Polymicrobial interactions stimulate resistance to host innate immunity through metabolite perception

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Bacteria in the human oral cavity often grow in an attached multispecies biofilm community. Members of this community display defined interactions that have an impact on the physiology of the individual and the group. Here, we show that during coculture growth with streptococci, the oral pathogen *Aggregatibacter actinomycetemcomitans* displays enhanced resistance to killing by host innate immunity. The mechanism of resistance involves sensing of the streptococcal metabolite hydrogen peroxide by *A. actinomycetemcomitans*, which stimulates a genetic program resulting in enhanced expression of the complement resistance protein ApiA. The oxidative stress response regulator OxyR mediates induction of *apiA* transcription, and this induction is required for coculture resistance to killing by human serum. These findings provide evidence that interaction between community members mediates prokaryotic resistance to host innate immunity and reinforce the need to understand how polymicrobial growth affects interaction with the host immune system.

Aggregatibacter | ApiA | complement | peroxide | *Streptococcus gordonii*

The Gram-negative bacterium *Aggregatibacter actinomycetemcomitans* is a common commensal of the human oral cavity and is a causative agent of localized aggressive periodontitis (1). *A. actinomycetemcomitans* inhabits the mammalian oral cavity beneath the gum line in an area between the tooth surface and the gingival epithelium known as the subgingival crevice (2). A consistent supply of nutrients is provided to the subgingival crevice by a serum exudate referred to as crevicular fluid (3) that passes through the gingiva and flows along the teeth (4–7). Oxygen levels within the subgingival crevice vary greatly, from microaerophilic conditions (2.1 kPa) in the "moderate" pockets (5–6 mm in depth) to near-anaerobic conditions (1.6 kPa) in the "deep" pockets (>6 mm in depth) (8). *A. actinomycetemcomitans* resides in the moderate pockets of the subgingival crevice and exhibits enhanced growth under microaerophilic conditions (9).

The mammalian oral cavity is home to a robust microbial community composed of many specialized microbes that are well adapted to growth in this environment. As with many complex communities, interactions between individual community members in the oral cavity have a significant impact on phenotypic aspects of the individuals as well as the group (10). Whether the subgingival crevice is healthy or diseased, *A. actinomycetemcomitans* often resides as a complex surface-associated (biofilm) microbial community, including several species from the genus *Streptococcus*, such as *S. oralis*, *S. sanguis*, *S. mitis*, and *S. gordonii* (10–13). These oral streptococci are typically nonpathogenic and rapidly consume sugars within the subgingival crevice, producing the metabolites lactic acid and hydrogen peroxide (H_2O_2). This physiological ability renders oral streptococci extremely competitive in the oral environment because they consume high-energy carbon sources and excrete metabolites that inhibit growth of neighboring microbes (14).

Our laboratory has pursued the idea that because it inhabits environments with oral streptococci (10–13), *A. actinomycetemcomitans* has adapted survival strategies for exposure to lactic acid and H_2O_2. Indeed, previous studies demonstrated that *A. actinomycetemcomitans* preferentially utilizes lactic acid over high-energy carbon sources, such as glucose, despite the fact that this bacterium grows significantly more slowly with lactic acid (15). The ability to preferentially use lactic acid not only eliminates caries-causing lactic acid from the oral environment but eliminates the need for *A. actinomycetemcomitans* to compete with the more numerous and rapidly growing oral streptococci for carbon (10). Instead, *A. actinomycetemcomitans* has evolved to use the streptococcal metabolic waste product lactic acid for carbon and energy.

Although our previous studies provided insight into the *A. actinomycetemcomitans* response to lactic acid, essentially nothing is known about how *A. actinomycetemcomitans* responds to the other primary metabolite of streptococci, H_2O_2. In this study, we examined the *A. actinomycetemcomitans* response to H_2O_2 by performing a transcriptome analysis of *A. actinomycetemcomitans* biofilms exposed to H_2O_2. In sharp contrast to other bacterial species, only 2 *A. actinomycetemcomitans* genes, *katA* and *apiA*, were differentially regulated on H_2O_2 exposure. In addition, these genes were regulated in a H_2O_2-dependent fashion during coculture with the oral bacterium *S. gordonii*. Induction of the outer membrane protein, ApiA, during coculture provided protection of *A. actinomycetemcomitans* from killing by human serum. Mechanistically, this enhanced protection was enacted by increased binding of the serum protein factor H by ApiA. These results indicate that bacterial resistance to killing by host innate immunity is enhanced during coculture and suggest that *A. actinomycetemcomitans* utilizes a streptococcal metabolite as a cue to an impending immune response.

Results

***A. actinomycetemcomitans* Transcriptional Response to H_2O_2.** For gene expression analyses, *A. actinomycetemcomitans* was grown in a liquid-phase once-flow-through biofilm flow cell (16) and a solid-phase membrane-associated colony biofilm (17). A custom Affymetrix GeneChip microarray (15) was used to monitor gene expression of *A. actinomycetemcomitans* biofilms in the presence or absence of a sublethal concentration of H_2O_2. Of the approximate 1,800 genes (>90% of the total genes in *A. actinomycetemcomitans*) that exhibited detectable expression on the GeneChip, only 2 showed statistically significant, reproducible changes in both models on H_2O_2 exposure (Fig. 1*A*), and these results were verified using RT-PCR (Fig. 1*B*). This is in stark contrast to similar studies with other bacteria, in which 140–520 genes were differentially expressed on H_2O_2 exposure (18–23).

The *katA* gene, which encodes a cytoplasmic catalase (KatA) that directly detoxifies H_2O_2 into O_2 and H_2O, was significantly upregulated in *A. actinomycetemcomitans* biofilms on exposure to H_2O_2. This was not surprising, because *katA* homologues in other bacteria

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A

Colony Biofilms

Fold Change	St. Dev.	ORF #	Description
7.2	1.6	AA0223	catalase *(katA)*
7.0	1.3	AA2485	adhesin invasin, putative *(apiA)*

Flowcell Biofilms

Fold Change	St. Dev.	ORF #	Description
11.1	1.5	AA0223	catalase *(katA)*
6.4	1.1	AA2485	adhesin invasin, putative *(apiA)*

B

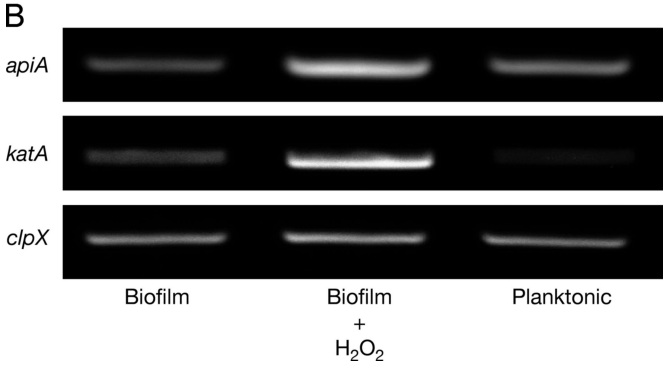

Fig. 1. *A. actinomycetemcomitans katA* and *apiA* are induced on H_2O_2 exposure. (*A*) A custom Affymetrix GeneChip was used to examine gene expression of *A. actinomycetemcomitans* colony and flow cell biofilms in the presence and absence of 1 mM exogenous H_2O_2. Fold changes were determined from 4 pairwise comparisons and determined to be statistically different for *katA* and *apiA* ($P < 0.05$) using GeneChip Operating Software version 1.4. (*B*) RT-PCR was used to verify *katA* and *apiA* induction in colony biofilms on following exposure to H_2O_2. The constitutively expressed gene *clpX* was used to standardize cDNA template levels, and planktonic-grown bacteria were used to assess the impact of enhanced aeration on basal transcript levels.

are induced on exposure to H_2O_2 (18, 20, 23). More surprising was the H_2O_2-mediated induction of *apiA*. ApiA is a 33-kDa trimeric outer membrane protein (also referred to as Omp100) (24) that is 55% identical [using pairwise (p) BLAST] to the nonfimbrial adhesin protein YadA in *Yersinia pestis* and *Yersinia enterolitica* (25). Similar to YadA function in *Yersinia*, ApiA stimulates *A. actinomycetemcomitans* autoaggregation, assists translocation into host cells, and binds the human serum protein factor H (26, 27). Binding factor H inhibits serum complement activity and protects *A. actinomycetemcomitans* from killing by the alternative complement pathway (26). Although similar in function, *yadA* and its homologues in other organisms have not been shown to be induced by H_2O_2 at this time.

***katA* and *apiA* Are Induced During Coculture with *S. gordonii*.** Although our results clearly show induction of *katA* and *apiA* by direct addition of the streptococcal metabolite H_2O_2, it was not clear if these genes were inducible during coculture with oral streptococci. To examine this, transcriptional fusions of the promoter regions of *katA* and *apiA* with the luminescence reporter genes *luxCDABE* were constructed. These reporter fusions allow the transcriptional activity of *katA* and *apiA* to be assessed by monitoring light production. To examine the impact of oral streptococci on *katA* and *apiA* transcription, *A. actinomycetemcomitans* carrying *katA-luxCDABE* and *apiA-luxCDABE* was spread on agar plates and light production was examined on exposure to disks containing the oral

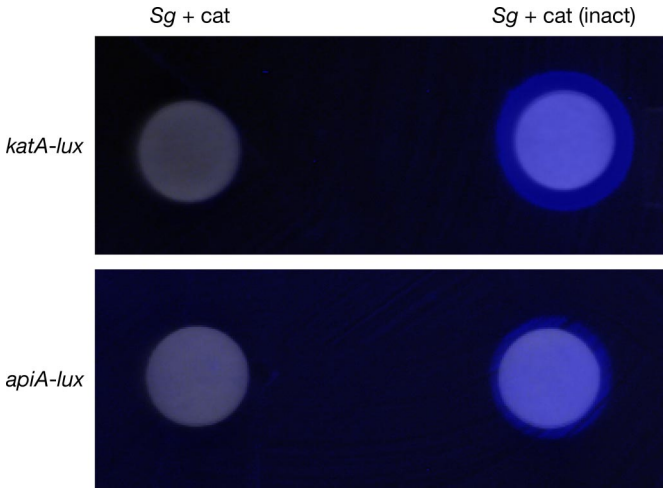

Fig. 2. *katA* and *apiA* are induced during coculture with *S. gordonii*. *A. actinomycetemcomitans* containing the *katA-luxCDABE* or *apiA-luxCDABE* reporter fusion was spread on agar Petri plates and exposed to *S. gordonii*–soaked disks containing active catalase (*Sg* + cat) or heat-inactivated catalase (*Sg*). Light production was examined using a Syngene imaging system. The image is a composite of visible light and luminescence (blue) photographs. Sterile paper disks did not induce light production by either reporter fusion (not shown).

streptococci *S. gordonii*. To ensure that any luminescence induction was attributable to *S. gordonii* H_2O_2 production, 10,000 units of exogenous catalase (or heat-inactivated catalase as a control) was added to each disk. The presence of *S. gordonii* elicited an increase in both *katA* and *apiA* expression by *A. actinomycetemcomitans* (Fig. 2). This induction was dependent on H_2O_2, because addition of exogenous active catalase mitigated this response (Fig. 2). Induction of each reporter was also observed in planktonic coculture [Fig. S1].

OxyR Is Required for H_2O_2-Mediated Induction of *katA* and *apiA*. Many H_2O_2-responsive promoters possess operator sequences that bind the LysR-type transcriptional regulator OxyR (28). OxyR exhibits DNA binding activity after oxidation of 2 reactive cysteine residues by H_2O_2 and can act as an inducer or a repressor of transcription. The *A. actinomycetemcomitans* genome contains an *oxyR*-like sequence (ORF AA1513) that putatively encodes a protein with 72% identity (assessed by pBLAST) to *Escherichia coli* OxyR and shows conservation of both reactive cysteine residues (C199 and C208). To determine if the *katA* and *apiA* promoter regions contain potential OxyR binding sequences, primer extension was used to map the transcriptional start sites of these genes. The results indicate that the promoters of *katA* and *apiA* possess sequences, centered 46.5 and 58 bp upstream of the transcriptional start sites, respectively (Fig. 3 *A* and *B*), with significant similarity to the consensus OxyR binding sequence (Fig. 3*C*) ATAG-n7-CTAT-n7-ATAG-n7-CTAT (29, 30). These sequences suggested that OxyR was a regulator of *katA* and *apiA* transcription. To test this, *oxyR* was insertionally inactivated in *A. actinomycetemcomitans* and transcript levels of *apiA* and *katA* were measured in response to H_2O_2. Inactivation of *oxyR* abrogated the induction of *apiA* and *katA* by H_2O_2, and this activation was restored by expression of *oxyR in trans* (Fig. 4 *A* and *B*, *Insets*).

OxyR Is Critical for Resistance to H_2O_2 and Human Serum. Based on the observation that OxyR regulates *katA* and *apiA*, we hypothesized that inactivation of *oxyR* would have a profound effect on *A. actinomycetemcomitans* survival on exposure to H_2O_2 or human serum. To test this hypothesis, we measured the survival of WT *A. actinomycetemcomitans* and the *oxyR*$^-$ mutant following exposure to H_2O_2 and human serum. Deletion of *oxyR* significantly decreased

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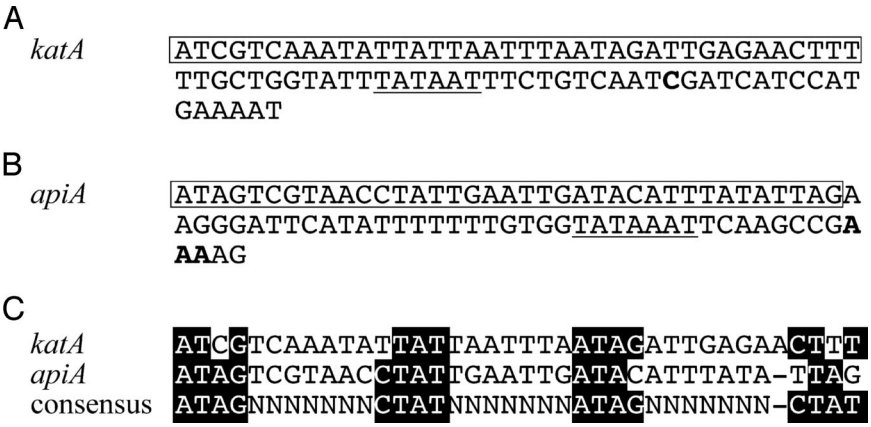

Fig. 3. DNA sequences of the *katA* (base pairs 154293–154374) (*A*) and *apiA* (base pairs 1724157–1724078) (*B*) promoter regions. Transcription start sites are in boldface, putative −10 regions are underlined, and *oxyR*-like binding elements are boxed. (*C*) Alignment of the putative *katA* and *apiA* OxyR binding sequences with the consensus binding sequence (29, 30).

survival of *A. actinomycetemcomitans* on exposure to H_2O_2 and human serum, and expression of *oxyR in trans* restored survival to WT levels (Fig. 4 *A* and *B*). Although it is clear that *katA* is a critical component of H_2O_2 resistance in numerous bacteria (18–23, 29), it was not known if the decrease in serum resistance was attributable to decreased levels of *apiA*. To test this possibility, *apiA* was constitutively expressed in the *oxyR*$^-$ mutant (27). Constitutive expression of *apiA* restored serum survival of the *oxyR*$^-$ mutant to WT levels (Fig. 4*B*), suggesting that *apiA* dysregulation was responsible for enhanced serum killing in this mutant. As expected, expression of *apiA* had no effect on the sensitivity of the *oxyR*$^-$ mutant to H_2O_2 (data not shown). These results demonstrate that the oxidative stress regulator OxyR is necessary for induction of a gene involved in complement defense in *A. actinomycetemcomitans*.

Coculture Enhances Resistance of *A. actinomycetemcomitans* to Alternative Complement Pathway Killing. Based on the observation that *apiA* transcription was enhanced during coculture, we hypothesized that coculture with *S. gordonii* would enhance *A. actinomycetemcomitans* resistance to serum killing. To test this, *A. actinomycetemcomitans* serum survival was assessed during coculture with S. *gordonii* in the presence of heat-inactivated or active catalase. Coculture with *S. gordonii* enhanced survival of *A. actinomycetemcomitans* to human serum by ≈7-fold (Fig. 5), clearly demonstrating enhanced survival of *A. actinomycetemcomitans* during coculture. The presence of active catalase mitigated this increase in survival, implicating *S. gordonii* H_2O_2 production as the key mediator of this phenotype (Fig. 5).

Coculture Enhances Factor H Binding to the *A. actinomycetemcomitans* Cell Surface. From a mechanistic standpoint, it was not clear how induction of *apiA* expression during coculture enhanced resistance to serum. Clues were provided by Asakawa *et al.* (26), who recently demonstrated that ApiA binds the human complement regulatory protein factor H. Factor H is a complement control protein that circulates in human serum, and when bound to cells, it inhibits the alternative pathway of complement activation. Because *apiA* is induced during coculture, we reasoned that the levels of factor H bound to the *A. actinomycetemcomitans* outer surface would be increased during coculture with *S. gordonii*. To examine this, immunofluorescence staining with an anti-factor H antibody was used to quantify the levels of factor H bound to the *A. actinomycetemcomitans* outer surface during monoculture and coculture growth. Our results revealed that ≈4-fold more factor H was bound to *A. actinomycetemcomitans* during coculture with *S. gordonii* as compared with monoculture conditions (Fig. 6). This enhanced binding of factor H during coculture was mitigated by the addition of active catalase, indicating that, as expected, H_2O_2 was the critical cue mediating increased binding.

Discussion

A. actinomycetemcomitans is a common commensal of the mammalian oral cavity, where it resides in a complex microbial community within the subgingival crevice. The subgingival crevice is distinct from the exposed tooth surface and poses several challenges for *A. actinomycetemcomitans* growth and survival, including competition with faster growing bacteria for nutrients; the presence of antimicrobial serum proteins such as immunoglobins, complement, and antimicrobial peptides (6, 31, 32); and the presence of high levels of metabolites produced by other members of the microbial community. Two of the most prominent microbial metabolites produced in the oral cavity are lactic acid and H_2O_2. As observed with lactic acid (15), our results demonstrate that *A. actinomycetemcomitans* displays a unique response to H_2O_2 exposure. It is intriguing that only 2 *A. actinomycetemcomitans* genes exhibited a significant change in gene expression on exposure to H_2O_2 (Fig. 1). This contrasts with transcriptome studies in many other aerobically or microaerophilically grown bacteria, which have shown large numbers of genes (140–520) differentially regulated in response to H_2O_2 (18–23). Many of the H_2O_2-responsive genes identified in these previous studies, such as DNA repair proteins and superoxide dismutases, are expressed by *A. actinomycetemcomitans* but are not responsive to H_2O_2, suggesting that these genes may be constitutively expressed in *A. actinomycetemcomitans* as an adaptation to frequent H_2O_2 exposure.

The H_2O_2 response was observed not only on exogenous addition of H_2O_2 but during coculture with the H_2O_2-producing oral bacterium *S. gordonii* (Fig. 2 and Fig. S1). Not surprisingly, *A. actinomycetemcomitans* displayed enhanced production of the H_2O_2-consuming enzyme catalase during coculture. This response has been observed previously in several bacterial species and serves to enhance resistance to H_2O_2 (18–23, 29). More intriguing was the finding that *A. actinomycetemcomitans* also induces the outer membrane protein ApiA during coculture. Because ApiA provides protection from killing by the alternative complement component of innate immunity (26), our data represent a demonstration of a metabolic cue produced by one bacterium mediating enhanced resistance to a component of host innate immunity by another. It should be noted that the mechanism of complement inhibition, namely, enhanced binding of the serum protein factor H (Fig. 6), not only protects the bacterium from the alternative complement pathway but signif-

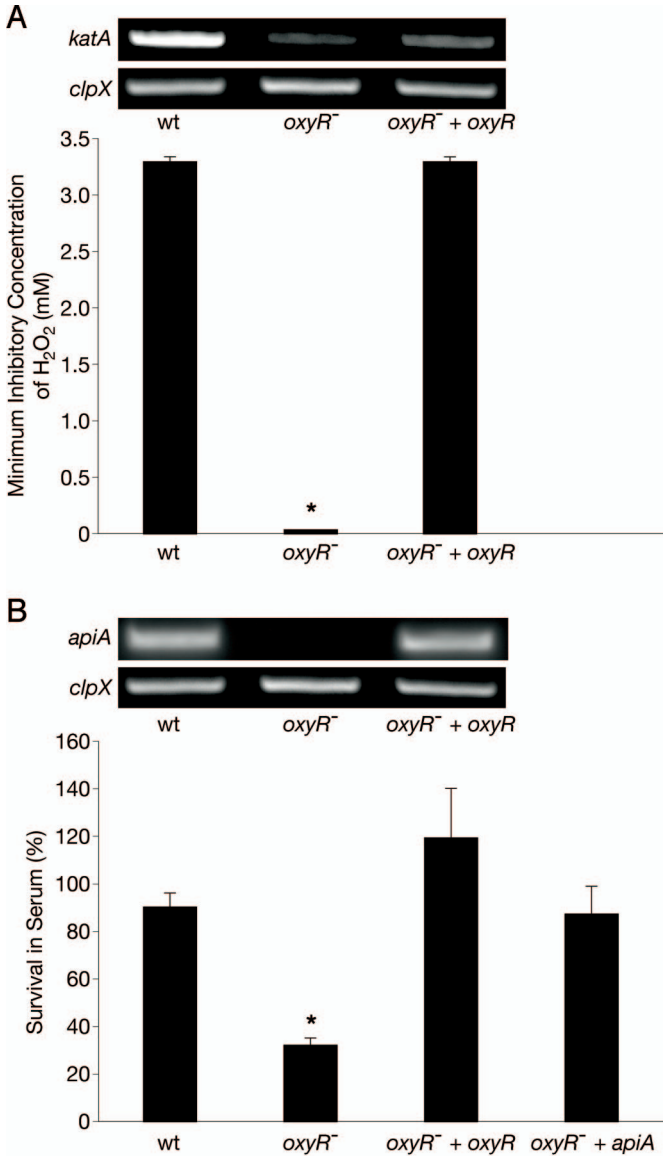

Fig. 4. The *A. actinomycetemcomitans oxyR*$^-$ mutant is hypersusceptible to killing by H_2O_2 and human serum. (*A*) The H_2O_2 minimum inhibitory concentration (lowest concentration necessary to inhibit visible growth of an organism) of WT *A. actinomycetemcomitans* (wt), the *A. actinomycetemcomitans oxyR*$^-$ mutant (*oxyR*$^-$), and the genetically complemented *A. actinomycetemcomitans oxyR*$^-$ mutant (*oxyR*$^-$ + *oxyR*). (*Inset*) RT-PCR analysis of mRNA levels of *katA* and the *clpX* constitutively expressed control in the wt *oxyR*$^-$, and *oxyR*$^-$ + *oxyR* after H_2O_2 exposure. (*B*) Survival of wt, *oxyR*$^-$, *oxyR*$^-$ + *oxyR*, and *oxyR*$^-$ constitutively expressing *apiA* (*oxyR*$^-$ + *apiA*) in the presence of 50% (vol/vol) normal human serum. Percent survival was calculated as follows: number of cells recovered from normal human serum treatment/number of cells recovered from heat-inactivated serum treatment. (*Inset*) RT-PCR analysis of mRNA levels of *apiA* and the *clpX* constitutively expressed control in the wt, *oxyR*$^-$, and *oxyR*$^-$ + *oxyR* after H_2O_2 exposure. As a control, exogenous catalase was added to serum to ensure that any phenotype observed was not attributable to endogenous H_2O_2 within serum. Error bars represent SEM. *$P < 0.004$ via Student's *t* test, $n = 3$.

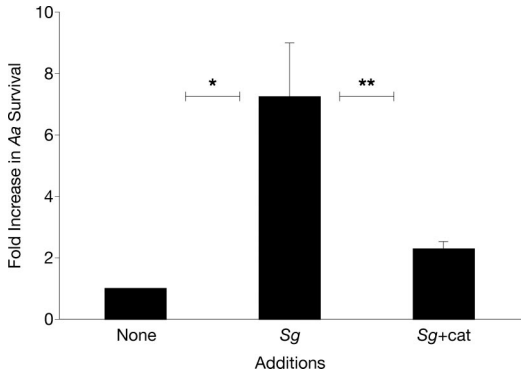

Fig. 5. Coculture with *S. gordonii* enhances *A. actinomycetemcomitans* resistance to killing by human serum. Fold increase in survival of *A. actinomycetemcomitans* on exposure to 50% (vol/vol) normal human serum when grown in monoculture (*Aa*), coculture with *S. gordonii* + heat-inactivated catalase (*Aa*+*Sg*), and coculture with *S. gordonii* + catalase (*Aa*+*Sg*+cat). Ratios were calculated as follows: colony-forming units present after serum treatment/colony-forming units present after heat-inactivated serum treatment. Error bars represent SEM. It is important to note that cell numbers were similar with and without catalase in the heat-inactivated complement cultures. *$P < 0.01$, **$P < 0.05$ via Student's *t* test, $n = 3$.

icantly decreases complement-mediated opsonic uptake and killing by host phagocytic cells (33). Thus, it is likely that the impact of coculture on *A. actinomycetemcomitans* resistance to innate immune effectors extends beyond our observations of protection from the alternative complement pathway.

Based on murine studies, it is likely that *A. actinomycetemcomitans* is exposed to H_2O_2 produced by streptococci in the oral cavity (14). Why would *A. actinomycetemcomitans* respond to H_2O_2 by enhancing resistance to complement killing? Although the answer is unknown, in vitro studies reveal that as oral streptococci numbers increase, the levels of streptococcal H_2O_2 increase because of the accumulation of lactic acid (34). This has been shown to have critical consequences in several nonoral in vivo models, because H_2O_2 produced by streptococci (35, 36), in combination with other factors such as lipoteichoic acids (37, 38), induces significant inflammation. Inflammation leads to increased vascular permeability, influx of serum, and recruitment of neutrophils to the subgingival crevice (3). In the context of these previous studies, we propose a model (Fig. 7) in which enhanced levels of streptococcal H_2O_2 produced during early inflammation stimulate a more robust immune response, followed by an influx of innate immune modulators. *A. actinomycetemcomitans* responds to rising H_2O_2 by enhancing resistance to innate immune effectors. In this sense, *A. actinomycetemcomitans* utilizes H_2O_2 as an anticipatory signal for an enhanced immune response.

Of course, streptococci are not the only source of H_2O_2 in the subgingival crevice. Another source is host tissues, which produce endogenous H_2O_2 from the mitochondria during aerobic respiration (39, 40). Based on the fact that host tissues produce significantly lower levels of H_2O_2 compared with *S. gordonii* (34, 39, 40) and that most of the host peroxide is likely scavenged by host catalase, we predict that endogenous H_2O_2 production has little impact on *A. actinomycetemcomitans* gene expression. This is likely not the case for neutrophils, which produce high levels of H_2O_2 on recruitment to the site of inflammation (41, 42). Thus, during gingival inflammation, *A. actinomycetemcomitans* also faces high levels of neutrophil-produced H_2O_2. Our model (Fig. 7) predicts that before significant neutrophil recruitment to the subgingival crevice, neutrophil H_2O_2 likely has little impact on *A. actinomycetemcomitans* gene expression. Instead, we predict that during early inflammation, streptococcal H_2O_2 will be the primary stimulant for enhanced production of ApiA and KatA. This initial stimulation by streptococcal H_2O_2 will augment the inflammatory response (35, 36) and stimulate recruitment of neutrophils to the subgingival crevice. We propose that the initial *A. actinomycetemcomitans* response to streptococcal H_2O_2 not only provides resistance to the influx of alternative complement during inflammation but likely provides resistance to neutrophil-produced H_2O_2. During these latter stages of inflammation, neutrophil H_2O_2 may also serve as an additional

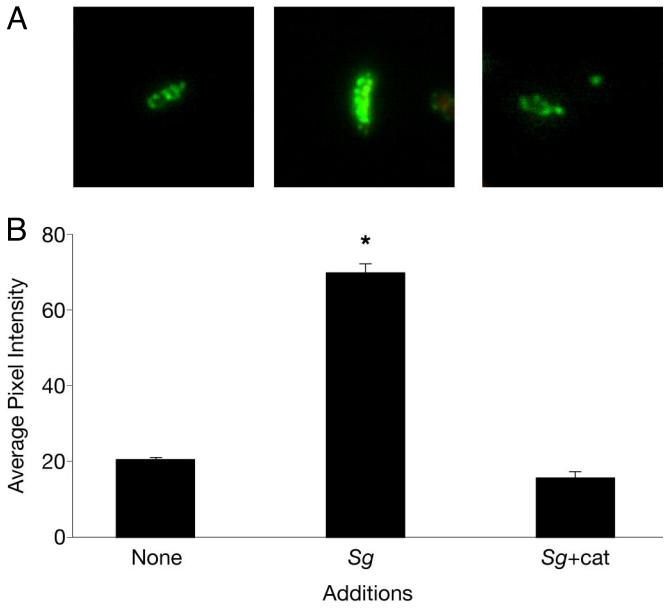

Fig. 6. Factor H displays enhanced binding to *A. actinomycetemcomitans* during coculture with *S. gordonii*. (*A*) Immunofluorescent micrographs of factor H attachment to the surface of *A. actinomycetemcomitans* during monoculture (*Aa*), coculture with *S. gordonii* + heat-inactivated catalase (*Aa*+*Sg*), and coculture with *S. gordonii* + catalase (*Aa*+*Sg*+cat). Images were recorded at magnification ×1,000. (*B*) Average green channel fluorescence intensity per cell. Averages were calculated from 40 independent measurements. Error bars represent SEM. *$P < 0.0001$ via Student's *t* test.

stimulus to *A. actinomycetemcomitans* to enhance expression of *katA* and *apiA*. Of course, this model requires vigorous in vivo testing in the future; however, these studies are significantly hampered by the lack of a robust primate model.

Our results demonstrate that *A. actinomycetemcomitans* uses the *S. gordonii* metabolite H_2O_2 as a cue to induce expression of *katA* and *apiA*, whose products aid in defense against host innate immunity. These findings suggest that *A. actinomycetemcomitans* may use H_2O_2 as an indicator of an impending host innate immune response in vivo and provide a description of a polymicrobial interaction that influences resistance to host innate immunity. There is considerable interest in understanding how pathogenic microbes evade host innate immunity. In fact, there is substantial effort aimed at developing therapeutics to enhance the effectiveness of the innate immune response (43). Such studies have focused on treatment of monoculture infections, despite the observation that many infections are polymicrobial. Our results clearly show that interaction between 2 prominent oral bacteria significantly affects killing by host innate immunity and reinforce the idea that understanding how polymicrobial interactions affect resistance to innate immunity is critical when examining interactions with the host immune system.

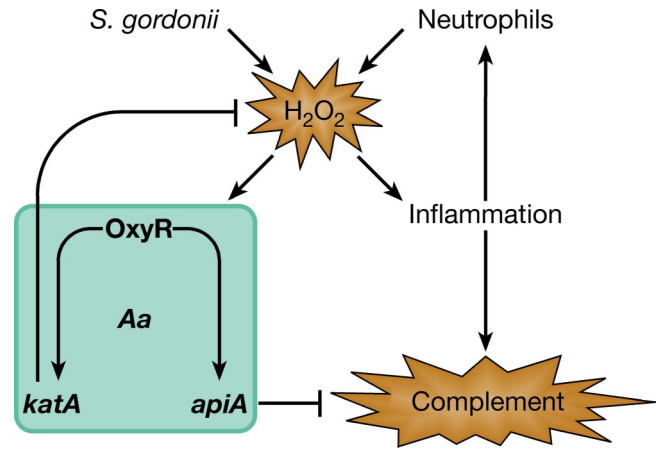

Fig. 7. Model for the role of H_2O_2 as a mediator of *A. actinomycetemcomitans* resistance to innate immunity. Enhanced levels of H_2O_2 produced by *S. gordonii* during plaque growth stimulate inflammation, leading to an influx of innate immune modulators, including complement and neutrophils. *A. actinomycetemcomitans* responds to rising H_2O_2 by induction of *katA* and *apiA*, which, in turn, enhance resistance to innate immune effectors. On recruitment to the site of inflammation, neutrophils increase the levels of H_2O_2 and further stimulate induction of *katA* and *apiA*.

Materials and Methods

Strains and Media. *A. actinomycetemcomitans* strains VT1169 (44), Y4 (45), *S.* strain Challis DL1.1 (ATCC 49818), *E. coli* DH5α, and *E. coli* SM10 were used in this study. *A. actinomycetemcomitans* strains were grown in brain heart infusion medium, tryptic soy broth + 0.5% yeast extract (TSBYE), or chemically defined medium (CDM) with 20 mM glucose (15). Culture conditions were at 37 °C in a 10% (vol/vol) CO_2 atmosphere with shaking at 165 rpm unless otherwise indicated. *E. coli* strains were grown on LB at 37 °C. Where applicable, antibiotics were used at the following concentrations: chloramphenicol at 2 μg/mL for selection and maintenance in *A. actinomycetemcomitans* and at 20 μg/mL for selection and maintenance in *E. coli*. In both *A. actinomycetemcomitans* and *E. coli*, spectinomycin was used at 50 μg/mL for selection and at 10 μg/mL for maintenance, and streptomycin was used at 50 μg/mL for selection and at 20 μg/mL for maintenance.

DNA and Plasmid Manipulations. DNA and plasmid isolations were performed using standard methods (46).

GeneChip and RT-PCR Analysis. For flow cell biofilm experiments, cells were grown in 20% (vol/vol) TSBYE medium in a once-flow-through biofilm flow cell as described (47). Biofilms were allowed to mature for 18 h, and TSBYE, with or without 1 mM H_2O_2, was then added for 30 min. To harvest biofilm cells, the coverslip was removed from the flow cell with a razorblade and vortexed for 1 min in 20 mL of RNALater to remove attached cells. For colony biofilms, 10^5 cells were spotted onto a UV-sterilized 0.2-μm polycarbonate membrane on 10 mL of solid CDM containing 1.5% (vol/vol) agarose in a 100-mm Petri dish. Cells were then grown for 32 h at 37 °C in a 10% (vol/vol) CO_2 atmosphere. The membranes were transferred to identical Petri plates and incubated for 2 h before being transferred to another Petri plate containing CDM with or without the addition of 1 mM H_2O_2. After 20 min, the membranes were transferred into 20 mL of RNALater. Cells were harvested from the membranes by gentle vortexing in the RNALater solution for ≈2 min until no cells visibly remained on the membrane. RNA isolation, preparation of labeled cDNA, and processing of the *A. actinomycetemcomitans* GeneChip microarrays were performed as described previously (48). Data analysis was performed using GeneChip Operating Software version 1.4 (Affymetrix). RT-PCR was performed as described (47) with the following changes: 100 ng of RNA was used for cDNA synthesis, 1 ng of cDNA was used as a template in the *katA* and *apiA* PCR reactions, and 5 ng of cDNA was used as a template in the *clpX* PCR. RT-PCR primers are included in *SI Text*. Planktonic *A. actinomycetemcomitans* was grown to the midexponential phase (OD_{600} = 0.4) and mixed 1:1 with RNALater before RNA purification.

Primer Extension. Primer extension was performed as previously described (49). Primers used were *apiA*-PE (5′-tctttagcccaatgcattgacaga-3′) and *katA*-PE (5′-catggtgttgtcattatcca-3′). The sizes of primer extension products were determined at the University of Oklahoma Health Science Center sequencing core facility.

Luminescence Reporter Assays. A total of 10^7 *A. actinomycetemcomitans* carrying either the *apiA-luxCDABE* or *katA-luxCDABE* reporter (reporter construction described in *SI Text*) was spread evenly over the surface of a TSBYE agar plate and grown overnight at 37 °C. Two 0.6-cm paper disks containing 10^7 *S. gordonii* were added to each *A. actinomycetemcomitans*–coated TSBYE agar plate. One disk received 10,000 U of bovine catalase (Sigma), and the other disk received 10,000 U of heat-killed catalase. The plates were incubated for an additional 4 h at 37 °C before imaging each for 15 min with a Syngene G:Box (Syngene) imaging system.

Construction and Complementation of an *oxyR*$^-$ Mutant in *A. actinomycetemcomitans.* Construction and complementation of the *oxyR*$^-$ mutant were performed as previously described (15) and are detailed in *SI Text*.

Serum Sensitivity Factor H Binding. *A. actinomycetemcomitans* serum sensitivity was determined as previously described (26) with minor modifications. Details of this method can be found in *SI Text*. *A. actinomycetemcomitans* and *S. gordonii* cocultures were grown as described for the serum sensitivity assay described previously. After 30 min of coculture, 100 μL of human serum was added to 900 μL of coculture and incubated at 37 °C for 30 min. Cells were collected by centrifugation at 5,000 $\times$ *g* for 10 min, and cell pellets were resuspended in 100% (vol/vol) ice-cold methanol and fixed at −20 °C for 2 h. After fixing, cells were stained as described (50) using 1:1,000 mouse anti-human factor H antibody (Santa Cruz Biotechnology) and 1:1,000 Alexa-488 goat anti-mouse (Invitrogen) secondary antibody. Images were captured at a magnification $\times$1,000 using a Nikon 50i microscope, 100 $\times$ 1.4NA PLAN APO lens, Nikon DS-2MBW digital camera, and Nikon NIS-Elements D 3.0 software.

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