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Role of de-N-acetylase PgaB from *Aggregatibacter actinomycetemcomitans* in exopolysaccharide export in biofilm mode of growth

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SUMMARY

Aggregatibacter actinomycetemcomitans, a Gram-negative bacterium, is the causative agent of localized aggressive periodontitis. Attachment to a biotic surface is a critical first step in the *A. actinomycetemcomitans* infection process for which exopolysaccharides have been shown to be essential. In addition, the *pga* operon, containing genes encoding for biosynthetic proteins for poly-N-acetyl glucosamine (PNAG), plays a key role in *A. actinomycetemcomitans* virulence, as a mutant strain lacking the *pga* operon induces significantly less bone resorption. Among the genes in the *pga* operon, *pgaB* codes for a de-N-acetylase that is responsible for the deacetylation of the PNAG exopolysaccharide. Here we report the role of PgaB in regulation of virulence genes using a markerless, scarless deletion mutant targeting the coding region of the N-terminal catalytic domain of PgaB. The results demonstrate that the N-terminal, catalytic domain of PgaB is crucial for exopolysaccharide export.

Keywords

Oral pathogen; biofilm; exopolysaccharide; deacetylation; export; virulence

INTRODUCTION

Surface exopolysaccharides are one of the major components of the microbial biofilm matrix and are made up of various sugars depending upon the associated bacterium.¹

Exopolysaccharides in a bacterial biofilm matrix hold cells tightly together, helping to evade immune defenses and facilitating resistance against antimicrobial agents.² Among the oral bacteria, *Aggregatibacter actinomycetemcomitans*, a bacterium associated with localized aggressive periodontitis,³ forms tenacious biofilms that can attach to abiotic as well as biotic

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CONFLICT OF INTEREST

Dr. Pier has numerous patents on the PNAG molecule licensed to Alopexx Vaccine and Alopexx Pharmaceuticals.

surfaces.⁴ The exopolysaccharide produced by *A. actinomycetemcomitans* is a homo-polymer of $\beta(1,6)$ -linked *N*-acetyl-D-glucosamine (GlcNAc; it will be referred to as PNAG (also referred to previously as PIA/PGA)) and is functionally and chemically closely related to exopolysaccharides produced by *Actinobacillus pleuropneumoniae*, *Escherichia coli*, *Staphylococcus aureus* and *Staphylococcus epidermidis*.⁵ In *A. actinomycetemcomitans*, the synthesis, modification and export of PNAG is controlled by proteins encoded in a four gene operon, *pgaABCD*.^{6, 7} The exopolysaccharide biosynthetic proteins are encoded by the *icaADBC* operon in staphylococcal species^{8, 9} and, as in *A. actinomycetemcomitans*, by *pgaABCD* in *E. coli* and other Gram-negative bacteria.¹⁰ These genes encode for a porin like molecule (PgaA), a de-N-acetylase (PgaB) and an N-acetylglucosaminyl transferase (PgaC/D) in *E. coli*.^{10, 11} Individually, each of the four genes in *A. actinomycetemcomitans* has been shown to be critical for the synthesis of PNAG.¹²

Among these four proteins, PgaB of both *E. coli* (*EcPgaB*) and *A. actinomycetemcomitans* (*AaPgaB*), and IcaB of *S. epidermidis* (*SeIcaB*), possess a de-N-acetylase domain,^{11, 13–15} which removes the N-acetyl groups from GlcNAc residues that make up the exopolysaccharide.^{10, 16} The proteins PgaB/IcaB are interesting since they confer positive charges to PNAG to promote PNAG association to the cell surface lipopolysaccharides in *A. actinomycetemcomitans*¹² or in *S. aureus*.¹⁷

The enzyme PgaB acts on the exopolysaccharide polymer either during secretion into the periplasm¹⁸ or after secretion when associated with the cell surface¹⁹ and alters its positive charge status.^{10, 16} Interestingly, the amount of deacetylation of the exopolysaccharide varies among bacteria, with a 3–5% deacetylation in *E. coli*,^{10, 20–22} 10% in *A. actinomycetemcomitans*¹² or as high 15–20% in *S. epidermidis*.¹¹

Recently, the effect of *pga* locus on the gene regulation at genome level of *A. actinomycetemcomitans* was investigated using RNA-Seq and the differentially expressed genes between mutant strain EA1002 that lacks the *pga* operon¹² and wild-type strain IDH781 were analyzed.^{23, 24} We demonstrated that the *pga* locus is involved in bacterial virulent gene regulation in *A. actinomycetemcomitans* and that the absence of PNAG production resulted in reduced colonization and bone loss in a rat model of periodontitis.²⁴ This was the first demonstration that virulence of *A. actinomycetemcomitans* was linked to the production of PNAG. However, the role of deacetylation of PNAG in virulence is unknown. Therefore, in this study, we focused on *pgaB* to address whether or not deletion of the DNA coding region for the catalytic activity of PgaB potentially affecting deacetylation will have an effect on the expression of the virulence genes, PNAG production and its export.

METHODS

Markerless in-frame deletion of the N-terminal catalytic domain coding region of *pgaB*

The markerless, isogenic *pgaB* N-terminal catalytic domain coding region (73–783 bp) deletion mutant (N_{pg}aB) in *A. actinomycetemcomitans* strain IDH 781 [(serotype d), a rough clinical isolate,²⁵ Table 1] was constructed by using a suicide plasmid pJT1 following the method previously described.²⁶ The flanking regions of the catalytic domain coding

region of the *pgaB* gene were amplified using the primer sets delta1F/delta1R and delta2F/delta2R, corresponding to the upstream and downstream flanking region, respectively (Table 2). The PCR products from upstream flanking region (1092bp; product-1) and the downstream flanking region (1093bp; product-2) were resolved on 1% TAE agarose gels and eluted from the gels using Qiagen gel extraction kit (Qiagen). The amplified products, product-1 and product-2, were cloned into the double-digested pJT1 vector (NotI and XhoI) using the Clontech In-Fusion technology (Clontech Laboratories, Inc.) following manufacturer's instructions. The recombinant product was transformed into NEB 10-beta cells (New England Biolabs, Inc.) and selected on spectinomycin (50 µg/ml) LB plates. The recombinant plasmid (pJT1 *NpgaB*) was isolated and the integrity of the nucleotide sequence was confirmed by sequencing. The pJT1 *NpgaB* plasmid was introduced into the IDH 781 strain by electroporation.²⁷ Electroporated *A. actinomycetemcomitans* cells were incubated in 5 ml of TSB medium for 5h at 37°C, 10% CO₂/90% air atmosphere conditions, spun down, suspended in 0.5 ml of TSB medium, and plated on BHI agar containing 35 µg/ml spectinomycin. Seventy colonies were selected and patched on to TSA plates without spectinomycin (2X) and screened for successful recombination by colony PCR using the primers (screen1F/screen1R; Table 2) outside of the flanking regions. One colony which was positive for the deletion was suspended in TSB (100 µl) and plated onto BHI agar supplemented with 1 mM IPTG and 10% sucrose and grown at 37°C, 10% CO₂/90% air atmosphere conditions. Ten colonies were further screened with the deletion screening primers (screen1F/screen1R; Table 2) to confirm the genomic deletion of the N-terminal catalytic domain coding region of PgaB. The amplified product was cloned into PCR2.1 and sequenced to confirm the presence of the C-terminal coding region (784 – 1917 bp) in frame with the signal sequence (1–72 bp) and the absence of the N-terminal catalytic domain coding sequence (73–783 bp). The mutant was named *NpgaB* and subjected to further characterization.

Bacterial strains and growth conditions

A. actinomycetemcomitans strain IDH 781, a *pga* operon deletion mutant (*pgaABCD* designated as EA1002¹²) and *NpgaB* were grown for two days at 37°C/10% CO₂/90% air atmosphere conditions on TSA plates in the presence of yeast extract (0.8%), sodium bicarbonate (0.4%) and glucose (0.75 %) with bacitracin (75 µg/ml) and vancomycin (5 µg/ml) added. Single colonies were scraped from the plates and suspended in TSB medium containing yeast extract (0.8%), sodium bicarbonate (0.4%) and glucose (0.75%). A hand-held homogenizer (Kimble Chase) was used to disperse the aggregated cells. After incubating on ice for two minutes, the cells in the top portion of the culture were collected. The OD₆₀₀ of the culture was adjusted to 0.50 (0.5×10^8 cells/ml) before further use in various assays described below.

Biofilm growth

For *in vitro* biofilm growth and cell harvest, inocula obtained from cultures grown as described above were used (3 µl for 45 ml of TSB medium). For biofilm formation experiments, 45 ml of inoculated medium was poured onto 15 cm polystyrene plates and incubated at 37°C, 10% CO₂/90% air atmosphere conditions.^{23,28} The spent medium was removed after 16 h and plates were washed with ice cold PBS. For RNA stabilization and

isolation experiments, ice cold saline was prepared as follows: citric acid saturated phenol mixture (0.5 ml), ethanol (9.5 ml) and 0.9 g of sodium chloride in a final volume of 100 ml. The attached biofilm cells were scraped, transferred with the saline solution and centrifuged at 8,000g (Eppendorf 5415C; Eppendorf) for 5 min at 4°C. Pelleted cells were frozen in liquid nitrogen and stored at -80°C until further use.

Quantitative RT-PCR

Isolation of total RNA and subsequent cDNA synthesis was carried out using the high capacity cDNA synthesis kit (Life technologies).²⁴ The cDNAs were diluted 1:10 to detect the mRNA of selected genes and 16SrRNA, used as control, was diluted 1:5000. The diluted cDNA samples were used to amplify the selected genes using specific primers listed in Table 2. These gene targets were amplified with SYBR green master mix (Roche) using the Light Cycler 480 system (Roche; Version 1.2.9.11). The differential gene expression between IDH 781 and NpgaB strains was calculated based on 2^{-CT} value compared to 16SrRNA. For each sample, three biological replicates with two technical replicates were used in the analysis. The results were subjected to Student's *t*-test for statistical significance ($P < 0.05$).

Biofilm adherence assay

Adherence of cells onto the surface of glass test tubes were assayed as follows: bacterial cells were prepared from the TSA agar plates as described above (bacterial growth) and the cells (15 µl, OD₆₀₀ = 0.5) were suspended in 5 ml of TSB medium, then added to glass test tubes, and incubated at 37°C, 10% CO₂/90% air atmosphere conditions and grown as a static culture. After 48 h, the cultures were subjected to vortexing for 10 s at high speed (Fisher Vortex Genie 2™) and washed with distilled water (2X) and the tubes were air dried²⁹. Five ml of 5 µg/ml ethidium bromide was added to the tubes, left for 10 min at room temperature and the tubes were washed with water and photographed in UV light.

Surface attachment, biofilm formation and staining assays

For assaying the surface attachment of the bacterial cells, 200 µl aliquots of cultures (IDH 781, NpgaB and EA1002) were transferred to the wells of a 96-well polystyrene microtiter plate (Falcon) and incubated for 24 h. The biofilm cells were washed with water, air-dried and stained with 100 µl of 1% Crystal violet (to estimate biofilm) or 100 µl (2 mg/ml) Congo red dye (to estimate exopolysaccharide) for 10 min at room temperature.^{23,29} The excess stain was washed thoroughly with water and the bound dye was solubilized in 200 µl of 100% DMSO (Congo red) or 200 µl of 33% acetic acid (Crystal violet). The optical density of the solubilized dye solution was measured using a Bio-Rad Benchmark microtiter plate reader set at 595 nm (Crystal violet) or at 415 nm (Congo red). All assays were performed in 9 wells (technical) per strain in triplicate (biological). When necessary, solubilized dye solutions were appropriately diluted before estimation of the optical density and the dilution factor was included in the comparison of means by one-way ANOVA ($P < 0.05$).

Glycogen staining using iodine

Bacterial cells (IDH 781, NpgaB and EA1002) grown on TSA plates for 2 days were collected by scraping and suspended in PBS buffer. After adjusting the OD₆₀₀ to 0.8, 25 µl

of the uniformly distributed cells was diluted to 25 ml using modified Kornberg medium (1.1% K_2HPO_4 , 0.85% KH_2PO_4 , 0.8% yeast extract (pH 6.8), 1% glucose and 0.4% sodium bicarbonate). Biofilms were grown as describe above in 96-well culture plates for 16 h in 10% CO_2 incubator. Biofilms were washed twice with prewarmed PBS buffer (37°C) and stained with 0.05 M iodine solution for 10 min, excess iodine was removed by washing in water ($\times 3$), dried and photographed. The iodine staining was performed in triplicate for each strain. Control wells had only the buffer with no inoculum.

Immunological detection of PNAG on the cell surface and in culture supernatants

Immunoblots were performed using each of the three strains grown as bacterial biofilms for 24 h as described above. The cultures were diluted in TSB to produce an OD_{600} of 1.5 and 10 ml of the cultures were centrifuged to recover the bacterial cells. The culture supernatant was passed through 0.22 μm syringe filters and saved for further analysis. The cell pellet was washed (3X, PBS) and treated with 500 μl of 0.5 M EDTA with gentle sonication, followed by boiling for 5 min. The extracts were cleared by centrifugation, and the solubilized cell extracts and culture supernatants were treated separately with 200 μg proteinase K and incubated at 60°C for 1 h and then at 85°C for 30 min to inactivate the protease. The cell surface extracts (100 μl of 1:10, 1:100, 1:500 and 1:1000 diluted in PBS) and culture supernatants (2 ml, 1 ml, 0.5 ml, 0.25 ml and 0.1 ml) were immobilized on to nitrocellulose using a vacuum manifold. Blots were blocked for 1 h with 1% bovine serum albumin (BSA) in PBS, probed with human IgG1 mAb F598³⁰ (high binding to both acetylated PNAG and deacetylated PNAG 50 $\mu g/ml$) diluted in PBS containing 1% BSA, washed thrice with PBS-T (0.05% Tween-20) for 10 min each and then incubated with HRP conjugated goat anti-human IgG secondary antibody (1:7000) diluted in PBS-T (0.05% Tween-20) containing 1% skimmed milk powder for 1 h at room temperature. The blots were washed thrice with PBS-T (0.05% Tween-20) for 10 min each. The chemiluminescent substrate (Super signal, ThermoFisher Scientific) was added and then the membrane was scanned with LICOR C-DiGit blot scanner (LICOR Biotechnology) to acquire the images.

Immunofluorescence microscopy

Bacterial strains were grown as biofilms for 16 h on plastic cover slips (Fisher Scientific Co.) as described above. The cover slips with biofilms were washed with PBS (3X) for 5 min. The cells were fixed with 2% (w/v) para-formaldehyde in PBS for 15 min at room temperature and then washed for 5 min with PBS (3X). Washed cover slips were incubated with blocking solution (5% normal goat serum, 0.5% BSA in PBS) for 1 h at room temperature. After washing with PBS (5 min; 3X), biofilm cells were incubated with primary antibody in blocking solution (5 $\mu g/ml$; human IgG1 mAb F598) at room temperature for 2 h. The cover slips were washed with PBS (3X) and incubated with the secondary antibody (1:1000 dilution, anti-human IgG, Alexa 488, ThermoFisher Scientific) in blocking solution at room temperature for 1 h. The cover slips were washed with PBS for 5 min (3X) and mounted with Gold™ mounting medium containing DAPI (ThermoFisher Scientific). Images were acquired using 20 $\times$ objective lens (Nikon Eclipse 80i microscope, Nikon Instruments Inc.) and processed using the Nikon Elements software version 4.13.04.

Confocal microscopy and cell viability

For confocal microscopy, the bacterial strains were grown on 35 mm glass bottom microwell culture dishes (MatTek Co.) as previously described.²⁴ For this study, biofilms were grown for either 16 h or 48 h. Images were acquired with a Nikon A1R-A1 confocal microscope (Nikon Instruments Inc.) using the objective lens Plan Apo VC 60× WI DIC N2. The images were processed (NIS Elements Software Version 4.2) and the percentage of cell death (dead/total cells × 100) was calculated from seven random zones and averaged.²⁴ The percentage of the mean cell death results, expressed as a ratio of dead/total cells × 100 for each strain, were subjected to Student's *t*-test for statistical significance ($P < 0.05$). For cell viability assay, biofilm cells were collected at 16 h or 48 h after growth and plated on TSA plates. Colonies were counted after each time interval and the means of the colony counts were subjected to Student's *t*-test for statistical significance ($P < 0.05$).

Statistical analysis

Quantitative assays were compared by one-way analysis of variance (ANOVA) or by Student's *t*-test using GraphPad Prism 6.0 software. All tests were performed with a confidence level of 95%.

RESULTS

Characterization of the *pga* operon in *A. actinomycetemcomitans* mutant strain

To determine whether or not the catalytic domain of PgaB is required for production of PNAG and export, we first generated a markerless, scarless chromosomal deletion of the N-terminal coding region of PgaB (NpgaB) (Fig. 1). After deletion of the N-terminal coding region, nucleotide sequencing was performed to ensure that the DNA corresponding to the coding region of the signal sequence and that of C-terminal domain of PgaB were in frame.

Evaluation of mRNA levels using qRT-PCR

Next, we evaluated the effect of the absence of the catalytic domain of PgaB on the expression level of genes involved in cell adhesion and virulence in the NpgaB strain by qRT-PCR and compared this to the corresponding levels in the wild type strain IDH 781 (Fig. 2). The results demonstrate that the genes involved in the PNAG synthesis (*pgaA*, *pgaB* and *pgaC*) are downregulated in the NpgaB strain (Fig. 2) although the *pgaA* gene is located upstream and *pgaC* is downstream of the *pgaB* gene. The downregulation of *pgaA* and *pgaC* clearly indicates that the transcription of the *pga* operon is affected in the NpgaB strain. Critical genes responsible for virulence such as *ltxA* and *cdtB* were also downregulated in NpgaB. The fold changes in the mRNA levels between IDH 781 and the NpgaB strains in genes such as *aae*, *apiA*, *flp-1*, which are involved in colonization, are significantly downregulated as well. Interestingly, among the immune evasion genes, *cdtB* was significantly downregulated to a greater extent than either *ltxA* or *katA*. Importantly, the crucial peptidoglycan precursor synthesis gene *murA* is unchanged in NpgaB strain ($P = 0.94$). To test whether or not the differential expression of these genes were linked to regulatory factors, we evaluated the gene *csrA* (a global regulator). Compared to the wild type, the mRNA expression level of *csrA* in NpgaB was significantly downregulated ($P <$

0.00001; Fig. 2). Significance in the fold changes was analyzed using Student's *t*-test and the P-values ranged from 0.01 (*katA*; fold change > 2) to 0.00001 (all others; fold change > 2). Two genes related to stress (*uspA* and *bolA*) and a gene involved in glycogen synthesis (*pgm*) were also analyzed to test for whether or not *NpgaB* retained characteristic phenotypes of EA1002. No significant change in the stress genes were observed (P=0.59) whereas the *pgm* was upregulated in both *NpgaB* and EA1002 compared to IDH 781 (2-fold; P<0.001).

Biofilm integrity in *A. actinomycetemcomitans*

To test the phenotypic variations between IDH 781 and *NpgaB* strains, we grew them on AAGM agar.⁴ Examination of colonies under a microscope showed that unlike the wild type colonies, the *NpgaB* mutant colonies lacked the internal star-shaped morphology (Fig. 3a). Also, the rugged edges characteristic of a rough phenotype in IDH 781 were less prominent in the *NpgaB* strain. However, the ruggedness was more prominent after 48 h (data not shown). We tested the *NpgaB* strain for biofilm formation in a 96-well tissue culture plate assay using Crystal violet. There was no difference in the ability of the two strains to form biofilms (data not shown). We then analyzed the biofilm-integrity of strains IDH 781 and *NpgaB* grown in glass tubes as static cultures (Fig. 3b). The wild type strain produced tenacious test-tube adhering biofilms whereas the *NpgaB* strain generated biofilms that were non-adherent to the walls of the test tubes.

Examination of PNAG production in *NpgaB*

The *A. actinomycetemcomitans* cells (wild type, EA1002 and *NpgaB*) were analyzed for the production of PNAG as follows. Typically, the cells were grown as biofilms on 96-well tissue culture plates for 24 h, washed with water and stained with Congo red (Fig. 4a). Clearly, the wells containing the IDH 781 cells showed greater intensity of the stain. The stained biofilms in wells were washed and the amount of the stain was quantified by measuring the absorbance at 415 nm using a spectrophotometer. The results showed that IDH 781 biofilm stained 9-fold higher than the *NpgaB* strain (Fig. 4b; P<0.008). The EA1002 strain, on the other hand, showed negligible staining as indicated by the absorbance values (Fig. 4b). Interestingly, *NpgaB* showed a significant increase in staining (2-fold higher; P<0.01) compared to EA1002. Comparison of the means of the absorbance (415 nm) values by one-way ANOVA showed significant difference among the strains (overall P<0.0001 while the P-value for the pair-wise comparisons with the wild type strain were <0.0001. To further clarify the apparent reduced level of exopolysaccharide production, we performed immunological detections for the presence of PNAG on the surface of the cells in each of the three strains (see below).

To evaluate whether the upregulation of *pgm* gene in *NpgaB* leads to accumulation of glycogen as observed in the EA1002 cells more than IDH781 cells, we grew the two strains under biofilm conditions in 96 well tissue culture plates and after 16 h of growth, the wells were stained using iodine (Fig. 4c). Both *NpgaB* and the EA1002 biofilm containing wells stain darker compared to IDH781 cells suggestive of the presence of excess glycogen. Control wells that were coated with medium alone did not stain with iodine.

Immunological detection of PNAG on the cell surface

The presence of PNAG on the cell surface or in the culture supernatants was tested by reacting human IgG1 mAb F598 with biofilm cells for each of the three strains grown for 24 h (Fig. 5). In the fractions derived from strain EA1002 cells devoid of *pga* operon, as expected, there was no detectable PNAG in either the cell fraction or the supernatant. Interestingly, in *NpgaB*, PNAG was found predominantly in the supernatant while only a minority was present on the cell surface. However, in the wild type IDH 781 cell cultures, both the cell surface extracts and culture supernatants contained significant amount of PNAG (Fig. 5). Taken together, although PNAG is present in the cell culture supernatants of IDH781 and *NpgaB*, when the cell surface extracts of these two strains are compared, most of the PNAG is associated with the cell surface in IDH 781 rather than in *NpgaB*. The absence of PNAG in the surface extracts of *NpgaB* strain suggests that PNAG produced by the *NpgaB* strain is unable to anchor to the cell surface as efficiently as it does in the wild type. Thus, the deletion of the catalytic domain in *PgaB* has led to a marked reduction in the production and/or export of the PNAG and also its ability to adhere to the cell surface.

To further evaluate the PNAG production in these strains (IDH 781, EA1002 and *NpgaB*), we performed immunofluorescent microscopic analysis. The three strains were grown as biofilms and stained with human IgG1 mAb F598. The immunofluorescence analysis revealed a strong signal in IDH 781 wild type cells whereas the signal from *NpgaB* strain was minimal (Fig. 6). Notably, the *NpgaB* mutant cells showed a punctate pattern suggesting that PNAG was not spread throughout the biofilm. In sharp contrast, the EA1002 cells showed no signal. Together, these results suggest that the *PgaB* catalytic domain is required for the optimal association of PNAG with the bacterial cell surface in IDH 781.

Confocal microscopy and cell viability assay

To evaluate whether the deletion of the catalytic domain coding region of *PgaB* affects the cell viability during biofilm growth, the *A. actinomycetemcomitans* strains were grown in biofilms for 16 h and 48 h and analyzed by confocal microscopy. The strain EA1002 was used as a control. In EA1002, we had shown earlier that the attenuated peptidoglycan synthesis due to down regulation of *murA* increases hydrostatic pressure which might lead to cell death. SYTO9/propidium iodide (Live/Dead) staining was used to compare the amount of cell death as previously described.^{23,24} The Live/Dead staining showed that the cell death (propidium iodide staining) at 16 h and at 48 h was comparable between the two strains and not significantly different (Fig. 7a). To confirm that the cell death occurring in these two strains is not significantly different, and to enumerate the viable cells present in these strains, we grew the two strains ensuring that equal number of cells were used for growth. After 16 h and 48 h, IDH 781 and *NpgaB* cells were enumerated by colony counting (Fig. 7b). Clearly, there was no significant difference between the numbers of viable cells either at 16 h ($P > 0.45$) or at 48 h ($P > 0.47$).

We also analyzed the biomass of the biofilms of *NpgaB* and the wild type strains using COMSTAT software. At 16 h, when the biofilm is still growing, the biomass values are 5.56 (2.13) $\mu\text{m}^3/\mu\text{m}^2$ for *NpgaB* and 2.01 (0.46) for the wild type IDH781. At 48 h, when the biofilms are mature, biofilm mass for *NpgaB* (20.06 (5.13) $\mu\text{m}^3/\mu\text{m}^2$) is comparable to the

wild type (18.91 (2.00) $\mu\text{m}^3/\mu\text{m}^2$). The means of the two biomasses are not significantly different using Student's *t* test ($P=0.59$).

DISCUSSION

The PNAG deacetylase enzyme PgaB of *A. actinomycetemcomitans* has been characterized previously in our laboratory and we showed that the N-terminal catalytic domain of PgaB is responsible for the deacetylase activity.¹⁵ The results reported here indicate that in the absence of the catalytic domain of PgaB, the *pga* operon transcription (*pgaA*, *pgaB* and *pgaC*) is downregulated which leads to less PNAG production. Also, the PNAG that is synthesized is not retained efficiently on the NpgaB cell surface. On the other hand, our results indicate that the presence of active catalytic domain of PgaB (wild type IDH 781) influences not only the amount of PNAG production but also the retention of PNAG on the cell surface (Fig. 5). Our results indicate that the isogenic catalytic domain mutant, NpgaB, retained very little or no PNAG at the cell surface and released most of the produced PNAG into the spent medium (Fig. 5). Because only little PNAG is present even in the spent medium compared to the wild type supernatant, it is highly likely that either the production of PNAG is defective (*pgaC* is down-regulated) and/or its export is impaired (*pgaA* is down-regulated) in NpgaB mutant.

In our earlier work with the EA1002 mutant strain, RNA-Seq transcriptome analysis showed that many genes that correspond to the attachment, communication, peptidoglycan synthesis and glycogen synthesis were significantly affected. We selected the same set of genes that were studied before for qRT-PCR analysis to test whether or not the NpgaB phenotype retains all characteristics of the EA1002 mutant. Our results indicate that the reduced level of PNAG production in NpgaB has a severe effect on many of the genes that are important for colonization and virulence (Fig. 2). There clearly is a down-regulation of both upstream and downstream genes of the *pga* operon. Previous studies have shown that the presence of each or all of the four genes are required for the production of PNAG.^{12,23,24} Our results further show that, in addition, the truncation of a part of a gene in this operon is also detrimental to the production of PNAG. The down-regulation of other virulent genes (Fig. 2) suggests that the *pga* operon might interact with a network of genes. It is possible that the affected genes could be due to global regulation. In this regard, PNAG production in *E. coli* has been shown to be post-transcriptionally repressed by CsrA, a global regulator.³¹ Evaluation of the mRNA level of *csrA* in the NpgaB mutant by qRT-PCR showed that the gene was downregulated (13-fold; P -value <0.0007). Whether or not this effect on the colonization and virulence genes is due to CsrA is not known at this time. Further experiments are needed to dissect the role of CsrA or other regulatory factors in *A. actinomycetemcomitans*. Interestingly, we noted that the reduction in PNAG production either through the deletion of the *pga* operon (EA1002) or by deletion of the N-terminal coding region of the *pgaB* gene (NpgaB) resulted in accumulation of glycogen (Fig. 4c).

Previous studies have reported that deacetylation is necessary for surface retention in other PNAG-like exopolysaccharide-producing bacteria such as *S. aureus*.³² Interestingly, deletion of *icaB* in *S. aureus*, a gene encoding a catalytic domain-similar to that in the *pgaB* gene in *A. actinomycetemcomitans*, did not prevent the synthesis of the exopolysaccharide. Most of

the PNAG (exopolysaccharide from *S. aureus*) was found in the cell culture supernatant. In *E. coli*, depending upon the condition of growth, PNAG was primarily cell bound (static growth condition) or in the culture supernatant (shaking growth condition) in the PNAG overexpressing strain.¹³ Irrespective of the condition of growth, the isogenic *pgaB* mutant of *E. coli* released little or no PNAG into the medium under either condition suggesting that PNAG secretion is defective in this mutant.¹³ Our results indicate that the isogenic catalytic domain mutant, NpgaB, retained very little or no PNAG at the cell surface and released most of the produced PNAG into the spent medium (Fig. 5). Because only little PNAG is present even in the spent medium compared to the wild type supernatant, it is highly likely that either the production of PNAG is defective or its export is impaired in NpgaB mutant. It should be noted that our study was performed under biofilm growth conditions whereas those in *E. coli* or *S. aureus* were performed in planktonic growth conditions.

The protein sequence of AaPgaB is very similar to that of EcPgaB and the structures are highly superimposable.¹⁵ In *E. coli*, the nonpolar *pgaB* strain was shown to be unable to export PNAG although they were able to produce PNAG.¹³ Furthermore, strains incorporating two catalytic site mutations, D115A and H184A, were also unable to export PNAG. Thus, *E. coli* strains expressing catalytically inactive PgaB protein, incapable of deacetylation, are unable to export PNAG when grown in broth culture.¹³ Isogenic deletion of any one of the genes in *E. coli*¹³ and in *A. actinomycetemcomitans*¹² resulted in no PNAG being produced. However, in sharp contrast to the results obtained in *E. coli*, the synthesis of PNAG in *A. actinomycetemcomitans* is minimized in the NpgaB mutant which lacks the coding region of the catalytic domain of PgaB. Collectively, the results obtained here for *A. actinomycetemcomitans*, and elsewhere for *E. coli*, indicate that PgaB enzyme activity is required for the export of PNAG.

Although PgaB is present in both Gram-negative as well as Gram-positive organisms, its role appears to be different in these organisms. In *S. epidermidis*, IcaB has been suggested to assist PNAG to attach to the cell surface¹¹ whereas in *E. coli*, it has been suggested to assist in the export of PNAG.¹³ In this regard, it should be pointed out that an adhesive exopolysaccharide (known as holdfast) of Gram-negative *Caulobacter crescentus*, tightly adheres to surfaces.^{33–35} While the disruption of the polysaccharide deacetylase gene (*hfsH*) does not abolish holdfast synthesis,³⁶ the holdfast of the mutant detaches from cells and binds weakly to surfaces. The observed shedding of holdfasts from the *hfsH* mutant cells is consistent with the phenotype caused by the loss of polysaccharide deacetylation in *S. epidermidis*.¹¹ We note that the sequence of AaPgaB is similar to that of SeIcaB (possessing a deacetylase domain) and also similar to EcPgaB (having two domains, a catalytic N-terminal domain and a carbohydrate binding C-terminal domain). Whereas SeIcaB³⁷ and the N-terminal domain of AaPgaB¹⁵ are catalytic active, the N-terminal domain of EcPgaB¹⁸ is not. Thus, functionally, significant difference in the catalytic activities of the N-terminal domains of the deacetylase enzyme among these bacteria exist. Collectively, results from this study suggest that AaPgaB integrates the properties of SePgaB (attachment to cell surface) and EcPgaB (export of PNAG) for its role in *A. actinomycetemcomitans*. Thus, we have shown that deacetylation might play an important role in bacterial adhesion by modulating adhesive and cohesive properties of the exopolysaccharide in *A. actinomycetemcomitans*.¹⁵

Confocal image analysis of biofilms of the NpgaB mutant showed that the cell death in the mutant strain was comparable at 16 h and 48 h to the IDH 781 strain (Fig. 7a). Our enumeration of viable cells after either 16 h or 48 h showed no significant difference in both the strains (Fig. 7b; $P > 0.45$). In contrast, at 48 h more cells are not viable in the EA1002 strain.²⁴ These studies indicate that although there are phenotypic changes occurring in

NpgaB mutant, overall there might not be significant metabolic changes in the NpgaB mutant. Absence of PNAG makes *A. actinomycetemcomitans* cells less virulent and less able to colonize resulting in no induction of bone loss in a rat model of periodontitis.²⁴ It is also likely that PNAG must be anchored to the cell surface to confer resistance to the immune system.³⁸ Clearly, surface associated PNAG is a requirement for such protection and in this regard, agents affecting PNAG synthesis or export could lead to potential therapeutic intervention strategies. In summary, taken in context with the results of immunoblotting, immunofluorescence, surface display of PNAG using Congo red staining, qRT-PCR analysis of the selected genes and the confocal analysis, our findings suggest that PgaB, in particular the deacetylation of PNAG by PgaB, promotes the production of PNAG as well as export from the cell.

CONCLUSIONS

The main conclusion that emerges from this study is that the deletion of the catalytic domain of PgaB in *A. actinomycetemcomitans* has a significant effect in the production, export and retention of PNAG on the cell surface. This effect could arise by any one of the following mechanisms: a) deletion of catalytic domain might have generated an export deficient phenotype where in the produced PNAG might be located in the periplasm; b) overall production of PNAG is reduced in the NpgaB mutant but the PNAG that is produced is exported but unable to adhere to the cell surface; and c) both production of PNAG and export are reduced in the mutant. The cell viability during biofilm growth, however, is not affected when compared to the wild type. In addition, deacetylation of PNAG appears to modulate the expression of genes involved in the attachment stage during biofilm growth, and virulence genes. Attenuation and absence of PNAG production also affects the carbon source regulator protein CsrA and leads to accumulation of glycogen. Thus, our study and the results of a previous study²⁴ strongly suggest that production of PNAG is essential for *A. actinomycetemcomitans* colonization and virulence. Importantly, inhibition of export or modification of PNAG might be therapeutically relevant in disease prevention.

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ABBREVIATIONS

AaPgaB	PgaB from <i>A. actinomycetemcomitans</i>
EcPgaB	PgaB from <i>E. coli</i>

EA1002	a mutant strain of <i>A. actinomycetemcomitans</i> lacking the exopolysaccharide producing operon <i>pga</i>
GlcNAc	N-acetyl-D-glucosamine
PNAG	homopolymer of β -(1,6)-N-acetyl-D-glucosamine

References

1. Watnick P, Kolter R. Biofilm, city of microbes. *J Bacteriol.* 2000; 182(10):2675–2679. [PubMed: 10781532]
2. Mah TF, O'Toole GA. Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol.* 2001; 9(1):34–39. [PubMed: 11166241]
3. Zambon JJ. *Actinobacillus actinomycetemcomitans* in human periodontal disease. *J Clin Periodontol.* 1985; 12(1):1–20. [PubMed: 3882766]
4. Fine DH, Furgang D, Kaplan J, Charlesworth J, Figurski DH. Tenacious adhesion of *Actinobacillus actinomycetemcomitans* strain CU1000 to salivary-coated hydroxyapatite. *Arch Oral Biol.* 1999; 44(12):1063–1076. [PubMed: 10669085]
5. Izano EA, Sadovskaya I, Vinogradov E, et al. Poly-N-acetylglucosamine mediates biofilm formation and antibiotic resistance in *Actinobacillus pleuropneumoniae*. *Microb Pathog.* 2007; 43(1):1–9. [PubMed: 17412552]
6. Kaplan JB, Velliyagounder K, Ragunath C, et al. Genes involved in the synthesis and degradation of matrix polysaccharide in *Actinobacillus actinomycetemcomitans* and *Actinobacillus pleuropneumoniae* biofilms. *J Bacteriol.* 2004; 186(24):8213–8220. [PubMed: 15576769]
7. Kaplan JB, Ragunath C, Velliyagounder K, Fine DH, Ramasubbu N. Enzymatic detachment of *Staphylococcus epidermidis* biofilms. *Antimicrob Agents Chemother.* 2004; 48(7):2633–2636. [PubMed: 15215120]
8. Gerke C, Kraft A, Sussmuth R, Schweitzer O, Gotz F. Characterization of the N-acetylglucosaminyltransferase activity involved in the biosynthesis of the *Staphylococcus epidermidis* polysaccharide intercellular adhesin. *J Biol Chem.* 1998; 273(29):18586–18593. [PubMed: 9660830]
9. Fluckiger U, Ulrich M, Steinhuber A, et al. Biofilm formation, icaADBC transcription, and polysaccharide intercellular adhesin synthesis by staphylococci in a device-related infection model. *Infect Immun.* 2005; 73(3):1811–1819. [PubMed: 15731082]
10. Wang X, Preston JF 3rd, Romeo T. The *pgaABCD* locus of *Escherichia coli* promotes the synthesis of a polysaccharide adhesin required for biofilm formation. *J Bacteriol.* 2004; 186(9):2724–2734. [PubMed: 15090514]
11. Vuong C, Voyich JM, Fischer ER, et al. Polysaccharide intercellular adhesin (PIA) protects *Staphylococcus epidermidis* against major components of the human innate immune system. *Cell Microbiol.* 2004; 6(3):269–275. [PubMed: 14764110]
12. Izano EA, Sadovskaya I, Wang H, et al. Poly-N-acetylglucosamine mediates biofilm formation and detergent resistance in *Aggregatibacter actinomycetemcomitans*. *Microb Pathog.* 2008; 44(1):52–60. [PubMed: 17851029]
13. Itoh Y, Rice JD, Goller C, et al. Roles of *pgaABCD* genes in synthesis, modification, and export of the *Escherichia coli* biofilm adhesin poly-beta-1,6-N-acetyl-D-glucosamine. *J Bacteriol.* 2008; 190(10):3670–3680. [PubMed: 18359807]
14. Little DJ, Poloczek J, Whitney JC, Robinson H, Nitz M, Howell PL. The structure- and metal-dependent activity of *Escherichia coli* PgaB provides insight into the partial de-N-acetylation of poly-beta-1,6-N-acetyl-D-glucosamine. *J Biol Chem.* 2012; 287(37):31126–31137. [PubMed: 22810235]
15. Parthiban C, Varudharasu D, Shanmugam M, et al. Structural and functional analysis of de-N-acetylase PgaB from periodontopathogen *Aggregatibacter actinomycetemcomitans*. *Mol Oral Microbiol.* 2016

16. Mack D, Fischer W, Krokotsch A, et al. The intercellular adhesin involved in biofilm accumulation of *Staphylococcus epidermidis* is a linear beta-1,6-linked glucosaminoglycan: purification and structural analysis. *J Bacteriol.* 1996; 178(1):175–183. [PubMed: 8550413]
17. Vergara-Irigaray M, Maira-Litran T, Merino N, Pier GB, Penades JR, Lasa I. Wall teichoic acids are dispensable for anchoring the PNAG exopolysaccharide to the *Staphylococcus aureus* cell surface. *Microbiology.* 2008; 154(Pt 3):865–877. [PubMed: 18310032]
18. Little DJ, Li G, Ing C, et al. Modification and periplasmic translocation of the biofilm exopolysaccharide poly-beta-1,6-N-acetyl-d-glucosamine. *Proc Natl Acad Sci USA.* 2014; 111(30):11013–11018. [PubMed: 24994902]
19. Amini S, Goodarzi H, Tavazoe S. Genetic dissection of an exogenously induced biofilm in laboratory and clinical isolates of *E. coli*. *PLoS Pathog.* 2009; 5(5):e1000432. [PubMed: 19436718]
20. Allignet J, Aubert S, Dyke KG, El Solh N. *Staphylococcus caprae* strains carry determinants known to be involved in pathogenicity: a gene encoding an autolysin-binding fibronectin and the ica operon involved in biofilm formation. *Infect Immun.* 2001; 69(2):712–718. [PubMed: 11159959]
21. Darby C, Hsu JW, Ghori N, Falkow S. *Caenorhabditis elegans*: plague bacteria biofilm blocks food intake. *Nature.* 2002; 417(6886):243–244. [PubMed: 12015591]
22. Parise G, Mishra M, Itoh Y, Romeo T, Deora R. Role of a putative polysaccharide locus in *Bordetella* biofilm development. *J Bacteriol.* 2007; 189(3):750–760. [PubMed: 17114249]
23. Shanmugam M, El Abbar F, Ramasubbu N. Transcriptome profiling of wild-type and pga-knockout mutant strains reveal the role of exopolysaccharide in *Aggregatibacter actinomycetemcomitans*. *PLoS One.* 2015; 10(7):e0134285. [PubMed: 26221956]
24. Shanmugam M, Gopal P, El Abbar F, et al. Role of exopolysaccharide in *Aggregatibacter actinomycetemcomitans*-induced bone resorption in a rat model for periodontal disease. *PLoS One.* 2015; 10(2):e0117487. [PubMed: 25706999]
25. Haubek D, Poulsen K, Asikainen S, Kilian M. Evidence for absence in northern Europe of especially virulent clonal types of *Actinobacillus actinomycetemcomitans*. *J Clin Microbiol.* 1995; 33(2):395–401. [PubMed: 7714199]
26. Juarez-Rodriguez MD, Torres-Escobar A, Demuth DR. Construction of new cloning, lacZ reporter and scarless-markerless suicide vectors for genetic studies in *Aggregatibacter actinomycetemcomitans*. *Plasmid.* 2013; 69(3):211–222. [PubMed: 23353051]
27. Sreenivasan PK, LeBlanc DJ, Lee LN, Fives-Taylor P. Transformation of *Actinobacillus actinomycetemcomitans* by electroporation, utilizing constructed shuttle plasmids. *Infect Immun.* 1991; 59(12):4621–4627. [PubMed: 1937823]
28. Kaplan JB, Kokeguchi S, Murayama Y, Fine DH. Sequence diversity in the major fimbrial subunit gene (flp-1) of *Actinobacillus actinomycetemcomitans*. *Oral Microbiol Immunol.* 2002; 17(6): 354–359. [PubMed: 12485326]
29. Kachlany SC, Planet PJ, Desalle R, Fine DH, Figurski DH, Kaplan JB. flp-1, the first representative of a new pilin gene subfamily, is required for non-specific adherence of *Actinobacillus actinomycetemcomitans*. *Mol Microbiol.* 2001; 40(3):542–554. [PubMed: 11359562]
30. Cerca N, Jefferson KK, Maira-Litran T, et al. Molecular basis for preferential protective efficacy of antibodies directed to the poorly acetylated form of staphylococcal poly-N-acetyl-beta-(1–6)-glucosamine. *Infect Immun.* 2007; 75(7):3406–3413. [PubMed: 17470540]
31. Heydorn A, Nielsen AT, Hentzer M, et al. Quantification of biofilm structures by the novel computer program COMSTAT. *Microbiology.* 2000; 146(Pt 10):2395–2407. [PubMed: 11021916]
32. Wang X, Dubey AK, Suzuki K, Baker CS, Babitzke P, Romeo T. CsrA post-transcriptionally represses *pgaABCD*, responsible for synthesis of a biofilm polysaccharide adhesin of *Escherichia coli*. *Mol Microbiol.* 2005; 56(6):1648–1663. [PubMed: 15916613]
33. Vuong C, Kocianova S, Voyich JM, et al. A crucial role for exopolysaccharide modification in bacterial biofilm formation, immune evasion, and virulence. *J Biol Chem.* 2004; 279(52):54881–54886. [PubMed: 15501828]

34. Li G, Smith CS, Brun YV, Tang JX. The elastic properties of the *Caulobacter crescentus* adhesive holdfast are dependent on oligomers of N-acetylglucosamine. *J Bacteriol.* 2005; 187(1):257–265. [PubMed: 15601710]
35. Merker RI, Smit J. Characterization of the adhesive holdfast of marine and freshwater caulobacters. *Appl Environ Microbiol.* 1988; 54(8):2078–2085. [PubMed: 16347718]
36. Tsang PH, Li G, Brun YV, Freund LB, Tang JX. Adhesion of single bacterial cells in the micronewton range. *Proc Natl Acad Sci U S A.* 2006; 103(15):5764–5768. [PubMed: 16585522]
37. Wan Z, Brown PJ, Elliott EN, Brun YV. The adhesive and cohesive properties of a bacterial polysaccharide adhesin are modulated by a deacetylase. *Mol Microbiol.* 2013; 88(3):486–500. [PubMed: 23517529]
38. Pokrovskaya V, Poloczek J, Little DJ, Griffiths H, Howell PL, Nitz M. Functional characterization of *Staphylococcus epidermidis* IcaB, a de-N-acetylase important for biofilm formation. *Biochemistry.* 2013; 52(32):5463–5471. [PubMed: 23866051]
39. Venketaraman V, Lin AK, Le A, Kachlany SC, Connell ND, Kaplan JB. Both leukotoxin and poly-N-acetylglucosamine surface polysaccharide protect *Aggregatibacter actinomycetemcomitans* cells from macrophage killing. *Microb Pathog.* 2008; 45(3):173–180. [PubMed: 18573331]

Figure 1.

Schematic diagram representing the *pga* operon before and after deletion. The bottom panel shows Δ NpgaB, the residual operon after the markerless deletion of the coding region of the N-terminal domain. NpgaB retains the signal sequence and C-terminal domain coding sequence in frame. Deletion of the gene segment coding for the N-terminal domain was confirmed by PCR amplification with primer sets screen1F/screen1R in colonies obtained with pJT1 suicide vector as described in Material and Methods.

Figure 2.

Expression of select genes in the *A. actinomycetemcomitans* strains. The relative levels of the gene transcripts critical in PNAG production and export (*pgaA*, *pgaB* or *pgaC*), in colonization (*aae*, *flp-1*, *tadA*, *apiA*) or immune evasion (*ltxA*, *cdtB*) and other genes (*katA*, *csrA*) were quantified by qRT-PCR as described in the text. Note that these genes are downregulated in Δ NpgaB indicating that disrupting *pga* operon has a broader effect on genes important to colonization and virulence of *A. actinomycetemcomitans*. Results are means $\pm$ standard deviations for biological triplicates normalized to 16SrRNA. The statistical significance of the fold changes between the strains in the expression levels of all represented genes were measured by Student's *t*-test. The P-value ranges are (*) - >0.01 ; (**) - >0.001 but <0.01 ; (***) - >0.0001 but <0.001 and (****) - <0.0001 .

Figure 3.

The role of the catalytic domain of PgaB in phenotypic variation and biofilm integrity. (a) Deletion of catalytic domain of PgaB (Δ NpgaB) results in a phenotype with attenuated rugged edges characteristic of IDH 781 but lacking the internal star shape. (b) In contrast to the phenotype characteristic observed in (a), Δ NpgaB does not produce a strong biofilm characteristic of wild type IDH 781.

Figure 4.

Production of PNAG by strains IDH 781, EA1002 and NpgaB strains. (a) Congo red staining; (b) Quantitative measure of the Congo red stain. Statistical analysis by one-way ANOVA showed there is significant difference in the amount of PNAG produced by these strains. The overall one-way ANOVA P-value was <0.0001 and the pair-wise comparison P-values were <0.0001 (IDH 781 vs. NpgaB), <0.0001 (IDH 781 vs. EA1002) and <0.007 (NpgaB vs. EA1002); (c) Accumulation of glycogen in *A. actinomycetemcomitans* strains shown using iodine staining. Shown are the control wells without any bacterial inoculum, IDH 781, NpgaB and EA1002 wells grown in 3×3 matrix and stained with iodine.

Figure 5.

The PgaB catalytic domain is required for the association of PNAG to the cell surface. Strain

$\Delta NpgaB$ produces minor amount of detectable PNAG, which is in the culture supernatant unlike the strain IDH 781 in which the PNAG is abundantly present both in the cell surface extract and in the culture supernatant. The strain EA1002, which lacks the *pga* operon does not produce detectable PNAG in either preparation. The ratios in the left panel refers to the dilution of the solubilized cell surface extracts while the volumes in the right panel refer to the volume of cell culture supernatants used in the blot³⁰.

Figure 6.

Immunofluorescence evaluation of biofilms for the production of PNAG. PNAG was visualized using the human mAb F598 and a secondary anti-human IgG conjugated to Alexa 488 (left panel). Images of the same field viewed by DAPI to stain DNA is shown in the middle panel. The last panel shows the merge of the two images. The scale bar is at 100 μ m.

Figure 7.

Confocal scanning laser microscopic image of biofilms. (a) Biofilm growth was imaged at seven different locations and averaged for analysis. The scale bar is at 20 μ m; (b) Cell viability for IDH 781 and Δ NpgaB strains at different time intervals of growth. The cell viability means were compared using Student's *t*-test. NS – not significant.

Table 1

Bacterial strains and plasmids

Strains/plasmids	Relevant characteristics	Reference or source
IDH 781	Clinical isolate, rough phenotype of <i>A. actinomycetemcomitans</i> , serotype d	25
EA1002	<i>pga</i> loci deletion mutant, <i>pgaABCD</i>	12
N _{pgaB}	N-terminal catalytic domain coding region deletion in <i>pgaB</i>	This study
pJT1	Suicide vector	26
pJT1 <i>N_{pgaB}</i>	Plasmid pJT1 containing <i>pgaB</i> gene minus the coding region for the N-terminal catalytic domain of PgaB	This study
NEB10	General cloning	New England Biolabs

Table 2

Primers used in the study.

PCR primers	Sequence (5'-3')	Orientation
Delta1F	ggg ccc aat taa tgg cca tta cgt caa aat gat tta ccg acg gcg	forward
Delta1R	cca cac cat caa gga aat ttt tag cag atg cta cac cgg tga taa ac	reverse
Delta2F	gtt tat cac cgg tgt agc atc tgc taa aaa ttt cct tga tgg tgt gg	forward
Delta2R	tac tag ttc gaa taa cct atg gtt tac ctg ctt gag tat tca gag ac	reverse
Screen1F	ccg acc aaa aat atg ttc g	forward
Screen1R	tgc cat gaa cca gta aat cg	reverse
<i>pgaA</i>	acc ctt gat gga gcg tta tg	forward
	tgc ggt ttt tgg tgt gta aa	reverse
<i>pgaB</i> C-terminus	ccg att ttg aag gcg tag aa	forward
	tag ggc ggg cgt act act ta	reverse
<i>pgaC</i>	atg gtt taa cgg ttg cca ag	forward
	acg cgg att acc tgt ggt ag	reverse
<i>csrA</i>	ccc gta aag ttg gtg aaa gc	forward
	tgc aca ccg att ttg act tg	reverse
<i>ltxA</i>	atc agc cct ttg tct ttc cta g	forward
	tga cca agt aaa cta tcgccg	reverse
<i>cdtB</i>	tgc att aag aca gga acc cg	forward
	tgc tct cga cgt ggt aaa tg	reverse
<i>aae</i>	tgg tgc tac ttc tgt ttctc	forward
	atc ttg aac cta gtg tgg ctg	reverse
<i>tadA</i>	tgg cag ccg ttt aaa tgt tg	forward
	att tcg cga gtc ata gaa cc	reverse
<i>flp-1</i>	tca aag cag ctg aag caa tc	forward
	gcg ata aaa cca ttg ttg ctg	reverse
<i>katA</i>	aat tca aat cat gcc gga ag	forward
	tgc gat tca gtt cca ata cg	reverse
<i>apiA</i>	ctc tac atc agc ctt atc cgc	forward
	tga tgg aaa tct gga agg cg	reverse
<i>murA</i>	aaa ccg gga cat tct tag tgg	forward
	cgt gat ggt atc ttc ggt gac	reverse
<i>pgm</i>	ccc cgg cag ata aac aaa ttg	forward
	gtt ctt tgg caa gtg aag cg	reverse
<i>bolA</i>	gga att tcg acc gca ctt tat tg	forward
	cga tcc cct caa ata cat cg	reverse
<i>uspA</i>	gaa acg caa ggc tat gat ttg g	forward
	gac gct gat gtt tcc agt cac	reverse
16SrRNA	aaa tgc gta gag atg tgg agg	forward

PCR primers	Sequence (5'-3')	Orientation
	tat cta atc ctg ttt gct ccc c	reverse

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