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## Porphyromonas gingivalis, the beast with two heads

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# Chapter 6

## Conserved Citrullinating Exoenzymes in *Porphyromonas* Species

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## Abstract

*Porphyromonas gingivalis* is one of the major oral pathogens implicated in the widespread inflammatory disorder periodontitis. Moreover, in recent years, *P. gingivalis* has been associated with the autoimmune disease rheumatoid arthritis. The peptidylarginine deiminase enzyme of *P. gingivalis* (PPAD) is a major virulence factor that catalyzes the citrullination of both bacterial and host proteins, potentially contributing to production of anti-citrullinated protein antibodies. Considering that these antibodies are very specific for rheumatoid arthritis, PPAD appears to be a link between *P. gingivalis*, periodontitis and the autoimmune disorder rheumatoid arthritis. PPAD was thus far considered unique among prokaryotes, with *P. gingivalis* being the only bacterium known to produce and secrete it. To challenge this hypothesis, we investigated the possible secretion of PPAD by eleven previously collected *Porphyromonas* isolates from a dog, two sheep, three cats, four monkeys, and a jaguar with periodontitis. Our analyses uncovered the presence of secreted PPAD homologues in eight isolates that were identified as *Porphyromonas gulae* (from a dog, monkeys and cats) and *Porphyromonas loveana* (from sheep). In all three PPAD-producing *Porphyromonas* species, the dominant form of the secreted PPAD was associated with outer membrane vesicles, while a minor fraction was soluble. Our results prove for the first time that the citrullinating PPAD exo-enzyme is not unique to only one prokaryotic species. Instead, we show that PPAD is produced by at least two other oral pathogens.

## Introduction

*Porphyromonas gingivalis* is a renowned oral pathogen<sup>1</sup>. This Gram-negative bacterium has been implicated in periodontitis<sup>2-4</sup>, a widespread disease of the oral cavity and one of the foremost causes of tooth loss worldwide<sup>1, 3</sup>. Notably, periodontitis is a polymicrobial disease in origin resulting from dysbiotic events, or perturbations of the oral microbiota<sup>5, 6</sup>. Nonetheless, *P. gingivalis* constitutes less than 0.01% of the oral microbiota<sup>7</sup>. In recent years, a different kind of interest has befallen this bacterium as the discovery of its citrullinating enzyme gave insights on the role that *P. gingivalis* seems to play in the etiology of rheumatoid arthritis (RA)<sup>2, 8-10</sup>. This autoimmune disease has been associated with periodontitis<sup>11-14</sup>.

Recent models suggest that RA is related to a loss of tolerance towards citrullinated proteins<sup>8-10</sup>. In this context it is noteworthy that a citrullinating enzyme has been discovered in *P. gingivalis*<sup>15, 16</sup>. This enzyme, a peptidylarginine deiminase (PAD), converts arginine residues into citrulline residues, which may cause drastic changes in the conformation and immunogenicity of the citrullinated protein<sup>15</sup>. PAD enzymes are commonly present and highly conserved among mammals. In contrast, the PAD of *P. gingivalis* (in short PPAD), was thus far the only known prokaryotic enzyme of this type<sup>15, 17</sup>. Of note, the production of PPAD is a strictly conserved feature of *P. gingivalis*<sup>18</sup>, where it is exported to the outer membrane and secreted into the host milieu. The secreted PPAD exists either in association with outer membrane vesicles (OMVs) or in a soluble state<sup>19, 20</sup>. These OMVs are nanostructures resulting from specific blebbing processes of the outer membrane and contain cargo of proteins implicated in virulence<sup>19, 21, 22</sup>. The outer membrane and OMV association of PPAD is probably facilitated through a modification with lipopolysaccharide A (A-LPS)<sup>20, 23</sup>.

*P. gingivalis* is known to share several traits with other species belonging to the genus *Porphyromonas*, each associated with periodontitis mainly in their own non-human host<sup>24</sup>. Despite its high host-specificity, there have been a few recorded cases of *P. gingivalis* isolated from animal hosts, especially beagle dogs<sup>25, 26</sup>. Other *Porphyromonas* species in livestock and pets have barely been studied so far, and the possible expression of PPAD by any of these species aside from *P. gingivalis* has remained unnoticed. However, considering the fact that PPAD is implicated in severe periodontitis and RA, it would be important to know whether this virulence factor

is conserved across *Porphyromonas* species and, if so, whether it has a similar subcellular localization. The presence of PPAD homologues in such species would, in fact, provide an opportunity to employ a wider array of animal models for studies on the role of this enzyme in RA in particular, and the systemic effects of oral diseases in general. Although RA has been studied in animal models, these investigations did not address the possible correlation with periodontitis<sup>27-32</sup>. Additionally, the identification of an expanded panel of PPAD-producing *Porphyromonas* species could help to shed more light on the proposed cooperativity between PPAD and the group of proteolytic enzymes generally referred to as gingipains. The objective of the present study was therefore to assess the presence of PPAD homologues in *Porphyromonas* species isolated from different animals with periodontitis.

## **Materials and methods**

### *Bacterial strains and culture conditions*

A total of 11 *Porphyromonas* species (Table S1) were previously isolated from diverse animal hosts (dog, cats, monkeys, sheep and jaguar) with periodontitis hailing from different countries<sup>33, 34</sup>. These isolates were included in the present analyses based on their availability. The *P. gingivalis* type strains W83 and ATCC 33277 were used as controls, together with their respective PPAD deletion mutants (W83  $\Delta$ PAD-A, ATCC 33277 3 $\Delta$ PAD-B)<sup>35</sup>. The *P. gingivalis* clinical isolate 20664 was collected in Groningen, the Netherlands, from a patient with severe periodontitis. Each strain was cultivated anaerobically for four days in Brain Heart Infusion (BHI) broth as previously described<sup>36</sup>.

### *LDS-PAGE and Western blotting*

Separation of proteins by lithium dodecyl sulphate (LDS) polyacrylamide gel electrophoresis (PAGE), Western blotting and immunodetection of PPAD with specific antibodies were performed as detailed in the Appendix.

### *Whole genome sequencing and phylogenetic analysis*

Total bacterial DNA was extracted and sequenced using a Miseq (Illumina) as detailed in the Appendix. The sequence reads were

submitted to the European Nucleotide Archive under the following project: PRJEB21305 with following accession numbers: ERS1790728 (Jaguar-1); ERS1790727 (I-433); ERS1790726 (I-372); ERS1790725 (G251); ERS1790724 (Chien5B); ERS1790723 (Chat2); ERS1790722 (3492); ERS1790721 (19X2-K1); ERS1790720 (157); ERS1827527 (TT1); ERS1825597 (TG1). Phylogenetic analyses were performed as detailed in the Appendix.

### *Outer membrane vesicles collection*

To collect OMVs, 2 mL fractions of bacterial cultures were centrifuged for 20 min at 16100 x *g* and 4°C in order to separate bacterial cells from the growth medium. Subsequently, 500 µL of the resulting supernatant was ultracentrifuged for two hours at 213000 x *g* and 4°C using an Optima MAX-XP ultracentrifuge (Beckman Coulter, Brea, CA, USA). Afterward, the ultracentrifugation pellet, enriched in OMVs, was resuspended in 500 µL of OMV buffer (5 mM MgCl<sub>2</sub> in PBS). Proteins in the supernatant and resuspended pellet fractions obtained upon ultracentrifugation were TCA-precipitated prior to analysis by LDS-PAGE and Western blotting, as described above.

### *Aqueous two-phase system protein purification*

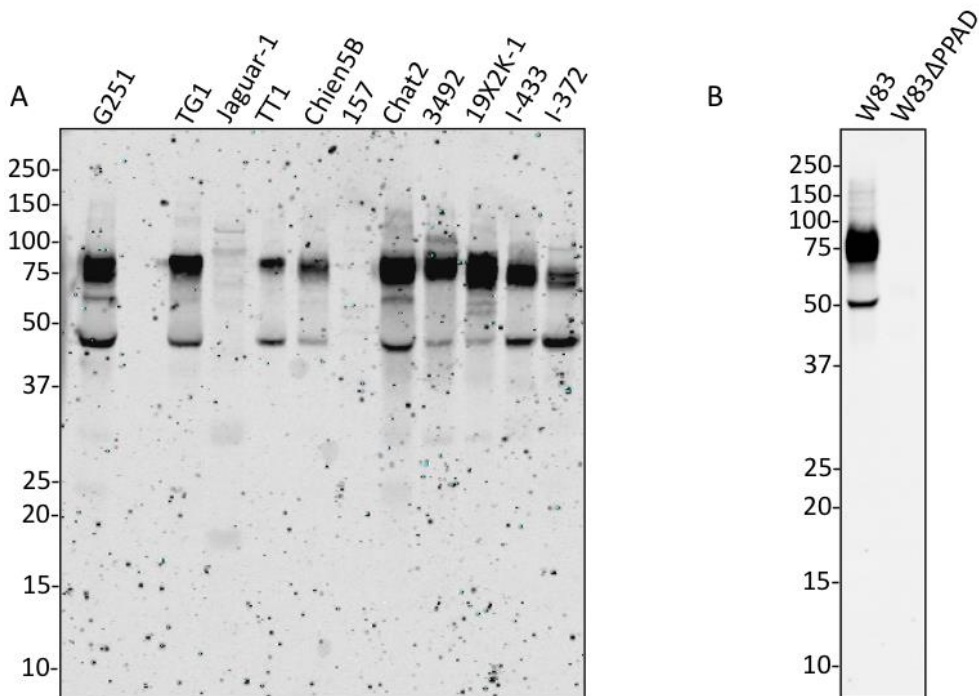
To explore the possible association of PPAD-like proteins with OMVs via a LPS modification, a protein phase separation was performed using the non-ionic detergent Triton X-114 (Sigma-Aldrich Co.) essentially as previously described<sup>37</sup>. For experimental details, see Appendix.

## **Results**

### *Detection of PPAD in Porphyromonas species from non-human hosts*

To investigate the occurrence of PPAD homologous enzymes beyond the *P. gingivalis* species boundaries, we screened an available previously established *Porphyromonas* collection<sup>33, 34</sup> including isolates from a dog (n=1), cats (n=3), sheep (n=2), monkeys (n=4) and a jaguar (n=1; Table S1). This search was initiated by Western blotting with PPAD-specific antibodies, and the results were subsequently verified by genome sequencing. Since substantial

amounts of PPAD of *P. gingivalis* are secreted, we focused our search on the secreted protein fraction of the cultured bacteria. Remarkably, Western blotting showed bands of proteins cross-reacting with the PPAD antibody for 9 of 11 isolates. Only the cat isolate 157 and the Jaguar-1 isolate produced no clearly cross-reactive bands, as was the case for the engineered PPAD deletion mutant of the *P. gingivalis* type strain W83 (Fig. 1).



**Figure 1.** Detection of a PPAD-like protein in *Porphyromonas* isolates from non-human hosts. *Porphyromonas* species isolates from non-human hosts were cultured for four days in BHI medium. Subsequently, bacterial cells were separated from the growth medium, and growth medium fractions were used for immunoblotting with PPAD-specific antibodies. (A) *Porphyromonas* species clinical isolates. (B) *P. gingivalis* reference strain W83 and the isogenic PPAD deletion mutant. Molecular weights of marker proteins are indicated.

The patterns of bands strongly cross-reacting with the PPAD antibody comprised a thick band of ~75-85-kDa and a sharper one of ~47-kDa, with the possible appearance of one or two faint bands of ~60-kDa. In the case of the monkey isolate I-372, the ~75-85-kDa band was separated into two closely migrating sharper bands. The latter finding suggests that the broad ~75-85-kDa band of the other isolates, including *P. gingivalis* W83, may actually be composed of

multiple closely migrating bands. Importantly, the observed banding patterns of secreted proteins of *Porphyromonas* isolates from non-human hosts closely resembled the pattern observed for the *P. gingivalis* type strain W83 (Fig. 1B). This implies that the proteins identified in this way are homologous to PPAD, or at least structurally related to PPAD. In fact, the observed PPAD banding pattern of *P. gingivalis* W83 and our *Porphyromonas* isolates from non-human hosts is consistent with literature data, where it was proposed that the ~75-85-kDa band represents the outer membrane-bound form of PPAD, and the ~47-kDa band represents the secreted soluble form<sup>15</sup>. The bands of ~60-kDa observed in samples of *P. gingivalis* W83, the monkey isolates G251 and 19X2K-1, and the cat isolate Chat2 could potentially correspond to unprocessed forms of PPAD.

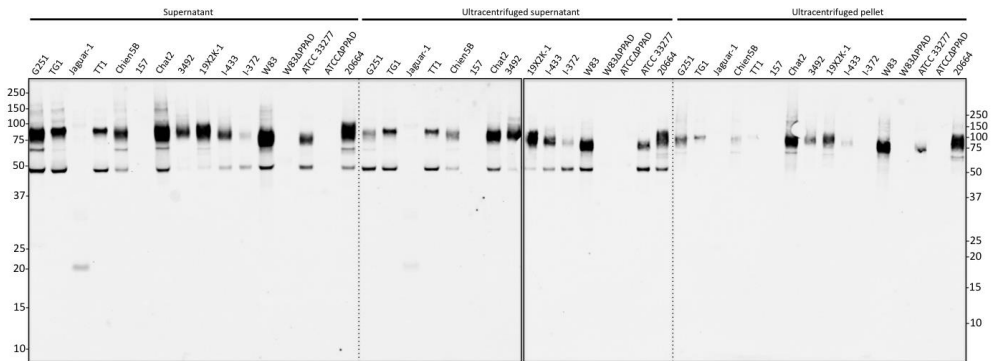
#### *Soluble and OMV-associated forms of potential PPAD homologues*

The similar banding patterns of PPAD from *P. gingivalis* W83 and the potential PPAD homologues prompted us to compare the localization of these proteins. Of note, PPAD is attached to the outer membrane of *P. gingivalis* and secreted OMVs, most likely by modification with A-LPS<sup>21, 22, 38</sup>. Accordingly, we assessed the possible OMV association of secreted proteins cross-reacting with the PPAD antibody by ultracentrifugation of supernatant fractions of *P. gingivalis* samples and the *Porphyromonas* isolates from non-human hosts. The resulting OMV-containing pellet and OMV-depleted supernatant fractions were subsequently analyzed *via* Western blotting with the anti-PPAD antibody (Fig. 2). Indeed, the soluble ~47-kDa PPAD species of the *P. gingivalis* strains W83, ATCC 33277 and 20664 fractionated exclusively with the supernatant fraction, as was the case for the ~47-kDa species of cross-reacting proteins in the *Porphyromonas* isolates from non-human hosts. In contrast, substantial amounts of the ~75-85-kDa PPAD species and similarly sized cross-reacting proteins were detectable in the pellet fraction upon ultracentrifugation. The latter is consistent with an association with large pelletable OMVs.

Importantly, interactions of proteins with OMVs can be validated using a temperature-dependent phase separation assay based on the detergent Triton X-114 where, at 37°C, LPS-modified hydrophobic proteins will fractionate with the detergent-rich phase, while hydrophilic proteins lacking lipid modifications fractionate with the



aqueous phase. We therefore applied this assay to *Porphyromonas* isolates from a dog (Chien5B), a cat (Chat2), and a monkey (G251). As a positive control, we used the *P. gingivalis* isolate 20664, which produces relatively large amounts of PPAD (Fig. 2). As shown by subsequent Western blotting, the ~75-85-kDa PPAD species localized prevalently to the detergent fraction (Figs. 3A, 3B), consistent with the proposed A-LPS attachment. Conversely, the ~47-kDa PPAD species appeared only in the lipid-free aqueous phase, as it would be expected of the secreted soluble form of PPAD (Fig. 3). Of note, the phase separation of the different protein species is not complete due to inevitable cross-contaminations between the aqueous and detergent phases.

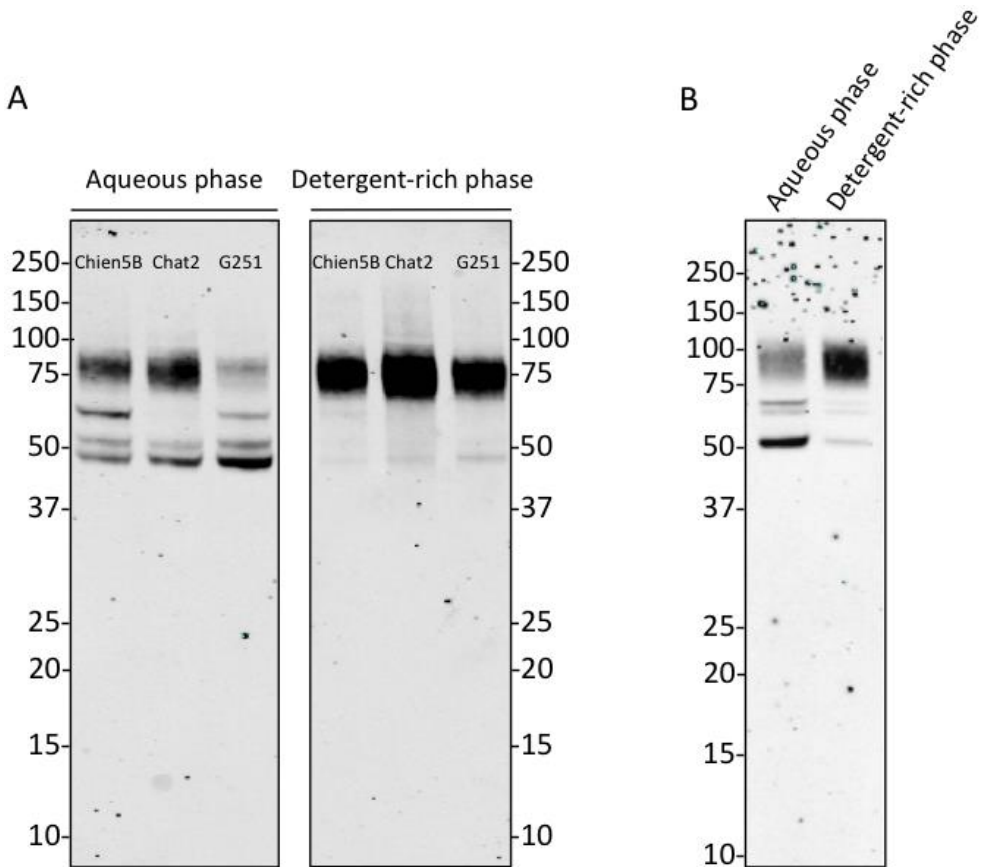


**Figure 2.** Association of PPAD-like proteins from non-human hosts with OMVs. Growth medium fractions (designated ‘supernatant’) of *Porphyromonas* species isolates were subject to ultracentrifugation. Subsequently, the supernatant and pellet fractions were analyzed by immunoblotting as indicated for Figure 1. Samples relating to the *Porphyromonas* species isolates from non-human hosts, the reference strains W83 and ATCC 33277 and their respective PPAD<sup>-/-</sup> mutants, and a *P. gingivalis* clinical isolate are listed. Molecular weights of marker proteins are indicated. Note that the W83 and respective PPAD mutant samples are identical to those used for Figure 1.

### Comparison of PPAD and its homologues in *Porphyromonas* species from non-human hosts

To identify genes encoding the potential PPAD homologues detected by Western blotting, we sequenced the different investigated *Porphyromonas* isolates from non-human hosts. This analysis identified homologous PPAD genes in the nine *Porphyromonas* isolates secreting proteins that cross-reacted with the PPAD antibody (Figs. 1-3), and it confirmed the absence of genes with similarity to

the PPAD gene from the cat isolate 157 and the Jaguar-1 isolate. All identified PPAD homologues contained the catalytic residues Asp130, His236, Asp238, Asn297 and Cys351, as well as the Arg152 and Arg154 residues allegedly involved in substrate specificity<sup>16</sup>. This implies that all identified PPAD homologues have a potential citrullinating activity. The highest overall amino acid sequence similarity to the PPAD reference protein from *P. gingivalis* W83 was observed for the homologous protein of the 19X2-K1 isolate (Fig. S1). Further, all PPAD proteins of the different *Porphyromonas* isolates from non-human hosts, including 19X2-K1, showed the amino acid substitutions G231N, E232T and N235D.



**Figure 3.** Detergent extraction of the ~75-85-kDa PPAD-like protein species. *Porphyromonas* species isolates were cultured and, subsequently, separated from the growth medium by centrifugation. Next, an aqueous two-phase system protein purification was performed, dividing each sample in an aqueous (lipid-free proteins) and a detergent-rich (lipid-bound proteins) phase. The resulting fractions of (A) three *Porphyromonas* isolates from non-human hosts and (B) the *P.*

*gingivalis* clinical isolate 20664 were analyzed by immunoblotting as indicated for Figure 1. Molecular weights of marker proteins are indicated.

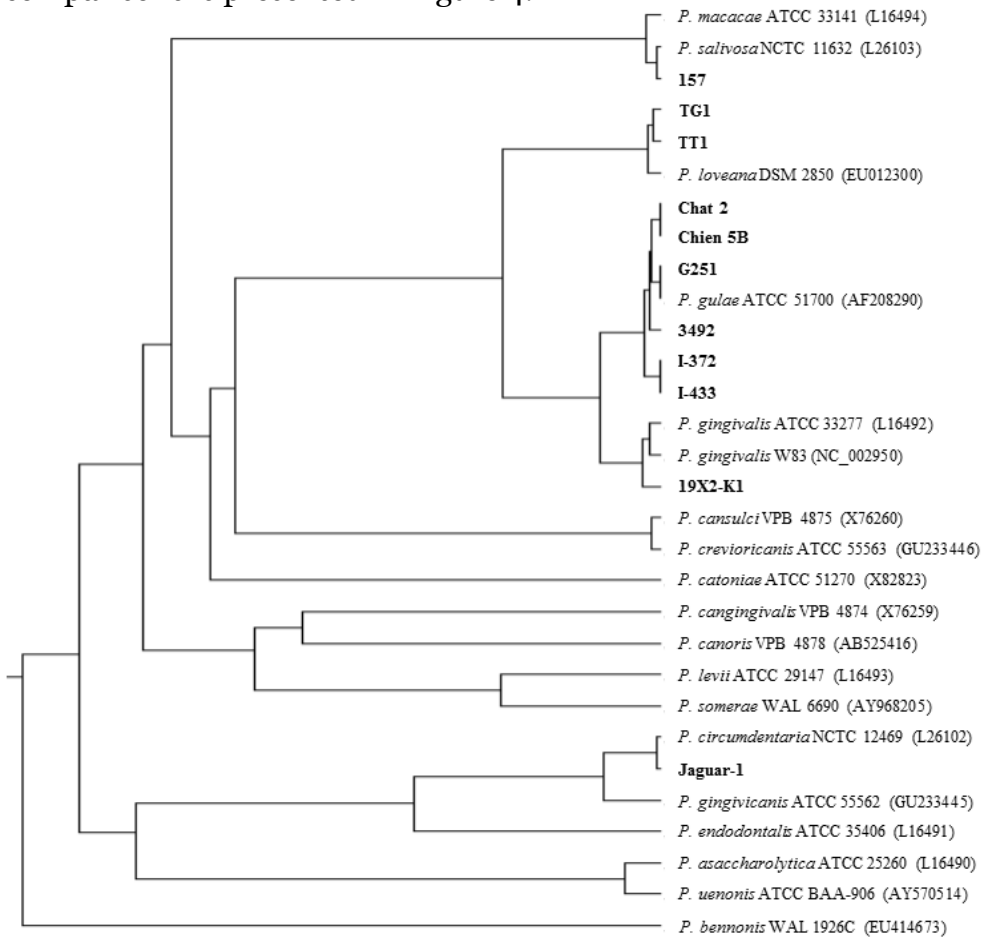
### *Presence of secreted gingipains in Porphyromonas species from non-human hosts*

To evaluate the possible secretion of the gingipains RgpA, RgpB and Kgp by *Porphyromonas* species from non-human hosts, we performed a Western blotting analysis with polyclonal rabbit antibodies that were raised either against the catalytic domain of RgpA or against Kgp. Of note, the catalytic domains of RgpA and RgpB are highly similar and, therefore, the antibodies raised against RgpA also bind to RgpB. As shown in Figure S2, this Western blotting analysis allowed the detection of RgpA/B and Kgp in the growth medium of most investigated isolates. In contrast, these gingipains were completely absent from the growth media of isolates 157, Jaguar-1 and TG1, whereas isolate TT1 lacked only RgpA. Importantly, these observations were fully supported by inspection of the respective genome sequences, showing the presence/absence of the respective gingipain genes. Intriguingly, the PPAD-producing isolate TG1 lacks the known gingipains, suggesting that the production of PPAD is not necessarily accompanied by the production of gingipains.

### *Identification of PPAD-producing Porphyromonas species*

The identification of PPAD homologues in nine *Porphyromonas* isolates from non-human hosts raised the question to which particular species these isolates belong. To this end, an accurate phylogenetic analysis was performed based on 16S rRNA-encoding sequences. This identified the monkey isolate 19X2-K1 as *P. gingivalis*, the cat isolate 157 as *Porphyromonas salivosa*, the Jaguar-1 isolate as *Porphyromonas circumdentaria*, and six other isolates from a dog, two cats, and three monkeys as *Porphyromonas gulae* (Table S1). The TG1 and TT1 isolates from sheep were regarded as a potentially novel species since no high sequence similarity match with 16S rRNA of known *Porphyromonas* species was found initially. However, additional 16S rRNA Blast searches showed a match of 99% sequence identity to the 16S rRNA gene of *Porphyromonas* UQD 444, which had been isolated from marsupials in Australia<sup>39</sup> (Table S1). This isolate recently received the name *Porphyromonas loveana*<sup>40</sup>. A phylogenetic tree of our *Porphyromonas* isolates and

representative reference species based on the 16S rRNA gene comparisons is presented in Figure 4.



**Figure 4.** *Phylogeny of Porphyromonas species isolated from non-human hosts.* A neighbor-joining phylogenetic tree based on the alignment of 16S rRNA genes was generated with ClustalW. Previously sequenced reference strains of different *Porphyromonas* species were included in the analysis and the respective access codes are indicated.

Of note, this tree is in agreement with a phylogenetic tree based on the housekeeping gene *gdh* for glutamate dehydrogenase (Fig. S3), and both trees confirm the species designations as presented in Table S1. Interestingly, Figure 4 unveils the evolutionary distance between the three *Porphyromonas* species in which we identified PPAD (i.e. *P. gingivalis*, *P. gulae* and *P. loveana*), and the two species lacking PPAD (i.e. *P. salivosa* and *P. circumdentaria*). Notably, while we have previously shown that *P. gingivalis* isolates invariably possess

PPAD<sup>18</sup>, this was so far not known for *P. gulae* or *P. loveana*. Our present investigations combined with publicly available sequencing data now show that all known *P. gulae* isolates contain a PPAD gene, while at least two of the three known *P. loveana* isolates have such a gene<sup>24</sup>. Consistent with the phylogeny based on the 16S rRNA and *gdh* genes, a phylogenetic tree based on the amino acid sequences of identified PPAD proteins groups these proteins according to the respective species (Fig. S4). The overall amino acid sequence identity between the newly identified PPAD homologues and PPAD of *P. gingivalis* is high (81% *P. loveana*, 93% *P. gulae* and 99% *P. gingivalis*; Fig. S1). Further, the comparison of amino acid sequences highlights particular amino acid substitutions in PPAD proteins from *P. gulae* and *P. loveana* (i.e. M27T, R31G, S95N, S109N, E159D, P259A, V385I) compared to PPAD proteins from *P. gingivalis* W83.

## Discussion

A correlation between oral health and rheumatoid arthritis has been suspected for centuries, dating back to Hippocrates and his treatment of arthritis by tooth extraction. It was therefore a natural consequence to investigate the oral microbiota, and specifically periodontal pathogens, to shed more light on the etiology of RA. Once the involvement of PADs in the onset of this disease became apparent, studies with focus on *P. gingivalis* and its apparently unique citrullinating enzyme PPAD were initiated. The present study shows for the very first time that PPAD homologous proteins are present in two *Porphyromonas* species from non-human hosts, namely *P. gulae* and *P. loveana*. All these PPAD homologues possess the residues implicated in the catalytic conversion of arginine to citrulline. This implies that these homologues qualify as *Porphyromonas* peptidyl-arginine deiminases, hereafter referred to as PPAD. We consider these findings important for future research on the precise function of PPAD as a virulence factor in animal models, especially because PPAD is a major virulence factor of *P. gingivalis*, implicated in severe periodontitis and RA in humans. Our findings show that the subcellular sorting of PPAD to the extracellular environment is conserved in *P. gingivalis*, *P. gulae* and *P. loveana*. Clearly, the extracellular PPAD protein is present in two major forms. The dominant form is OMV-associated, probably via an A-LPS modification. In addition, a smaller fraction of the

extracellular PPAD is present in soluble secreted state. The fact that this sorting pattern is conserved across species suggests that it has biological and/or clinical relevance. For example, soluble secreted PPAD would easily reach cell surface-exposed targets on host cells or tissues. The OMV association of PPAD could serve two purposes, namely protection from degradation by proteases of *Porphyromonas* (e.g. the gingipains) or proteases of the host, and delivery to destinations within phagocytic host cells. The latter would be consistent with the notion that OMVs of various Gram-negative bacterial pathogens have been implicated in the intracellular delivery of virulence factors<sup>19, 41</sup>.

Whether the newly identified PPAD homologues from *P. gulae* and *P. loveana* are actually involved in pathogenesis in the non-human hosts remains to be demonstrated. In this context, it will also be important to accurately characterize the catalytic activity and substrate specificity of the newly identified PPAD homologues since, despite the compelling similarities, it is conceivable that the different PPAD homologues have evolved subtle differences.

To date, various functions of PPAD in pathogenesis have been proposed, including the protection of *Porphyromonas* proteins against degradation by gingipains through citrullination, the citrullination of important host proteins, and neutralization of acidic environments through the production of ammonia as a byproduct from the citrullination reaction. All these functions would be relevant for *Porphyromonas* pathogenesis, not only in humans but also in other non-human vertebrates. Intriguingly, the *P. loveana* isolates TT1 and TG1 were isolated from New Zealand sheep with “broken mouth periodontitis”, a common as yet untreatable ovine disease where sheep lose the incisor teeth needed to consume pasture. Eventually, this will cause malnutrition, weight loss and poor production, which has a serious impact on flock productivity and represents a significant economic burden in sheep farming<sup>42</sup>. As previous studies indicate that the bacteria responsible may be periodontal pathogens and *Bacteroides* spp.<sup>43, 44</sup>, it seems plausible that *P. loveana* could be involved in this disorder, possibly in combination with other oral pathogens.

Lastly, the presently investigated 19X2-K1 isolate represents one of the few documented cases of *P. gingivalis* isolated from a non-human host, a monkey in this case. The remaining samples, with the exception of 157 and Jaguar-1, are instead the first reported cases of non-*P. gingivalis* species producing a PPAD homologue. This proves

for the first time that expression of the citrullinating PPAD enzyme is not a feature unique to only one species among all prokaryotes, as it was thus far believed.

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The authors declare that they have no financial and non-financial competing interests in relation to the documented research. The present research has no particular ethical implications.

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