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Tobacco-induced alterations to *Porphyromonas gingivalis*-host interactions

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SUMMARY

Smokers are more susceptible than non-smokers to persistent infection by *Porphyromonas gingivalis*, a causative agent of periodontitis. Patients who smoke exhibit increased susceptibility to periodontitis and are more likely to display severe disease and be refractory to treatment. Paradoxically, smokers demonstrate reduced clinical inflammation. We show that *P. gingivalis* cells exposed to cigarette smoke extract (CSE) induce a lower pro-inflammatory response (TNF- α , IL-6, IL12 p40) from monocytes and PBMCs than do unexposed bacteria. This effect is reversed when CSE-exposed bacteria are subcultured in fresh medium without CSE. Using microarrays representative of the *P. gingivalis* genome, CSE-exposure resulted in differential regulation of 6.8% of *P. gingivalis* genes, including detoxification and oxidative stress-related genes; DNA repair genes; and multiple genes related to *P. gingivalis* virulence, including genes in the major fimbrial and capsular operons. Exposure to CSE also altered the expression of outer membrane proteins, most notably by inducing the virulence factors RagA and RagB, and a putative lipoprotein co-transcribed with the minor fimbrial antigen. Therefore, CSE represents an environmental stress to which *P. gingivalis* adapts by altering gene expression and outer membrane proteins. These changes may explain, in part, the altered virulence and host-pathogen interactions that have been documented *in vivo* in smokers with periodontal disease.

Introduction

Cigarette smokers, and those exposed to secondhand smoke, are more susceptible to multiple infectious diseases than unexposed individuals (Arcavi and Benowitz, 2004; Kum-Nji et al., 2006; Polanska et al., 2006; Keskinoglu et al., 2007; Araco et al., 2008; Jeppesen et al., 2008). Tobacco smoking is considered to be a major environmental risk factor for periodontitis, a common infectious oral disease that results in detachment of the gingival tissue cuff, destruction of the collagen attachment to the root surface of the tooth, and resorption of the supporting alveolar bone. Patients who smoke exhibit not only increased susceptibility to periodontitis but are also more likely than non-smokers to display severe disease and to be refractory to treatment (Palmer *et al.*, 2005). Although periodontitis is caused by a polymicrobial infection of the gingiva, the Gram negative, anaerobic bacterium, *Porphyromonas gingivalis*, has been strongly associated with diseased sites in the oral cavity and is considered to be a causative agent of chronic periodontitis (Holt and Ebersole, 2005; Socransky and Haffajee, 2005). In addition, *P. gingivalis* infection elicits systemic

inflammation (Dye *et al.*, 2005) and may increase susceptibility to systemic vascular diseases (Gibson *et al.*, 2006; Pussinen *et al.*, 2007). Multiple studies have shown that smokers are more likely to be infected with *P. gingivalis*, to harbor higher numbers of *P. gingivalis*, and to exhibit more persistent infection (Zambon *et al.*, 1996; Kamma *et al.*, 1999; Eggert *et al.*, 2001; Haffajee and Socransky, 2001; Grossi *et al.*, 2007) relative to non-smokers.

Despite increased *P. gingivalis* infection rates and susceptibility to disease progression, smokers consistently display reduced clinical inflammation, measured as angiogenesis, edema, inflammatory index, and/or gingival bleeding compared to non-smokers (Kinane and Chestnutt, 2000; Rezavandi *et al.*, 2002; Nair *et al.*, 2003; Scott and Singer, 2004; Palmer *et al.*, 2005). Smokers also exhibit lower levels of pro-inflammatory cytokines at diseased sites (Bostrom *et al.*, 1999; Shirodaria *et al.*, 2000; Erdemir *et al.*, 2004; Goutoudi *et al.*, 2004; Petropoulos *et al.*, 2004). This represents a clinical conundrum to oral health professionals who must diagnose periodontal diseases, and a scientific enigma to those attempting to understand the mechanisms underlying tobacco-induced and / or exacerbated periodontitis.

It is known that tobacco smoke and specific smoke components and/or metabolites can exert a profound negative influence on the host response (Barbour *et al.*, 1997; Kinane and Chestnutt, 2000; Scott *et al.*, 2001; Johnson and Hill, 2004; Mullally, 2004; Palmer *et al.*, 2005; Rehani *et al.*, 2008). For example, in myelocytic cells we have recently shown that nicotine and/or its primary metabolite, cotinine, suppresses the pro-inflammatory cytokine response to *P. gingivalis* while promoting the release of the anti-inflammatory cytokine IL-10 (Rehani *et al.*, 2008). Nicotine also suppresses the production of reactive oxygen species in response to Gram negative stimuli, concomitant with a reduced capacity to kill phagocytosed *P. gingivalis* cells (Xu *et al.*, 2008).

In contrast, there are essentially no data available on how components in cigarette smoke may influence the organisms that comprise the oral microbial biofilm. Thus, it is possible that smoking not only influences the host response but also the organisms in the dental biofilm and that the increased severity of periodontitis in smokers may be attributed to both of these effects. We hypothesized that cigarette smoke extract (CSE) represents an environmental stress to *P. gingivalis* and that the organism adapts to this stress by altering its pattern of gene expression. In this report, we show that the pro-inflammatory response of primary human peripheral blood mononuclear cells (PBMCs) against *P. gingivalis* is significantly reduced when the bacteria are pre-treated with CSE and that this effect was reversed when CSE treated bacteria were sub-cultured in fresh growth medium without CSE. The response of *P. gingivalis* to CSE exposure was examined using both biochemical and molecular biologic approaches. Numerous genes, including those encoding outer membrane proteins and virulence determinants were differentially expressed upon CSE exposure. These results may explain at least in part the altered virulence and host-pathogen interactions associated with cigarette smoking.

Results

P. gingivalis growth in CSE-conditioned medium

To determine if a physiologically relevant dose of CSE (500 ng ml⁻¹ nicotine equivalents [see Experimental Procedures]; (McGuire *et al.*, 1989; Chen *et al.*, 2001; Fraser *et al.*, 2001; Scott *et al.*, 2001) was overtly toxic to *P. gingivalis* W83 cells, we compared the growth rates of bacteria in CSE-conditioned and non-conditioned medium. As shown in Figure 1, similar growth characteristics were observed, suggesting that *P. gingivalis* W83 tolerates this level of CSE. Similar results were obtained for bacteria grown in CSE with 4000 ng ml⁻¹ nicotine equivalents. CSE is not overtly toxic at this high concentration. These data are

in agreement with Cogo *et al*, who have recently reported that nicotine and cotinine, the primary nicotine metabolite in humans, does not reduce or stimulate *P. gingivalis* growth (Cogo et al., 2008).

Induction of TNF- α and other pro-inflammatory cytokine release from innate cells by CSE-exposed *P. gingivalis*

Evidence from *in vivo* studies in humans that have shown that smokers exhibit reduced clinical inflammation in response to pathogenic plaque bacteria (Scott and Singer, 2004; Palmer et al., 2005) and exhibit decreased gingival crevicular fluid (GCF) concentrations of major pro-inflammatory mediators, such as TNF- α and IL-1 (Bostrom et al., 1999; Shirodaria et al., 2000; Erdemir et al., 2004; Goutoudi et al., 2004; Petropoulos et al., 2004) and increased GCF levels of anti-inflammatory cytokines including IL-10 and TGF- β 1 (Goutoudi et al., 2004; Stein et al., 2004). To determine if exposure of *P. gingivalis* to CSE alters its inflammatory potential, primary human monocytes were challenged with control and CSE-treated bacteria. As shown in Figure 2a, the release of TNF- α was significantly lower when monocytes were incubated with CSE-treated bacteria. Interestingly, when CSE-treated organisms were sub-cultured in fresh growth medium without CSE, their inflammatory potential increased back to the level of untreated bacteria (Figure 2b), suggesting that the CSE-induced effect is reversible. Thus, *P. gingivalis* appears to reversibly respond to CSE as an environmental stress. The production of multiple pro-inflammatory cytokines (TNF- α , IL-6 and IL-12 p40) by CSE-exposed and control *P. gingivalis* was also screened in PBMC's. Production of TNF- α , IL-6 and IL-12 p40 was reduced in PBMCs stimulated with CSE-exposed bacteria compared to untreated *P. gingivalis* controls and, again, the inflammatory potential of *P. gingivalis* was restored on reconditioning in fresh GAM (Figure 3).

P. gingivalis genes up-regulated on CSE-exposure

In order to further characterize the response of *P. gingivalis* to CSE-exposure, we performed microarray analyses to identify genes that are differentially expressed in CSE-treated versus control bacteria. A total of 104 genes (approximately 4.7% of the *P. gingivalis* genome) were found to be differentially expressed by 2-fold or more ($p < 0.05$); 58 genes were induced and 46 were suppressed upon CSE treatment (see Figure 4). Of the 58 genes that were induced by CSE treatment, multiple genes within several predicted operons (based upon the gene annotation of the *P. gingivalis* genome sequence by TIGR) were identified, including the major fimbrial operon (2 genes), an operon encoding outer membrane antigenic lipoproteins (4 genes); the transfer (*tra*) gene cluster (4 genes) and an operon encoding an ABC transporter of unknown function [PG0682 – PG0685; 3 genes]. Within the CSE-induced genes, several functional families predominate. One group of genes encode proteins that may be involved in DNA replication and repair (e.g. *ruvC* [PG1324, holliday junction endonuclease]; PG0817 encoding a putative integrase; and *radC* [PG0894, DNA repair protein]) or in the insertion and transposition of genetic material (e.g., *traE* [PG1483]; *traF* [PG1482]; and *traK* [PG1478], each encoding proteins associated with a conjugative transposon). Several other transfer genes (*traG* [PG1481] and *traQ* [PG1473]) from this same region of the *P. gingivalis* genome were differentially expressed but fell just below the 2-fold limit used in the analysis. In addition, several of the insertion sequences that are present in the *P. gingivalis* genome were differentially expressed (ISPg3 [PG0299]; ISPg5 [PG0943]; ISPg6 [PG1061]).

A second group of CSE-induced genes encode proteins involved in potential pathogen-host interactions and virulence. These include several proteases (encoded by *tpr*, *trpT* and PG0317), an efflux transporter (PG0283) related to the bacterial secretion system I protein HlyD, and a putative polysaccharide transport protein related to O-antigen flippase

(PG0912). In addition, two genes encoding putative lipoproteins (PG2133 and PG2134) that are required for the assembly of the major fimbriae of *P. gingivalis* (Wang *et al.*, 2007) were induced by CSE. These genes are co-expressed with *fimA* encoding the fimbrial subunit protein. Several other genes encoding cell surface or outer membrane polypeptides were also differentially expressed including the co-expressed major immunoreactive 61 and 63 KDa surface antigens encoded by PG2102 and PG2100, respectively, putative lipoproteins (PG1233, PG1234 and PG0722), and an outer membrane TonB-dependent receptor (PG2008). Other genes were differentially expressed but fell just below the 2-fold limit used in the analysis, including the *lpt-1* (*ptp1*, PG1641) gene that has recently been shown to be a multifunctional regulator of virulence (Maeda *et al.*, 2008).

To verify the results of the microarray experiments, the expression of select genes from the array dataset were analyzed by RT-PCR. As shown in Table 2, the RT-PCR results of the genes that were tested were consistent with the increased expression of these genes observed in the microarray analyses of CSE-treated cells.

***P. gingivalis* genes down-regulated on CSE-exposure**

Many of the 46 genes (2.1% of the *P. gingivalis* genome) that were suppressed by two-fold or more ($p < 0.05$) encode hypothetical proteins (see Figure 4) whose functions have yet to be determined. However, two genes in the capsular biosynthesis locus (Aduse-Opoku *et al.*, 2006) were down-regulated - a putative capsular polysaccharide synthesis gene (*capK*, PG0111) and a polysaccharide transport protein related to a flippase (PG0117). The expression of two other genes in the capsular biosynthesis locus, encoding a glycosyltransferase (PG0118) and a UDP-N-acetyl-D-mannosaminuronic acid dehydrogenase (*wecC*, PG0108) were down-regulated just below the 2-fold limit used in the analysis. Also down-regulated in response to CSE was a sensor histidine kinase (*fimS*) which together with the co-expressed response regulator FimR has been reported to regulate the expression of the major and minor fimbrial operons of *P. gingivalis* (Wu *et al.*, 2007). Lastly, the expression of *hmuR* (PG1552) encoding a tonB-dependent hemoglobin receptor HmuR was reduced by approximately 2-fold by CSE treatment. Select down-regulated genes were analyzed by RT-PCR (see Table 1) with consistent results.

CSE alters expression of *P. gingivalis* outer membrane proteins RagA and RagB

In addition to the microarray analysis described above, a membrane fraction that was enriched for *P. gingivalis* outer membrane proteins was isolated from CSE-treated and control cells and analyzed by SDS gel electrophoresis. As shown in Figure 5, the Triton X-100 insoluble membrane fraction contained approximately ten major polypeptide bands, of which three were clearly overrepresented in the extract from CSE-treated bacteria. These bands were excised from the gel and analyzed by MALDI-MS after digestion with trypsin. Tryptic peptides were identified for the RagA (43% sequence coverage), RagB (66% coverage) and a putative lipoprotein (PG0179, 53% coverage) that is likely co-expressed with the minor fimbrial antigen of *P. gingivalis*, based on the TIGR (J. Craig Venter Institute) annotation of the *P. gingivalis* genome sequence. Interestingly, western blots of RagB proteins revealed that reconditioning of *P. gingivalis* in GAM, following culture in GAM-CSE, resulted in RagB expression levels returning close to control levels (106%), as determined by densitometry (Figure 6).

Discussion

Cigarette smoking increases vulnerability to *P. gingivalis* infection and increases susceptibility to periodontitis, but reduces clinical signs of overt inflammation. The mechanisms underlying this dichotomy remain to be determined. We show that *P. gingivalis*

exposed to CSE induces a significantly lower pro-inflammatory response from monocytes and PMBCs than control bacteria grown without CSE. Furthermore, this effect is reversed when CSE-treated bacteria are sub-cultured in medium without CSE, suggesting that CSE exposure may represent an environmental stress that *P. gingivalis* is able to specifically respond to. These results are in keeping with several *in vivo* reports of reduced levels of pro-inflammatory cytokines at diseased sites (Bostrom et al., 1999; Shirodaria et al., 2000; Erdemir et al., 2004; Goutoudi et al., 2004; Petropoulos et al., 2004).

Our results clearly show that the response of *P. gingivalis* to CSE exposure is varied and genome wide. Approximately 7% of the genes in the *P. gingivalis* genome were found to be differentially expressed upon exposure to CSE. A number of the differentially expressed genes have been associated with aspects of *P. gingivalis* virulence. Several functionally-related genes were dysregulated, including multiple genes in the major fimbrial and capsular polysaccharide operons, as well as genes encoding transcriptional regulators; efflux pump and transport proteins; proteases and cell envelope proteins. The major fimbriae have been shown to be important for numerous aspects of *P. gingivalis* virulence, including bacterial adhesion and invasion of epithelial and endothelial cells (Lamont & Jenkinson, 1998 and 2000). PG2133 and PG2134 encode putative lipoproteins that are co-expressed with FimA, the major fimbrial subunit protein. Wang et al. recently showed that inactivation of these genes results in the expression of significantly shorter fimbriae, suggesting that these putative lipoproteins play an essential role in fimbrial biogenesis. The mutant strains also exhibited reduced invasion and intracellular persistence in macrophages and were less virulent in a mouse model of periodontitis (Wang *et al.*, 2007).

There are at least six different capsular serotypes (K1 to K6) expressed by *P. gingivalis* and altered virulence has been associated with K⁺ and K⁻ strains (van Winkelhoff et al., 1993; Laine and van Winkelhoff, 1998; Lamont and Jenkinson, 1998; Califano et al., 1999). While the contribution of *P. gingivalis* capsule to inflammation is poorly understood, recent evidence shows that capsular polysaccharide from several strains of *P. gingivalis*, including W83 elicits a substantial pro-inflammatory cytokine response from murine innate cells (d'Empaire et al., 2006). The down regulation of several genes in the capsular biosynthesis operon (PG0111 and PG0117) suggests that capsule synthesis may be reduced upon exposure to CSE, consistent with the reduced inflammatory potential of CSE-treated bacteria.

In addition to the differential expression of genes described above, several other cell surface or outer membrane proteins, i.e., RagA, RagB and PG0179 were shown by biochemical approaches to be present at higher levels after CSE treatment. Interestingly, the genes encoding for these components were not identified as being differentially expressed in the microarray experiments. This suggests that post-transcriptional events may also be involved in the response to CSE, or alternatively may reflect inherent limitations in the microarray approach. Each of the differentially expressed proteins has been associated with aspects of *P. gingivalis* virulence. The *ragAB* locus was likely acquired by horizontal gene transfer (Curtis *et al.*, 1999). RagA is a putative tonB-dependent outer membrane receptor whereas RagB is an immunodominant lipoprotein. Both genes have reported to be up-regulated under conditions of thermal stress (Bonass *et al.*, 2000) and both represent significant *P. gingivalis* virulence factors (Hall et al., 2005; Shi et al., 2007), although their specific contribution to periodontal disease remains to be determined. However, it is interesting that the RagA homolog (designated OmpA) from the closely related bacterium *Bacteroides caccae* has been associated with inflammatory mucosal (bowel) disease (Wei *et al.*, 2001). PG0179 is a gene that is co-expressed with the minor fimbrial antigen (*mfaI*). The minor fimbriae play a key role in *P. gingivalis* autoaggregation (Lin *et al.*, 2006) and are critical mediators of the interspecies interactions between *Porphyromonas gingivalis* and oral streptococci that

facilitate biofilm formation (Lamont et al., 2002; Daep et al., 2006; Daep et al., 2008). Thus, stimulation of the minor fimbrial operon by CSE may facilitate increased *P. gingivalis* colonization of the periodontia.

Lastly, a number of genes that were differentially expressed encoded proteins involved in DNA replication, DNA repair and the transfer or mobilization of genetic material. Comparison of the recently completed genome sequence of *P. gingivalis* ATCC 33277 with that of strain W83 indicates that mobile genetic elements have contributed greatly to genomic diversity among *P. gingivalis* strains (Naito et al., 2008). The up regulation of transfer genes and several insertion sequence elements in CSE-exposed cells suggests that the environmental stress imposed by cigarette smoke may stimulate genetic rearrangements. In addition, considering the highly carcinogenic potential of cigarette smoke, the up-regulation of DNA-repair and -control genes is not surprising. Cigarette smoke is also a major oxidative stressor (Yamaguchi et al., 2007; Lagente et al., 2008). A single cigarette is estimated to contain 10^{16} oxidant molecules (Pryor *et al.*, 1986). Thus, the up regulation of ferritin [PG1286], an iron sequestrator, may provide protection against free-iron-related oxidative stress (Ratnayake *et al.*, 2000). In addition, the response regulator, RprY (also up regulated after CSE treatment) has recently been shown to be responsive to reactive oxygen species and iron (Duran-Pinedo *et al.*, 2007). It is also interesting to note that several other anti-oxidant genes were differentially expressed ($p < 0.05$) when the threshold for induction in the array experiment was reduced from 2-fold to 1.5-fold. In addition, the up-regulation of efflux pump and other ABC transport systems may confer resistance to potentially harmful chemicals present in CSE. Taken together, these results suggest that CSE may represent a potent environmental stressor that induces a protective response from *P. gingivalis*.

In summary, smokers are more prone to infection with *P. gingivalis* and to develop periodontitis, yet exhibit reduced clinical inflammation. Consistent with this, we have found that CSE-exposed *P. gingivalis* exhibits a reduced capacity to illicit an inflammatory response from immune cells. *P. gingivalis* responds globally to CSE exposure and multiple virulence associated genes are differentially expressed. These results may explain in part the altered virulence and host-pathogen interactions that occur in smokers with periodontal disease and provide some of the first information illustrating how *P. gingivalis* responds at the molecular level to cigarette smoke.

Experimental procedures

Materials

P. gingivalis W83 was purchased from the American Type Culture Collection, Manassas, VA; medium supporting *P. gingivalis* growth (Gifu Anaerobe Medium [GAM]) was bought from Nissui Pharmaceutical, Tokyo; Histopaque 1077, lysozyme and protease inhibitor cocktail came from Sigma, St. Louis, MO; RNA Midi and PCR purification kits from Qiagen, Valencia, CA; random hexamers were from Biosynthesis, Lewiston, TX; RT buffer, DTT and ABI Power SYBR Green Master Mix were from Applied Biosystems, Foster City, CA; 2:3 aminoallyl dUTP:dTTP ratio labeling mix was purchased from Amersham Biosciences, Piscataway, NJ; HRP-linked anti-Rabbit IgG was from Cell Signaling Technology, Danvers, MA; while BCA protein assay kit and SuperSignal West Pico Chemiluminescent Substrate kits were bought from Pierce, Rockford, IL. ELISA kits were purchased from eBioscience, Inc. (San Diego, CA; IL-6 and TNF- α) or BD Biosciences (San Jose, CA; IL-12 p40). RPMI 1640, fetal bovine serum, L-glutamine, HEPES, penicillin G and streptomycin were bought from Invitrogen Life Technologies, Carlsbad, CA. *P. gingivalis* W83 microarrays were obtained from The J. Craig Venter Institute through the NIDCR Oral Microbe Microarray Initiative (NOMMI). The E38 monoclonal anti-RagB

antibody was a kind gift of Dr. Mike A. Curtis of The London, Queen Mary's School of Medicine and Dentistry, UK.

Bacterial culture and *in vitro* modeling of tobacco exposure

P. gingivalis W83 was maintained as a frozen stock culture. The use of individual tobacco components/metabolites (e.g. nicotine, cotinine, benzopyrene, pyridines, free radicals, etc) in *in vitro* experimental models is comparably straight-forward, but may not reflect *in vivo* complexities. Cigarette smoke contains more than 4000 different compounds, many of which are toxic and/or bioactive. Cigarette smoke extract- (CSE) conditioned GAM (GAM-CSE) was prepared using standard reference cigarettes (2R1, Kentucky Tobacco Research and Development Center). Using a three-way stopcock, cigarette smoke was drawn through GAM by syringe and expelled before a new volume of smoke was drawn through the media. Constituents of tobacco smoke change dramatically at differing combustion temperatures (Bernard, 2005). Therefore, each cigarette was "smoked" in 35 ml "drags." A "drag" was performed over two seconds, and one was performed every 20 seconds. CSE-conditioned medium was filtered (0.22 μm) and diluted to the required concentration in nicotine equivalents [determined by GLC as we reported previously (Fraser *et al.*, 2001)] and adjusted to pH 7.2. We utilized GAM-CSE at a dose physiologically relevant to *P. gingivalis in situ* in the sub-gingival environment of the oral cavity, 500 ng ml⁻¹ to 4000 ng ml⁻¹ nicotine equivalents (McGuire *et al.*, 1989; Chen *et al.*, 2001; Fraser *et al.*, 2001; Scott *et al.*, 2001). To examine the response of *P. gingivalis* to CSE, cells were grown to mid- to late exponential phase (O.D._{600nm} = 1.0; $\approx 1 \times 10^9$ cells ml⁻¹) in GAM or in GAM-CSE under anaerobic conditions (80% N₂, 10% H₂, 10% CO₂) at 37°C in a Coy Laboratories anaerobic chamber.

Isolation and culture of primary human monocytes and PBMCs

Whole, citrated (10%) venous blood was obtained from healthy donors (University of Louisville, Institutional Review Board, Human Subjects Protection Program, study number 503.05). Primary monocytes were isolated by an indirect magnetic monocyte isolation kit (Miltenyi Biotec, Auburn, CA), as we have previously reported (Martin *et al.*, 2003b; Martin *et al.*, 2005). This procedure routinely results in >95% pure CD14⁺ cells, as shown by flow cytometry, with > 98% viability, as determined by trypan blue exclusion. PBMCs were isolated by separation and collection of the buffy coat and elimination of erythrocyte contamination with Histopaque 1077 density gradients, as previously described (Martin *et al.*, 2003a). This procedure routinely results > 98% PBMC viability, as determined by trypan blue exclusion. Human monocytes and PBMCs were cultured at 37°C and 5% CO₂ atmosphere, in complete RPMI (RPMI 1640 supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 10 mM HEPES, 100 U/ml penicillin G, 100 $\mu\text{g}/\text{ml}$ streptomycin) plus or minus stimulating agents, as described below.

Pro-inflammatory cytokine release from *P. gingivalis*-stimulated innate cells

Primary human monocytes (0.5×10^6 cells per well) were stimulated with $10^6 - 10^9$ *P. gingivalis* grown in GAM or GAM-CSE, as detailed in Figure 2a and b. Cell-free supernatants were harvested by centrifugation ($3,200 \times g$, 8 mins) and TNF- α cytokine levels were determined by ELISA. The production of multiple pro-inflammatory cytokines (TNF- α , IL-6 and IL-12 p40) by CSE-exposed and control *P. gingivalis* was also screened in PBMC's, as presented in Figure 3.

***Porphyromonas gingivalis* W83 microarrays**

P. gingivalis W83 microarrays were obtained from The J. Craig Venter Institute through the NIDCR Oral Microbe Microarray Initiative (NOMMI). To determine how exposure to CSE

influences the activity of the *P. gingivalis* genome, we compared gene expression in *P. gingivalis* W83 grown in control (GAM) and conditioned (CSE-GAM) media. Microarrays were performed on triplicate GAM and GAM-CSE cultures. Total RNA from control and experimental cultures was isolated by extraction from washed cells with hot phenol followed by chloroform extraction and isopropanol precipitation. RNA was purified and genomic DNA was removed using a Qiagen Midi kit with on-column DNase treatment followed by further digestion with RNase-free DNase to remove any trace contamination of genomic DNA. The synthesis of labeled cDNA was carried out by methods established by The J. Craig Venter Institute (www.jcvi.org). Briefly, 2-2.5 µg of total RNA was mixed with 500 ng of random hexamers, incubated at 70°C for 10' and transferred to ice. A 50 × 2:3 aminoallyl dUTP:dTTP ratio labeling mix containing 25 mM remaining nucleotides, 5 × RT buffer, 3 µl of 0.1 M DTT and 2 µl Superscript II (200U/µl) were added. Samples were then incubated for 10' at room temperature and transferred to 42°C, overnight. Reactions were stopped with 10 µl of 1N NaOH and 10 µl of 0.5 M EDTA for 15' at 65°C and neutralized with 25 µl 1M Tris, pH 7.4. Unincorporated aa-dUTP and free amines were removed using a Qiagen QIAquick PCR purification kit protocol with phosphate buffers. The cDNA was then dried down in a speed vacuum and resuspended in 4.5 µl of 0.1 M sodium carbonate buffer, pH 9.0. The appropriate NHS-ester Cy3 or Cy5 dye in DMSO was added (4.5 µl) and the reaction incubated at room temperature in the dark for 2 hours. The reaction was neutralized by adding 35 µl of 100 mM sodium acetate, pH 5.2. The labeled cDNA was purified to remove any uncoupled dye using the Qiagen PCR purification kit with Minelute columns and an extra wash. The labeled cDNA was quantified and evaluated by spectrophotometry and equivalent pmol of Cy3 and Cy5 samples were combined, dried down, and stored at -80°C. Hybridization and subsequent analysis of the arrays will be carried out following protocols established by The J. Craig Venter Institute, essentially as described by (Hegde *et al.*, 2000). Arrays were pre-hybridized at 42°C for 1 hr in 5 × SSC/0.1%SDS/ 1% BSA and washed several times with MilliQ water. Washed arrays were then dipped into isopropanol and dried by centrifugation at 1000 rpm for 10 minutes. For hybridization, Cy3/Cy5 probe mixtures were suspended in 50 µl hybridization buffer (50% formamide, 5 × SSC, 0.1%SDS, 0.1 mM DTT, 0.56 µg/ml sheared salmon sperm DNA), heated then applied to the array under a Lifter™ cover slip. The array was incubated at 42°C in a sealed chamber for 15-20 hr. After hybridization, the array was washed twice in 2 × SSC/0.1% SDS at 55°C, twice in 0.1 × SSC/0.1%SDS, once with 0.1 × SSC, water rinsed, and dried. Arrays were then scanned using an Axon slide reader, scanning in the Cy5 channel and the Cy3 channel. Identification and calculation of spots was carried out using the TIGR Spotfinder software (The J. Craig Venter Institute; <http://www.jcvi.org/cms/research/software/>). Subsequent normalization of fluorescent data was conducted using TIGR-MIDAS (also freely available from The J. Craig Venter Institute). Subtraction of local background and integration of Cy5 and Cy3 fluorescent intensities was calculated using Excel. Array experiments were carried out in triplicate using independently isolated RNA samples. Up- and down-regulated genes (>1.5, < 0.5, p < 0.01) are presented in Figure 4.

Validation of array data

Differentially expressed genes of interest were INDIVIDUALLY confirmed by quantitative real-time reverse transcription-PCR using an Applied Biosystems 7500 Real-Time PCR system by standard methodology (James *et al.*, 2006). Briefly, forward and reverse primers (1.25 µl, 50 pmol each), ABI Power SYBR Green Master Mix (12.5 µl), and cDNA (10 µl, 20 ng per reaction) were added to a 96 well reaction plate. Samples were quantified using standard cycling conditions (50°C, 2 mins; 95°C 10 mins; followed by forty cycles of 95°C, 15 secs and 60 °C, 1 min). Primer details are provided in Table 1.

***Porphyromonas gingivalis* W83 outer membrane protein profiling**

Outer membrane preparations were obtained from *P. gingivalis* cells grown in GAM or GAM-CSE. Briefly, the cell pellet was suspended in 10mM Tris, pH 7.5, 1mM EDTA (TE) containing 100 µg/ml lysozyme and protease inhibitor cocktail and was incubated for 15 minutes at 25°C. The cell suspension was sonicated three times on ice and cellular debris was removed by centrifugation at 1000 × *g* for 10 minutes. The supernatant was subsequently centrifuged at 100,000 × *g* for 60 minutes. The membrane pellet was suspended in 5 mls TE containing 0.5M NaCl and centrifuged again at 100,000 × *g* for 60 minutes. After discarding the supernatant, the membrane pellet was suspended in 5 ml MgCl₂ containing 1% Triton X-100. Triton X-100 insoluble material was then collected by centrifugation at 100,000 × *g*. The resulting outer membrane fraction was suspended in TE containing 0.5% SDS and analyzed by PAGE. Outer membrane preparations (20 µg protein) were electrophoresed through 4-15% gradient SDS-PAGE gels and protein bands were visualized by staining with Coomassie brilliant blue R-250. Gel image analysis and densitometry were performed using the Kodak 4000MM Image Station system (Eastman Kodak, New Haven, CT). Protein identification was achieved by excision of the protein bands of interest (see Figure 5) from SDS-PAGE gels, followed by in-gel trypsin digestion, peptide preparation, MALDI-MS analysis, and bioinformatic identification by peptide mass fingerprinting using the core facilities at the University of Louisville core proteomics laboratory.

Expression of RagB in response to CSE

P. gingivalis cells were sequentially passaged in GAM, GAM-CSE and GAM, respectively. Bacterial cells were lysed (10mM Tris, 100mM NaCl and 1mM EDTA) and probed by western blot (20 µg protein per lane). Total protein estimated using a BCA protein assay kit. MAbE38, an anti-RagB antibody, was a kind gift from Mike A. Curtis. Anti-Rabbit HRP-linked IgG was used as the secondary antibody. Probing and visualization of immunoreactive bands was performed by chemiluminescence using SuperSignal West Pico Chemiluminescent Substrate kit as per the manufacturer's protocol. Gel image analysis and densitometry were performed using the Kodak 4000MM Image Station system.

Statistical analyses

All experiments were repeated a minimum of three times. The statistical approach to the analysis of the microarray data is presented in Figure 4. For all other data, statistical significance between groups was evaluated by ANOVA and the Tukey multiple-comparison test using the InStat program (GraphPad Software, San Diego, CA). Differences between groups were considered significant at the level of $p < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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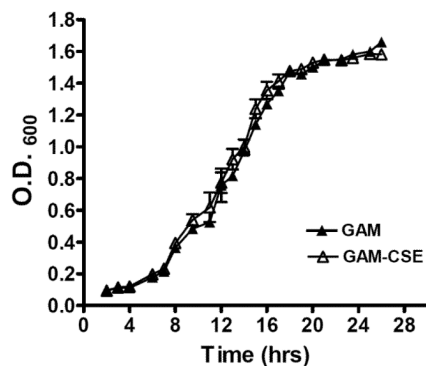


Figure 1. Typical growth curves of *P. gingivalis* W83 in CSE-conditioned and unconditioned medium

Growth of *P. gingivalis* was compared in GAM medium and GAM conditioned with CSE (500 ng ml⁻¹ nicotine equivalents). Closed triangles represent growth in GAM. Open triangles represent growth in GAM-CSE. Error bars represent the mean (s.d.) of 3 experiments. There were no significant differences in the growth characteristics of *P. gingivalis* cultured in GAM or GAM-CSE ($p > 0.05$).

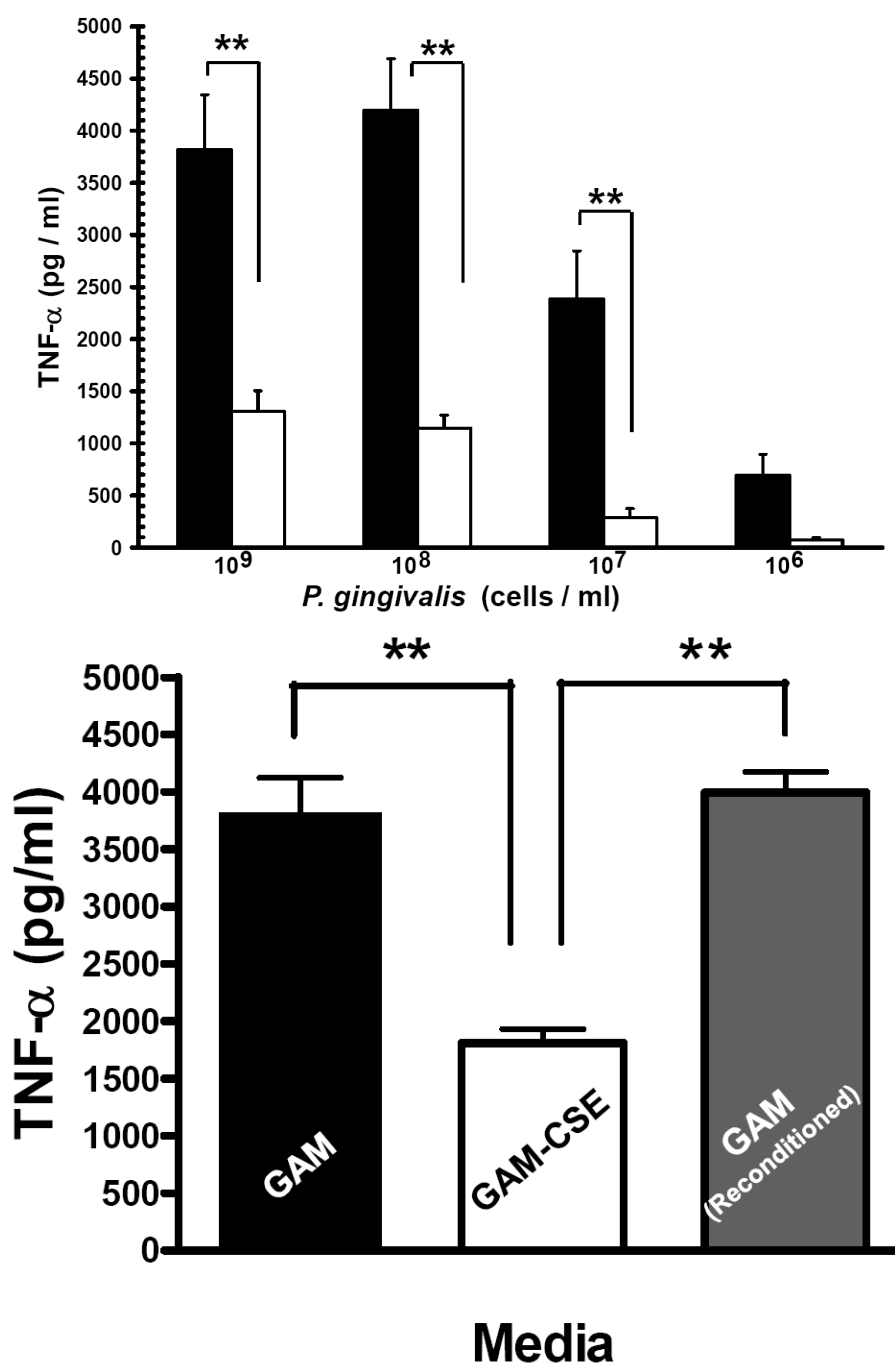


Figure 2. Reversible suppression of TNF- α release by human monocytes stimulated with CSE-treated *P. gingivalis*

(a) Reduced TNF- α release by human monocytes stimulated with CSE-treated *P. gingivalis*. Primary human monocytes (0.5×10^6) were stimulated with 10^6 to 10^9 cells of control *P. gingivalis* (black bars) or GAM-CSE (grey bars) grown *P. gingivalis* cells (20h). Cell-free supernatants were harvested by centrifugation and levels of TNF- α were determined by ELISA. Error bars represent the mean (s.d.) of 3 experiments.

** Indicates statistical significance at $p < 0.01$.

(b) Reconditioning of *P. gingivalis* in GAM rescues pro-inflammatory potential.

Primary human monocytes (0.5×10^6) were stimulated for 20 h with GAM cultured *P. gingivalis*; CSE-treated *P. gingivalis*; or *P. gingivalis* cells that were first grown in GAM-CSE for two passages then reconditioned in untreated GAM for 2 passages (10^7 *P. gingivalis* cells). Monocytes stimulated with reconditioned *P. gingivalis* produced levels of TNF- α that were similar to monocytes that were stimulated with bacteria from control cultures (not exposed to CSE). Error bars represent the mean (s.d.) of 3 experiments.

** Indicates statistical significance at $p < 0.01$.

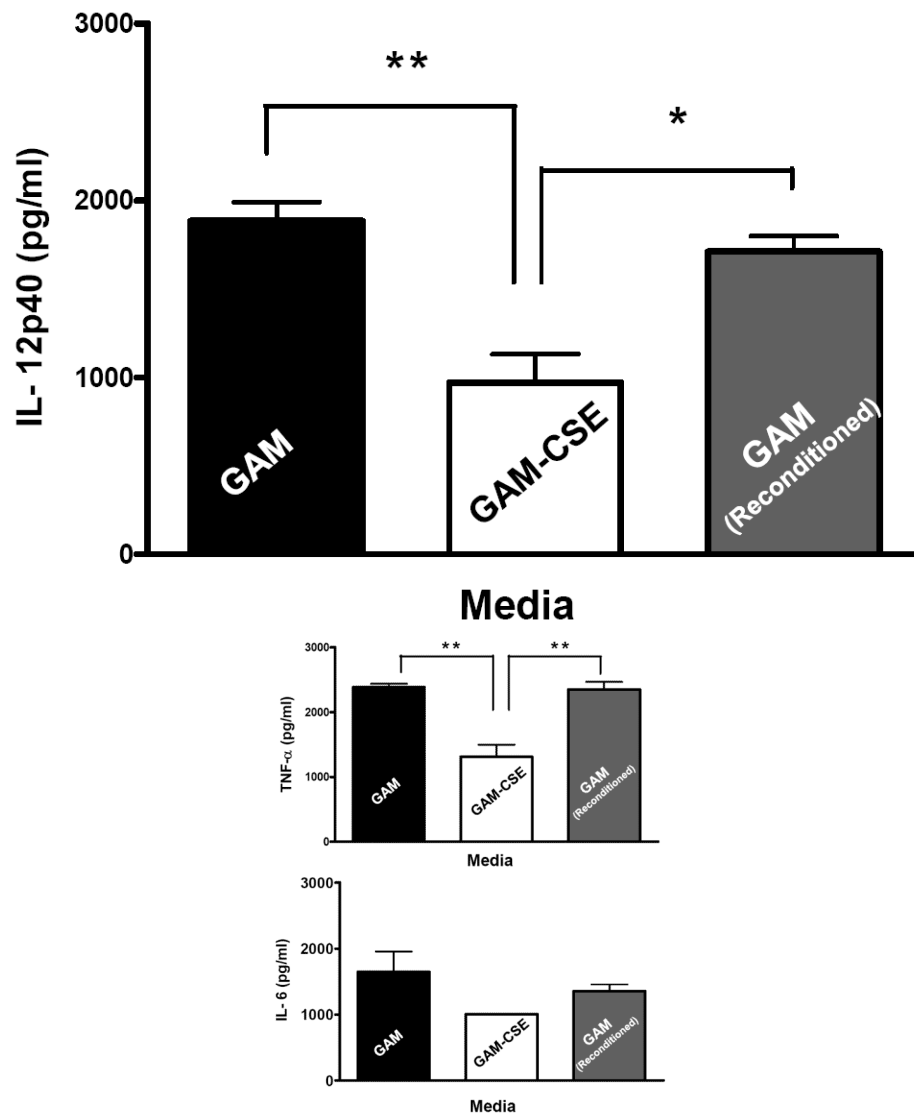


Figure 3. Reversible suppression of multiple pro-inflammatory cytokines in human PBMCs stimulated with CSE-treated *P. gingivalis*

Primary human PBMCs (0.5×10^6) were stimulated for 20 h with GAM cultured *P. gingivalis*; CSE-treated *P. gingivalis*; or *P. gingivalis* cells that were first grown in GAM-CSE for two passages then reconditioned in untreated GAM for 2 passages (10^7 *P. gingivalis* cells). PBMCs stimulated with reconditioned *P. gingivalis* produced levels of TNF- α that were similar to PBMCs that were stimulated with bacteria from control cultures (not exposed to CSE). Error bars represent the mean (s.d.) of 3 experiments.

* Indicates statistical significance at $p < 0.05$.

** Indicates statistical significance at $p < 0.01$.

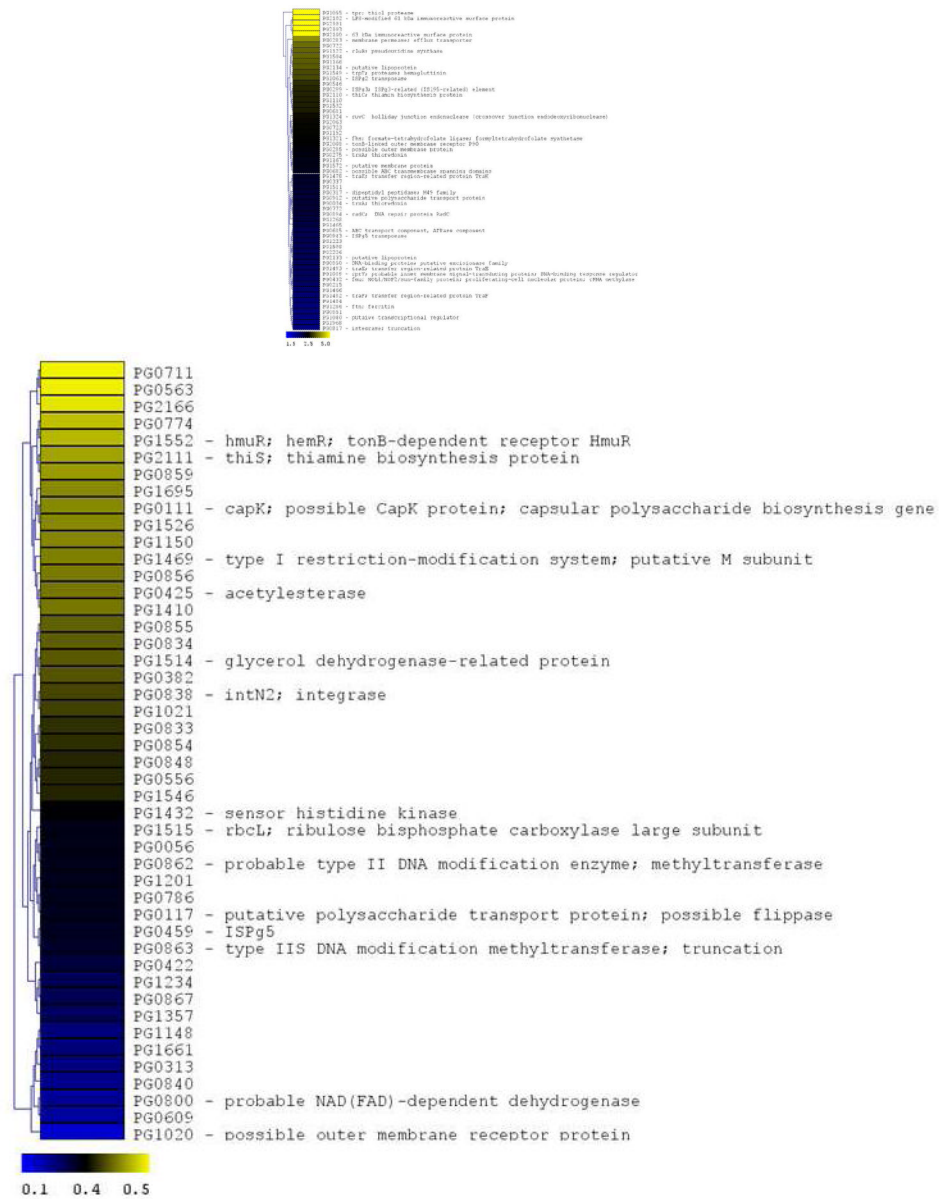


Figure 4. *P. gingivalis* genes differentially expressed following CSE exposure

The microarray data of Log2 ratios were uploaded onto MultipleExperiment viewer 4.1v (January 8, 2008 release) software and evaluated by t-test with p value based on 't' distribution with significance (alpha cut-off) set at $p < 0.01$. The subset of significant genes were divided into two groups based on i) upregulated genes (>1.5 fold increase) and ii) down regulated genes (<0.5 fold). The distance was calculated based on Euclidean distance with average linkage and the tree was generated based on hierarchical clustering. CSE-induced genes are shown in Figure 4a (significantly induced genes >1.5 fold compared to baseline; $p < 0.01$; genes are represented in yellow to blue colour with the lower limit = 1.5; midpoint value = 2.5; and upper limit = 5). CSE-suppressed genes are shown in Figure 4b (significantly down-regulated genes < 0.5 fold compared to baseline; $p < 0.01$; genes are represented in yellow to blue colour with the lower limit = 0.1; midpoint value = 0.4; and upper limit = 0.5).

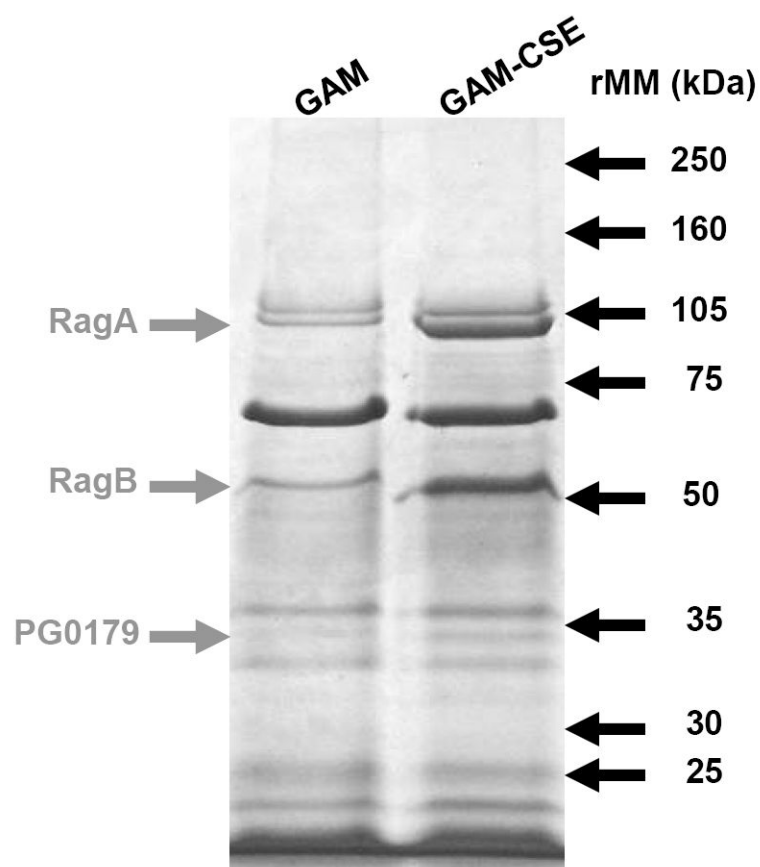


Figure 5. Up-regulation of specific outer membrane protein (OMP) in CSE-exposed *P. gingivalis* Outer membrane proteins of *P. gingivalis* grown in GAM and GAM-CSE were isolated by differential extraction with Triton X-100. 20 μ g total OMP extracts were run through a 4-15% gradient gel. MALDI-MS of excised bands followed by comparison with the ORF database of *P. gingivalis* W83 from The J. Craig Venter Institute identified the major CSE up-regulated bands, as shown.

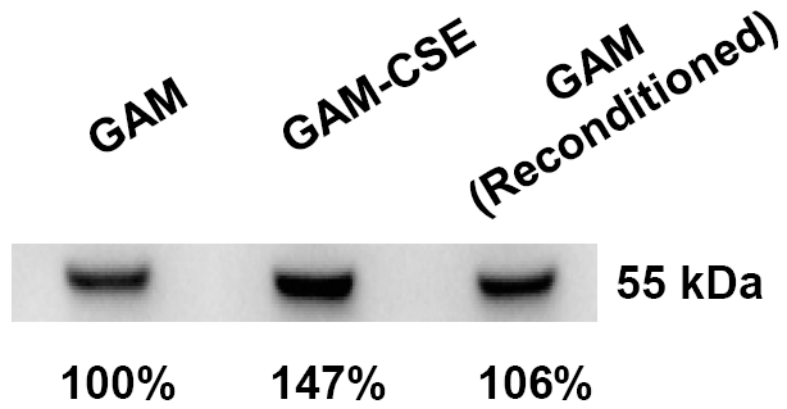


Figure 6. CSE-induced RagB upregulation in *P. gingivalis* is reversible

A typical western blot of RagB in lysates of cells sequentially passaged in GAM, GAM-CSE and GAM, respectively, is shown. Culture in GAM-CSE resulted in an increase in relative RagB expression levels (147%). Relative RagB expression returned to near control levels on reconditioning in GAM.

Table 1

Primers employed in real time PCR analysis of selected CSE-regulated *P. gingivalis* genes

Gene	Forward primer	Reverse primer
PG1055; Tpr	CCTACAGATTGGAGGTGGCT	ATAGGCATGGTATGCTGCAA
PG2102; 61kDa antigen	ATCGTTTGGTCTGATACGCA	CTGCACGTCAGCCTGTATT
PG2100; 63 kDa antigen	ATTACAAGATGGCTGTGGCA	TGCTGTCATGACTGTCCAAA
PG2008; TonB-linked receptor	ATTCTTAGGAACGAGCGCAT	GGGATTCCCTTGATCGAGTA
PG1286; Ferritin	AGCGATCAATGACCAAATCA	CACGGCATCGATAGCTTCT
PG1552; HemR	GATTTGAAGCCGGAGAAGAG	TAAGGCAGTACCACATTCGC
PG0111; CapK	AGGCAACGGAGAAGTATCGT	AAAGCACCATCAATGACGAA
PG1432; Sensor histidine kinase	ACAGCTGAACTGCATCAAC	CTGCATATAAGTGCGGGCTA
PG0117; polysaccharide transport protein	TCTCGAACGACCAATAGTGC	CATTCTCTGCAATCGGCTTA

Primers were designed using the real-time PCR primer design software provided by GenScript (www.genscript.com/ssl-bin/app/primer). T_m of primers was 60 ±2°C (PG1286 forward primer, T_m = 56°C).

Table 2Real time PCR analysis of selected CSE-regulated *P. gingivalis* genes

Locus name *	Putative identification in TIGR and/or Oralgene database	Microarray (fold [mean, s.d.])	RT_PCR (fold [mean, s.d.])
PG1055	Thiol protease (Tpr)	13.93, 4.73	108.69, 21.43
PG2102	LPS-modified immunoreactive 61 kDa antigen	10.58, 1.47	36.62, 1.44
PG2100	Immunoreactive 63 kDa antigen	6.00, 1.23	30.79, 1.47
PG2008	tonB-linked outer membrane receptor P90	2.50, 2.00	1.74, 0.09**
PG1286	Ferritin (ftn)	2.02, 0.85	2.23, 0.21
PG1552	tonB-dependent receptor (HmuR, HemR)	0.47, 0.29	0.72, 0.02***
PG0111	Putative capsular polysaccharide biosynthesis gene (CapK)	0.45, 0.04	0.78, 0.02***
PG1432	Sensor histidine kinase	0.39, 0.10	0.62, 0.05**
PG0117	Putative polysaccharide transport protein	0.36, 0.14	0.50, 0.08

* The J. Craig Venter Institute annotations

** >1.5, <2.0 fold change in gene activity

*** Reduced activity not reaching threshold (<1.5 fold)