTI-VEFVKLIAETVEKFIKK). An additional terminal lysine residue was manually added to the δ-toxin entries, to reflect what was actually observed in the samples. The search was performed with "no enzyme specificity," in "thorough" mode, with biological modifications allowed. Spectral matches were manually confirmed in PeakView v.2.2 (AB SCIEX).

Intradermal testing

Five dogs used in this study were healthy and of mixed age, sex and breed. They had no history of skin disease. Animal ethics approval was obtained (Kaiawhina Animal Ethics Committee 164 013/15). The dogs were sedated using IV 10 µg/kg medetomidine hydrochloride (Domitor; Pfizer Animal Health, Auckland, NZ), placed in lateral recumbency, and an area on the lateral thorax was clipped using a number 40 Oster blade. Intradermal injections of 0.10 mL of each of the four supernatants and controls were performed using a 0.5 mL syringe with a 1/2 inch 29 G needle (BD Ultrafine; Becton Dickinson Ltd., Scoresby, Australia), at sites 30 mm apart. Ten minutes after the supernatant injections, analgesia was provided using 1 mL of 2% lignocaine hydrochloride (Lopaine; Ethical Agents, Auckland, NZ) SC at each site. Biopsy was performed 15 minutes after the supernatant injections near the centre of each site using a 6-mm punch (Biopunch; Fray Products Corp, New York, NY, USA) but avoiding the needle puncture. The 30 biopsies (six from each dog) were placed in individual vials of 10% buffered formalin solution. The biopsy sites were closed with a simple interrupted braided cable nylon suture (Rivermid, Portland, OR, USA). Reversal of sedation with SC 50 µg/kg atipamezole (Antisedan; Pfizer, Auckland, NZ) was unnecessary in all but two dogs at the end of the procedure. All dogs recovered well with no adverse effects and were checked daily by a veterinary technician. Dogs and biopsy sites were examined by a veterinarian after 10 days, at which time the sutures were removed.

Histopathology

Biopsies were routinely processed and standard staining procedures were followed (Prophet 1994). Toluidine blue (Carson 1997) was used to stain sections of approximately 3–4 µm thickness. Mast cells were identified by their morphology and distinct metachromatic granules. Degranulation was defined as mast cells surrounded by scattered free granules (> 5–6) or represented by a cloud of free granules. Sections were examined at 400 x magnification (a field of view of approximately 0.5 mm diameter), and mast cells were enumerated and examined for degranulation at two different levels of the dermis. These regions were as defined by Shipstone *et al*. (1999), with the dermo-epidermal region being approximately half a 400 x field (approximately 0.25 mm) below the basal layer of the epidermis, and the mid-dermal region being a 400-x field (approximately 0.5 mm diameter area) at the level of the apocrine glands. For each biopsy, mast cells were counted in 4–6 sections per region. This amounted to 20–30, 400-x fields/region. Total mast cells per ten 400-x fields and degranulated mast cells per ten 400-x fields were then recorded, and percentage degranulation was calculated for each set of the four supernatants, positive control and negative control.

Analysis of RNAIII gene expression in response to increasing culture density

To assess whether δ-toxin expression in the *S. pseudintermedius* isolates was controlled by a quorum sensing mechanism, *RNAIII* gene expression was measured using quantitative PCR as cultures of the isolates increased in density.

S. pseudintermedius isolates from the four atopic dogs were grown overnight in TSB at 37°C with shaking. Bacterial cells were washed twice with PBS, then diluted 1:100 in fresh TSB and incubated at 37°C with shaking. At indicated time points (0, 1, 2, 4, 7 and 8 hours), 100 µL of each culture was mixed with an equal volume of RNAprotect Bacteria Reagent (QIAGEN, Venlo, Netherlands) and stored at −80°C for RNA analysis. Optical density of the culture was measured at the same time points. These experiments were performed in triplicate independently.

Total RNA was extracted using E.Z.N.A. Bacterial RNA Kit (Omega Bio-tek, Norcross, GA, USA) according to the manufacturer's protocol. The isolated RNA was quantified using a NanoDrop2000 spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Complementary DNA was synthesised using the High Capacity RNA-to-DNA Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Quantitative PCR was conducted according to standard protocols using SYBR Premix Ex TaqTM II (Takara Bio, Shiga, Japan) and RNAIII primers (S.inter_-RNA3_f: 5′-GCAGCAGATATCATTAGC-3′ and S.inter_-RNA3_r: 5′-TGCTACAATGGCTTC-3′; Sung *et al*. 2006), on the StepOne Real-time PCR machine (Applied Biosystems). The expression of *RNAIII* was normalised to that of *gyrB* (reaction performed as above with *gyrB* primers: S.inter_gyrB_f:5′-GCGTCCGTTGATTGAAGCG-3′ and S.inter_gyrB_r: 5′ AACGTCACTTGCAACATCGC-3′; Crawford *et al*. 2014) and relative expression calculated by the 2-ΔΔCt method. *gyrB* is a house-keeping gene whose expression is stable over time in a TSB culture of staphylococci and thus reflects the density of the culture (Jiang *et al*. 2019).

Results

Whole genome sequencing

Whole genome sequencing of the four *S. pseudintermedius* strains from atopic dogs identified 2,009 core genes and 1,045 strain-specific accessory genes. Core genes were defined as those present in all four isolates. The ROARY plot provides a visual

comparison of the pangenomes of the four isolates alongside the reference genome (Figure 2). This comparison of the pan-genomes showed isolates 1 and 4 to be most similar. An alignment of nucleotide sequences of *agrC, agrD* and δ-toxin (*hld*) from the *agr* locus of the four isolates and a reference strain is shown in Supplementary Figure 1. The *agrC* and *agrD* sequences of isolates 1 and 3 were the same as the reference strain HKU10-13, while isolates 2 and 4 had a different subtype. In contrast, the *hld* sequences of isolates 3 and 4 were the same as that of the reference subtype HKU10-13, while those of isolates 1 and 2 were of the reference subtype ED99.

Degranulation of murine MC by synthetic S. pseudintermedius

The cultured mouse mast cells degranulated when incubated with synthetic *S. pseudintermedius* δ-toxin. Figure 3 shows that the β-hexosaminidase concentrations, as a proportion of the freeze/thaw control, rose with increasing concentrations of δ-toxin, and exceeded those of *S. aureus* δ-toxin at each point (Figure 3). The efficacy of mast cell degranulation by the isolates was comparable despite the genetic variations described above.

Degranulation of mast cells in vivo

Optical densities of TSB cultures before obtaining the supernatant ranged from 2.36 to 2.78. N-formylated δ-toxin was detected in the filtered culture supernatants of all four isolates by LC-IDA-MS/MS with peptide scores > 99% and mass errors of < 2 ppm. Ionised fragments of δ-toxin were compared with those expected from its structure, with a compatibility ranging from 99% for the major fragments to > 50% (Supplementary Figure 2). This experiment was carried out in triplicate.

Injection of the filtered culture supernatants into the skin of five normal dogs resulted in sharply defined but irregular wheals of 15–20 mm diameter within 15 minutes. No reaction was observed at the sites of the negative control. The injection of positive control (compound 48/80) resulted in a well-demarcated, regular wheal of approximately 12 mm. No clinical evidence of inflammation or necrosis at the injection sites became evident in the following 10 days.

Histopathology

Examples of normal and degranulating toluidine-stained mast cell morphology are presented in Figure 4. Normal mast cells and degranulating mast cells were identified in the mid dermis and dermo-epidermal region. Degranulation was identified by extracellular granules that were scattered or in clouds. Nuclei of intact mast cells were often obscured by densely stained intracytoplasmic granules (Figure 4(c, d)). Histopathology revealed no evidence of cytolysis of non-professional phagocytes (e.g. keratinocytes) in the biopsy sections.

The median percentage of mast cell degranulation in biopsies from the five dogs injected with purified supernatants of the four isolates ranged from 84 (min 38, max 100)% for Isolate 1 to 95 (min 97, max 100)% for Isolate 4 in the mid dermis and 62 (min 55, max 78)% for Isolate 2 to 85 (min 45, max 92)% for Isolate 4 at the dermo-epidermal region. Median degranulation percentage for biopsies injected with the negative control (PBS) was 28 (min 11, max 41)% in the mid dermis and 12 (min 8, max 15)% at the

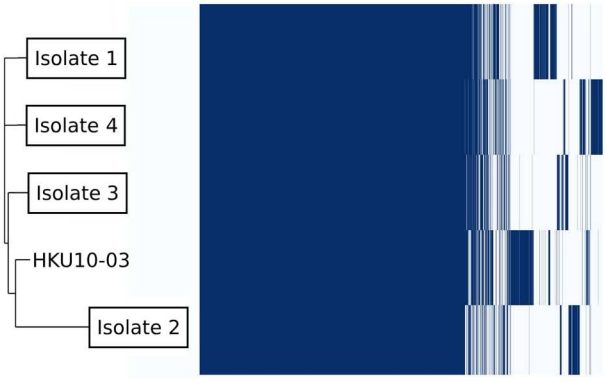

Figure 2. Pan-genome analysis of *Staphylococcus pseudintermedius* isolates from atopic dogs, performed using ROARY software. The matrix plot identifies presence or absence of 3,054 gene clusters as solid lines or spaces, respectively. This shows that the pan-genomes of the four isolates were dissimilar despite similar ability of their culture supernatants to degranulate mast cells *in vitro* and *in vivo*.

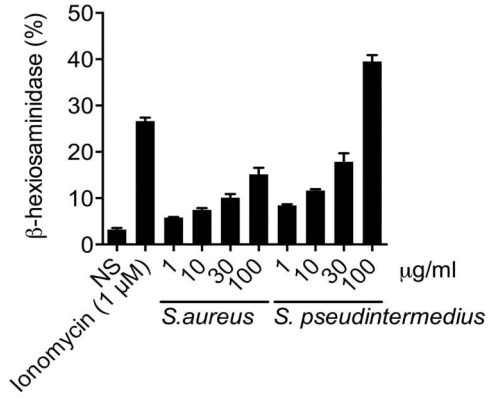

Figure 3. Mean (SE) hexominidase activity released by degranulation of cultured mouse mast (MC9) cells following incubation with synthetic δ-toxin from *Staphylococcus aureus* and *S. pseudintermedius* or with positive control (ionomycin) or normal saline (NS). Data are expressed as a proportion of the hexominidase activity released after freezing and thawing the cultured cells and are the mean of three independent experiments.

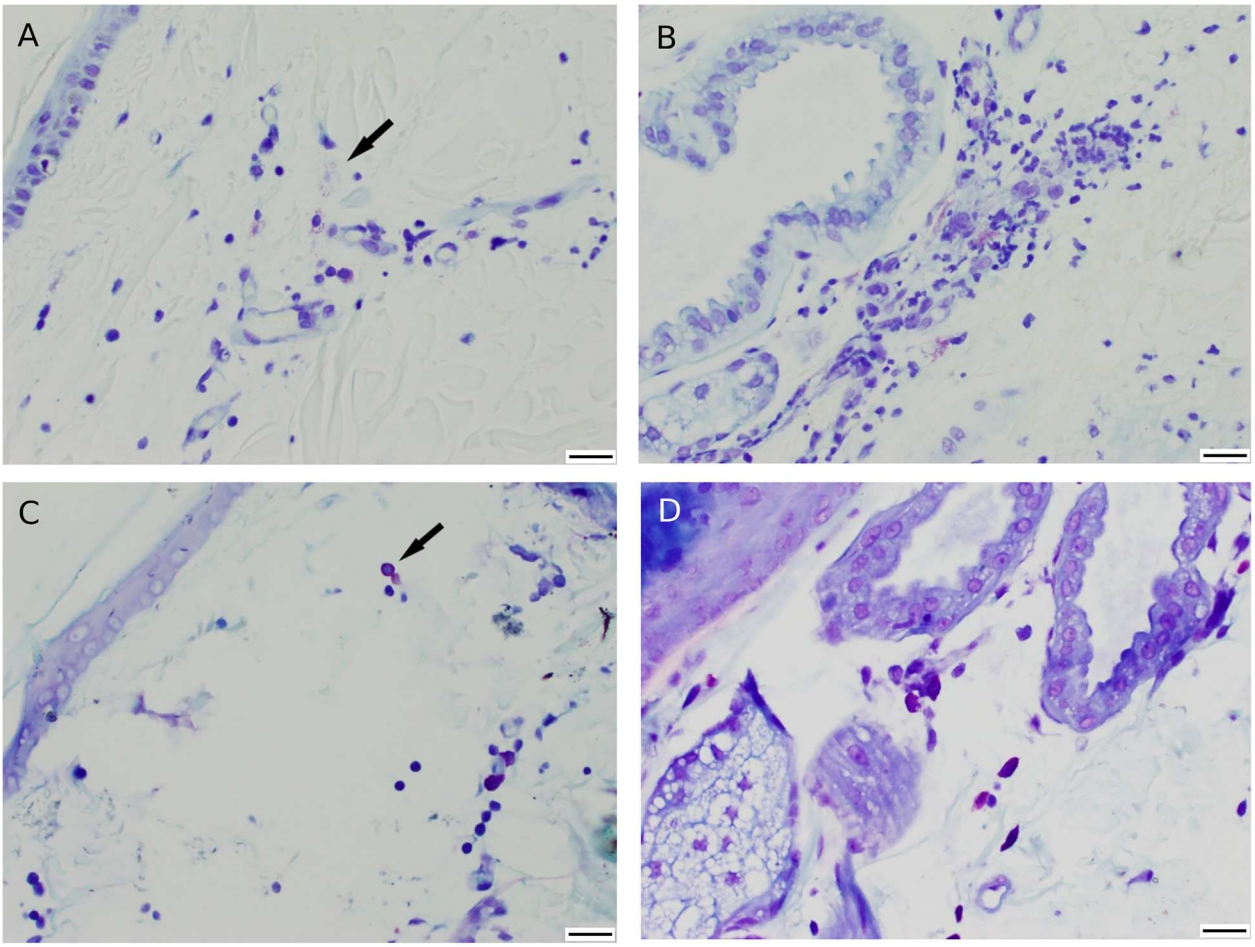

Figure 4. Histopathology of mast cell degranulation and intact mast cells in a normal dog injected with a purified culture supernatant from *Staphylococcus pseudintermedius* Isolate 2 obtained from an atopic dog or with a negative control (phosphate buffered saline) (toluidine blue staining, bar = 20 µm). (A) Dermo-epidermal region (arrow indicates degranulated mast cell); (B) mid-dermis; (C) negative control, dermo-epidermal (arrow indicates intact mast cell); (D) negative control, mid-dermis. Nuclei of intact mast cells in the negative controls were often obscured by densely stained cytoplasmic granules.

dermo-epidermal region. The median degranulation percentage for biopsies injected with the positive control was 62 (min 50, max 80)% in the mid dermis and 42 (min 35, max 50)% at the dermo-epidermal region (Figure 5 and Supplementary Table 1). Thus four genetically different *S. pseudintermedius* isolates from atopic dogs caused similar degrees of mast cell degranulation *in vivo*.

Analysis of RNAIII *gene expression in response to increasing culture densities*

The density of cultures of the *S. pseudintermedius* isolates from atopic dogs, as measured by optical density, increased up to 7 hours of incubation (Figure 6). As cell density increased, *RNAIII* expression increased relative to expression of the reference gene *gyrB*. This suggests that *RNAIII* expression may be controlled by a quorum-sensing mechanism. As the δ-toxin gene *hld* is encoded in the *RNAIII* transcriptional unit, its expression may then also be controlled by quorum sensing.

Discussion

Degranulation of mast cells in the skin of five normal dogs was evidenced by the rapid appearance of wheals at the sites of injection of filtered culture supernatant (containing δ-toxin) from *S. pseudintermedius* and by histopathology. The lack of immediate histopathological and subsequent clinical evidence of inflammation and/or necrosis would suggest that if δ-toxin were the responsible agent, it was not present in the higher concentrations associated with cytolysis (Wang *et al*. 2007). The single injection of filtered culture supernatant into the skin of normal dogs may have been inadequate to stimulate IgE production as was observed in mice (Nakamura *et al*. 2013).

Synthetic *S. pseudintermedius* δ-toxin degranulated mast cells from a mouse-derived line with a range of concentrations consistent with that of δ-toxin expected from TSB cultures of *S. aureus* (Nakamura *et al*. 2013).

Expression of *RNAIII* increased relative to reference gene expression disproportionally in TSB cultures of

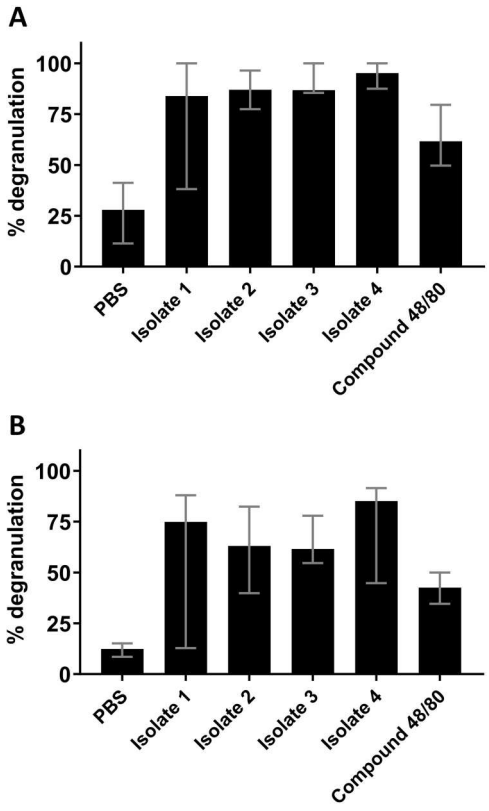

Figure 5. Median (min, max) percentage of mast cell degranulation in the mid-dermis (A) and dermo-epidermal junction (B) following intradermal injection of purified culture supernatant from four *Staphylococcus pseudintermedius* isolates, and the positive control compound 48/80 or phosphate buffered saline (PBS), in five normal dogs.

all four *S. pseudintermedius* isolates as the culture increased in density. This supported the presence of an *agr* quorum-sensing mechanism switching the culture from growth to exotoxin production. The known strong correlation of *RNAIII* and δ-toxin expression (Chen *et al*. 2012; Gomes-Fernandes *et al*. 2017) supports the use of *RNAIII* as a surrogate for δ-toxin production.

While the genomic analysis identified two types of *agr* elements among the four *S. pseudintermedius* isolates from atopic dogs, expression levels of *RNAIII* (containing mRNA for δ-toxin) were similar. This suggests *agr* differences were not important in this study.

Limitations

This was a study with a small number of *S. pseudintermedius* isolates obtained from lesions of atopic dogs. Nevertheless, whole genome sequencing has revealed no significant differences between isolates from normal dogs and those with cAD (Hodille *et al*. 2016). However, normal dogs were used in the *in vivo* study rather than those with cAD, whose results might have varied from those reported here. A recent study (Di Nardo *et al*. 2023) found that the normal skin microbiome suppresses human and murine dermal mast cells via dermal fibroblasts. If this mechanism is replicated in dogs, the degree of degranulation may have been greater had we used cAD dogs. It would have been preferable to have a second negative control in the *in vivo* study using TSB in case the broth itself contained an element that affected the observed mast cell degranulation. Although this was planned, the TSB solution was discarded after thawing due to concerns with the colour of the thawed solution. Nevertheless, TSB is unlikely to degranulate mast cells (Nakamura 2013) and has functioned as a negative control in a number of mast cell activation experiments (Nakamura 2013; Starkl *et al*. 2020; Di Nardo *et al*. 2023). We do not believe the culture solution is a likely cause of degranulation in the biopsies injected with bacterial filtrates, but nevertheless acknowledge this omission.

The nature of the inflammation following mast cell degranulation was not investigated in this study.

Quantitation of δ-toxin production was not performed in the culture filtrates because there is a dearth of evidence supporting a relationship between the *in vivo* and *in vitro* production of the toxin (Hodille *et al*. 2016). While no other staphylococcal exotoxins have been reported to degranulate mast cells and other PSM have been specifically excluded (Nakamura *et al*. 2013), the possibility of some other agent in the supernatant causing *in vivo* mast cell degranulation remains. This, together with measurement of *in vivo* production of δ-toxin in atopic dogs, should be explored in subsequent studies.

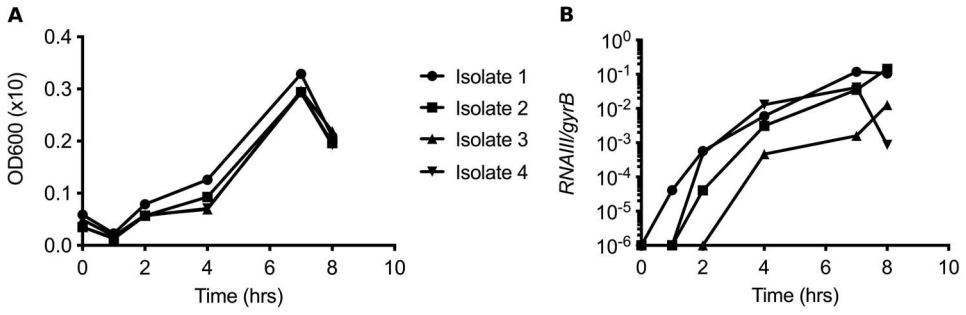

Figure 6. Mean (A) optical density (OD600) and (B) expression of mRNA encoding δ-toxin (*RNAIII*) relative to that of the housekeeping gene *gyrB*, measured by quantitative real-time PCR, for triplicate liquid cultures of four isolates of *Staphylococcus pseudintermedius* from the skin of atopic dogs.

Tissue handling can cause tissue damage in biopsies. However, no histopathologic evidence of changes in any cell type other than mast cells was observed, and handling could not account for the differences between control and treated degranulation.

Clinical significance

δ-toxin, an exotoxin of *S. pseudintermedius,* has been shown to degranulate mast cells *in vitro.* Four filtered culture supernatants known to contain δ-toxin caused degranulation of dermal mast cells in five normal dogs. The degranulating agent in the supernatants is suspected, but not proven, to be δ-toxin. This agent may provide a mechanistic relationship between *S. pseudintermedius* and cAD by impairing skin barrier function and promoting the dermal inflammation that follows degranulation.

cAD is a common condition in dogs (Outerbridge and Jordan 2021). *S. pseudintermedius*-associated cutaneous dysbiosis and pyoderma frequently accompany the pruritus and inflammation of cAD. These can be resolved only transiently, with antibiotics. Our findings may also be relevant to those dogs who are usually clinically normal but become pruritic when *S. pseudintermedius* pyoderma occurs. 'Bacterial hypersensitivity' is a concept used to describe these dogs. The emergence of antibiotic resistance demands the urgent development of non-antibiotic treatment strategies. Prevention of *S. pseudintermedius* reaching quorum sensing numbers on the skin and staphylococcal antitoxin treatments are potential strategies should δ-toxin be shown to have clinical significance in dogs with cAD associated with dysbiosis and/or pyoderma.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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