Arginine deiminase inhibits *Porphyromonas gingivalis* surface attachment

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The oral cavity is host to a complex microbial community whose maintenance depends on an array of cell-to-cell interactions and communication networks, with little known regarding the nature of the signals or mechanisms by which they are sensed and transmitted. Determining the signals that control attachment, biofilm development and outgrowth of oral pathogens is fundamental to understanding pathogenic biofilm development. We have previously identified a secreted arginine deiminase (ADI) produced by *Streptococcus intermedius* that inhibited biofilm development of the commensal pathogen *Porphyromonas gingivalis* through downregulation of genes encoding the major (*fimA*) and minor (*mfa1*) fimbriae, both of which are required for proper biofilm development. Here we report that this inhibitory effect is dependent on enzymic activity. We have successfully cloned, expressed and defined the conditions to ensure that ADI from *S. intermedius* is enzymically active. Along with the cloning of the wild-type allele, we have created a catalytic mutant (ADIC399S), in which the resulting protein is not able to catalyse the hydrolysis of L-arginine to L-citrulline. *P. gingivalis* is insensitive to the ADIC399S catalytic mutant, demonstrating that enzymic activity is required for the effects of ADI on biofilm formation. Biofilm formation is absent under L-arginine-deplete conditions, and can be recovered by the addition of the amino acid. Taken together, the results indicate that arginine is an important signal that directs biofilm formation by this anaerobe. Based on our findings, we postulate that ADI functions to reduce arginine levels and, by a yet to be identified mechanism, signals *P. gingivalis* to alter biofilm development. ADI release from the streptococcal cell and its cross-genera effects are important findings in understanding the nature of inter-bacterial signalling and biofilm-mediated diseases of the oral cavity.

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INTRODUCTION

Porphyromonas gingivalis is an oral pathobiont, i.e. a natural member of the human microbiota, that under certain perturbations to the host and/or microflora can cause pathology. This Gram-negative, highly proteolytic anaerobe is regarded as the primary aetiological agent of adult periodontal disease, leading to chronic inflammation and destruction of both the soft and hard tissues supporting the teeth (Choi *et al.*, 1990; Dzink *et al.*,

1988; Grossi *et al.*, 1994; Lamont & Jenkinson, 2000; Moore *et al.*, 1991). As a pathobiont, the ability to proliferate and express virulence determinants is central to its shift to pathogenicity, thus determining the mechanisms that control the emergence of its pathogenic state are of fundamental importance.

P. gingivalis is a strict anaerobe that preferentially utilizes protein or peptide substrates for growth. Although studies have shown that *P. gingivalis* may utilize free amino acids or dipeptides, the uptake and growth rates on these substrates are limited and highly variable among strains (Seddon *et al.*, 1988; Takahashi *et al.*, 2000; Tang-Larsen *et al.*, 1995). In general, single amino acids do not support planktonic growth; however, arginine appears to be an amino acid of particular importance (Masuda *et al.*, 2001; Seddon *et al.*, 1988; Takahashi *et al.*, 2000; Tang-Larsen

Abbreviations: IMAC, immobilized metal affinity chromatography; MWCO, molecular weight cut off; SUMO, small ubiquitin-like modifier.

The GenBank/EMBL/DBJ accession number for the arginine deiminase sequence of *Streptococcus intermedius* is JX534201.

Two supplementary figures are available with the online version of this paper.

et al., 1995). *P. gingivalis* produces proteolytic enzymes that specifically cleave at arginine residues within peptides, as well as enzymes dedicated to releasing an arginine molecule from the carboxy-terminus of a peptide (Chen *et al.*, 1992; Masuda *et al.*, 2001, 2002). Although the mechanism is unknown, some data indicate preferential uptake and utilization of arginine by *P. gingivalis* (Masuda *et al.*, 2001, 2002; Pike *et al.*, 1994; Takahashi *et al.*, 2000). What remains unclear is why arginine is such an important amino acid. Within the oral cavity (i.e. in saliva, plaque or gingival crevicular fluid) of either healthy or diseased individuals the levels of arginine are relatively low (0.007–220 µM) and in comparison with other amino acids, arginine is considered a limiting nutrient (Brand *et al.*, 1997; Higham & Edgar, 1989; Syrjänen *et al.*, 1990; Téllez *et al.*, 2008; Van Wuyckhuysse *et al.*, 1995). There is a positive correlation between arginine levels and the increased rates of *P. gingivalis* uptake by murine macrophages (Sosroseno, 2004). Finally, it is also important to note that the metabolic fate of arginine in mammalian cells has a significant effect on host cell physiology. Arginine is not only an immediate precursor for protein synthesis; it is also catabolized to critical cell-signalling molecules, such as nitric oxide and glutamate (Wu & Morris, 1998). Thus, we hypothesize that there is likely to be a complex interplay between host and microbiota for this substrate and that the utilization of this resource is tightly regulated.

In our previous studies, we discovered an extracellular arginine deiminase (ADI) produced by the oral bacterium *Streptococcus intermedius* that inhibited *P. gingivalis* biofilm formation, without affecting its planktonic growth (Christopher *et al.*, 2010). The canonical role of ADI is to catalyse the intracellular hydrolytic conversion of free L-arginine to L-citrulline and ammonia (Fig. 2a) (Cunin *et al.*, 1986). This catalysis represents the first step of the anaerobic arginine dihydrolase pathway, which is utilized by a number of organisms, including not only a number of oral streptococci but also *Pseudomonas aeruginosa* and *Mycoplasma* species, for the synthesis of ATP (Abdelal, 1979; Casiano-Colón & Marquis, 1988; Rahman *et al.*, 1980). Within the oral biofilm community, the release of ammonia from this first reaction plays an important role in inhibiting tooth decay and in modulating the microbial composition of the community through neutralization of acid end products that are produced by a variety of oral bacteria via fermentation of sugars (Burne & Marquis, 2000; Casiano-Colón & Marquis, 1988; Nascimento *et al.*, 2009). In contrast with its canonical role in anaerobic ATP synthesis within the cytoplasm, ADI's function as an extracellular enzyme is unclear.

Our studies have shown that exposure of *P. gingivalis* strain 381 to ADI secreted by *S. intermedius* results in a decrease in the expression of fimbrial subunits (encoded by *fimA* and *mfa1*), which are both key virulence determinants and essential to biofilm development (Amano *et al.*, 2004; Christopher *et al.*, 2010; Hamada *et al.*, 1998; Lamont & Jenkinson, 1998; Xie *et al.*, 2007). This interspecies

inhibition is consistent with reports by Xie and colleagues that showed the effect of a cell-wall-associated *Streptococcus cristatus* arginine deiminase on *P. gingivalis* strain 33277, with the only distinction being that in strain 33277 expression of *fimA*, but not *mfa1*, is altered (Wu & Xie, 2010; Xie *et al.*, 2007). *P. gingivalis* strain variation could contribute to these differences in gene expression and/or variation in gene regulation. In addition, using chemical inhibitors, they obtained data that indicated that enzymic activity was not required for ADI signalling and our early studies showed that ADI, which appeared to be inactive (unable to convert arginine to citrulline in a standard ADI assay), could inhibit *P. gingivalis* biofilm formation (Christopher *et al.*, 2010; Xie *et al.*, 2007). However, we have since determined (data presented here) that enzymic activity of ADI is sensitive to the redox state. We therefore postulate that a seemingly inactive ADI protein could become reactivated when tested in our anaerobic biofilm system, confounding our previous analysis. To determine definitively if enzymic activity is required, we generated a catalytically inactive form of the *S. intermedius* ADI (ADIC399S), using an *Escherichia coli* expression system. Here, we show that enzymic activity is necessary and sufficient for ADI to inhibit biofilm formation. Moreover, we show that the addition of arginine (the substrate removed by ADI) enhances biofilm formation by *P. gingivalis*. Our working model is that *P. gingivalis* can sense arginine levels and that availability of this amino acid is a critical cue in modulating expression of virulence determinants and commitment to a sessile lifestyle.

METHODS

Strains and culture conditions. Bacterial strains and plasmids are listed in Table 1. *P. gingivalis* strain 381 and *S. intermedius* strain F0413, upon revival from –80 °C DMSO stocks, were maintained on trypticase soy broth (TSB) agar plates containing 1 µg haemin ml⁻¹, 1 µg menadione ml⁻¹, 20% defibrinated sheep's blood and 1.5% agar at 37 °C under anaerobic conditions. *E. coli* strains were maintained on LB 1.5% agar plates amended with the appropriate antibiotics for plasmid maintenance, 150 µg ampicillin ml⁻¹ or 30 µg kanamycin ml⁻¹ at 37 °C.

Broth cultures of *P. gingivalis* were grown in Todd–Hewitt broth (THB) containing 1 µg haemin ml⁻¹ and 1 µg menadione ml⁻¹ (designated THBHK) at 37 °C under anaerobic conditions. For standard *P. gingivalis* biofilm assays, cells were grown in chemically defined medium (CDM) plus 1% tryptone as previously described (Christopher *et al.*, 2010; Milner *et al.*, 1996).

Arginine-free RPMI 1640 (Sigma Aldrich, R1780) was used as a defined medium to study the effect of arginine on *P. gingivalis* biofilm formation. Unlike the complete RPMI 1640 that contains all essential amino acids, the arginine-free RPMI lacks not only arginine but also L-leucine and L-lysine; therefore RPMI 1640 was supplemented with these nutrients to the concentrations indicated for RPMI: specifically L-leucine (0.05 g l⁻¹) and L-lysine–HCl (0.04 g l⁻¹) were added. The medium was also amended with additional nutrients utilized by *P. gingivalis*, specifically the dipeptide L-alanyl-L-glutamine (0.43 g l⁻¹), citric acid (2 mM), trace metals (Christopher *et al.*, 2010), haemin (1 µg ml⁻¹) and menadione (1 µg ml⁻¹). This supplemented defined medium was designated arginine-free RPMI-S. L-Arginine–HCl was

Table 1. Strains and plasmids

Strains	Genotype	Source or reference
<i>P. gingivalis</i> strain 381	Wild-type	Dr Howard Kuramitsu, State University of Buffalo, NY
<i>S. intermedius</i> strain F0413	Clinical isolate	Lab collection (Christopher <i>et al.</i> , 2010)
<i>E. coli</i> DH5 α	F'/ <i>endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1 (lacZYA-argF) U169 deoR [80 dlac (lacZ)M15 recA1]</i>	Lab collection
<i>E. coli</i> JM109	<i>endA1 recA1 gyrA96 thi hsdR17 (r_k, m_k⁺) relA1 supE44 Δ(<i>lac-proAB</i>) [F' <i>traD36 proAB laqI</i>^qΔM15]</i>	Promega
<i>E. coli</i> 10G Chemically Competent Cells	<i>mcrA Δ(mrr-hsdRMS-mcrBC) endA1 recA1 ϕ80dlacZΔM15 ΔlacX74 araD139 Δ(<i>ara,leu</i>)7697 galU galK rpsL nupG λ^- tonA</i>	Lucigen
<i>E. coli</i> BL21(DE3)	F ⁻ <i>ompT gal dcm lon hsdS_B(r_B m_B) λ(DE3 [<i>lacI lacUV5-T7 gene 1 ind1 sam7 nin5</i>])</i>	Lab collection
Plasmids		
pGEM-T Easy	Cloning vector	Promega
pGEM-ADI	pGEM-T Easy containing full-length <i>S. intermedius</i> arginine deiminase	This study
pET16b	Expression vector	Novagen
pET16-ADI	pET16b containing full-length <i>S. intermedius</i> arginine deiminase	This study
pET16-ADIC399S	pET16b containing full-length <i>S. intermedius</i> arginine deiminase harbouring a cysteine to serine mutation at amino acid position 399	
pRham N-His SUMO	Expression vector	Lucigen
pRham N-His SUMO-ADI	pRham N-His SUMO containing full-length <i>S. intermedius</i> arginine deiminase	This study
pRham N-His SUMO-ADIC399S	pRham N-His SUMO containing full-length <i>S. intermedius</i> arginine deiminase harbouring a cysteine to serine mutation at amino acid position 399	This study

added at the concentrations indicated. All stock solutions of supplements were prepared in arginine-free RPMI 1640.

Cloning of *arcA* and site-directed mutagenesis. The coding region of the *arcA* gene from *S. intermedius* was PCR amplified and cloned into pGEM-T Easy (Invitrogen), generating pGEM-ADI. The insert was sequenced using M13 forward and reverse primers provided by Genewiz, and verified to match the protein sequence obtained for the purified ADI previously isolated from the culture supernatant of *S. intermedius* (Christopher *et al.*, 2010). The *arcA* gene was then liberated by *NcoI* and *XhoI* (New England Biolabs) digestions (these restriction sites were encoded in the 5' end of the *arcA* PCR primer set), ligated to pET16b; generating pET16b-ADI. The plasmid was then transformed into *E. coli* strains JM109 and BL21(DE3).

To create the catalytic mutant allele, primers were designed to mutate the nucleotides encoding the cysteine at position 399 to a serine. PCR was performed using the mutagenesis primers (ADI Cys-Ser SDM F and ADI Cys-Ser SDM R), pET16b-ADI and *Pfu* polymerase (Phusion, New England Biolabs), with cycling at 95 °C for 3 min, 95 °C for 30 s, 64 °C for 1 min and 72 °C for 15 min, repeated for 30 cycles; followed by a final extension of 72 °C for 15 min. PCR products were incubated with 2 μ l *DpnI* (New England Biolabs) per 50 μ l reaction volume at 37 °C for 24 h. A portion of the digest (5 μ l) was used to transform *E. coli* DH5 α cells (Invitrogen) according to the manufacturer's instructions. The plasmids from the resulting clones were sequenced using T7 and T7-term primers (Genewiz) and the sequences were verified to encode the protein sequence containing the mutated amino acid at position 399.

To facilitate purification and removal of the purification tag, ADI and ADIC399S were cloned into the *E. coli* expression vector pRham N-His SUMO (Lucigen). Previously sequenced pET16-ADI and pET16-ADIC399S plasmids were used as the template for PCR. The primer design and cloning protocols provided by the manufacturer were used without exception. Plasmids were transformed to *E. coli* 10G Chemically Competent Cells (Lucigen), which allows for further protein induction. Using SUMO forward primer and pETite reverse primer, both provided by the manufacturer, the clones were screened by PCR and sequenced to verify that no mutations were introduced.

Protein purification. LB broth cultures were amended with 0.5 % L-arginine, 30 μ g kanamycin ml⁻¹, 0.2 % L-rhamnose and 0.05 % D-glucose and inoculated with *E. coli* containing either pRham N-His SUMO-ADI or pRham N-His SUMO-ADIC399S (Burne *et al.*, 1989). Cultures were grown at 37 °C, on a platform shaker at 250 r.p.m., for 6 h. Cells were then pelleted at 6000 g for 5 min and the supernatants were discarded. Pellets were frozen at -20 °C. To purify the proteins, 5 ml lysis buffer (6 M guanidine hydrochloride, 100 mM Na₂PO₄, 10 mM Tris/HCl pH 8.0) per 1 g wet weight of the pellet was added and cells were lysed by shaking for 20 min at room temperature. Lysate was centrifuged at 10 500 g for 10 min at 4 °C. Centrifuge columns (Pierce) were equilibrated with 1 ml HisB buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 8.0); followed by addition of Ni-NTA-agarose (1 ml per 250 ml culture; HisPur Ni-NTA, ThermoFisher) to the column, which was then equilibrated with 10 ml HisB buffer. Lysates were transferred to the column and incubated for 30 min on ice, with mixing every 10 min. The agarose was washed three times with 10 ml HisB buffer. Ni-bound protein was eluted with 1 ml HisC buffer (50 mM NaH₂PO₄, 300 mM NaCl,

Table 2. Primers used in the study

Primer name	Sequence (5'–3')	Orientation*	Purpose
SiADIIncoIF	GCCATGGATGTCTACACAT CCAATTC	F	Amplify <i>S. intermedius arcA</i> to insert into pET16b at the <i>NcoI</i> site
SiADIxhoI3R	GCCTCGAGTTAGATTTCTTCACGTTC	R	
ADI Cys-Ser SDM F	GTGGTGGACCTCGTAGTATGTCAATGCCATTTG	F	Create a Cys to Ser mutation in <i>arcA</i>
ADI Cys-Ser SDM R	CAAATGGCATTGACATACTACGAGGTCCACCAC	R	
ADI-SUMO F	CGCGAACAGATTGGAGGTATGTCTACACATCCAATTCAT	F	Amplify <i>S. intermedius arcA</i> to insert into the Lucigen Expresso/SUMO plasmid
ADI-SUMO R	GTGGCGGCCGCTCTATTAGATTTCTTCACGTTCAAATGG	R	

*F, Forward primer; R, reverse primer.

250 mM imidazole, pH 8.0). Proteins were dialysed in cassettes [Thermo Scientific Slide-A-Lyser Dialysis Cassettes, 10K molecular weight cut off (MWCO)] overnight at 4 °C in 20 mM Tris/HCl (pH 8.0), 150 mM NaCl, 10 % glycerol buffer. Proteins were stored at –20 °C or digested immediately to remove the purification tag.

Small ubiquitin-like modifier (SUMO)–His-tag removal. Protein concentrations were quantified by Bradford assay (QuickStart Bradford reagent, Bio-Rad) using a BSA standard curve. One unit of protease per 100 µg fusion protein (Lucigen, SUMO Express Protease) was added and tubes were incubated overnight at 4 °C. Tag-less proteins were purified as described above over immobilized metal affinity chromatography (IMAC) columns with the exception that the proteins are released in the washes while the SUMO–His tag and protease theoretically remain bound to the Ni-NTA. The wash fractions were collected, dialysed overnight at 4 °C in PBS and 10 % glycerol buffer, and then concentrated with Amicon spin columns (MWCO 10 000). Protein samples, before and after protease treatment, were run on SDS-polyacrylamide gels and stained with Coomassie blue to assess purity of the protein (see Fig. S1, available with the online version of this paper, for post-protease treatment). Protein concentrations were quantified by Bradford assay using a BSA standard curve. Protein samples were adjusted to 100 µg ml^{–1} in PBS, 10 % glycerol buffer, 2 mM DTT, 0.1 % BSA and stored at –20 °C.

Arginine deiminase activity. Enzymic activity was determined as previously described with slight modifications (Christopher *et al.*, 2010; Xie *et al.*, 2007). In brief, 100 µl of the 100 µg protein ml^{–1} samples in PBS, 10 % glycerol buffer, 2 mM DTT, 0.1 % BSA prepared above was added to a 96-well microtitre plate. Samples were mixed with 50 µl 0.1 M L-arginine HCl and incubated at 37 °C for 2 h. To terminate the reaction, 50 µl 20 % sulfuric acid was added to the wells. To detect the presence of L-citrulline, 50 µl 3 % 2,3-butanedione monoxime was added and the reaction was incubated in the dark for 1 h at 56 °C. Colour development was quantified at 492 nm using a plate reader and was compared to an L-citrulline standard curve to ensure samples were within the range of detection.

Biofilm assays. To test the effect of ADI on biofilm formation, a *P. gingivalis* strain 381 culture was grown for 24–36 h anaerobically in THBHK. Cultures (1 ml) were pelleted, the supernatants were carefully removed, and pellets were resuspended into 5 ml 2 × CDM/1 % tryptone prior to addition to a 96-well plate (BD Falcon, no. 353936) (Christopher *et al.*, 2010; Milner *et al.*, 1996). Each well consisted of a total volume of 200 µl, containing 100 µl of the cell

resuspension and the appropriate protein and/or buffer. The resulting final cell concentration was 10-fold less than that of the THBHK culture, thus providing a starting OD₆₀₀ of 0.2–0.3. The assays were repeated a minimum of three times, representing different biological replicates; each biological replicate was represented at least three times on a 96-well plate for technical replicates. Where appropriate a Student's *t*-test was performed to generate *P*-value to verify statistical significance. Biofilm assays were incubated anaerobically for 24 h at 37 °C. Commercially available *Mycoplasma arginini* ADI was purchased from PeproTech (catalogue no. 150-12) and reconstituted as per the manufacturer's instructions.

To test biofilm formation in the presence or absence of L-arginine, 1 ml of a 24 h culture of *P. gingivalis* grown in THBHK was pelleted, the supernatant was removed and the cells were resuspended in 10 ml arginine-free RPMI-S with or without L-arginine at the concentrations indicated. An aliquot of the resuspended culture (200 µl) was then pipetted into each well of a 96-well microtitre plate or to a Millicell EZ slide (PEZGS0816) well (for microscopic observation). The assays were repeated a minimum of three times, representing different biological replicates; each biological replicate was represented at least three times on a 96-well plate or two times on a Millicell EZ slide for technical replicates. Biofilm assays were incubated anaerobically for 24 h at 37 °C.

Safranin staining for semi-quantification of biofilm biomass.

Culture supernatants were removed by inversion and shaking and the plates were washed twice by immersion in distilled water and then allowed to air dry overnight. Wells were stained with 0.1 % safranin (250 µl per well) for 15 min. The excess safranin was removed; plates were washed twice by immersion in distilled water and allowed to air dry. The amount of biofilm was assessed macroscopically and semiquantitatively. To quantify the amount of biofilm, 200 µl per well of 95 % ethanol:1 % SDS was added for 5 min to solubilize the safranin; the A₄₉₂ was determined using a plate reader.

Live/dead staining. Culture supernatants were removed by aspiration and wells were washed twice with PBS. The Invitrogen live/dead BacLight Bacterial Viability kit (L7012) was used as per the manufacturer's instructions. Briefly, Components A and B were mixed in equal volume, added to a saline solution (9.9 g to 1 l distilled water) with 3 µl dye in 1 ml, and 200 µl of the solution was added to each well. Slides were incubated in the dark for 15 min. The dye mixture was removed from the wells, the wells were broken away from the slide, and the slide surface was gently washed in dH₂O to remove excess dye and any unbound cells. Slides were visualized on an Olympus FSX100 under phase-contrast and fluorescence.

Growth of rate analysis of *P. gingivalis* in RPMI. To determine the ability of RPMI-S supplemented with arginine to support exponential growth, cultures of *P. gingivalis* were grown for 24 h in THBHK. The cultures were then pelleted, the supernatants were carefully removed, and the pellets were resuspended in arginine-free RPMI-S or arginine-free RPMI-S supplemented with L-arginine at a concentration of 0.04 % or 0.4 %. The resulting final cell concentration was 10-fold less than that of the THBHK culture, thus providing a starting OD₆₀₀ of 0.2–0.4. These growth experiments were repeated three times and each of the three biological replicates was tested five times per condition to provide technical replicates. Test tubes were incubated in an anaerobic chamber at 35 °C. OD₆₀₀ was measured at the times indicated; cells were continuously maintained in the chamber and prior to each reading cells were resuspended with a pipette.

RESULTS

Cloning of ADI and catalytic mutant

We previously demonstrated that *S. intermedius*-derived ADI purified from culture supernatants was capable of inhibiting *P. gingivalis* biofilm formation (Christopher *et al.*, 2010). To facilitate protein purification and to eliminate the potential for co-purification of other *S. intermedius* proteins, a tag-less ADI was cloned and expressed in *E. coli*. ADI sequences vary at the C terminus with isoleucine or valine being the last translated amino acid. Using the sequence obtained from mass spectrometry, it was determined that the last amino acid in the *S. intermedius* sequence is an isoleucine, similar to what was published for *Streptococcus gordonii* (Christopher *et al.*, 2010). Primers were designed with an isoleucine before a stop codon; the *arcA* gene was amplified, cloned and sequenced (Fig. 1). *S. intermedius* ADI displayed up to 92 % amino acid sequence identity to other oral *Streptococcus* species outside of the *milleri* group (*Streptococcus anginosus*, *Streptococcus constellatus* and *S. intermedius*) (BLASTP, NCBI). Additionally, *S. intermedius* ADI displayed 37 % and 34 % identity and 54 % and 55 % similarity of amino acids with *M. arginini* and *P. aeruginosa* ADIs, respectively (BLASTP, NCBI).

Separating effects of arginine catabolism from potential signalling effects of ADI independent of enzymic activity is essential in order to determine the mechanism by which ADI affects biofilm formation. A catalytic mutant was generated to address the importance of enzymic activity in the phenotype observed. Catalytic sites, previously determined for ADI from *P. aeruginosa* and *M. arginini*, were mapped to the *S. intermedius* sequence and all known major and minor sites that contribute to catalytic activity or substrate binding of the protein were found to be present (Fig. 1) (Das *et al.*, 2004). Of particular importance is the conservation of the Cys residue at position 399 (GRGGPRCM), which is responsible for the nucleophilic attack within the catalytic site consisting of the Cys–His–Glu triad (Fig. 1) (Lu *et al.*, 2004; Ni *et al.*, 2009; Wang *et al.*, 2007; Wei *et al.*, 2007). To investigate whether enzymic activity is required for the reduction of biofilm formation, we used site-directed mutagenesis to create an

ADI protein containing a Cys to Ser mutation at amino acid 399 (ADIC399S). The mutation has previously been shown to abolish enzymic activity in other ADIs and, importantly, the protein with this mutation was stable and retained the same conformation as the wild-type allele while still binding arginine (Das *et al.*, 2004; Galkin *et al.*, 2005; Lu *et al.*, 2004, 2006).

ADI purification and enzymic activity

The addition of arginine to the growth medium greatly improved the yield of protein expressed in *E. coli*, in agreement with what has been reported by others (Burne *et al.*, 1989). The addition of glycerol and BSA were critical to maintaining the protein at –20 °C without degradation or the loss of activity, and, most importantly, the addition of 2 µM DTT greatly increased enzymic activity. Interestingly, the mutated form of the protein was less prone to degradation and we were able to achieve high levels of recovery, possibly due to its lack of enzymic activity. It was noted that ADI activity is sensitive to redox as it was rapidly lost in a non-reducing environment and a seemingly inactive ADI protein could easily become reactivated upon the addition of DTT. It follows that addition of ‘inactive’ ADI to an anaerobic culture also provides the conditions to regain activity. We propose that reactivation of the enzyme under reducing conditions misled us to previously think that an ‘inactive’ protein can signal *P. gingivalis* (Christopher *et al.*, 2010).

To verify that the tag-less ADI derived from *E. coli* maintained enzymic activity we determined whether it was capable of producing citrulline, using the ADI colorimetric assay described above. Wild-type ADI hydrolysed L-arginine to L-citrulline, while the ADIC399S mutant was unable to hydrolyse the substrate (Fig. 2).

P. gingivalis biofilm formation in the presence of ADI

Previously, it has been reported that ADI's effect on *P. gingivalis* biofilm formation was independent of enzymic activity (Xie *et al.*, 2007). To further explore this finding, we tested the effect of the inactive protein (ADIC399S). As shown in Fig. 3(a), inhibition of *P. gingivalis* biofilm formation is dependent on enzymic activity. When *P. gingivalis* was grown in the presence of the tag-less *E. coli*-derived ADI, there was a 55 ± 2 % reduction in biofilm formation. On average across biological replicates, there was a 62 ± 17 % (*P*-value 0.03) decrease in biofilm formation in the presence of the enzyme (data not shown). This is in contrast with cells grown in the presence of the catalytic mutant (ADIC399S) where the biofilm was indistinguishable from that in control wells (Fig. 3a).

We also took advantage of a commercially available *M. arginini* ADI, which was guaranteed by the manufacturer to have enzymic activity, to determine if there was an effect on *P. gingivalis* biofilm formation by an ADI produced by

Since our results indicated that enzymic activity is necessary for ADI's inhibitory effect on biofilm formation, we proceeded to test the hypothesis that arginine was a

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Fig. 2. Arginine deiminase activity of the native and mutant proteins. Preparations of the *S. intermedius* ADI and the catalytic mutant (ADIC399S) were tested for their ability to hydrolyse arginine. Shown on the left is the reaction catalysed by ADI. The assay detects the end product citrulline. No activity was detected with ADIC399S. Error bars represent the SD of technical replicates (run three times per biological replicate).

biofilm formation (data not shown). There was a more modest increase in the presence of 0.04 % L-arginine with a larger range of induction of 102–293 % (average 149 ± 75 %; *P*-value 0.09) (data not shown). These media conditions were not able to support exponential growth (Fig. S2); however, the cells that formed the biofilm were viable (Fig. 5). These data indicate that L-arginine promotes aggregation and attachment of *P. gingivalis* to the surface.

DISCUSSION

Although there is substantial evidence showing that arginine catabolism plays a central role in the switch to anaerobic and biofilm growth in many host-associated bacteria, there are few data in regard to arginine availability directing biofilm formation (Beenken *et al.*, 2004; Müsken *et al.*, 2010; Zhu *et al.*, 2010, 2007). In agreement with data presented here, arginine has been shown to stimulate biofilm formation of *P. aeruginosa* (Bernier *et al.*, 2011; Caiazza & O'Toole, 2004). The data show that this amino

acid in particular acts as a distinct signal, completely repressing swarming motility over semi-solid surfaces while enhancing biofilm formation (Bernier *et al.*, 2011). To our knowledge, these reports are the only examples of arginine being a biofilm-promoting environmental cue.

P. gingivalis is highly proteolytic; among its battery of extracellular proteases are a number that specifically target arginine. The peptidyl-arginine deiminase citrullinates both free arginine and terminal or internal peptide-associated arginine, and select gingipains are arginine-specific endopeptidases (RgpA and RgpB) that specifically target arginine residues within proteins. (Chen *et al.*, 1992; Endo *et al.*, 1989; McGraw *et al.*, 1999; Rodríguez *et al.*, 2009). Working coordinately with the gingipains is a carboxypeptidase that preferentially cleaves at terminal arginine residues (Masuda *et al.*, 2001, 2002). Hence, arginine appears to be an important amino acid for *P. gingivalis*. The ability of *P. gingivalis* to synthesize arginine has yet to be determined. It does appear to have the ability to make the necessary precursors and a limited number of enzymes that are known in other organisms to participate

Fig. 3. Effect of ADI on *P. gingivalis* biofilm formation. (a) Biofilm assays were performed with the addition of buffer control, ADI, or the catalytic mutant ADIC399S. Biofilm was quantified with safranin staining (reported as A₄₉₂). The addition of ADI reduced the amount of biofilm biomass; however ADIC399S had no effect on biofilm formation. This shows that enzymic activity is required for inhibition. The graph represents one biological replicate. Error bars represent the SD of technical replicates. (b) Effect on biofilm formation of 0.2 mg *M. arginini* ADI ml⁻¹ was compared with buffer control. A reduction in biofilm biomass was detected in wells containing *M. arginini* ADI. This shows that *M. arginini* ADI, a commercially available enzyme, also inhibits biofilm formation. Error bars represent the SD of technical replicates (run three times per biological replicate).

Fig. 4. Biofilm formation by *P. gingivalis* in the absence and presence of L-arginine. Overnight cultures of *P. gingivalis* were pelleted, the supernatant was removed and the cells were resuspended in arginine-free RPMI-S or arginine-free RPMI-S with the addition of 0.04 or 0.4% L-arginine and allowed to incubate for an additional 24 h. Biofilm was quantified with safranin staining (reported as A_{492}). The addition of arginine promoted biofilm formation at both concentrations tested. The graph represents three biological replicates. Error bars represent the SD of technical replicates.

in arginine biosynthesis I (acetyl cycle) are present in the genome, specifically acetylornithine aminotransferase (PG1271) and the carbamoyl-phosphate synthase small and large subunits (PG0529 and PG0530, respectively) (Lewis *et al.*, 2009; Nelson *et al.*, 2003; Xu *et al.*, 2007). Interestingly, using bioinformatics and publicly available databases, genes encoding the enzymes that complete the synthesis of L-arginine from L-citrulline or L-argininosuccinate do not appear to be present. Further studies are required to determine if *P. gingivalis* has a biosynthetic pathway for arginine synthesis. Therefore, by extension, if the bacterium is unable to synthesize its own arginine, sensing and responding to the presence of this amino acid in the environment could be critical to its survival in the oral cavity.

Arginine as a critical signal for biofilm formation, and by extension for commitment to a sessile life style, is an important resource for host-associated bacterial communities. Within the human body, as a whole, arginine is considered to be a limiting nutrient, and the oral cavity is no exception (Brand *et al.*, 1997; Higham & Edgar, 1989; Syrjänen *et al.*, 1990; Téllez *et al.*, 2008; Van Wuyckhuysen *et al.*, 1995). The low levels of L-arginine could be due in part to the activity of ADI once taken up by the oral community. The function of ADI in the oral cavity is

Fig. 5. Images showing the effect of L-arginine addition on *P. gingivalis* biofilm formation. Overnight cultures of *P. gingivalis* were pelleted, the supernatant removed and the cells resuspended in arginine-free RPMI-S or arginine-free RPMI-S with the addition of 0.04 or 0.4% L-arginine. Cell resuspensions were added to Millicell EZ Slide wells and slides were incubated for 24 h. The wells were washed and stained with BacLight live/dead stain. The results show that biofilm formation was promoted by addition of arginine and the cells were viable.

well-documented with regard to its ability to contribute to intracellular ATP pools for the cells and for protection from acidic conditions due to ammonia production (Burne & Marquis, 2000). The gene encoding ADI (*arcA*) is found in the genome of a diverse array of oral bacteria, including *Streptococcus sanguinis*, *S. gordonii*, *Granulicatella elegans*, *Treponema denticola*, *Filifactor alocis*, *Parvimonas micra*, *Actinomyces* sp. and *Lactobacillus brevis*, generally those species that are considered to be less acid tolerant (Casiano-Cólon & Marquis, 1988). With the exception of *G. elegans*, it is not known whether these organisms secrete an extracellular form of ADI. We hypothesize that due to the ubiquity of ADI, as well as of streptococcal species, the *S. intermedius* strain isolated is not unique in its ability to produce extracellular ADI, but the biofilm activity was observed because of the absence of other inhibitory factors being produced by this isolate, such as peroxides and antimicrobial peptides (Christopher *et al.*, 2010). The question of whether these strains secrete ADI is made complicated because protein export in Gram-positive bacteria is not as well understood as that of Gram-negative, and it is still not clear how ADI is brought to the cell surface as there is no canonical LPXTG domain (Navarre & Schneewind, 1999). A notable example is with the heavily studied *Streptococcus pyogenes*. ADI is found on the surface of the cell, however the mechanism by which it is transported and presented is not readily apparent (Cole *et al.*, 2005).

Our studies confirm that enzymic activity is required for the inhibitory effect of ADI on *P. gingivalis* biofilm formation. We also show that addition of arginine enhances biofilm formation, indicating that arginine availability may be an underlying signal controlled by ADI. The role of arginine and the sensing of this amino acid by *P. gingivalis* is an unexplored area of study, but an important one as we start to appreciate the role of arginine and its precursors in the oral cavity in particular as how they relate to uptake and utilization by cells of the host immune system.

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