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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 anticitrullinated 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 11 previously collected *Porphyromonas* isolates from a dog, 2 sheep, 3 cats, 4 monkeys, and a jaguar with periodontitis. Our analyses uncovered the presence of secreted PPAD homologues in 8 isolates that were identified as *Porphyromonas gulae* (from a dog, monkeys, and cats) and *Porphyromonas loveana* (from sheep). In all 3 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 exoenzyme is not unique to only 1 prokaryotic species. Instead, we show that PPAD is produced by at least 2 other oral pathogens.

Keywords: periodontal disease(s), periodontitis, microbiology, PPAD, citrullination, OMVs

Introduction

Porphyromonas gingivalis is a renowned oral pathogen (Bostanci and Belibasakis 2012). This Gram-negative bacterium has been implicated in periodontitis (de Smit et al. 2011; Petersen and Ogawa 2012; How et al. 2016), a widespread disease of the oral cavity and one of the foremost causes of tooth loss worldwide (Bostanci and Belibasakis 2012; How et al. 2016). Notably, periodontitis is a polymicrobial disease in origin resulting from dysbiotic events, or perturbations of the oral microbiota (Jiao et al. 2014; Lamont and Hajishengallis 2015). Nonetheless, *P. gingivalis* constitutes <0.01% of the oral microbiota (Hajishengallis et al. 2012). 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) (Pischon et al. 2008; Detert et al. 2010; de Smit et al. 2011; Quirke et al. 2014). This autoimmune disease has been associated with periodontitis (Mercado et al. 2000; Chen et al. 2013; de Smit et al. 2015; Fuggle et al. 2016).

Recent models suggest that RA is related to a loss of tolerance toward citrullinated proteins (Pischon et al. 2008; Detert et al. 2010; Quirke et al. 2014). In this context, it is noteworthy that a citrullinating enzyme has been discovered in *P. gingivalis* (Konig et al. 2014; Montgomery et al. 2016). This enzyme, a peptidilyarginine deiminase (PAD), converts arginine residues into citrulline residues, which may cause drastic changes in the conformation and immunogenicity of the citrullinated protein (Konig et al. 2014). 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 (McGraw et al. 1999; Konig et al. 2014). Of note, the production of PPAD is a strictly conserved feature of *P. gingivalis* (Gabarrini et al. 2015), 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 (Sato et al. 2013; Veith et al. 2014). These OMVs are nanostructures resulting from specific blebbing processes of the outer membrane and contain cargo of proteins implicated in

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A supplemental appendix to this article is available online.

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virulence (Veith et al. 2014; Gui et al. 2015; Xie 2015). The outer membrane and OMV association of PPAD is probably facilitated through a modification with lipopolysaccharide A (A-LPS) (Shoji et al. 2011; Sato et al. 2013).

P. gingivalis is known to share several traits with other species belonging to the genus _Porphyromonas_, each associated with periodontitis mainly in their own nonhuman host (Coil et al. 2015). Despite its high host specificity, there have been a few recorded cases of _P. gingivalis_ isolated from animal hosts, especially beagle dogs (Madianos et al. 1994; Menard and Mouton 1995). 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 (Pedersen et al. 1976; Lipman et al. 1991; Narayan et al. 1992; Hanna 2005; Lemetayer and Taylor 2014; Riggio et al. 2014). In addition, 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 (Appendix Table) were previously isolated from diverse animal hosts (dog, cats, monkeys, sheep, and jaguar) with periodontitis hailing from different countries (Laliberte and Mayrand 1983; Loos et al. 1993). 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 ΔPAD-A, ATCC 33277 3ΔPAD-B) (Wegner et al. 2010) . The _P. gingivalis_ clinical isolate 20664 was collected in Groningen, the Netherlands, from a patient with severe periodontitis. Each strain was cultivated anaerobically for 4 d in brain heart infusion (BHI) broth as previously described (Stobernack et al. 2016).

Lithium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis and Western Blotting

Separation of proteins by lithium dodecyl sulfate (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 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), and 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 $16,100 \times g$ and 4°C to separate bacterial cells from the growth medium. Subsequently, 500 µL of the resulting supernatant was ultracentrifuged for 2 h at 2 13,000 $\times g$ and 4°C using an Optima MAX-XP ultracentrifuge (Beckman Coulter). Afterward, the ultracentrifugation pellet, enriched in OMVs, was resuspended in 500 µL OMV buffer (5 mM $MgCl_2$ in phosphate-buffered saline [PBS]). Proteins in the supernatant and resuspended pellet fractions obtained upon ultracentrifugation were trichloroacetic acid (TCA)–precipitated prior to analysis by LDS-PAGE and Western blotting, as described above.

Aqueous 2-Phase System Protein Purification

To explore the possible association of PPAD-like proteins with OMVs via an LPS modification, a protein phase separation was performed using the nonionic detergent Triton X-114 (Sigma-Aldrich) essentially as previously described (Bordier 1981). For experimental details, see Appendix.

Results

Detection of PPAD in Porphyromonas Species from Nonhuman Hosts

To investigate the occurrence of PPAD homologous enzymes beyond the _P. gingivalis_ species boundaries, we screened an available previously established _Porphyromonas_ collection (Laliberte and Mayrand 1983; Loos et al. 1993), including isolates from a dog ($n = 1$), cats ($n = 3$), sheep ($n = 2$), monkeys ($n = 4$) and a jaguar ($n = 1$; Appendix Table). 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). The patterns of bands strongly cross-reacting with the PPAD antibody comprised a thick band of ~75 to 85 kDa and a sharper one of ~47 kDa, with the possible appearance of 1 or 2 faint bands of ~60 kDa. In the case of the monkey isolate I-372, the ~75- to 85-kDa band was separated into 2 closely migrating sharper bands. The latter finding suggests that the broad ~75- to 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 nonhuman 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 nonhuman hosts is consistent with literature data, where it was proposed that the ~75- to 85-kDa band represents the outer membrane–bound form of PPAD, and the ~47-kDa band represents the secreted soluble form (König et al. 2014). 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 (Shoji et al. 2002; Gui et al. 2015; Xie 2015). 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 nonhuman 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 nonhuman hosts. In contrast, substantial amounts of the ~75- to 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
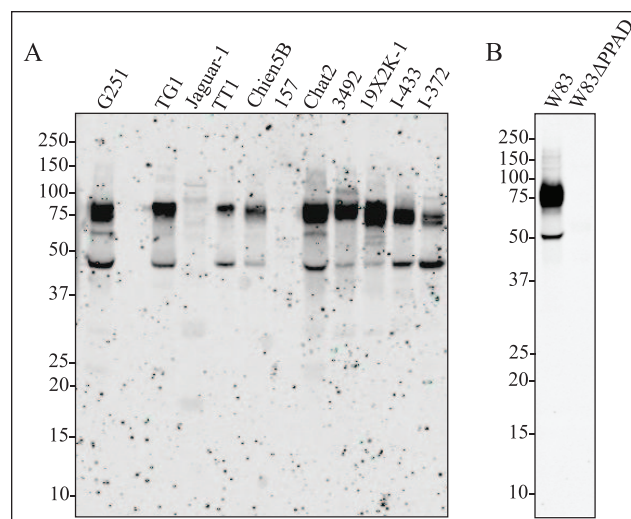

Figure 1. Detection of a PPAD-like protein in *Porphyromonas* isolates from nonhuman hosts. *Porphyromonas* species isolates from nonhuman hosts were cultured for 4 d in brain heart infusion medium. Subsequently, bacterial cells were separated from the growth medium, and growth medium fractions were used for immunoblotting with PPAD-specific antibodies. (**A**) *Porphyromonas* species clinical isolates. (**B**) *Porphyromonas gingivalis* reference strain W83 and the isogenic PPAD deletion mutant. Molecular weights of marker proteins are indicated. PPAD, peptidylarginine deiminase enzyme of *P. gingivalis*.

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- to 85-kDa PPAD species localized prevalently to the detergent fraction (Fig. 3A, B), 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.

Comparison of PPAD and Its Homologues in Porphyromonas *Species from Nonhuman Hosts*

To identify genes encoding the potential PPAD homologues detected by Western blotting, we sequenced the different investigated *Porphyromonas* isolates from nonhuman hosts. This analysis identified homologous PPAD genes in the 9 *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 (Montgomery et al. 2016). This implies that all identified PPAD homologues have a potential citrullinating activity. The highest overall amino
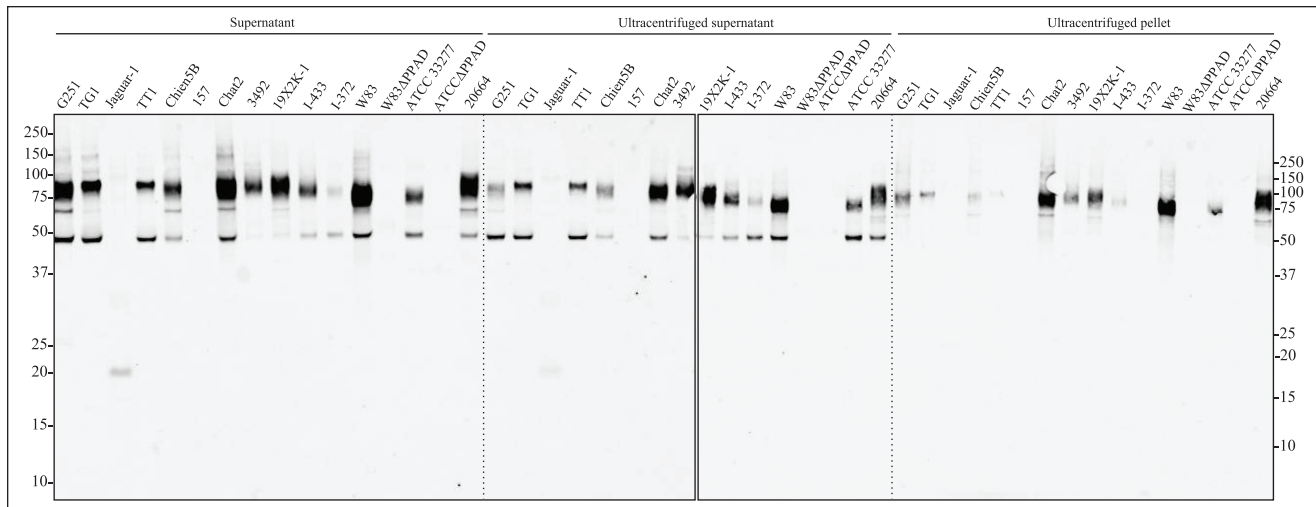
Figure 2. Association of PPAD-like proteins from nonhuman hosts with outer membrane vesicles (OMVs). Growth medium fractions (designated "supernatant") of *Porphyromonas* species isolates were subject to ultracentrifugation. Subsequently, the supernatant and pellet fractions were analyzed by immunoblotting as indicated for Figure 1. Samples relating to the *Porphyromonas* species isolates from nonhuman hosts, the reference strains W83 and ATCC 33277 and their respective PPAD^{−/−} mutants, and a *Porphyromonas 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. PPAD, peptidylarginine deiminase enzyme of *P. gingivalis*.
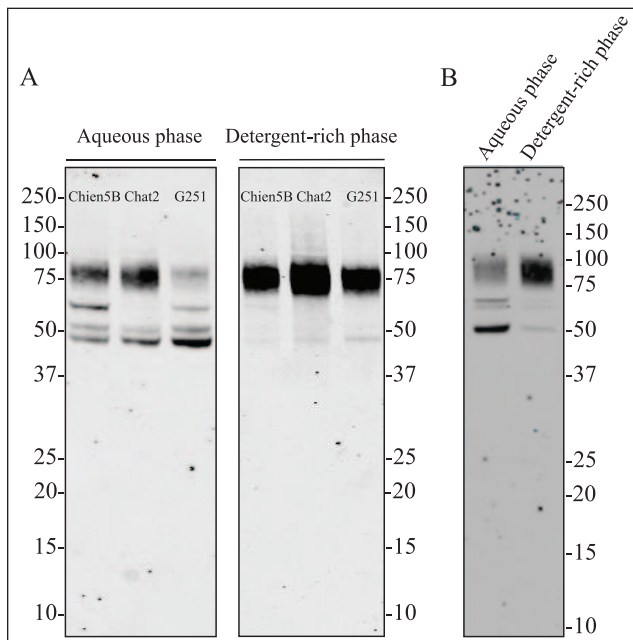

Figure 3. Detergent extraction of the ~75- to 85-kDa PPAD-like protein species. *Porphyromonas* species isolates were cultured and, subsequently, separated from the growth medium by centrifugation. Next, an aqueous 2-phase system protein purification was performed, dividing each sample into an aqueous (lipid-free proteins) and a detergent-rich (lipid-bound proteins) phase. The resulting fractions of (**A**) 3 *Porphyromonas* isolates from nonhuman hosts and (**B**) the *Porphyromonas gingivalis* clinical isolate 20664 were analyzed by immunoblotting as indicated for Figure 1. Molecular weights of marker proteins are indicated. PPAD, peptidylarginine deiminase enzyme of *P. gingivalis*.

acid sequence similarity to the PPAD reference protein from *P. gingivalis* W83 was observed for the homologous protein of the 19X2-K1 isolate (Appendix Fig. 1). Furthermore, all PPAD

proteins of the different *Porphyromonas* isolates from nonhuman hosts, including 19X2-K1, showed the amino acid substitutions G231N, E232T, and N235D.

Presence of Secreted Gingipains in *Porphyromonas* Species from Nonhuman Hosts

To evaluate the possible secretion of the gingipains RgpA, RgpB, and Kgp by *Porphyromonas* species from nonhuman 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 Appendix Figure 2, 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 9 *Porphyromonas* isolates from nonhuman hosts raised the question to which particular species these isolates belong. To this end, an accurate phylogenetic analysis was performed based on 16S
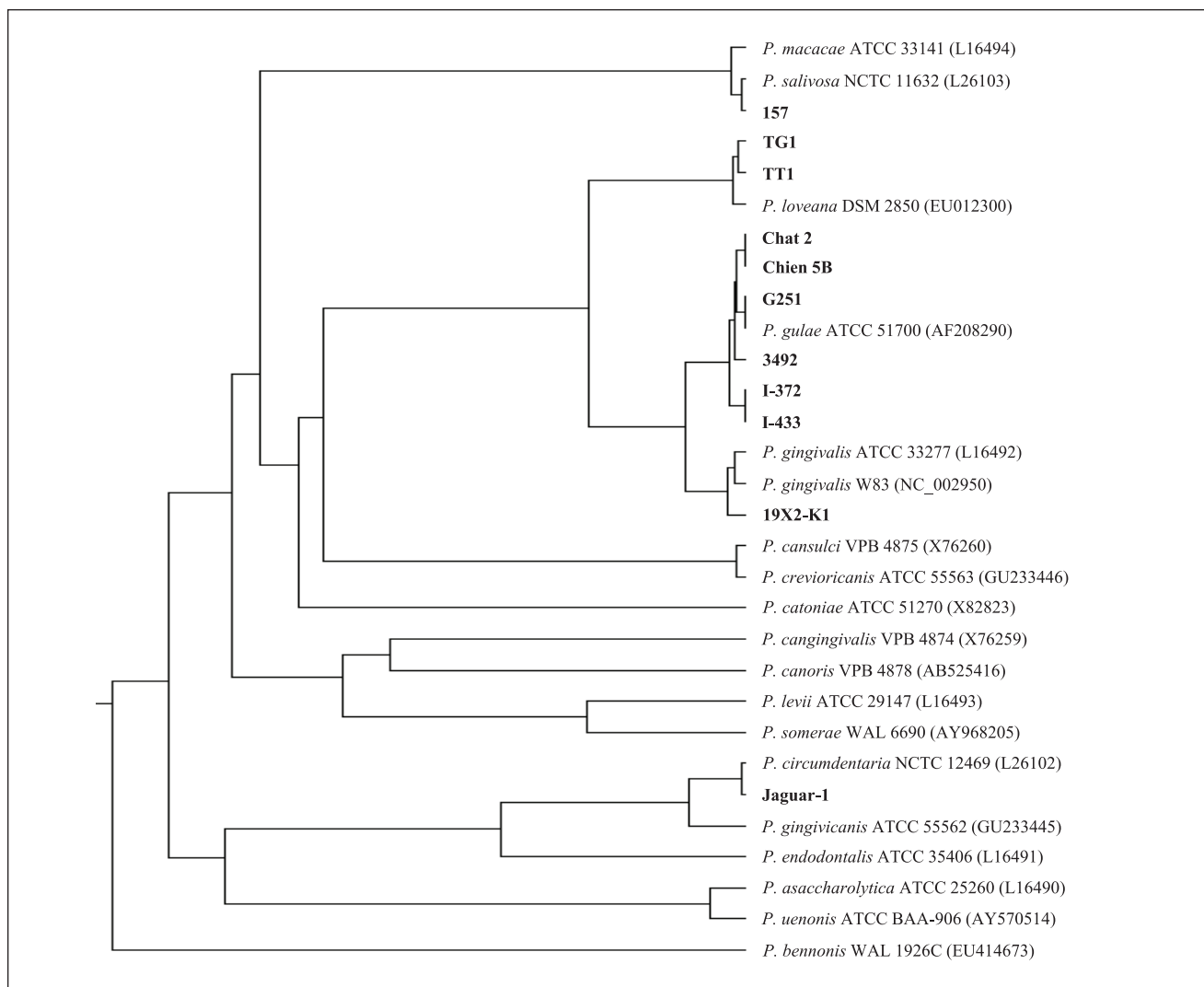
Figure 4. Phylogeny of *Porphyromonas* species isolated from nonhuman hosts. A neighbor-joining phylogenetic tree based on the alignment of 16S ribosomal RNA genes was generated with ClustalW. Previously sequenced reference strains of different *Porphyromonas* species were included in the analysis and the respective access codes are indicated.

ribosomal RNA (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 6 other isolates from a dog, 2 cats, and 3 monkeys as *Porphyromonas gulae* (Appendix Table). 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 (Mikkelsen et al. 2008) (Appendix Table 1). This isolate recently received the name *Porphyromonas loveana* (Bird et al. 2016). A phylogenetic tree of our *Porphyromonas* isolates and representative reference species based on the 16S rRNA gene comparisons is presented in Figure 4. Of note, this tree is in agreement with a phylogenetic

tree based on the housekeeping gene *gdh* for glutamate dehydrogenase (Appendix Fig. 3), and both trees confirm the species designations as presented in Appendix Table. Interestingly, Figure 4 unveils the evolutionary distance between the 3 *Porphyromonas* species in which we identified PPAD (i.e., *P. gingivalis, P. gulae*, and *P. loveana*), and the 2 species lacking PPAD (i.e., *P. salivosa* and *P. circumdentaria*). Notably, while we have previously shown that *P. gingivalis* isolates invariably possess PPAD (Gabarrini et al. 2015), 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 2 of the 3 known *P. loveana* isolates have such a gene (Coil et al. 2015). 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 (Appendix Fig. 4).

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*; Appendix Fig. 1). Furthermore, 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 RA 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 2 *Porphyromonas* species from nonhuman 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 2 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 a 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 2 purposes: 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 (Veith et al. 2014; Jan 2017).

Whether the newly identified PPAD homologues from *P. gulae* and *P. loveana* are actually involved in pathogenesis in the nonhuman 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 by-product from the citrullination reaction. All these functions would be relevant for *Porphyromonas* pathogenesis, not only in humans but also in other nonhuman vertebrates. Intriguingly, the *P. loveana* isolates TT1 and TG1 were isolated from New Zealand sheep with "broken mouth periodontitis," a common 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 (Riggio et al. 2013). As previous studies indicate that the bacteria responsible may be periodontal pathogens and *Bacteroides* spp. (McCourtie et al. 1990; Yamasaki et al. 1990), it seems plausible that *P. loveana* could be involved in this disorder, possibly in combination with other oral pathogens.

Last, the presently investigated 19X2-K1 isolate represents one of the few documented cases of *P. gingivalis* isolated from a nonhuman 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 1 species among all prokaryotes, as it was thus far believed.

Author Contributions

G. Gabarrini, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; M.A. Chlebowicz, J.W.A. Rossen, H.J.M. Harmsen, contributed to data analysis, critically revised the manuscript; M.E. Vega Quiroz, A.C.M. Veloo, M.L. Laine, contributed to data acquisition, critically revised the manuscript; J.M. van Dijl, A.J. van Winkelhoff, contributed to conception and data interpretation, critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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