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Original Article

Identification of a Czc-like operon of the periodontal pathobiont *Porphyromonas gingivalis* involved in metal ion efflux

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ABSTRACT

Objective: The study aimed to investigate the role of the *PGN2012* gene of the periodontitis contributing pathobiont *Porphyromonas gingivalis*. *PGN2012* is a homolgue of TolC and is a gene our group previously showed was overexpressed in hyperinvasive cells.
Methods: The study used a combination of bioinformatics, knockout mutagenesis, growth experiments, biofilm assays and human cell invation assays to investigate *PGN2012* function.
Results: Bioinformatics identified that PGN2012 is part of one of four TolC containing gene loci in *P. gingivalis* that we predicted may encode a metal resistance RND family tripartite pump, similar to those present in other Gram-negative bacteria, but which are not well understood in anaerobic bacteria. A *ΔPGN2012* deletion displayed slightly reduced growth in liquid culture but did not effect biofilm formation or human cell invasion. When metal ions were included in the medium the mutant displayed significantly increased sensitivity to the divalent metal ions Zn^{2+} (500 μM), Co^{2+} (2 mM), and Cd^{2+}(0.1 mM) but not Cu^{2+}.
Conclusions: We propose to rename the *PGN2012-2014* genes *czcCBA*, which we suggest plays a role in intracellular stress resistance where zinc is often employed by host cells in antibacterial defence with implications for chronic infection in humans.

1. Introduction

The Gram negative anaerobe, *Porphyromonas gingivalis* is considered to be a keystone pathogen in human periodontitis [1], a condition with a global incidence affecting 10% of the world's population [2]. Periodontitis encapsulates a range of conditions characterised by inflammation of the gingiva, gum recession and bleeding as well as influencing turnover of jaw alveolar bone [3]. This can ultimately lead to tooth loss and is also increasingly associated with a range of systemic inflammatory conditions such as cardiovascular disease, arthritis and Alzheimer's disease [4–6].

In the sub-gingival pockets that form during periodontal disease, heterogenous biofilms develop in which bacteria interact with the subgingival tooth surfaces, with each other and with human epithelial cells, as well as with underlying layers and immune infiltrates [7]. During these interactions it is the cell surface and functions therein that influence both how the bacteria live under these conditions but also how they stimulate responses from cells within the gingival tissue. Bacterial cells in this sub-gingival niche, including *P. gingivalis*, are exposed to an ever-changing array of environmental compounds, chemicals and conditions, with the understanding of these being key to understanding disease [8].

In this context, our group identified a range of bacterial genes that were altered in their expression levels in a subset of the *P. gingivalis* population that show a 'hyper-invasive' phenotype, i.e. invade and internalise in human epithelial cells at an increased rate [9]. We hypothesised that a number of these genes might be key to interactions with human cells, e.g. OmpA [10]. Many of these genes encode cell surface proteins present or which seem to be involved in transport of metal ions which may have an impact on responses to toxic compounds in their environment and also be related to the ability of metal ions to generate oxidative stress in bacterial cells [11].

One of these genes was *PGN2012* (previously annotated as *PG0063*), which was >6-fold upregulated in the hyperinvasive subtype [9]. *PGN2012* encodes a putative outer membrane protein that is predicted to be the periplasmic channel component of a putative HME (Heavy Metal Efflux) type RND (Resistance Nodulation Division) transport system in *P. gingivalis* [9]. In Gram-negative bacteria

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these are used for the export of a range of compounds including toxic molecules like divalent metal ions [12], ethidium bromide [13], but also have a key role in antibiotic resistance to beta-lactams and macrolides [14], while also having the ability to secrete proteinaceous toxins such as hemolysins [15]. They are often referred to as tripartite transport systems, made up of a TolC-like trimeric channel protein embedded in the outer membrane that spans the periplasmic space while also interacting with an inner membrane transporter and a fusion protein to form an active transport complex (Fig. 1A) [16]. They are present in a range of species that includes prominent pathogens such as *E. coli* and *Pseudomonas aeruginosa* as well as soil bacteria (e.g. *Rhizobia* spp.) where they are involved in influencing the nodulation of leguminous plants [17]. For the RND-family, energy for the process is thought to derive from Proton motive force [18] while ATP is used in TolC-dependent hemolysin type systems that require an ATP binding cassette (ABC) [19]. Of these RND family systems, it is the antibiotic resistance AcrAB-TolC and MexAB-OprM pumps of *E. coli* and *P. aeruginosa* that are the most intensely studied [20]. In addition to the known substrates, many of these pumps have roles in transport of compounds affecting virulence, e.g. bile salts, peptides, that seem to influence pathogenicity in animal models [21].

Given their importance in many bacterial pathogens, we investigated the role of one of these TolC-like pump systems in the periodontal pathobiont *Porphyromonas gingivalis*. Specifically, the *PGN2012* gene cluster, which we previously showed to be associated with a hyperinvasive bistable subtype of *P. gingivalis* [9]. In this paper we show that the *PGN2012* gene is part of a gene locus involved in resistance to a selection of heavy metal cations and discuss the implications on host-pathogen interactions and the biology of *P. gingivalis.*

2. Materials and methods

2.1. Bacterial growth conditions

Porphyromonas gingivalis ATTC 33277 was routinely cultured on fastidious anaerobe agar (FAA) plates supplemented with 10% horse blood (Oxoid) or in liquid broth (BHI-A broth supplemented with 0.5% yeast extract, hemin (1 mg/mL), cysteine (250 μg/mL) and vitamin K (1 mg/mL)) in an anaerobic cabinet (Don Whitley Scientific miniMACs, 10% CO_2, 10% H_2 and 80% N_2) at 37 °C. Culture medium was supplemented with erythromycin (5 μg/mL) as required. *Escherichia coli* DH5α (cloning strain) was cultured at 37 °C on Luria-Bertani Broth (LB) agar plates or in Luria Bertani broth at 37 °C (250 rpm) and supplemented with ampicillin (50 μg/mL) as required.

Antibiotic sensitivity to Amoxicillin, Metronidazole, Ampicillin and Vancomycin was estimated using E-test strips from Biomerieux according to manufacturer's instructions on FAA plates (see Fig. S4 for details).

2.2. Construction of *P. gingivalis ΔPGN2012* deletion strain

A deletion construct where the coding sequence of PGN2012 was replaced by an erythromycin (Ery) cassette was designed *in silico* and produced by GeneArt (ThermoFisher) and flanked by 500bp of PGN2012 flanking DNA. This construct was then blunt-cloned into the plasmid pJET1.2 (Ap^R) in *E. coli* DH5-alpha, before being transferred into *P. gingivalis* ATTC 33277 via natural competency with selection on FAA-Ery plates (FAA agar with erythromycin (5 μg/mL), essentially as described by Ref. [10]. Colonies were initially checked for the correct insertion of the Ery cassette via PCR using primers flanking the insertion site (PGN2012_FOR:GGGCAATGACTGTATTACAGA and PGN2012_REV: GCTGCTTTTCAATCAGTTCCG) in concert with primers internal to the Ery cassette(Ery_FOR:ATGACAAAAAAGAAATTGCCC and Ery_REV:CTACGAAGGATGAAATTTTTC).

Mutants were further confirmed using Nanopore sequencing to establish not only the correct insertion site, but also to probe for any other rearrangements in the genome. In brief, flow-cells were mounted in a MinION in concert with a MinIT device (Oxford Nanopore Technologies). Extracted DNA was purified, quantified (Qubit, Invitrogen) using the ligation sequencing kit and Flow cell

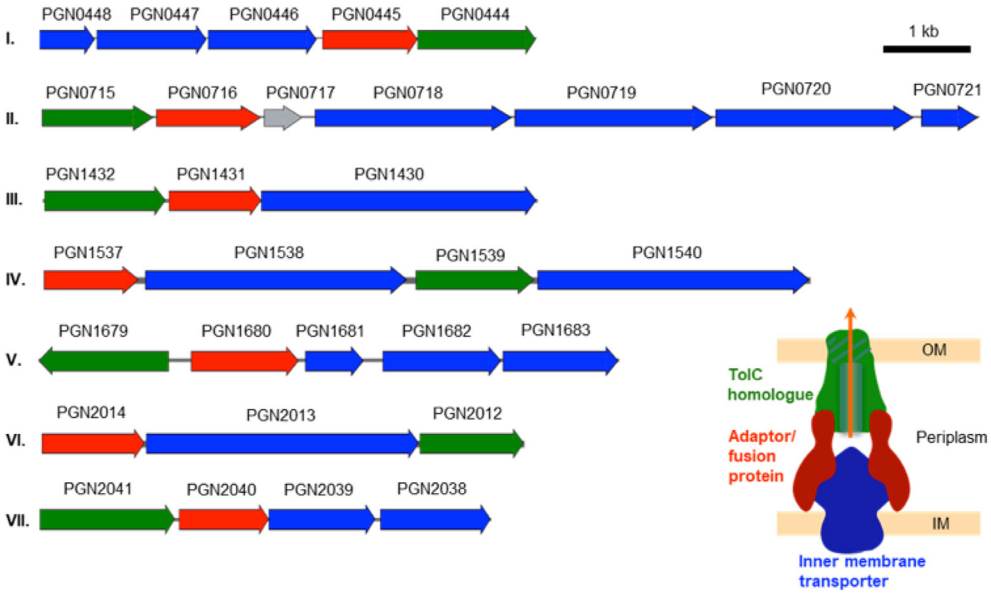

Fig. 1. TolC homologues and their operons in *P. gingivalis*. Seven TolC homologues and their related operons (to scale, bar = 1 kb) were identified by a Pfam screening of the full genome of *P. gingivalis* ATTC33277. TolC channel protein and gene homologues are colour coded green, Adaptor/membrane fusion (RND) proteins Red, and inner membrane transporters blue. IM= Inner Membrane, OM= Outer Membrane. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

priming kit as per manufacturer's instructions. Extracted fastQ files were aligned to the genome of *P. gingivalis* ATCC33277 and viewed using IGV viewer. The nanopore data was also assembled into contigs using CANU and RACON, CGView (https://cgview.ca/); was used for whole genome BLAST comparisons and genome diagrams generated with SnapGene (SnapGene software (from Insightful Science; available at snapgene.com). The sequence of *P. gingivalis* ATCC 33277 was analysed using the InterProScan-PFAM search tool at Galaxy-EU (https://usegalaxy.eu/root?tool_id=interproscan).

2.3. Protein and Western blot analysis

The total protein profiles of *P. gingivalis* and the PGN2012 strain were analysed by resuspending pellets from liquid cultures ($OD_{600} = 1.0$) in 2x SDS loading buffer before SDS_PAGE analysis by Coomassie Blue staining (FastBlue) or Western blot. Western blot analysis was performed by transferring the proteins to nitrocellulose paper (GE healthcare life science) by semi-dry transfer using a Biorad Trans-blot semi dry transfer cassette (Biorad). The transferred proteins were probed for using the gingipain specific mab1b5 gingipain primary antibody [22], alongside an anti-mouse IgG horse radish peroxidase (HRP) conjugated secondary antibody. The HRP was activated using enhanced chemiluminescence (ECL) solution (Thermo Scientific), and fluorescence visualised using a LICOR luminescence scanner (LICOR).

2.4. Biofilm growth and assessment

P. gingivalis overnight liquid cultures (BHI-A) were diluted to OD_{600} 0.05 and innoculated into a 96 well plate (Greiner). The bacteria were then grown in BHI-A under anaerobic conditions for 72 h at 37 °C with total growth assessed using a TECAN infinite M200 plate reader. The wells were gently washed with PBS (0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4) 3 times and stained with 100 μL 0.1% crystal violet for 20 min. The excess stain was then removed, and the stained biofilms were washed until the PBS became clear before being left to air-dry. The crystal violet in the biofilms was solubilised using 100 μL ethanol: acetone (80:20) solution and transferred to a new 96 well plate. The crystal violet was quantified by measuring the absorbance at 575 nm using the TECAN plate reader. The biofilm formation was standardised against growth by dividing the crystal violet absorption by the OD_{600} [23].

2.5. Anaerobic metal ion sensitivity growth experiments

Overnight cultures of *P. gingivalis* were adjusted to OD_{600} 0.1 in BHI-A or BHI-A supplemented with different concentrations of metal salts that were determined experimentally during this study ranging from 0.01 mM to 5 mM as indicated (all from Sigma-Aldrich). The samples were prepared in triplicate for each metal concentration and placed in the wells of a 96 well plate (Greiner) and conducted on at least three separate occasions. The 96 well plate was placed inside a Cerillo Stratus plate reader (Cerillo, Virginia) within an anaerobic incubator. The plate was incubated anaerobically, and the samples blanked, the OD_{600} readings were taken every 30 min for up to 5 days. Statistical difference was measured using a multiple comparisons ANOVA of the growth curves and Students t-test of specific time points in GraphPad Prism.

2.6. Antibiotic protection assay

All antibiotic protections assays were carried out as in Ref. [10]. Briefly, H357 tongue OSCC cells were seeded at 8×10^4 cells/mL in 1 mL of DMEM (Dulbecco's Modified Eagle's Medium) per replicate and incubated in two 24 well-plates (Greiner) at 37 °C, 5% CO_2 until 70–80% confluent (24–48 h) and gently washed with PBS before blocking with 1 mL DMEM supplemented with 2% bovine serum albumin (BSA) for 60 min at 37 °C, 5% CO_2. 3-day old *P. gingivalis* were taken from agar plates and resuspended in DMEM before counting to achieve a multiplicity of infection (MOI) 1:100. Bacteria were added to OSCC cells for 90 min at 37 °C (5% CO_2). Control wells with bacteria (Wild type or mutant) only were used as controls to enumerate total bacteria. To assay invasion, bacteria and media were removed, cells washed and DMEM supplemented with 200 μM metronidazole (previously established to kill all *P. gingivalis* [9]) for 1 h before washing again. Cells were then lysed by addition of 200 μL of deionised water and scraping before serial dilution of released bacteria and enumeration by Miles and Misra serial dilution spot (5 μL) plating on FAA plates anaerobically for 7 days [24]. The viability wells only containing bacteria were also serially diluted and spotted onto FAA plates in the same manner without the addition of water or scraping. Adhered but uninvaded bacteria were enumerated by subtraction of the invaded from the total bacteria. Data were analysed in GraphPad Prism.

2.7. Whole cell gingipain protease activity assay

The activity of the two different types of gingipain proteases; arginine and lysine were investigated using either an arginine or lysine substrate attached to the fluorescent 7-amido-4-methylcourmarin, the fluorescence of which can be measured once cleaved from the substrate. Overnight cultures of *P. gingivalis* were adjusted to 0.01 with BHI-A. 1 mL of OD_{600} before resuspension in PBS. To assay activity 50 μl of resuspended cells was incubated with either 1) Arginine gingipain: 100 μLPBS containing 1 mM L-cysteine, 200 μM αN-benzoyl-L-arginine-7-amido-4-methylcourmarin (room temperature) for 5 min in triplicate in a 96 well plate. The reaction was then quenched with the addition of 50 μLof 200 μM N-α-tosyl-L-phenylalanine chloromethyl ketone (TPCK), before the fluorescence of the released methylcoumarin measured with the excitation and emission 365 nm and 460 nm. Or 2) Lysine gingipain: 100 μLPBS containing 1 mM L-cysteine, 10 μM D-Ala-Leu-Lys-7-amido-4-methylcoumarin (Merck) at 37 °C for 5 min and quenched with TLCK and substrate release measured with excitation and emission 365 nm and 460 nm.

3. Results & discussion

3.1. PGN2012 as a putative HME-family member in P. gingivalis

In our study by Suwannakul et al. (2010) we identified a set of genes that were differentially regulated in a hyper-invasive subpopulation of *P. gingivalis*. One of these was *PGN2012* (formerly annotated at *PG0063*), located within a predicted RND-superfamily gene operon (Fig. 1). Within the *P. gingivalis* ATCC 33277 genome there are 7 predicted RND-family loci, compared to enteric bacteria such as *E. coli* which have only one TolC homologue, but which contain several putative inner membrane adaptor/transporter partners (e.g. *emrAB*, *acrAB*, *hly* genes) [25]. However, other gram-negative bacteria, for example *P. aeruginosa,* have 18 putative TolC like outer membrane efflux proteins with specific functions and substrates [26].

Upon further analysis, the seven operons (I-VII, Fig. 1) predicted to encode TolC-like homologues (Pfam, Galaxy-EU) are located with loci that also contain predicted permeases transporters (blue) and membrane fusion protein homologues (red) and in some cases ABC (ATP-Binding Cassette) proteins (Orange). Of these seven loci, three contain homologues of Hemolysin export type (HlyD) genes (red,

locus II, V and VII), suggesting the presence of putative Type-I protein secretion systems that *P. gingivalis* may utilise to secrete proteinaceous substrates, such as toxins [15] or enzymes [27]. It is likely that locus III (PGN1430-2/*xepCAB*), which contains an AcrB/D-TolC-permease triad is involved in export of a range of toxic compounds as it was found by Ref. [28] to be overexpressed under excess hemin conditions [28], while [29] showed that mutants in the genes of this locus had altered sensitivity to ethidium bromide and a range of antibiotics, including ampicillin, rifampicin and norfloxacin [29,30]. Similar findings were shown for locus V mutant also (PNG_1680, TolC-RND-Permease-permease), while mutants in locus VI only showed a mild alteration in Tetracycline sensitivity [30]. Of the other loci, locus I contains a fusion protein with homology to both CusC (copper efflux) and HlyD, alongside two MacB-like permeases, and an ABC protein (suggesting small molecule periplasmic substrates), while cluster IV contains homologues of the AcrB/D proteins, again suggesting potential small molecular substrates, but currently there is no knowledge of substrates for any of these.

In contrast, locus VI (containing PGN2012) is predicted to be a member of the HME-subfamily, potentially involved in heavy metal transport, and it is this gene that was up-regulated by > 6-fold in our original dataset [9]. Of this family, probably the best understood are those involved in efflux of cadmium, zinc and cobalt ions, known as czc operons [12]. In these systems the TolC-like pump (CzcC) forms a channel that interacts with the membrane fusion partner (CzcB) and an inner membrane ion transporter (CzcA) [31], forming part of a locus with the genes in the order, PGN2014-2012. We assume that these are transcribed as a unit given their operon structure and since the preceding and subsequent genes at both ends are unrelated and that no internal promoters are present in the sequence.

In addition, the PGN2013 gene has strong homology to CzcA type proteins with the presence of conserved residues with members of the HME3 and 5 groups (AIG-X3-DDA-X3-VEN) [12]. Finally, a structural alignment of PGN2012 using the Phyre2 structural prediction server produces a high scoring match with TolC homologues (not shown) [32]. From these analyses, we predicted that PGN2012 is a TolC like channel protein that interacts with PGN2013 the CusA/CzcA pump via further interactions with PGN2014.

3.2. Growth and host-cell interactions of a PGN2012 mutant strain

In order to establish the role of *PGN2012* (Locus VI) in virulence and physiology of *P. gingivalis* we performed deletion mutagenesis of the *PGN2012* gene. We chose the TolC component of the cluster as it is essential for efflux in other systems, and as it was the third gene of the predicted operon it is unlikely to cause any polar effects on the genes preceding it. As outlined above, we synthesized a construct (Supplementary Fig. S1), where the *PGN2012* gene was replaced by the *ermF* erythromycin resistance gene as transformed cells using natural competency as in previous work [10]. The correct insertion of the *ermF* gene was checked by PCR (Supplementary Fig. S1) as well as via nanopore sequencing. Nanopore sequencing was performed with chromosomal DNA from *ΔPGN2012* and reads were mapped onto the reference genome of *P. gingivalis* (ATCC3277, NC01729.1) (Supplementary Fig. S2); indicating a clean knockout with no reads mapping to the absent PGN2012 gene, while mapping onto the *ermF* cassette. In addition, a *de novo* assembly was performed using Canu, showed that no erroneous recombination had occurred in this region, but 10 contigs which mapped to regions of the reference genome. Having established that this mutagenesis was achieved we moved on to assess the phenotype of this mutant.

As many TolC-RND systems transport a range of substrates, i.e. are promiscuous transporters, including intracellular toxic compounds [33], we first tested the fitness of the Δ*PGN2012* strain in standard BHI-A media (Fig. 2a). A 72-h growth experiment revealed that Δ*PGN2*012 has a marginally slower exponential phase growth rate (m = 311 min) compared to wild type (m = 305 min, $P < 0.05$, n = 3). There was also a statistically significant decrease in optical density for Δ*PGN2012* between 20 and 44.5 h (<0.05).

Efflux pumps have also been shown to influence biofilm formation in other bacteria through the efflux of quorum sensing inducers and or EPS substrates [34]. As biofilms are also essential for the progression of periodontitis, we investigated the effect of knocking out *PGN2012* on biofilm formation of *P. gingivalis* on polystyrene surfaces. As illustrated in Fig. 2b, biofilm formation of Δ*PGN2012* did not change quantitatively or microscopically (Supplementary Fig. S3) when compared to the wild type.

One of the most well-studied virulence aspects of *P. gingivalis* are the secreted gingipain proteases. Given the observed alterations in growth characteristics and their role in nutrient acquisition [35] we next tested whether the gingipain levels may be altered in the *PGN2012* mutant strain. We performed whole cell protein profiling, gingipain assays as well as Western blots using an anti-gingipain antibody (mab1B5, [22]); illustrating that the PGN2012 mutant had no alterations in gingipain expression or activity compared to the Wild type parent (Fig. 2c and d).

In our study by Suwannakul et al. (2010) *PGN2012* was one of the genes found to be overexpressed in a hyper invasive subpopulation, suggesting a role in the invasive capabilities of *P. gingivalis*. To investigate this antibiotic protection assays were performed. Fig. 2e shows the results of the antibiotic protection assays, demonstrating no significant change in total association, adhesion, or invasion. Therefore, these results suggest that Δ*PGN2012* does not have a specific role in the adhesive and cell internalisation abilities of *P. gingivalis* but do not exclude a role in epithelial intracellular survival or resistance to other intracellular toxic compounds.

3.3. PGN2012 knockout mutation reveals no changes in sensitivity to clinically relevant antibiotics

As RND efflux systems are well documented for their role in multidrug resistance, we first assessed the antibiotic sensitivity profile of our wild-type and *PGN2012* mutant (except for erythromycin, used to make the *PGN2012* strain). Given the previous work by Ref. [30]; which investigated the knockout of *PGN2014* (third gene in this operon) and its effect on antibiotic resistance to tetracycline, ampicillin, rifampicin and norfloxacin, we did not expect large changes to be observed. As a preliminary screen we used E-test strips (0.016−256 μg/mL), which would be followed up if any changes were observed with liquid MIC assays. For these tests we used the most clinically relevant antibiotics used in the UK in dentistry, namely Metronidazole, Amoxicillin, Ampicillin and as a negative control Vancomycin [36]. As shown in Supplementary Fig. S4, our data show no differences in sensitivity to these drugs, with high levels of sensitivity (<0.016μg/mL) to Metronidazole, Amoxicillin and Ampicillin in these tests and higher levels of 2 μg/mL for Vancomycin. In summary, *PGN2012* does not play a role in resistance to the most commonly used dental antibiotics in the UK.

3.4. The PGN2012 gene plays a role in altered sensitivity to metal ions

Given the predicted function of the *PGN2010-2012* operon we next assessed whether this operon may represent a novel metal resistance operon in *P. gingivalis*.

To investigate the effect of this mutation on efflux, minimal

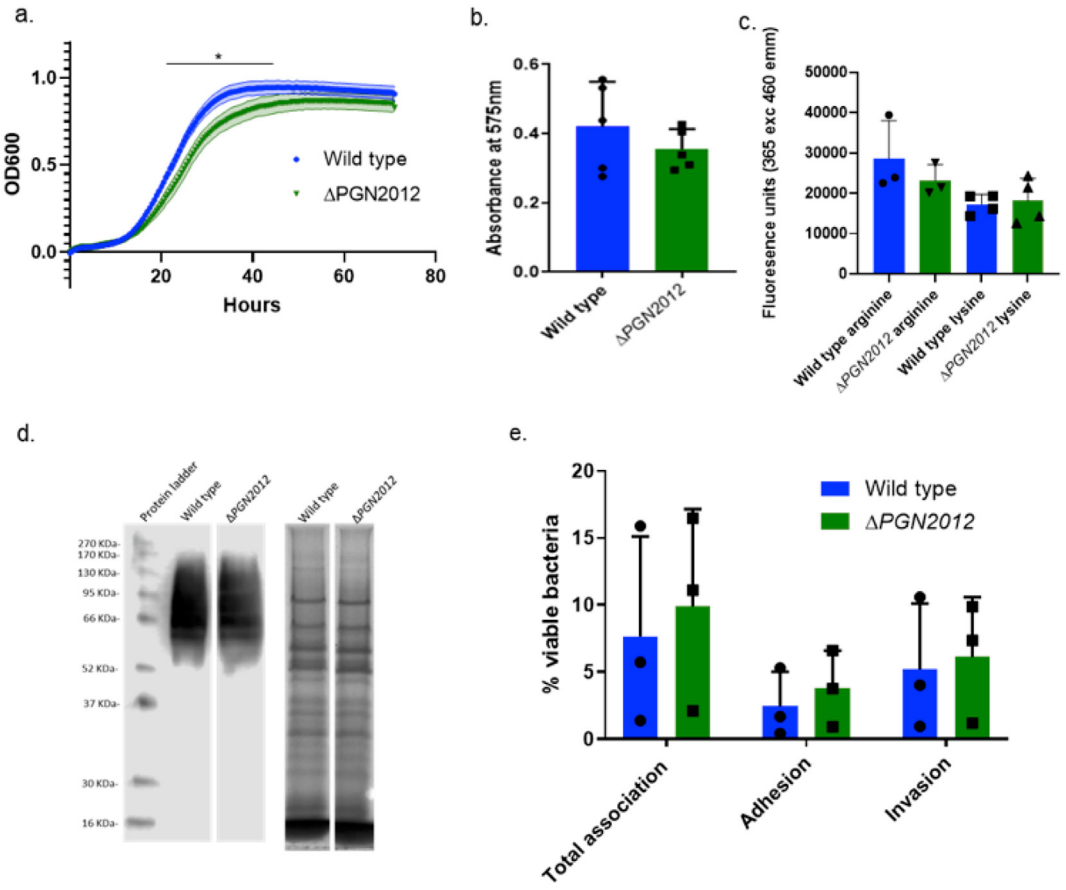

Fig. 2. Characterisation of the Δ*PGN2012* mutant. a. Anaerobic growth comparison of wild type and Δ*PGN2012 P. gingivalis*. Wild type and Δ*PGN2012 P. gingivalis* were grown in a Cerillo Stratus anaerobically with samples taken every 30 min. N = 3 with 9 technical replicates per biological repeat, shaded areas represent±SEM made in Graphpad prism. Statistical analysis was analysed by 2-way ANOVA comparing the difference of each time point. *p < 0.05. **b. Biofilm formation assay.** Biofilms were grown in polystyrene 96-well plate anaerobically at 37 °C for 5 days. The OD_{600} was measured using a TECAN plate reader before the biofilms were washed and stained with crystal violet. The crystal violet was measured at 575 nm using a TECAN plate reader and normalised to growth. Statistical significance was determined by a Students t-test. **c. Gingipain activity.** Overnight cultures of *P. gingivalis* adjusted to OD_{600} 0.01 in PBS, were incubated with arginine or lysine bound methylcoumarin, for 5 min before the reaction was quenched in the linear phase and the fluorescence read at 365 excitation and 460 emission. Error bars represent±SEM. Statistical analysis was performed using a t-test. N = 3 for arginine and N = 4 for lysine each including triplicates of technical repeats. **d. Western blot for gingipains.** Overnight liquid cultures were pelleted and boiled in SDS-lysis buffer before analysis by SDS-PAGE and transferred to nitrocellulose and probed with the mab1b5 antibody and HRP-anti-mouse IgG. Visualisation was via ECL reagent (Thermofischer) and a LICOR luminescence scanner. **e. Antibiotic protection assays**. Performed as described in methods, N = 3 (3 repeats per experiments). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

inhibitory concentration experiments were performed over 3 days to reveal a complete growth curve and investigate whether Δ*PGN*2012 had become more susceptible to different metal ions. Since our bioinformatic analysis revealed that *PGN2012* was homologous to members of the HME family of RND tripartite systems we tested susceptibility to ions relevant to both Czc and Cus systems [12,37], namely copper (Cu^{2+}), cadmium (Cd^{2+}), cobalt (Co^{2+}), zinc (Zn^{2+}), all in the form of chloride salts.

As shown in Fig. 3 we performed these analyses in two ways, first by measuring growth by OD_{600} after 3 days at concentrations of the metal ions calibrated around experimentally derived estimated MICs. These data were probed further by performing growth curves of *P. gingivalis* and the mutant under these same conditions. In all cases data are from three independent experiments and indicate increased sensitivity of the *PGN2012* mutant to zinc, cadmium, and cobalt, but showed no altered response to copper in the rangs tested, with estimated MIC reductions corroborating this.

In the case of zinc Chloride, the data (Fig. 3a) showed a statistically significant increase in sensitivity to zinc ions at 0.5 mM, with an estimated 2-fold higher MIC for Zn in the wild-type of 1 mM vs 0.5 mM for the mutant strain. Similarly, growth curves performed at 0.5 mM showed that the wild type had a mean generation time of 17.9 h vs over 100 h for the mutant and reached a final OD_{600} of 0.45 whilst Δ*PGN2012* does not grow above 0.11. Detailed analysis shows a statistically significant difference in growth between 91 h and 132 h ($P < 0.05$). For cobalt at 2 mM, the growth curves (Fig. 3c) illustrate a clear and statistically significant reduction in growth in Δ*PGN2012*, with no growth of Δ*PGN2012* until 105 h. When tested at 1 mM (below MIC) there was still evidence of increased sensitivity (Supplementary Fig. S5), with the exponential growth significantly slower in Δ*PGN2012* ($P < 0.05$) with a generation time of 11.3 h in comparison to the wild types 4.6 h.

Next, sensitivity to cadmium was assessed (Fig. 3b), here the sensitivity of the mutant for Cd toxicity was increased over a range of concentrations, and significantly at 0.1 mM, with estimated MIC of approximately 0.2 mM vs < 0.05. Again, growth curves at different concentrations were used to probe more deeply, revealing that at both 0.05 mM (μ(WT) = 7.95 vs 17.5) and 0.1 mM (μ = 9.5 vs 65 h) growth of the mutant was significantly impaired vs the wild type. Taken together these data all indicate increased sensitivity to metal ions of Co, Cd and Zn, but also illustrate the need for detailed growth curve analysis vs simple one point MIC experiments.

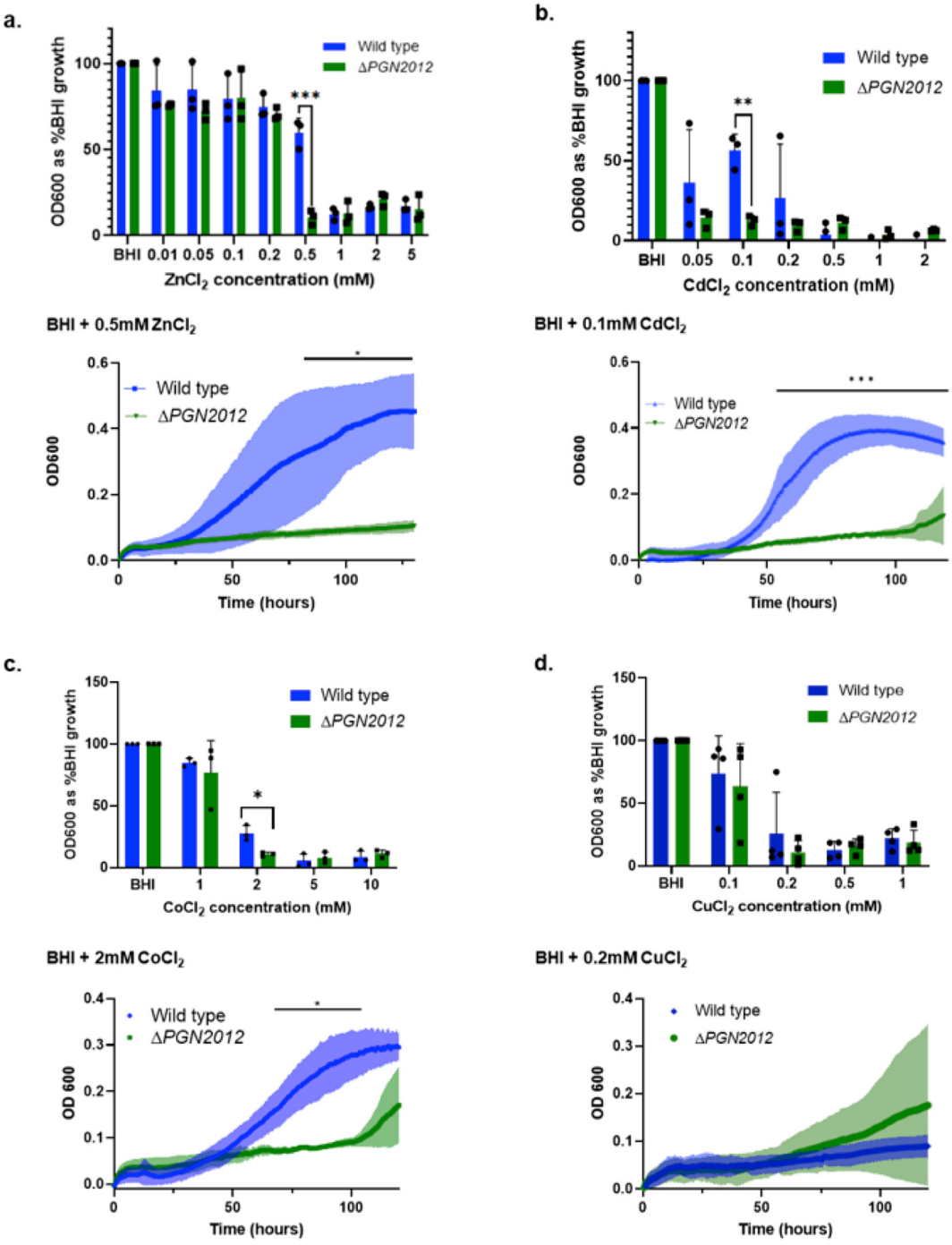

Fig. 3. Growth of wild-type and Δ*PGN2012* in media containing metal ion salts. In all cases overnight cultures of wild type and Δ*PGN2012* were pelleted and resuspended at a starting OD_{600} of 0.1 into BHI Supplemented with the concentration of metal salt indicated. Cultures were incubated anaerobically at 37 °C, with the OD_{600} being measured every 30 min by a cerillo stratus plate reader. For each of **a.** $ZnCl_2$, **b.** $CdCl_2$, **c.** $CoCl_2$ and **d.** $CuCl_2$; we show growth comparisons (akin to MIC assays) at 72 h (upper) as well as a growth curve of the two strains at the concentration around the MIC. In all cases N = 3 or 4 with 3 technical repeats to each biological repeat with shaded areas representing±SEM. Statistical analysis was performed by *t*-test (upper) and multiple comparisons ANOVA (lower). P < 0.05*.

Finally, given that the conserved region of the PGN2012 partner transporter, *PGN2013* contained signature residues in common with the copper transporter CusA as well as CzcA, we also tested sensitivity to copper ions (in the form of $CuCl_2$). In contrast to the data on Co, Cd and Zn, copper had no differential effect on the PGN2012 mutant in either MIC or growth curve experiments (Fig. 3d). In addition, since all experiments were also conducted in the presence of chloride salts, these data also confirm that this is not causing any of the effects observed.

This is the first paper that has investigated an HME-RND efflux pump in *P. gingivalis* in the context of metal ion transport and resistance. However, heavy metal efflux pumps have been well documented in other Gram negative bacteria such as *R. metallidurans* [31], *E. coli* [38], *Salmonella typhimurium* [39], and

Pseudomonas aeruginosa [40,41]. Our data suggest that the *PGN2012* system is most similar to that of CzcCBA originally identified in *R. metallidurans,* with resistances to zinc, cadmium and cobalt ions. The greatest sensitivity came from Zn^{2+} and Cd^{2+} with sensitivities of 0.5 mM and 0.1 mM respectively. Whereas the sensitivity to cobalt was only seen at the higher concentration of 2 mM, indicating a greater sensitivity to zinc and cadmium than cobalt in *P. gingivalis*. In contrast to this, the initial studies of CzcCBA by Mergeay et al. [42] found that for *R. metallidurans* the resistance provided by the two plasmids containing CzcCBA and Cnr were highest for zinc and cobalt, with cadmium having the least resistance.

The question that follows here is what the role of a heavy-metal ion efflux pump of this type has in *P. gingivalis* and its environment in the oral cavity and during its pathogenesis. The first query relates to what the concentration of zinc might be in plaque and the oral cavity. Several studies have estimated that the zinc concentration in plaque is between 0.1 and 0.8 mM [43,44] while that in saliva is in a similar range of 0.6 mM [45]. Of course, during intake of foodstuffs, the levels of zinc in the mouth and local niches in which *P. gingivalis* inhabits might fluctuate meaning the ability to resist these changes in concentration might impart a competitive advantage. In the context of this study, one might ask why transcription of a czc operon was upregulated in a hyper-invasive subpopulation. While we have no evidence at present, in other organisms *czc* operon expression is induced by increased levels of zinc in the intracellular environment [46–48]. In addition, there is a body of evidence that zinc is part of the human bodies line of defence, with zinc being released intracellularly as an innate immune response of macrophages and neutrophils as well as present in increased levels at mucosal surfaces during infection in what is sometimes called nutritional immunity [49–52]. Hence one might hypothesise a situation where increased levels of czc gene expression occur as a response to intracellular release of zinc as is the case for *S. typimiurium* and UPEC *E. coli* during infection [48,53]. In fact, *czc* efflux systems are one of a myriad of bacterial efflux proteins that also includes Gram positive CzcD and Gram negative ZntD pumps [51].

When efflux gene lacking mutants of bacteria such as *E. coli* K12 and *Streptococcus pneumoniae* and *S. pyogenes* have been tested in virulence assays they have not only shown increased sensitivity to Zn ions but also reduced virulence [48,54–56]. Such antibacterial effects of Zn may be direct via replacement of other ions such as Manganese, or indirect via generation of oxidative stress [49]. There is evidence that RND pump expression does respond to oxidative stress such as the case of SoxS induction of AcrAB in *E. coli,* and RND pumps are in some cases involved in oxidative stress resistance [57–59]. One then can thus postulate that *Porphyromonas* may also employ czc operon-based Zn efflux as a strategy to improve survival at the mucosal interface or during challenge by neutrophils or during the intracellular part of its life cycle inside epithelial cells. This is an area for future work and presents the exciting opportunity of zinc supplementation to reduce bacterial loads in subgingival plaque. Several studies have indicated that this may be a successful strategy with inhibition of *P. gingivalis* epithelial cell interaction and damage reduced by zinc [60] as well as effects on biofilm formation in vitro [61].

3.5. Conclusion

In this paper we report the investigation of the *PGN2014-2012* containing operon, which we propose here to rename *czcCBA* and specifically study a mutant lacking the outer membrane efflux component. This strain had increased sensitivity to zinc, cadmium, and cobalt ions, with its resistance to zinc being the most physiologically relevant in terms of survival in the human body and reinforces possibilities for use of zinc as a potential adjunct to periodontal therapy.

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Author contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Dr Ashley Gains. The first draft of the manuscript was written by Prof Graham Stafford and Dr Ashley Gains and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Declaration of competing interest

We have no conflicts of interest to declare.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.anaerobe.2023.102696.

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