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Community signaling between *Streptococcus gordonii* and *Porphyromonas gingivalis* is controlled by the transcriptional regulator CdhR

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Summary

Interspecies signaling between *P. gingivalis* and *S. gordonii* serves to constrain development of dual species communities. Contact with *S. gordonii* propagates a tyrosine phosphorylation dependent signal within *P. gingivalis* that culminates in reduced transcription of adhesin and signaling genes. Here we demonstrate the involvement of the *P. gingivalis* orphan LuxR family transcription factor PGN_1373, which we designate CdhR, in this control pathway. Expression of *cdhR* is elevated following contact with *S. gordonii*; however, regulation of *cdhR* did not occur in a mutant lacking the tyrosine phosphatase Ltp1, indicating that CdhR and Ltp1 are components of the same regulon. Contact between *S. gordonii* and a CdhR mutant resulted in increased transcription of *mfa*, encoding the subunit of the short fimbriae, along with higher levels of Mfa protein. Expression of *luxS*, encoding AI-2 synthase, was also increased in the *cdhR* mutant after contact with *S. gordonii*. The Mfa adhesive function and AI-2-dependent signaling participate in the formation and development of dual species communities, and consistent with this the *cdhR* mutant displayed elevated accumulation on a substratum of *S. gordonii*. Recombinant CdhR protein bound to upstream regulatory regions of both *mfa* and *luxS*, indicating that CdhR has a direct effect on gene expression. LuxS was also found to participate in a positive feedback loop that suppresses CdhR expression. Interaction of Mfa fimbriae with *S. gordonii* is necessary to initiate signaling through CdhR. These results reveal CdhR to be an effector molecule in a negative regulatory network that controls *P. gingivalis*-*S. gordonii* heterotypic communities.

Keywords

biofilm; fimbriae; luxS; signaling

Introduction

Bacteria on surfaces tend to accumulate into complex communities known as biofilms. Community development proceeds through distinct stages involving attachment, accretion, proliferation and dispersal (Stoodley *et al.*, 2002, O'Toole *et al.*, 2000). The temporal and spatial progression of communities is associated with the expression of distinct sets of

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genes, resulting in phenotypic differences not only between biofilm and planktonic cells, but also between cells in different stages of community development (Stanley and Lazazzera, 2004, Davey and O'Toole, 2000). On human mucosal surfaces such as in the oral cavity and lower gastrointestinal tract, bacterial biofilms comprise multiple species (Kuboniwa and Lamont, 2010, Macfarlane, 2008). Assembly of these complex communities involves interbacterial attachment and signaling, and organisms tend to accumulate in groupings that are physiologically compatible (Jenkinson and Lamont, 2005, Kolenbrander *et al.*, 2002, Kuramitsu *et al.*, 2007).

Dental plaque is a complex multispecies biofilm community that develops on tooth surfaces and is a direct precursor of periodontal disease, one of the most common infectious diseases in developed countries (Rosan and Lamont, 2000). While over 700 species or phylotypes of bacteria can inhabit mature dental plaque (Aas *et al.*, 2005), a smaller subset of these are considered potential pathogens in periodontal disease. Foremost among these pathogenic biofilm constituents is the gram-negative anaerobe *Porphyromonas gingivalis* (Ximenez-Fyvie *et al.*, 2000b). This organism possesses an array of virulence factors that enable colonization and persistence in dental biofilms, and mediate destruction of the periodontal tissues in susceptible hosts (Lamont and Jenkinson, 1998, Hajishengallis, 2009). *P. gingivalis* is a later or secondary colonizer of biofilm communities, relying on antecedent organisms to provide attachment sites and physiological support (Kuboniwa and Lamont, 2010, Socransky *et al.*, 1999). In dental plaque, *Streptococcus gordonii* is one of the early colonizers that initiate biofilm formation, and *P. gingivalis* is capable of accumulating into a heterotypic community with *S. gordonii* and related oral streptococci (Demuth *et al.*, 2001, Kuboniwa *et al.*, 2006, Simionato *et al.*, 2006). *P. gingivalis* can bind directly to *S. gordonii* without the need for bridging organisms (Kuboniwa and Lamont, 2010) and *P. gingivalis* and *S. gordonii* demonstrate mutualistic growth (Periasamy and Kolenbrander, 2009). Colonization of the oral cavity by *P. gingivalis* is facilitated by the presence of antecedent colonizers such as *S. gordonii* (Holt and Ebersole, 2005), and indeed introduction of *P. gingivalis* into the mouths of human volunteers results in almost exclusive localization in areas of streptococcal-rich supragingival plaque (Slots and Gibbons, 1978).

Community formation between *P. gingivalis* and *S. gordonii* ensues from initial co-adhesion that is mediated by at least two sets of adhesin-receptor pairs. The *P. gingivalis* long fimbriae, comprised of the FimA structural subunit, bind to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) present on the streptococcal surface (Maeda *et al.*, 2004). In addition, the *P. gingivalis* short fimbriae, comprised of the Mfa structural subunit, engage the streptococcal SspA/B (Antigen I/II) adhesins (Park *et al.*, 2005) through an approximately 27 aa binding epitope of SspA/B termed BAR (Demuth *et al.*, 2001). Within the BAR domain, binding specificity is determined by amino acid motifs that resemble the consensus nuclear receptor (NR) box protein-protein interacting domain sequence of eukaryotes (Daep *et al.*, 2008). The crystal structure of SspB shows that the BAR domain protrudes from the protein, resembling a handle that is available for attachment (Forsgren *et al.*, 2010). Community development with *P. gingivalis* does not occur with streptococci that lack the BAR motif, such as *S. mutans* and *S. intermedius* (Kuboniwa and Lamont, 2010). In this manner, pioneer organisms such as the oral streptococci can influence the composition and pathogenic potential of the multispecies plaque biofilm.

Once attached to a streptococcal substratum, *P. gingivalis* executes a complex regulatory pathway that culminates first in the formation of heterotypic microcolonies, and ultimately in limitation of excessive microcolony development. Initially, community accumulation requires signaling mediated by AI-2 that can be derived from either *P. gingivalis* or *S. gordonii* (McNab *et al.*, 2003). Dampening this process is a regulatory pathway involving Ltp1, a Low Molecular Weight Tyrosine Phosphatase. Levels of Ltp1 are increased

following contact with *S. gordonii* (Maeda *et al.*, 2008), and Ltp1 phosphatase activity downregulates transcription of *luxS* and of several genes involved in exopolysaccharide synthesis and transport. Ltp1 also positively regulates transcription of the *hmu* operon involved in hemin uptake and dephosphorylates the gingipain proteases. Thus Ltp1 may be part of a more general pathway that controls the pathogenic potential of *P. gingivalis*. However, the mechanisms by which Ltp1 effectuates control of gene transcription related to community development remain to be established.

The genome of *P. gingivalis* contains a number of transcriptional regulators, including members of the LuxR family. However, *P. gingivalis* does not produce acyl homoserine lactones (AHLs) and thus its LuxR regulators are considered orphans (or solos) in that they lack a cognate LuxI AHL synthase. Unpaired LuxR regulators are becoming increasingly recognized and studied, and they can control properties as diverse as type IV secretion, antibiotic production, exopolysaccharide synthesis and biofilm formation (Patankar and Gonzalez, 2009, Subramoni and Venturi, 2009). The *P. gingivalis* 33277 LuxR orphan PGN_1373 has been found to control transcription of the *hmu* operon in a cell density dependent manner (Wu *et al.*, 2009), properties consistent with participation in the regulatory network that could also involve Ltp1. In this study we characterize the community associated role of PGN_1373, which we designate CdhR (Community Development and Hemin Regulator). Expression of *cdhR* was elevated by *S. gordonii* and this regulation requires the presence of Ltp1. Mutants lacking CdhR exhibited enhanced heterotypic community formation with *S. gordonii*. CdhR bound to the upstream regulatory regions of *mfa* and *luxS* resulting in reduced expression and thus contributing to restricted community development.

Results

Transcriptional organization of PGN_1373 (CdhR)

The *P. gingivalis* orphan LuxR transcriptional regulator PGN_1373 (hereinafter called CdhR) is flanked by PGN_1372 upstream and transcribed in the same direction, and by PGN_1374 downstream and transcribed in the opposite direction (Fig. 1A). PGN_1372 is annotated as a hypothetical protein with a hemerythrin cation binding domain; and PGN_1374 is annotated as ribosomal large subunit pseudouridine synthase D. The end of the PGN_1372 coding region and the beginning of *cdhR* coding region overlap by 4 bases, and cotranscription of PGN_1372 and *cdhR* was confirmed by RT-PCR (Fig. 1B). PCR was carried out by using two different forward primers recognizing regions 250 bp upstream or 60 bp downstream of initiation codon of PGN_1372 (primers are underlined in Fig. 1C). The reverse primer was designed to bind to *cdhR* coding region. Genomic DNA template confirmed the fidelity of the primers which amplified the predicted 1.5 kb or 1.2 kb products (Fig. 1B). However, only the forward primer in the PGN_1372 coding region was able to amplify the 1.2 kb fragment with cDNA as a template (Fig 1B, right side). This result suggested that PGN_1372 and *cdhR* are in the same operon and the transcriptional start site is located within 250 bp from ATG initiation codon of PGN_1372.

Next we focused on the upstream region of PGN_1372 as a 5′-RACE target to define the transcriptional start site (tss) for *cdhR*. In 6 independent RACE reactions the tss was mapped to an A residue 22 bp upstream of the ATG start codon of PGN_1372 (Fig. 1C) The tss of PGN_1372 is thus closer to the translational initiation site than has been reported for other *P. gingivalis* genes such as *kgp* (−170), *rgpA* (−109) and *rgpB* (−131) (Jackson *et al.*, 2000). However, transcriptional initiation closer to the translational start occurs with the fimbrial genes: *fimA* has a tss located at −41 (Park *et al.*, 2007), while the tss for *mfa* is at −44 (Park *et al.*, 2006). Putative −10 and −35 elements for PGN_1372 were centered at −11/12 and −32/33 from the tss. The −10 sequence TATACT has a 5/6 match with the P3″ consensus

element identified for a subset of *P. gingivalis* promoters (Jackson *et al*., 2000). There is also a putative P5′ element (Jackson *et al*., 2000) of sequence GGGATT centered at −39/40, however a P3′ element was not identified in this regulatory region.

The *cdhR* gene was replaced with *ermF* and quantitative RT-PCR (qRT-PCR) performed on total mRNA from parental and Δ*cdhR* strains. *cdhR* message was absent in Δ*cdhR*, and levels of PGN_1372 were similar in parental and mutant strains demonstrating that the mutation did not have an effect on the adjacent cotranscribed gene, and that *cdhR* is unlikely to be autoregulated (not shown).

CdhR is regulated in *P. gingivalis-S. gordonii* heterotypic communities

On streptococcal substrata *P. gingivalis* cells are recruited from the fluid phase and accrete into rudimentary biofilm microcolonies. Contact and short range signaling occurs between *P. gingivalis* and *S. gordonii*, and *P. gingivalis* regulates its transcriptome and proteome to adapt to the community environment (Simionato *et al.*, 2006, Kuboniwa *et al.*, 2009). This initial increase in biomass of *P. gingivalis* does not require exogenous nutrients and is independent of growth and division (Kuboniwa *et al.*, 2009, Kuboniwa *et al.*, 2006, Maeda *et al.*, 2008). These signaling and response interactions also occur in mixed bacterial aggregates (Park *et al.*, 2006), and proteins that are regulated in aggregates have been found to play a role community development (Maeda *et al.*, 2008, Kuboniwa *et al*., 2009). Pelleted aggregates of organisms thus provide a facile means to study regulatory circuitry (Kreth *et al*., 2005). Hence, to begin to assess the role of CdhR in *P. gingivalis-S. gordonii* community development we employed qRT-PCR of total RNA to examine expression levels in heterotypic aggregates of *P. gingivalis* with *S. gordonii. S. cristatus* which does not support *P. gingivalis* accumulation was employed as a control. In addition, the relationship of CdhR to Ltp1, a key component of the community constraint pathway, was investigated with an *ltp1* deletion mutant.

Contact with *S. gordonii*, but not with *S. cristatus*, increased the amount of *cdhR* mRNA in *P. gingivalis* in a time dependent manner (Fig. 2A). As the tyrosine phosphatase Ltp1 is also upregulated in *P. gingivalis-S. gordonii* communities (Maeda *et al.*, 2008), these results suggested that CdhR and Ltp1 may be components of the same regulatory circuit. We tested this hypothesis by measuring *cdhR* transcription in a Ltp1 deficient mutant. Transcriptional activity of *cdhR* did not increase in the *ltp1* mutant when *P. gingivalis* cells were in contact with *S. gordonii* (Fig 2A), supporting the contention that CdhR is downstream of Ltp1 in a regulatory circuit that restricts community development. To further investigate the hierarchical arrangement of Ltp1 and CdhR, expression of *ltp1* in the CdhR deficient mutant was tested (Fig. 2B). Both parental and CdhR mutant strains showed equivalent amounts of *ltp1* mRNA when in contact with *S. gordonii*. Taken together, these results indicate that a community induced increase in Ltp1 subsequently leads to an increase in the amount of the transcriptional regulator CdhR.

CdhR constrains *P. gingivalis* heterotypic community development

To challenge the hypothesis that CdhR functions as a negative regulator of community development, the ability of the *cdhR* mutant to develop into communities on a streptococcal substratum was examined. In order to diminish the influence of possible pleiotropic effects of the mutation on the observed phenotypes, the wild-type *cdhR* allele was introduced into the Δ*cdhR* mutant using the *E. coli-Bacteroides* shuttle vector pT-COW, to create CΔ*cdhR*. This complemented strain should thus display a phenotype equivalent to the parental strain. The accumulation of *P. gingivalis* parent and mutant strains on a substratum of *S. gordonii* was examined using confocal microscopy with fluorescently labeled bacteria. The CdhR deficient mutant displayed greater accumulation on *S. gordonii* as compared to the wild-type

and complemented strains (Fig. 3A-C). Several quantitative measures, using ImageJ software, confirmed the enhanced community phenotype of the Δ*cdhR* mutant. Total area covered by *P. gingivalis* (Fig. 3D), average thickness of the *P. gingivalis* accumulations (Fig. 3E), and total *P. gingivalis* volume (Fig. 3F) were all elevated for the CdhR deficient mutant in comparison with parental or complemented strains. To ensure that different amounts of *P. gingivalis* were not the result of variation in the coverage of the streptococcal substratum, the ratio of *P. gingivalis* to *S. gordonii* was measured (Fig. 3G). Ratios of green (*P. gingivalis*) to red (*S. gordonii*) fluorescence confirmed the enhanced community phenotype of the CdhR mutant strain. In contrast, parental and CdhR mutant strains accumulated to the same extent in monospecies biofilms in microtiter plates (not shown). Collectively, these results demonstrate that CdhR makes an important contribution to the community restraint mechanisms operational in *P. gingivalis* in mixed species accumulations.

CdhR controls production of Mfa and LuxS

Accumulation of *P. gingivalis* on streptococcal substrata requires adhesins such as Mfa, and signaling molecules such as the AI-2 family of compounds produced by the enzymatic action of LuxS (McNab *et al*., 2003, Park *et al.*, 2005). Mfa binds to the streptococcal Ssp receptors to initiate community formation; however, within 20 h of contact the activity of the *mfa* promoter is reduced, and community development is constrained (Park *et al*., 2006). Mfa fimbriae remain present however, and the communities do not dissociate. LuxS-dependent signaling stimulates the initial development of microcolonies (McNab *et al.*, 2003). We reasoned, therefore, that CdhR could control community development through negative regulation of *mfa* and *luxS*. Quantitative RT-PCR was utilized to determine the transcriptional activity of *mfa* and *luxS* following 20 h of contact between *P. gingivalis* and *S. gordonii*. As shown in Fig. 4, mRNA levels for both *mfa* and *luxS* were increased in the absence of CdhR, when *P. gingivalis* was in contact with *S. gordonii* but not when in contact with *S. cristatus*. Moreover, the complemented CΔ*cdhR* strain displayed a similar phenotype to the wild type strain. These findings indicate that CdhR is a negative regulator of *mfa* and *luxS*, which in turn contributes to restricted community development. To test whether CdhR regulation of *mfa* and *luxS* is direct or acts through another *P. gingivalis* regulator, we purified recombinant CdhR as a His_6 fusion. Recombinant CdhR has been shown to be functional and bind to the promoter of the *hmuY* gene (Wu *et al.*, 2009). The ability of recombinant CdhR protein to bind to the upstream regulatory regions of *mfa* and *luxS* was investigated using EMSA. The promoter region for *mfa* has been defined as proximal to the gene (Park *et al*., 2006), and the promoter that drives *luxS* transcription is adjacent to the upstream *pfs* gene (Chung *et al.*, 2001). 200 bp fragments upstream of the initiation codons for *mfa* and *pfs* were generated for the EMSA analysis. Fig. 5 shows that purified rCdhR directly binds to the promoter regions that control both *mfa* and *luxS*. The promoter fragments were almost completely shifted with 2 μg of protein, similar to the binding affinity of CdhR for the *hmuY* promoter. The mobility shifts were inhibited by the addition of unlabelled target DNA, but not by the promoter fragment of *fetB*, indicating that the binding of CdhR to these regions is specific. Furthermore, CdhR was unable to shift a 200 bp fragment containing the promoter for the *fimA* gene (not shown). These data indicate that CdhR directly regulates expression of *mfa* and *luxS*.

Next, we sought to determine whether the transcriptional activity controlled by CdhR is reflected at the protein level. To accomplish this we used Mfa antibodies to probe western blots of cell lysates of parental and CdhR mutant cells that were in contact with *S. gordonii*. As shown in Fig. 6A, the CdhR deficient mutant expressed higher levels of Mfa protein compared to parental cells, a result consistent with the mRNA data. In contrast, there was no change in the expression level of FimA (Fig. 6B). Collectively, these results demonstrate

that transcriptional regulation is mediated directly by CdhR and is accompanied by changes in phenotypic properties that impact dual species community development.

LuxS and Mfa participate in control of CdhR

Planktonic cells of *P. gingivalis* express *luxS*, and AI-2 signaling activity negatively regulates transcription of the *hmu* operon (James *et al*., 2006). As CdhR activates *hmu* transcription, we investigated whether AI-2 can regulate CdhR. As shown in Fig 7A, the amount of *cdhR* mRNA was elevated in a *luxS* mutant, indicating that AI-2 negatively regulates CdhR. Chemical complementation of the *luxS* mutant with exogenous DPD, the precursor of AI-2, reduced expression of *cdhR*. Hence, the impact of AI-2 on *hmu* levels may be mediated by downregulation of the transcriptional activator CdhR. Furthermore, these results suggest that as the number of *P. gingivalis* cells increase and AI-2 levels are elevated, CdhR is down regulated and the organism expresses more Mfa fimbriae and LuxS enzyme. This positive feedback loop can be predicted to facilitate the transition to a community environment. Moreover, initial close proximity to *S. gordonii* and to streptococcal AI-2 may also contribute to suppression of CdhR.

In addition to chemical communication, fimbrial engagement of a receptor can be the trigger for subsequent changes in gene expression (Schwan *et al.*, 2005, Zhang and Normark, 1996). Hence we examined the extent to which the Mfa fimbriae themselves contribute to regulation of *cdhR*. mRNA levels for *cdhR* and *ltp1* were quantitated by qRT-PCR in a *mfa* mutant strain. Contact with *S. gordonii* did not induce upregulation of either *ltp1* or *cdhR* in the absence of the Mfa fimbriae (Fig. 7B and C). These results suggest that interaction of Mfa with the streptococci is necessary for stimulation of the Ltp1-CdhR pathway.

Discussion

Communities of bacteria on solid surfaces typically contain multiple, physiologically diverse bacterial taxa. Study of interspecies signaling and response interactions in communities is an emerging field that provides insights into the behaviour of bacteria in their natural habitats. For example, quorum sensing regulated functions are important microbial competition factors in dual species communities consisting of *Pseudomonas aeruginosa* and *Agrobacterium tumefaciens* (An *et al.*, 2006) or of *Serratia plymuthica* and *E. coli* (Moons *et al*., 2006). The bacterial communities that accumulate on surfaces in the oral cavity can comprise hundreds of species, and cell to cell communication shapes community composition, composite metabolic activity and pathogenic potential. Colonization of dental communities by *P. gingivalis* is influenced by the composition of the antecedent microbiota. While in general colonization of this anaerobe is favored by bacterial metabolism that reduces the oxygen tension, specific streptococcal species can either promote or antagonize *P. gingivalis* attachment. Contact with *S. cristatus* propagates a signal in *P. gingivalis* that causes downregulation of *fimA* expression with resultant failure of these organisms to develop into a dual species community (Xie *et al.*, 2000, Xie *et al.*, 2007). In contrast, *S. gordonii*, and related oral streptococci that possess a BAR motif in their Ag I/II family adhesins, allow *P. gingivalis* attachment through the shorter Mfa fimbriae, and promote two species community development (Daep *et al.*, 2008, Demuth *et al.*, 2001). Signaling mediated by AI-2 family molecules also stimulates dual species bacterial accumulation (McNab *et al.*, 2003). These interspecies interactions will thus elevate the pathogenic potential of oral communities; however, realization of this potential requires that other host and bacterial factors be conducive to the onset of disease. Indeed, many individuals harbor *P. gingivalis* and remain healthy (Ximenez-Fyvie *et al.*, 2000a, Mayanagi *et al*., 2004), indicating that mechanisms of virulence restraint are operational. We have found that the accumulation of *P. gingivalis* on streptococcal substrata is tightly controlled (Maeda *et al.*,

2008, Simionato *et al.*, 2006), which may be an adaptation to prevent reduced nutrient availability or exposure to elevated oxygen levels.

As various adhesins and signaling systems are required for *P. gingivalis-S. gordonii* community development, *P. gingivalis* requires a system to co-ordinately regulate these effectors. One component of this community regulatory pathway is the tyrosine phosphatase Ltp1. Ltp1 is upregulated following contact with *S. gordonii*, and tyrosine phosphatase-dependent signal transduction culminates in reduced transcription of *luxS* and of genes involved in exopolysaccharide production (Maeda *et al.*, 2008). Herein we show that the LuxR family orphan transcription regulator, which we designate CdhR, is a component of the same regulatory pathway as Ltp1. CdhR is upregulated following contact with *S. gordonii*, but only in the presence of Ltp1, indicating that CdhR is downstream of Ltp1. In the absence of CdhR, dual species *P. gingivalis-S. gordonii* community formation is enhanced, also consistent with CdhR operating distal to Ltp1. CdhR was previously identified as a positive regulator of the *hmu* hemin uptake locus in planktonic cells of *P. gingivalis* (Wu *et al.*, 2009). This activator function of CdhR also occurs in communities, as the *cdhR* knockout strain had less *hmuR* mRNA as compared to the parent (not shown). In contrast, planktonic cells of a *P. gingivalis* CdhR deficient strain did not exhibit differential regulation of *mfa* or of *luxS* (Wu *et al*., 2009). Hence the repressor functions of CdhR appear to require expression above basal levels, such as is induced following contact with *S. gordonii*.

P. gingivalis is one of the few organisms in which transcriptional control of *luxS* has been reported (James *et al.*, 2006), although other bacteria transcriptionally control *pfs* (Beeston and Surette, 2002) which in *P. gingivalis* is part of an operon with *luxS*. The results from the current study show that loss of CdhR increases expression of *luxS*, and that CdhR can bind to the upstream regulatory region of the *pfs-luxS* operon. Hence, while lacking an AI-1 system, *P. gingivalis* appears to have utilized a LuxR homolog to control LuxS and AI-2. The GppX hybrid Two Component System (TCS) has also been implicated in regulation of *luxS* transcription (James *et al.*, 2006) and we are currently investigating the relationships among GppX, Ltp1, CdhR and LuxS.

As dual species *P. gingivalis-S. gordonii* communities develop, the *mfa* gene is downregulated, one aspect of processes that regulate the level of *P. gingivalis* accumulation (Park *et al.*, 2006). Control of Mfa production requires propagation of a signal from *S. gordonii* or related oral streptococci that are capable of supporting *P. gingivalis* microcolony development. The results of this study indicate that CdhR is the effector molecule of streptococcal-dependent *mfa* downregulation. However, transcriptional activity of *mfa* is also positively regulated by the FimS/FimR TCS (Lo *et al.*, 2010, Wu *et al.*, 2007), and Mfa expression is negatively regulated by the Clp proteolytic chaperone system components ClpXP (Capestany *et al.*, 2008). Thus, *P. gingivalis* fine tunes production of Mfa according to the environmental situation.

Control of community or biofilm development through LuxR family transcriptional regulators has also been established in *Vibrio cholerae*. The transcriptional factor VpsT controls exopolysaccharide synthesis and biofilm formation (Yang *et al.*, 2010). The regulatory network in which VpsT operates is complex and expression of VpsT is positively autoregulated, and also decreased through quorum-sensing dependent signaling (Yang *et al.*, 2010). Moreover, VpsT directly detects cyclic di-guanosine monophosphate (c-di-GMP) to negatively regulate biofilm formation (Krasteva *et al.*, 2010). A cognate kinase for VpsT has not been identified and c-di-GMP sensing results in a change in oligomerization (Krasteva *et al.*, 2010). The mechanisms by which CdhR is regulated remain to be fully defined. Similar to VpsT, quorum sensing, in this case mediated by LuxS, decreases CdhR expression,

indicating that planktonic cells become primed for community development when AI-2 reaches certain levels. CdhR also lacks a cognate kinase and is unlikely that Ltp1 directly dephosphorylates CdhR, as CdhR lacks a predicted tyrosine phosphotransferase domain and a two hybrid screen for targets of Ltp1 did not detect CdhR (unpublished). An upstream initiator of the Ltp1-CdhR pathway is the Mfa fimbriae themselves. Levels of CdhR, therefore, may be held in balance by positive and negative feedback loops involving LuxS and Mfa.

Bacterial pathogens are often adapted to colonize and persist in the absence of clinical disease in immunologically competent hosts, and bacteria that colonize host surfaces can develop mechanisms to minimize pathogenic outcomes and constrain increases in biomass and biofilm development (Galan and Zhou, 2000, Tierrez and Garcia-del Portillo, 2005, Whiteley *et al.*, 2001, Belotserkovsky *et al.*, 2009, Monds *et al.*, 2007). It is becoming apparent that complex circuitry within *P. gingivalis* limits community development on a streptococcal substratum. Our previous results (Maeda *et al.*, 2008, Simionato *et al.*, 2006), along with the data in this study are consistent with the model depicted in Fig. 8. Upon encountering a streptococcal surface, a streptococcal derived signal propagating through Mfa elevates Ltp1 and which acts indirectly through a phosphorylation/dephosphorylation cascade to raise CdhR. Subsequently, CdhR reduces transcription of *luxS* and *mfa*, ultimately restricting further community development. Lower levels of LuxS, and consequently AI-2, will propagate the signaling event throughout the developing community as AI-2 is a negative regulator of CdhR. Mfa fimbriae are thought to penetrate the peptidoglycan (Murakami *et al.*, 2004) and could thus initiate signal transduction directly. Alternatively, conformational changes in the fimbrial structure upon receptor binding may be transmitted to other outer membrane signaling initiators.

The processes that control the assembly, function and evolution of heterotypic communities are not as fully understood as those governing homotypic community development. However, local interactions between component organisms play key roles. Contact dependent signaling has emerged as an important means by which bacteria in close proximity can modulate the growth and the behaviour of synergistic and antagonistic species (Blango and Mulvey, 2009). We have modeled an early stage in the assembly of oral biofilms, the recruitment and accretion of cells of *P. gingivalis* onto a streptococcal substratum prior to increases in biomass due to growth and division. This model facilitates the identification of interspecies signaling interactions that are independent of nutritional support. Components of this communication system, such as CdhR, may be of central importance in establishing the potential pathogenicity of complex oral communities.

Experimental procedures

Bacteria, plasmids and culture conditions

Bacterial strains and plasmids are listed in Table S1. *P. gingivalis* strains were cultured in Trypticase Soy Broth (TSB), supplemented with hemin and menadione, anaerobically at 37°C. When necessary, erythromycin (10 μg ml^{-1}), tetracycline (1 μg ml^{-1}), or gentamicin (100 μg ml^{-1}) were incorporated into the medium. For AI-2 complementation, *P. gingivalis* Δ*luxS* cultures at OD_{600} 0.2 were supplemented with10 μM DPD (OMM Scientific, Dallas, TX) and grown to OD_{600} 0.5. *S. gordonii* and *S. cristatus* were cultured anaerobically at 37°C in TSB. *Escherichia coli* strains were grown aerobically at 37°C in LB media containing, when necessary, ampicillin (100μg ml^{-1}) or kanamycin (50 μg ml^{-1}).

Construction of complemented strain

DNA sequence containing 300 bp upstream of the PGN_1372 initiation codon and 100 bp downstream of the *cdhR* (PGN_1373) termination codon was amplified from *P. gingivalis* chromosomal DNA, using primers 1372-3f and 1372-3r (Table S2). The amplified product was cloned into shuttle vector plasmid pT-COW (Gardner *et al.*, 1996). The resulting plasmid pT1373, was introduced into the Δ*cdhR* mutant by conjugation (Simionato *et al.*, 2006) from *E. coli* TOP10, creating strain CΔ*cdhR*. The conjugation reaction mixture also contained helper *E. coli* J53 containing R751, an IncP plasmid used to mobilize vectors from *E. coli* to a *Bacteroides* recipient (Shoemaker and Salyers, 1987). Transconjugants were selected with erythromycin and tetracycline and the presence of plasmids was confirmed by PCR and Southern hybridization. Plasmids were sequenced to confirm the construct. The conjugated strain produced similar levels of CdhR protein to the parental strain.

RT-PCR and quantitative real time RT-PCR

Primers were designed by Primer3 software. Total RNA was extracted from *P. gingivalis* cultures and digested with DNaseI using the Qiagen (Valencia, CA) RNeasy mini-kit. 16S and 23S rRNAs were depleted by MICROB*E*xpress kit (Ambion, Austin, TX). The iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) was used to generate cDNA from RNA (1 μg) templates using gene specific primers (Table S2): 1372f (forward primer binding to PNG_1372); 1371f (forward primer binding to PGN_1371); and 1373r (reverse primer binding to *cdhR*). PCR conditions were 95°C for 6 min and 30 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 3 min, followed by a final extension at 72°C for 7 min. PCR products were examined on 1.0% agarose gels. For real time quantitative RT-PCR, specific DNA standards for the genes under investigation were synthesized from chromosomal DNA using standard PCR and were quantified using an Eppendorf BioPhotometer. Real time RT-PCR was performed on a Bio-Rad iCycler using SYBR Green Supermix (Bio-Rad). Results were analyzed with the iCycler iQ Optical System software version 3.0a. The melt curve profiles were examined to verify a single peak for each sample. Transcript levels for the following genes were determined with the corresponding gene-specific primer pairs (Table S2): *mfa*, MFAf and MFAr; *luxS*, LUXf and LUXr; *ltp1*, LTPf and LTPr; *cdhR*, CDHf and CDHr; 16S, 16Sf and 16Sr. Transcript levels were normalized to 16S. In control experiments these primers did not amplify *S. gordonii* DNA sequences.

5′ RACE

The transcriptional start site of *cdhR* was determined by using the FirstChoice RNA ligase-mediated rapid amplification of cDNA ends (RACE) kit (Ambion). RNA was isolated and digested with DNaseI as described for the RT-PCR. A 45-base 5′ RNA adapter oligonucleotide was ligated to the 5′ ends of the total RNA (10 μg) using T4RNA ligase. RT was performed by using MLV reverse transcriptase with randon decamers provide by Ambion. Nested PCR was performed by first using the 5′RACE outer primer and CDHO (Table S2) a *cdhR* specific outer primer. Inner PCR was then conducted with the PCR product generated from the outer primers as template using the 5′ RACE inner primer that anneals to the adapter oligonucleotide, and CDHI (Table S2), a *cdhR* specific inner primer. The PCR product was TA cloned into the pCR4-TOPO vector and transformed into *E. coli* Top10. Cloning of nested PCR product was confirmed by colony PCR with M13 primers and the PCR products of six-independent clones were sequenced with an ABI capillarysequencer (Perkin-Elmer, Covina, CA).

P. gingivalis *heterotypic communities*

Heterotypic *P. gingivalis-S. gordonii* communities were generated as described previously (Kuboniwa *et al.*, 2006). *S. gordonii* cells cultured for 16 h in TSB with rocking in a

CultureWell coverglass system (Grace Biolabs, Bend, OR), washed three times with pre-reduced PBS and labeled with hexidium iodide (15 μg ml^{-1}; Invitrogen, Carlsbad, CA). *P. gingivalis* cells were stained with 5-(and-6)-carboxyfluorescein, succinimidyl ester (4 μg ml^{-1}; Invitrogen), and 2×10^6 cells in pre-reduced PBS reacted with the *S. gordonii* biofilm for 24 h anaerobically at 37°C in the dark with rocking. After washing, the resultant communities were examined on a Yokogawa spinning disc confocal scanning laser microscope system with a 60× 1.4 N.A. objective. Images were digitally reconstructed (3D image; x-y-z section), with Imaris software (Bitplane, Zurich), and quantitation of fluorescence measures was determined with ImageJ. Biofilm assays were repeated independently three times witheach strain in triplicate and analyzed with a Student's unpaired two-tailed t test.

For gene expression studies a cell pellet assay was adopted (Kreth *et al*., 2005). Bacteria were cultivated to mid-exponential phase, harvested, washed and resuspended in pre-reduced PBS. *P. gingivalis* strains (10^8 cells) were mixed with an equal number of *S. gordonii* or *S. cristatus* cells in PBS. The cell mixtures were pelleted by centrifugation (10,000 g for 1 min) and incubated at 37°C anaerobically for 20 hours (except where indicated). RNA isolated from the consortia had negligible levels of streptococcal RNA as determined by RT-PCR with streptococcal specific 16S rRNA primers.

Expression of recombinant CdhR protein

The *cdhR* coding region was amplified using primers RCDf and RCDr (Table S2). The amplicon was confirmed by sequencing, and cloned into pCR4-TOPO (Invitrogen). For protein expression, the *cdhR* gene was cloned into pET30b to create pET30b-1373 which was transformed into *E. coli* BL21(DE3) (Novagen, Madison WI). His-tag protein was purified with a BioLogic DuoFlow chromatography system loaded with a Nickel-NTI column. Purity was greater than 97%, as determined by SDS-PAGE Coomassie staining, and the identity of the band confirmed by LC MS/MS sequencing.

EMSA

Electrophoretic mobility shift assays (EMSA) were performed using the LightShift chemiluminescent EMSA kit (Pierce, Rockford, IL) as described previously (Wu *et al.*, 2009). Biotin-labeled DNA fragments were generated by PCR using 5′ biotin-incorporated primers: for *mfa*, EMFf and EMFr; for *luxS* ELUf and ELUr (Table S2). Binding reactions contained 20 fmol biotin-labeled DNA, 10 mM Tris (pH 7.5), 50 mM KCl, 1 mM dithiothreitol, 10 ng/μl poly(dI-dC), 2% glycerol, 0.05% NP-40, and 2 mM MgCl with varying amounts of CdhR protein and dH_2O up to a total volume of 20 μl. Binding reactions were at room temperature for 30 min and samples were then loaded and run into a 5% nondenaturing polyacrylamide gel in 0.5× Tris-borate-EDTA buffer. The DNA and protein complexes were transferred to a positively charged nylon membrane (380 mA, 30 min) and the biotin end-labeled DNA was detected with streptavidin-horseradish peroxidase conjugate and chemiluminescent substrate. To test the specificity of binding, specific competition reactions were carried out with 200 fold excess of unlabeled target DNA (*mfa* or *luxS* promoter fragments). In addition, specificity was tested using 200 fold excess of a non specific promoter fragment of *fetB* generated by PCR using primers FETf and FETr (Table S2).

Western immunoblotting

P. gingivalis cells were lysed in SDS-PAGE sample buffer and proteins (5 μg) were separated by SDS-PAGE and transferred to nitrocellulose membranes. Blots were blocked with 5% BSA in TBS (PBS containing 0.1% Tween 20) for 1 h at room temperature and probed with antibodies to Mfa or FimA protein diluted 1:10,000, at 4°C overnight. Antigen–

antibody binding was detected with anti-rabbit IgG peroxidase conjugate (1:5,000, Cell Signaling, Danvers, MA) and the Pierce ECL Substrate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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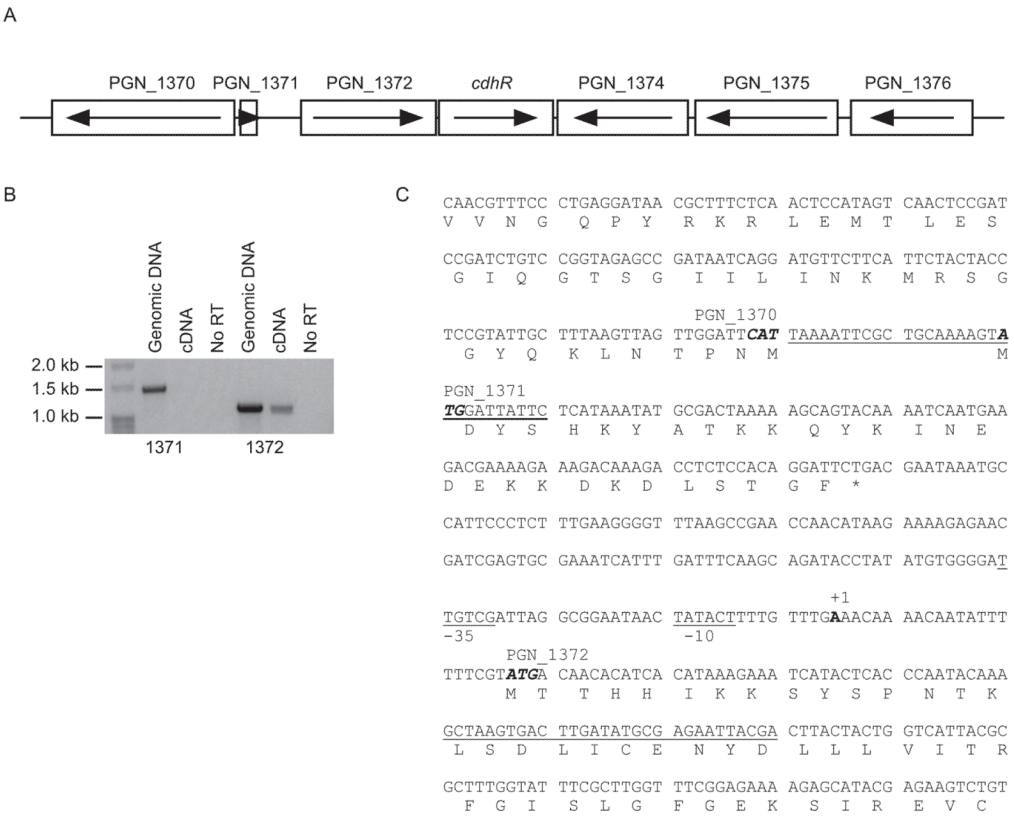

Fig. 1.

Physical and transcriptional organization of the *cdhR* region in the *P. gingivalis* 33277 chromosome. (A) Genetic organization of the *cdhR* region. Arrows indicate the direction of transcription. (B) RT-PCR of genomic or cDNA with primers spanning *cdhR* and PGN_1371 (left side) or PGN_1372 (right side). The presence of an amplicon with cDNA demonstrates cotranscription of PGN_1372 and *cdhR*. The No RT lanes are controls without reverse transcriptase. (C) Based on 5′ RACE, the transcriptional start site for *cdhR* and PGN_1372 was mapped to an A (bold and labeled +1) 22 bp upstream of the ATG start codon of PGN_1372. Primers used in the RT-PCR for (B) are underlined. Putative −10 and −35 promoter regions are labeled and underlined. Initiation codons are in bold italics, and termination codon is marked with a *.

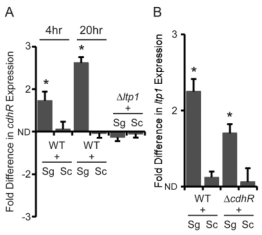

Fig. 2.

Contact with *S. gordonii* induces *cdhR* expression when Ltp1 is present. Quantitative RT-PCR of mRNA from *P. gingivalis* strains in contact with *S. gordonii* (Sg) or *S. cristatus* (Sc). Fold difference is expressed relative to *P. gingivalis* alone. ND is no difference. (A) *cdhR* mRNA is increased in *P. gingivalis* 33277 (WT) by contact with *S. gordonii* but not with *S. cristatus*, at 4 h and 20 h. In contrast, *cdhR* expression in the *P. gingivalis ltp1* mutant strain (Δ*ltp1*) is unaffected by *S. gordonii* after 20 h. (B) Expression of *ltp1* is elevated by *S. gordonii*, but not by *S. cristatus* in the WT and CdhR mutant (Δ*cdhR*) strains at 20 h. Asterisk denotes a significant difference at $P < 0.01$ (t test) in levels of mRNA in *P. gingivalis* in contact with *S. gordonii* in comparison to *P. gingivalis* in contact with *S. cristatus*. Results are means with standard deviation of 3 independent experiments performed in duplicate.

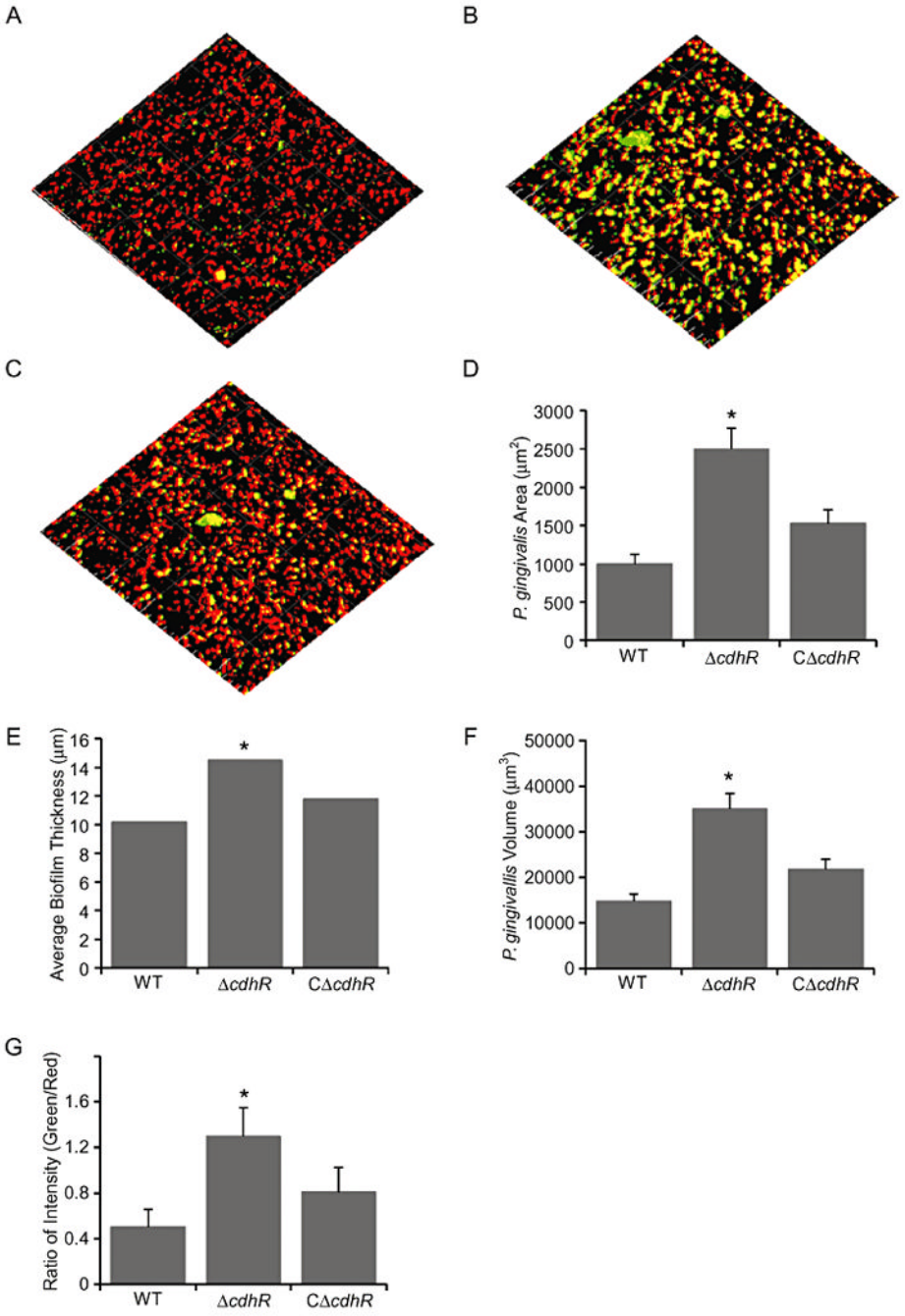

Fig. 3.

CdhR constrains heterotypic *P. gingivalis-S. gordonii* community development. Analysis of dual species communities by confocal laser scanning microscopy. Substrata of *S. gordonii* cells (red) were reacted with (A) *P. gingivalis* 33277 (WT), (B) mutant (Δ*cdhR*) or (C) complemented mutant (CΔ*cdhR*) strains (green) for 24 h. A series of 0.2 μm deep optical fluorescent *x-y* sections (102 × 102 μm) were collected to create digitally reconstructed 3D images of the two species with Imaris software. Colocalized bacteria appear yellow. Images were analyzed by Image J for: (D) Total area of *P. gingivalis* fluorescence accumulation; (E) Average thickness of *P. gingivalis* accumulation; (F) *P. gingivalis* biovolume; and (G) Ratio of *P. gingivalis* to *S. gordonii* fluorescence. Images are representative of 3 independent

experiments. Quantitative results are means with standard deviation of 3 independent experiments performed in triplicate. Asterisk denotes a significant difference at $P < 0.01$ compared to WT.

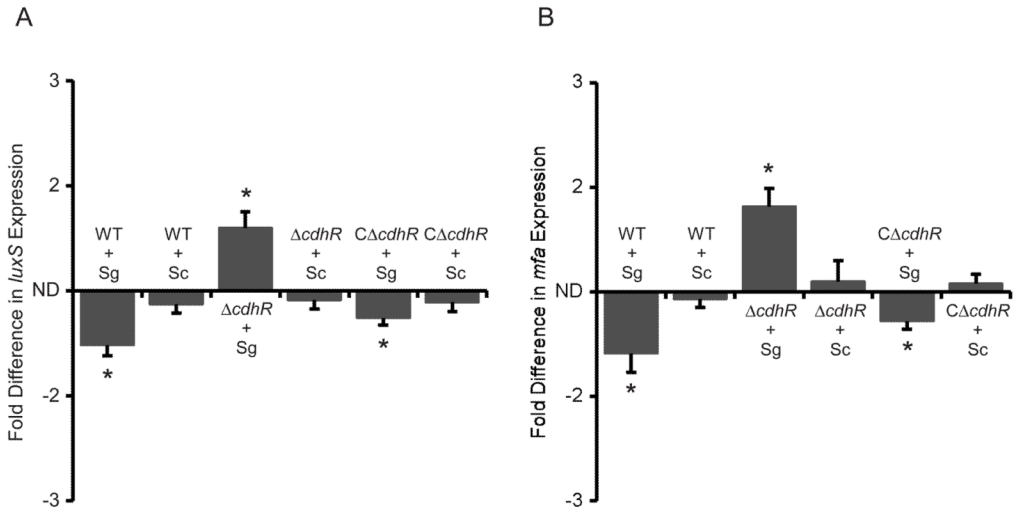

Fig. 4.

CdhR negatively regulates expression of *luxS* (A) and *mfa* (B). mRNA levels in *P. gingivalis* wild type (WT), Δ*cdhR* and CΔ*cdhR* strains were measured by quantitative RT-PCR. Fold difference is expressed mRNA levels after contact with *S. gordonii* (Sg) or *S. cristatus* (Sc) relative to *P. gingivalis* alone. ND is no difference. Data are means with standard deviation of 3 independent experiments performed in duplicate. Asterisk denotes a significant difference at $P < 0.01$ (t test).

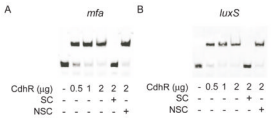

Fig. 5.

CdhR protein binds to the promoter regions of *mfa* (A) and *luxS* (B). EMSA was performed with 20 fmol biotin-labeled DNA fragments containing the promoter regions and rCdhR. Non-biotinylated specific competitor oligonucleotide (SC) and non-specific competitor (*fetB* promoter, NCS) were used at 200-fold excess. Images are representative of three independent experiments.

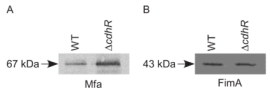

Fig. 6.

CdhR regulates protein levels of Mfa. Western immunoblotting of Mfa (A) or FimA (B) expression in *P. gingivalis* wild type (WT) and Δ*cdhR* strains. Whole cell lysates were separated by SDS-PAGE and probed with antibodies to the Mfa (67 kDa) or FimA (43 kDa) subunit proteins. Images are representative of three independent experiments.

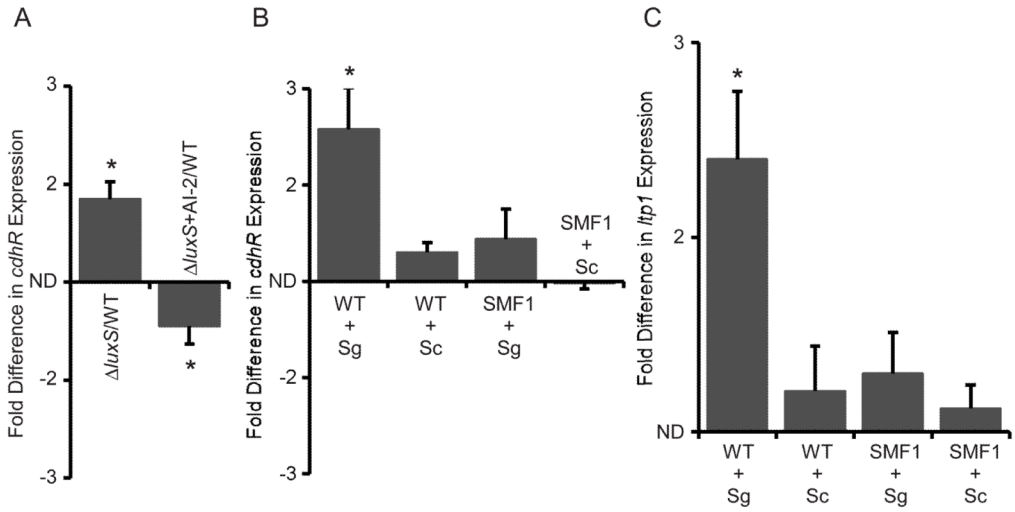

Fig. 7.

(A) LuxS/AI-2 controls *cdhR* expression in *P. gingivalis*. Quantitative RT-PCR of *cdhR* mRNA in the Δ*luxS* mutant strain cultured with or without exogenous AI-2. Asterisk denotes a significant difference between WT and mutant at $P < 0.01$ (t test). (B) Mfa is necessary for regulation of *cdhR* in heterotypic communities with *S. gordonii* (Sg). Quantitative RT-PCR of *cdhR* mRNA in *P. gingivalis* wild type (WT) or *mfa* mutant (SMF1) strains in contact with *S. gordonii* or *S. cristatus* (Sc). Asterisk denotes a significant difference at $P < 0.01$ (t test) compared to *P. gingivalis* alone. (C) Mfa is necessary for regulation of *ltp1* in heterotypic communities with *S. gordonii*. Quantitative RT-PCR of *ltp1* mRNA in *P. gingivalis* WT or SMF1 strains in contact with *S. gordonii* or *S. cristatus*. Asterisk denotes a significant difference at $P < 0.01$ (t test) compared to *P. gingivalis* alone. Data are means with standard deviation of 3 independent experiments performed in duplicate.

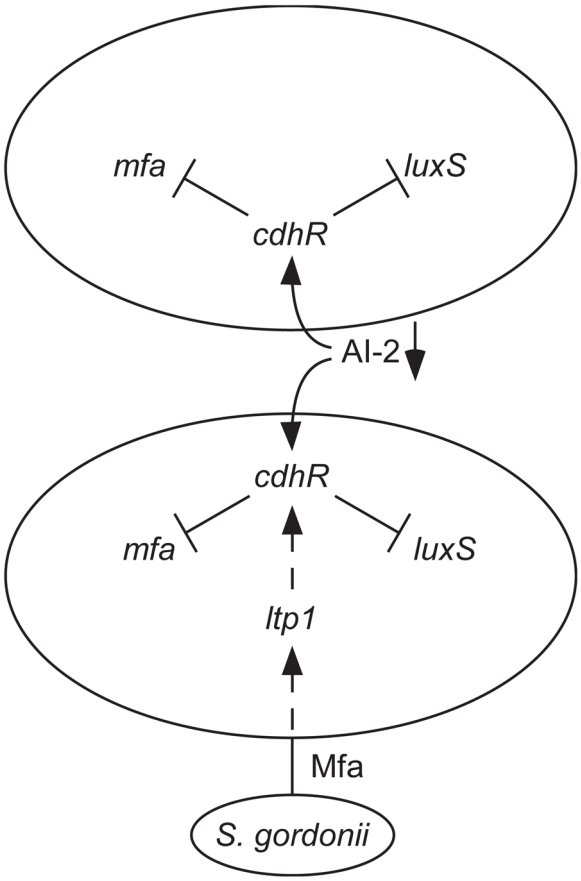

Fig. 8.

Model of circuitry governing heterotypic community formation between *P. gingivalis* and *S. gordonii*. *P. gingivalis* expressing Mfa and LuxS can bind and accumulate on a substratum of *S. gordonii*. Communication between *S. gordonii* and *P. gingivalis* requires Mfa and is transduced through Ltp1 indirectly to CdhR. Higher levels of CdhR decrease the amount of Mfa and LuxS and consequently restrain *P. gingivalis* accumulation. Loss of AI-2 will further increase *cdhR* expression which may compensate for loss of signal due to the reduction of the Mfa fimbriae.