A novel human antimicrobial factor targets *Pseudomonas aeruginosa* through its type III secretion system

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*Pseudomonas aeruginosa* is an important opportunistic bacterial pathogen. Despite its metabolic and virulence versatility, it has not been shown to infect articular joints, which are areas that are rarely infected with bacteria in general. We hypothesized that articular joints possess antimicrobial activity that limits bacterial survival in these environments. We report that cartilages secrete a novel antimicrobial factor, henceforth referred to as the cartilage-associated antimicrobial factor (CA-AMF), with potent antimicrobial activity. Importantly, CA-AMF exhibited significantly more antimicrobial activity against *P. aeruginosa* strains with a functional type III secretion system (T3SS). We propose that CA-AMF represents a new class of human antimicrobial factors in innate immunity, one which has evolved to selectively target pathogenic bacteria among the beneficial and commensal microflora. The T3SS is the first example, to the best of our knowledge, of a pathogen-specific molecular target in this antimicrobial defence system.

INTRODUCTION

*Pseudomonas aeruginosa* is an important opportunistic bacterial pathogen. Despite aggressive antibiotic therapy, the morbidity and mortality rates associated with *P. aeruginosa* infection are extremely high due to the intrinsic resistance of *P. aeruginosa* to many antibiotics and the emergence of multi-drug resistant strains (Koch, 2002; Engel, 2003). *P. aeruginosa* is a highly versatile pathogen, capable of infecting various human tissues so long as the tissue defences are compromised in some manner (Yamaguchi & Yamada, 1991; Zahm et al., 1991; Tsang et al., 1994; de Bentzmann et al., 1996; Engel, 2003; Daas et al., 2009; Lin et al., 2009; Shigemura et al., 2009). Articular joints, however, are rarely infected with bacterial pathogens, including *P. aeruginosa*. We hypothesized that articular cartilages possess antimicrobial activity that limits *P. aeruginosa* survival within joint environments.

Using an ex vivo explant culture model (Pascual Garrido et al., 2009), we examined the capacity of *P. aeruginosa* to proliferate in the presence of human articular cartilages obtained from asymptomatic organ and tissue donors. We report that cartilages from both ankle and knee joints secrete a novel antimicrobial factor(s) referred to as the cartilage-associated antimicrobial factor (CA-AMF), with potent microbicidal activity against *P. aeruginosa*. Importantly, CA-AMF required *P. aeruginosa* to possess a functional type III secretion system (T3SS) in order to exert its cytotoxicity, indicating that the T3SS played a crucial role in pathogen recognition by CA-AMF and/or its antimicrobial activity. The T3SS is a highly conserved needle-like secretion apparatus that is absent in commensal bacteria but is present in many important animal and plant Gram-negative bacterial pathogens and is essential for their pathogenesis (Engel, 2003; Stavrinides et al., 2008; Hauser, 2009; Veesenmeyer et al., 2009). We postulate that CA-AMF may represent a new class of human antimicrobial factors in innate immunity, one which has evolved to selectively target pathogenic bacteria amongst the beneficial and commensal microflora.

METHODS

Articular cartilage culture and conditioned media preparation. We have approval from the Rush University Medical Center.
Institutional Review Board (ORA no. 08082803-IRB01) to obtain cartilage samples of human knee and ankle joints from normal asymptomatic human donors through the Gift of Hope Organ & Tissue Donor Network of Illinois within 48 h of the donor’s death. The articular cartilages used in these studies are listed in Table S1, available in JMM Online. Articular cartilage cultures were prepared as described previously (Pascual Garrido et al., 2009). Briefly, using a 4 mm biopsy punch, four full-depth explant pieces from each cartilage were generated and placed in DMEM+ F12 (Gibco, Invitrogen), which is referred to as regular medium (RM), augmented with 10% serum and antibiotics (100 U penicillin ml\(^{-1}\), 100 μg streptomycin ml\(^{-1}\), 0.25 μg fungizone (amphotericin B) ml\(^{-1}\) and 100 μg gentamicin ml\(^{-1}\)). After incubation at 37 °C and 5% CO\(_2\) for 1 day to reach the steady state, cartilage explants were washed three times (twice with pre-warmed 1.0 ml PBS and once with serum and antibiotic-free RM). Cartilage explants were then incubated in 1 ml RM without serum or antibiotics at 37 °C and 5% CO\(_2\) for 24 h prior to the addition of bacteria or cartilage-conditioned medium (CM) collection.

Synovial fluids (SFs) were collected by surgical aspiration with patients’ consent (IRB=08082803-IRB01.a.m.01) using the arthrocentesis procedure. SFs were diluted threefold with PBS to reduce viscosity, prior to bacteria challenge.

**Bacterial strains and media.** All bacterial strains used in these studies and their sources are listed in Table S2. Bacteria were obtained in Luria–Bertani (LB) medium at 37 °C in a non-shaking incubator. Approximately 1 × 10\(^5\) bacteria were added to cartilage explants or the indicated medium and bacterial growth was monitored by serial dilution and the determination of c.f.u. by plating on LB agar, followed by 48 h incubation at 37 °C.

**Construction of expression vectors harbouring pscJ, exoT and exoT(R149K, AAA).** The pscJ gene was amplified from PA103 genomic DNA by PCR using primers pscJ-F1-Sac containing a SacI restriction site (5’-ttttgagctcATGAGGCGAACGGTGAAAGG-3’) and pscJ-R1-XbaI containing an XbaI restriction site (5’-tttttttctagtcT-CAGCCCGTGCGGGTGCC-3’), using a PCR program previously described (Shafikhani, 2002). Primer bases that are shown in lower case type above are not homologous to the chromosomal sequences and contain the engineered restriction sites. The pUCP20 plasmid and PCR product were digested with SacI and XbaI and the PCR product was directionally cloned into the pUCP20 plasmid. The pUCP20:: pscJ was transformed into PA103 pscJ::Tn5 bacteria which were made competent as described by Chuanchuen et al. (2002). pUCP20::exoT and pUCP20::exoT(R149K, AAA) have been described previously described (Garry-Ryan et al., 2004).

**Enzymes and reagents.** We obtained enzymes and reagents from the following sources: β-defensin 2 neutralizing antibody was from Santa Cruz Biotechnology; proteinase K, papain, lipase (human pancreas), DNase, RNase, chondroitinase and heparinase were from Sigma-Aldrich; trypsin was from Gemini Bio-Products; and 2-mercaptoethanol was from Acros Organics.

Time-lapse microscopy was performed as previously described (Shafikhani & Engel, 2006; Shafikhani et al., 2008). Briefly, HeLa cells were seeded in a 24-well dish (1 × 10\(^5\) per well) in RM augmented with 10% FCS and incubated overnight at 37 °C and 5% CO\(_2\). The next day, bacteria were added at a m.o.i. of ca 10. The time-lapse video microscopy was performed 1.5 h after the addition of bacteria, using an Axio Observer Z1 inverted microscope (Zeiss).

**Live/dead viability assays.** Chondrocyte viability within cartilage explants was determined by immunofluorescent (IF) microscopy using the Live/Dead Assay kit (Invitrogen), as previously described (Pascual Garrido et al., 2009). Bacterial viability in the presence or absence of CM was determined by Live/Dead staining using the ViaGram Red+ viability kit (V-7023, Invitrogen), according to the supplier’s protocol. Briefly, bacteria grown in CM or RM were spun at 3000 r.p.m. (1600 g) for 5 min and resuspended in BSA–saline. SYTOX and DAPI were added and bacteria were incubated for 10 min. Bacteria were either directly mounted on a slide or first adhered to a 0.2 μm polycarbonate filter (GTBP01300, Millipore) with a Swinney syringe and then mounted on a slide and imaged by IF microscopy.

**CM-AMF structure analyses.** To determine the size of CA-AMF, conditioned medium from three different cartilages was fractionated by passage through Amicon fractionation columns. As a control, RM was also subjected to fractionation. We collected four fractions, CM-F1 (>30 kDa), CM-F2 (>10 kDa, <30 kDa), CM-F3 (>3 kDa, <10 kDa) and CM-F4 (<3 kDa), and tested them for antimicrobial activity against PA103, as described in the text. The enzymic treatments were performed as follows. Enzymes were added to 1 ml RM or CM at the indicated concentrations in Table 1. Three hours after treatment, samples were heated to 100 °C to inactivate enzymes and cooled to room temperature prior to the addition of bacteria.

**Statistical analyses.** Statistical analyses were performed by one-way analysis of variance, Bartlett’s test for equal variances or Student t-test, using the Prism statistics software. Data are presented as means ± SEM, and P-values less than or equal to 0.05 were taken as significant.

**RESULTS**

**Articular cartilages possess antimicrobial activity against P. aeruginosa**

We used an ex vivo cartilage explant culture model (Pascual Garrido et al., 2009) to study the impact of human cartilages obtained from asymptomatic organ donors (Table S1) on PA103, an important lung P. aeruginosa isolate which is extensively studied due to its high virulence and cytotoxicity (Feltman et al., 2001; Geiser et al., 2001; Lee et al., 2005). Approximately 1 × 10\(^5\) wild-type PA103 bacterial cells were added to 1 ml RM or to 1 ml RM containing four full-depth cartilage explant discs from a cartilage (4 mm diameter) and were incubated at 37 °C in 5% CO\(_2\). Bacterial growth was monitored by serial dilution and plating on LB agar plates at the time of bacterial addition (T0) and 24 h after incubation (T24). As shown in Fig. 1(a), while P. aeruginosa exhibited substantial growth in RM (1.3 × 10\(^4\) ± 3.8 × 10\(^3\) c.f.u. ml\(^{-1}\), n=10), growth of PA103 was inhibited by at least five-log orders in the presence of cartilages (1.4 × 10\(^4\) ± 1.2 × 10\(^5\) c.f.u. ml\(^{-1}\), n=7, different cartilages, P<0.0001). In fact, no bacteria could be detected at T24 in 70% of cartilage-containing media tested. These results indicated that the cartilages possessed a factor(s) with potent anti-P. aeruginosa activity, CA-AMF. Cartilage tissue viability was assessed at T0 and T24 with a Live/Dead assay, as described previously (Pascual Garrido et al., 2009), to ensure that chondrocytes within tissue explants remained viable throughout these experiments (Fig. S1).

To determine whether CA-AMF was secreted or if it was associated within cartilages, CM supernatants (the
supernatant discs from RM in which four full-depth cartilage explant discs were grown for 24 h) were collected from various cartilages and tested for their antimicrobial activity against PA103. CM supernatants also exerted potent antimicrobial activity against P. aeruginosa, reducing the bacterial number by at least 4 log orders (2.1 × 10^4 ± 4.2 × 10^4 c.f.u. ml⁻¹, n=17, different cartilages, P<0.0001), indicating that CA-AMF was secreted into the medium (Fig. 1a). Normal SFs, collected from asymptomatic patients, also exhibited substantial anti-Pseudomonas activity (2.5 × 10^4 ± 4.4 × 10^4 c.f.u. ml⁻¹, n=4, P<0.001).

### CA-AMF is bactericidal in nature

CA-AMF in CM could be bacteriocidal, causing bacteria to lyse, or it could convert metabolically active bacteria into a viable but non-culturable state, as is the case for many environmental bacteria (Jones et al., 1991; Oliver, 1995). To distinguish between these possibilities, we added 1 × 10^7 bacterial cells to CM or RM media and assessed the impact of CA-AMF exposure on bacterial viability after 24 h (Fig. 1b) using a Live/Dead viability assay, imaged by IF microscopy. While there were numerous live bacteria in RM, very few bacterial cells could be detected after 24 h incubation in CM (Fig. 1b). Growth in CM for even 10 min reduced the total number of PA103 bacteria per field of view by 57 ± 6% and increased the percentage of dead bacteria by 43% compared with growth in RM (Fig. S2, n=8, P<0.01). These data indicated that CA-AMF is bactericidal in nature.

### CA-AMF is a small and tightly structured glycolipid or glycopeptide

Human chondrocytes in cartilages have been shown to express human β-defensin 2 (hBD-2), an antimicrobial peptide with activity against Gram-negative bacteria, including P. aeruginosa (Harder et al., 1997; Varoga et al., 2004). hBD-2 is a small peptide with three disulfide bonds which are required for its activity (De Smet & Contreras, 2005). Treatment with the reducing agent β-mercaptoethanol or addition of neutralizing antibody against hBD-2, both of which have been shown to protect against hBD-2 microbicidal activity (De Smet & Contreras, 2005; Nakatsuji et al., 2010), did not protect PA103 from the antimicrobial activity contained in CM (Table 1), indicating that CA-AMF is not hBD-2. Moreover, treatments with heat (100 °C for 1 h) or cold (−80 °C overnight) did not suppress the antimicrobial activity in CM, indicating that CA-AMF is highly stable (Table 1).

Hog pancreatin has many different enzymic activities (Marchis-Mouren et al., 1960; Keller & Allan, 1967; Klüh, 1981). Treatment of CM with hog pancreatin for 3 h followed by 1 h heat inactivation at 100 °C, while having no adverse effect on bacterial growth in RM, abrogated CA-AMF antimicrobial activity in a dose-dependent manner (Fig. 2a). To gain further insight into the structure of CA-AMF, we treated CM with various enzymes known to be present in hog pancreatin, including protease, DNase, RNase, lipase and amylase (Marchis-Mouren et al., 1960; Keller & Allan, 1967; Klüh, 1981). These treatments included three different proteases (protease K, trypsin or papain), two different lipases (human pancreas lipase or porcine pancreas lipase type II) and DNase1, RNase1 or z amylase. Enzyme treatments were for 3 h at 37 °C followed by heating at 100 °C for 1 h to inactivate the enzymes. While having no adverse effect on bacterial growth in RM, treatments with proteases, lipases, DNase1 and RNase1 did not block the antimicrobial activity of CA-AMF on PA103 in CM (Table 1). It is important to note that although CA-AMF is resistant to various proteases, it may still possess a peptide moiety, as a number of small but tightly structured

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**Table 1. Structural analyses of CA-AMF**

One millilitre of medium, from at least three different cartilages, was subjected to the indicated conditions or treated with the indicated enzymes. Enzyme treatment was for 3 h followed by heat inactivation of the enzyme at 100 °C for 1 h. After cooling to room temperature, samples were tested for their antimicrobial activity against PA103, as described in the text.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration</th>
<th>CA-AMF activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-defensin 2 (neutralizing Ab)</td>
<td>4 or 40 µg ml⁻¹</td>
<td>Active</td>
</tr>
<tr>
<td>2-Mercaptoethanol</td>
<td>1 or 2 µM</td>
<td>Active</td>
</tr>
<tr>
<td>Heat</td>
<td>100 °C for 1 h</td>
<td>Active</td>
</tr>
<tr>
<td>Cold</td>
<td>−80 °C for 24 h</td>
<td>Active</td>
</tr>
<tr>
<td>Proteinase K</td>
<td>100 or 200 µg ml⁻¹</td>
<td>Active</td>
</tr>
<tr>
<td>Papain</td>
<td>100 or 500 µg ml⁻¹</td>
<td>Active</td>
</tr>
<tr>
<td>Trypsin</td>
<td>0.05 %</td>
<td>Active</td>
</tr>
<tr>
<td>Lipase (human pancreas)</td>
<td>10 units ml⁻¹</td>
<td>Active</td>
</tr>
<tr>
<td>Lipase type II (porcine)</td>
<td>100 µg ml⁻¹</td>
<td>Active</td>
</tr>
<tr>
<td>DNase</td>
<td>1.8 µg ml⁻¹</td>
<td>Active</td>
</tr>
<tr>
<td>RNase</td>
<td>100 µg ml⁻¹</td>
<td>Active</td>
</tr>
<tr>
<td>Chondroitinase</td>
<td>2.5 units ml⁻¹</td>
<td>Active</td>
</tr>
<tr>
<td>Heparitinase</td>
<td>2.5 units ml⁻¹</td>
<td>Active</td>
</tr>
</tbody>
</table>

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peptides have been previously shown to be highly resistant to proteolysis (Timpl et al., 1983; Selvaggini et al., 1993; Beck et al., 2009). Interestingly, amylase was able to suppress the antimicrobial activity in CM in a dose-dependent manner (Fig. 2b).

**Fig. 1.** Articular cartilages possess antimicrobial activity against *P. aeruginosa*. (a) Approximately $1 \times 10^7$ wild-type PA103 bacterial cells were added to RM ($n=17$) and RM + cartilage ($n=7$, different cartilages) or CM ($n=17$, different cartilages). Bacterial growth was determined 24 h after bacterial addition to the indicated media and the results were plotted as means ± SEM. Note that both cartilages and CM potently inhibit PA103 growth, while PA103 exhibits substantial growth in RM ($^* P<0.0001$). (b) Approximately $1 \times 10^7$ wild-type PA103 bacterial cells were added to RM or CM. Bacterial viability was assessed by IF microscopy 24 h after exposure, using a Live/Dead viability kit. (Red denotes dead bacteria while green represents live bacteria.) Note that exposure to CM results in a significant decline in the total number of bacteria in the field of view, indicating that CA-AMF is bactericidal in nature. Bars, 20 μm.

**Fig. 2.** Structural analyses of the antimicrobial activity (CA-AMF) in CM. CM was treated with pancreatin (mg ml$^{-1}$) (a) or amylase (b) at the indicated concentrations for 3 h, followed by enzyme inactivation by heating at 100 °C for 1 h. Wild-type PA103 bacterial cells ($1 \times 10^7$) were added to CM, pancreatin- or amylase-treated CM medium after cooling. Bacterial growth was determined as described in Fig. 1 and the data from three independent experiments were plotted as means ± SEM ($n=CM$ from three different cartilages, $P<0.001$). Note that both pancreatin and amylase abrogate CA-AMF activity in a dose-dependent manner. (c) CM was fractionated by passage through Amicon fractionation columns. Four fractions, F1 (>30 kDa), F2 (>10 kDa, <30 kDa), F3 (>3 kDa, <10 kDa) and F4 (<3 kDa), were collected and examined for antimicrobial activity against PA103 as described in Fig. 1. Data are from at least four independent experiments and error bars represent SEM. Note that the antimicrobial activity in CM is primarily in F4 and to a lesser extent in F3.
The matrix of articular cartilage consists of many glycosylated components (Heinegard, 2009). For example, hyaluronic acid forms a coating around chondrocytes in articular cartilage and, together with the most-abundant proteoglycan aggrecan, is responsible for the uptake and retention of water. The two major glycan components of aggrecan are chondroitin sulfate and heparan sulfate. Treatment with chondroitinase or heparitinase had no impact on the antimicrobial activity of CA-AMF in CM (Table 1), ruling out chondroitin sulfate or heparan sulfate as the antimicrobial agent in CM and the possibility that contaminating chondroitinase or heparitinase in the amylose treatment might have been responsible for abrogating the antimicrobial activity of CA-AMF in CM.

To hone in on the size of CA-AMF, we fractionated CM by passage through Amicon fractionation columns, as described in Methods. We collected four fractions, CM-F1 (>30 kDa), CM-F2 (>10 kDa, <30 kDa), CM-F3 (<3 kDa, <10 kDa) and CM-F4 (<3 kDa), and tested them for antimicrobial activity against PA103, as described in the text. The data indicated that CA-AMF microbicidal activity was primarily in fraction 4 (F4), which had compounds less than 3 kDa, although fraction 3 (F3; >3 kDa, <10 kDa) also exhibited some activity against PA103 (Fig. 2c). As expected, pancreatin and amylase treatments abrogated the antimicrobial activity in F4 (data not shown). Collectively, these data suggested that CA-AMF may be a small complex sugar or a tightly structured protease-resistant glycopeptide or a lipase-resistant glycolipid.

**CA-AMF antimicrobial activity requires a functional T3SS**

While studying CA-AMF antimicrobial activity on various mutant forms of PA103 (Table S2), we made a surprising observation. We found that PA103 pscJ::Tn5 (pscJ), the isogenic mutant strain which lacks the T3SS apparatus, was significantly more resistant to CA-AMF killing compared with wild-type PA103 (Fig. 3a). Complementing the pscJ mutant with the pscJ gene, expressed on the pUCP20 plasmid (pPscJ), rendered the pscJ+pPscJ strain sensitive to CA-AMF-mediated killing, indicating that the T3SS was required for CA-AMF to exert its cytotoxicity. Using a Live/Dead viability assay, we further confirmed the importance of the T3SS apparatus in CA-AMF-mediated *Pseudomonas* killing (Fig. 3b, compare with Fig. 1b).

PA103 has two T3SS effectors, ExoT and ExoU (Feltman et al., 2001). Interestingly, the effectorless mutant (PA103 AUΔT) was also significantly more resistant to CA-AMF but not the exoU-deleted (AU) or the exoT-deleted (AT) mutant strains (Fig. 3a). ExoU and ExoT are potent cytotoxins that are inactive inside the bacterium but become activated by host factor 14-3-3 and superoxide dismutase, respectively, upon entry into the host’s cytoplasm (Sato et al., 2006; Maresso et al., 2007). These data suggested that either the ExoU or the ExoT activity was required to mediate CA-AMF killing of the bacterium from inside the bacterium, or
Alternatively, in the absence of effector proteins passing through this apparatus, the T3SS may be inaccessible to the CA-AMF entry into the bacterium. To distinguish between these possibilities, we complemented PA103 ΔUAT with a pUCP20 expression vector harbouring either the wild-type ExoT or its inactive form ExoT(R149K, AAA) that has been shown to be secreted through the T3SS (Garrity-Ryan et al., 2004). PA103 ΔUAT + pExoT and PA103 ΔUAT + pExoT (R149K, AAA) strains were both highly sensitive to CA-AMF antimicrobial activity in the CM (Fig. 3a), indicating that CA-AMF killing does not depend on ExoT or ExoU activities; rather the T3SS may be inaccessible to CA-AMF in the absence of substrate passing through it. It is worth noting that the growth of the T3SS mutant strains was reduced by 2 log orders in CM (9.3 \times 10^6 \pm 4.5 \times 10^6 \text{ ml}^{-1}) compared with growth in the control medium (1.2 \times 10^7 \pm 9.8 \times 10^6 \text{ ml}^{-1}), suggesting that the enhanced resistance of the T3SS mutant strains to the antimicrobial activity in CM may be due to reduced permeability to CA-AMF, which supports the notion that the T3SS may act as a conduit through which CA-AMF may gain access inside the bacterium.

PAK, a flagellated strain of \textit{P. aeruginosa}, which expresses ExoS, ExoT and ExoY effector toxins (Feltman et al., 2001), was also highly sensitive to CA-AMF antimicrobial activity, while its T3SS isogenic mutant (PAK \textit{pscJ}: Tn5) exhibited substantial growth in CM (Fig. 3a), indicating that T3SS-specific CA-AMF antimicrobial activity was not limited to the PA103 strain. CA-AMF was also highly effective in killing other T3SS-possessing bacteria including \textit{Salmonella enterica} serovar \textit{typhimurium}, enterohaemorrhagic \textit{Escherichia coli} (EHEC) and enteropathogenic \textit{E. coli} (EPEC) (Fig. S3), some of which are known to have more than one T3SS (Galán & Wolf-Watz, 2006; Bulgin et al., 2009; Hauser, 2009).

Unlike \textit{P. aeruginosa} whose T3SS is encoded by genes located on its chromosome, \textit{Versinia pseudotuberculosis} possesses its T3SS genes on a virulence plasmid (pYV) for the delivery of the well-studied T3SS-secreted Yop effectors (Matsumoto & Young, 2009). As expected, the T3SS mutant \textit{Y. pseudotuberculosis} strain PAN100 was able to grow substantially in CM (Fig. 4a). Interestingly, the wild-type \textit{Y. pseudotuberculosis} strain PAN177 also exhibited substantial growth in CM after 24 h (Fig. 4a). Since bacteria frequently lose their non-essential plasmids to reduce the metabolic burden (Sobeczyk et al., 1992), we reasoned that CA-AMF may have selected for \textit{Y. pseudotuberculosis} bacteria that have lost their pYV plasmid and are therefore T3SS mutant. To determine the status of the T3SS activity in bacteria colonies emerging from the PAN177 wild-type culture following growth in CM or RM after 24 h, we performed a functional assay in which the isolated colonies were tested for their ability to induce the T3SS and YopE-dependent disruption of the actin cytoskeleton and cell rounding in HeLa cells (Andor et al., 2001). As expected, and in line with previous reports (Black & Bliska, 2000; Andor et al., 2001; Viboud & Bliska, 2001; Viboud et al., 2006), prior to growth in CM or CM, wild-type PAN177 induced cell rounding in >90% of HeLa cells within 2 h, while the T3SS mutant PAN100 failed to cause cell rounding (Fig. 4b, upper and lower panels, and video clips S1 and S3). Consistent with our hypothesis, all ten resistant colonies emerging from the wild-type culture in CM failed to induce cell rounding in HeLa cells and exhibited a phenotype identical to that of the T3SS mutant strain PAN100, suggesting that CA-AMF selected for T3SS mutant bacteria that probably lost their pYV plasmid (Fig. 4b, middle panel and video clip S2). In contrast, only one isolate out of ten from the culture of wild-type PAN177 grown in RM failed to induce cell rounding in HeLa cells, indicating that after 24 h of growth in RM, the frequency of plasmid loss is ~10%. Combined, these data indicated that T3SS plays a crucial part in CA-AMF bacteria recognition and/or its microbicidal activity.

### DISCUSSION

Uncompromised articular joints are rarely infected with bacterial pathogens, including \textit{P. aeruginosa}, prompting us to hypothesize that cartilages possess antimicrobial activity that limits \textit{P. aeruginosa} survival in these environments. The data in this report confirm our hypothesis and demonstrate that cartilages secrete a novel antimicrobial factor(s), CA-AMF, with potent bactericidal activity against this pathogen and others (Figs 1 and S3). Although, CA-AMF appears to be a single small molecule of less than 3 kDa (Fig. 2c), we cannot rule out the possibility that it may be a complex structure, composed of several small subunits, forming in the presence of a target bacterium. Some of the more remarkable features of CA-AMF include its extreme resistance to heat, cold and reducing agents, as well as its ability to maintain full activity in the presence of proteases, lipases, RNase, DNase, heparitinase and chondroitinase (Table 1).

Importantly, CA-AMF was far more active against \textit{P. aeruginosa} strains with a functional T3SS (Fig. 3), indicating that T3SS is required for CA-AMF activity. In line with this interpretation, CA-AMF was highly effective against \textit{Salmonella}, EHEC and EPEC, which also possess T3SS and exerted a strong selection against the T3SS in \textit{Y. pseudotuberculosis} (Fig. 4. and video clips S1–S3). The involvement of the T3SS in CA-AMF antimicrobial activity raises an intriguing possibility that the T3SS may be recognized as a pathogen-specific molecular target by innate immunity. Because the T3SS is highly conserved and since it is primarily found in pathogenic bacteria, T3SS would be an ideal target for innate immunity. This notion is supported by the findings that \textit{P. aeruginosa} lung isolates obtained from chronically infected cystic fibrosis patients are predominantly T3SS mutants (Wu et al., 2004; Li et al., 2005; Yahr & Wolfgang, 2006), indicating that T3SS may also be selected against in the lung environment as well. Moreover, Franchi et al. (2012) recently reported that intestinal phagocytes produce massive amounts of pro-inflammatory cytokines only in response to pathogenic \textit{Salmonella}, but not when...
they encounter commensal bacteria. Interestingly, this phenomenon was also dependent on the presence of a functional T3SS in this pathogen, further supporting the notion that the T3SS may be recognized as a pathogen-specific molecular target by innate immunity, albeit its recognition leads to a different response. More studies are needed to determine whether the T3SS is required for CA-AMF bacterial recognition/binding, or alternatively, if it acts as a conduit through which CA-AMF gains entry inside the bacterium by retrograde transport.

While the market potential for new antibacterial drugs is estimated to be in the billions of dollars, antibacterial drug discovery and development has slowed considerably (Devasahayam et al., 2010). Currently, there are no antibiotics on the market with the specificity to distinguish and target bacteria based on their virulence and pathogenicity. Consequently, antibiotic treatment often has dramatic and deleterious effects on normal microflora and can lead to the development and spread of resistance among bacteria and enhanced vulnerability to new infections (Wlodarska &
Finlay, 2010). The evolutionary design of CA-AMF in innate immunity is extremely clever in that maintaining the T3SS virulence apparatus results in the pathogen’s demise. On the other hand, resistance to this antimicrobial agent most likely requires inactivation of this apparatus, which would result in the loss of virulence and domestication of the pathogen. Identification of CA-AMF will likely advance our knowledge of innate immunity. It will enhance our understanding of the T3SS structure, assembly and function. It could also have a significant impact on antimicrobial therapy and the treatment of infectious diseases, through the development of a new class of antibiotics with specificity to pathogenic bacteria.

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REFERENCES


