Published in final edited form as:
Mol Oral Microbiol. 2013 April ; 28(2): 83–101. doi:10.1111/omi.12012.

Microbial interactions in building of communities

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SUMMARY

Establishment of a community is considered to be essential for microbial growth and survival in the human oral cavity. Biofilm communities have increased resilience to physical forces, antimicrobial agents, and nutritional variations. Specific cell-to-cell adherence processes, mediated by adhesin-receptor pairings on respective microbial surfaces, are able to direct community development. These interactions co-localize species in mutually beneficial relationships, such as streptococci, veillonellae, *Porphyromonas gingivalis* and *Candida albicans*. In transition from the planktonic mode of growth to a biofilm community, microorganisms undergo major transcriptional and proteomic changes. These occur in response to sensing of diffusible signals, such as autoinducer molecules, and to contact with host tissues or other microbial cells. Underpinning many of these processes are intracellular phosphorylation events that regulate a large number of microbial interactions relevant to community formation and development.

Keywords

biofilm; *Streptococcus*; *Porphyromonas*; *Candida*; phosphorylation; signaling

INTRODUCTION

In the natural world, microorganisms are mostly organized into communities, and these in turn are found assembled upon abiotic or living substrates as biofilms. A typical biofilm forms at an interface of two phases and comprises microbial cells enclosed within a matrix consisting of polysaccharides, proteins, nucleic acid and lipids (Flemming & Wingender, 2010), derived from microbe and environmental sources. Mono-species biofilm formation often proceeds through distinct developmental stages, as exemplified by *Pseudomonas aeruginosa* (Sauer *et al*., 2002) and by *Candida albicans* (Chandra *et al*., 2001). The process is initiated through low affinity attachment of planktonic cells to a substrate, followed by high affinity adhesion mediated by specific receptors. Microcolonies develop upon growth and division of attached cells, sometimes referred to as a linking film. Subsequently, recruitment of planktonic cells (from the fluid phase) leads to the further development of the community. Moreover, recruitment of heterotypic bacterial species, and/or initial adhesion

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by multiple species in close proximity leads to the formation of multi-species (polymicrobial) communities, which represents the most common situation in nature. The integrity of the biofilm community is maintained by inter-microbial adhesion, cell signaling by means of cell-to-cell contact, and metabolic communication including quorum sensing (Swift et al., 2001; Blango & Mulvey, 2009).

The advantages for microorganisms growing in biofilm communities over remaining in planktonic conditions are numerous. The inherent matrix of the biofilm, such as extracellular polymeric substances (EPS), and the presence of persister cells surviving at low metabolic rates, contribute to the widely described phenomenon of reduced sensitivity to antimicrobial agents (Hoyle & Costerton, 1991). Biofilms are also more resilient to mechanical removal and to killing by the host immune system (Leid *et al.*, 2005). More recently, extracellular DNA (eDNA) has been shown to play an important structural role in stabilizing biofilms (Barnes *et al.*, 2012). In addition, this eDNA may be a source for potential transfer of antibiotic resistance or virulence genes between species within the communities (Roberts & Mullany, 2010).

GENERAL CHARACTERISTICS OF ORAL BIOFILMS

The organization of oral microorganisms into dental plaque biofilms plays an essential role in their survival (Jakubovics & Kolenbrander, 2010). The microorganisms are continually subjected to environmental challenges in the oral cavity including variations in oxygen and nutrient availability, pH fluctuations and the antimicrobial properties of saliva (Abiko & Saitoh, 2007). Microorganisms that fail to attach to host surfaces, or to adhered antecedent organisms, are unable to participate in community development and are subjected to eventual displacement due to the flow of saliva and other mechanical shearing forces (Scannapieco, 1994).

Oral bacteria bind to accessible host or bacterial surfaces and form complex communities in an orderly fashion. Mature dental plaque on teeth contains about 10^9 bacteria per gram and anywhere up to about 200 microbial species or phylotypes (Dewhirst et al., 2010). Certain species initiate community formation by interacting directly with the salivary pellicle that is deposited on newly available tooth surfaces. Notable pioneer organisms include many species of oral streptococci (Nyvad & Kilian, 1990), *Actinomyces spp. Granulicatella adiacens, Abiotrophia defectiva, Gemella* spp. and *Rothia* (Jenkinson, 2011). These early colonizers are all components of the natural microbiota (Aas *et al.*, 2005), and few are known to be directly responsible for the development of a diseased state. However, early colonizers such as streptococci (Palmer *et al.*, 2001) can alter the pathogenic potential of the oral biofilm through both their influence on how the biofilm community develops and by elevating the pathogenic potential of other bacteria (Whitmore & Lamont, 2011).

Streptococcus adhesion molecules

Streptococci are facultatively anaerobic and adhere to an array of salivary molecules including mucins, proline-rich proteins, statherin, salivary agglutinin (gp-340), and α-amylase (Nobbs *et al.*, 2009). They also bind a wide variety of oral microorganisms, leading to development of complex microbial networks that stabilize communities. Accordingly, streptococci express a diversity of cell surface molecules that enable adherence to host or bacterial receptors. For example, long thread-like structures termed pili are produced by *Streptococcus sanguinis*, and by other *Streptococcus* species, and are composed of polymers of three different protein subunits (PilA, PilB, PilC). These promote attachment to host receptors (Okahashi *et al.*, 2010) and in *Streptococcus pneumoniae* are required for full virulence virulence (Barocchi *et al.*, 2006). Pili and many other surface proteins are found covalently-linked to the cell wall peptidoglycan through their C-terminal anchorage

sequences (Nobbs *et al.*, 2009). Other cell-wall anchored polypeptides identified in oral streptococci functioning as adhesins include: CshA (and CshB) which forms surface fibrils that interact with fibronectin and other oral microbes (Holmes *et al.*, 1996; McNab *et al.*, 1999); Hsa (and GspB) that interact with salivary pellicle, epithelial cells and blood platelets (Kerrigan *et al.*, 2007); PadA which interacts with blood platelets (Petersen *et al.*, 2010) and salivary pellicle components; Fap1 which binds salivary pellicle and mediates biofilm formation (Ramboarina *et al.*, 2010); BapA1, which represents a new family of streptococcal adhesins involved in biofilm formation (Liang *et al.*, 2011); AbpA which binds α-amylase (Nikitkova *et al.*, 2012); and glucan-binding proteins (GbpB, GbpC) that promote adhesion of bacteria to polysaccharide matrix (Mattos-Graner *et al.*, 2006; Biswas *et al.*, 2007). Genomic sequencing has revealed that some streptococcal strains may carry up to 30 or more genes encoding proteins with predicted cell-wall anchorage (Nobbs *et al.*, 2009). Therefore it is likely that in future more functional adhesins will be characterized that play a role in streptococcus colonization and biofilm development.

Antigen I/II (AgI/II) family protein adhesins are produced by most oral streptococci. The sequences and structures are well-conserved, but they have a diverse range of functions in mediating adhesion to host surfaces, and coaggregation with other oral microorganisms (Brady et al., 2010). The AgI/II protein expressed by *S. mutans* (variously designated SpaP, Pac or AgB) is responsible for adhesion of bacteria to salivary pellicle. *S. gordonii* produces two AgI/II family proteins termed SspA (172 kDa) and SspB (164 kDa). These polypeptides mediate coaggregation of streptococci with *Actinomyces oris* (Egland *et al.*, 2001) but with strain specificities (Jakubovics *et al.*, 2005). SspB also interacts directly with *Porphyromona gingivalis* through a C-terminal region designated BAR that is recognized by the shorter fimbriae on the surface of *P. gingivalis* (Daep *et al.*, 2008).

Microbial interactions between earlier and later colonizers

Streptococci and other precursor organisms provide unique receptor sites for later, more pathogenic colonizers such as *Fusobacterium nucleatum* (He *et al.*, 2012), *Tannerella forsythia, Treponema denticola*, and *Porphyromonas gingivalis* (Fig. 1) (Kuboniwa & Lamont, 2010; Periasamy & Kolenbrander, 2010), which are closely associated with the development of periodontitis (Haffajee & Socransky, 1994; van Winkelhoff *et al.*, 2002). Adherence of *P. gingivalis* to antecedent bacteria promotes initial colonization, and ultimately facilitates periodontal destruction (Slots & Gibbons, 1978). A well understood interspecies interaction is between that of *P. gingivalis* and the oral commensal *Streptococcus gordonii*. This occurs through two sets of adhesin-receptor pairs (Fig. 2). *P. gingivalis* cells display preferential binding to oral surfaces coated with certain streptococci, such as *S. gordonii* and other members of the oralis group (Lamont *et al.*, 1992). It is likely that this interaction begins primarily on the supragingival tooth surface (Ximenez-Fyvie *et al.*, 2000; Mayanagi *et al.*, 2004; Haffajee *et al.*, 2008). From here, *P. gingivalis* may spread laterally to the subgingival region via an increase in biomass or by cell dispersal as the result of active cellular release or passive mechanical shearing of the supragingival biofilm. *P. gingivalis*, and some other secondary colonizers such as *Fusobacterium nucleatum* can also provide bridging functions by expressing multiple adhesins that bind other later colonizers (Kolenbrander *et al.*, 2002). Both attachment-based and physiological interactions between late colonizers and compatible precursor organisms can promote progression of the plaque biofilm towards a more pathogenic state (Kuboniwa & Lamont, 2010; Whitmore & Lamont, 2011).

Interspecies recognition and co-adhesion contributes to community formation and to the success of the participating bacteria. Bacterial growth within a community can bring metabolic advantages and access to nutrients that would be unavailable to planktonic organisms. As certain species flourish within the community they release metabolites that

can be utilized by other community inhabitants. In such a heterotypic biofilm, bacteria will often co-localize with other constituents that are metabolically compatible (Jenkinson & Lamont, 2005). This metabolic synergy within the community can allow the development of a more complex microbiota. One example of metabolic synergy occurs between *T. denticola* and *P. gingivalis*. When grown in a dual species biofilm, these organisms produce a significantly larger biomass than the total of the individual monospecies biofilms (Grenier, 1992). *P. gingivalis* produces isobutyric acid which stimulates growth of *T. denticola*, while *T. denticola* produces succinic acid that enhances growth of *P. gingivalis* (Fig. 1). The chymotrypsin-like proteinase produced by *T. denticola* also stimulates formation of a dual species biofilm with *P. gingivalis* (Cogoni *et al.*, 2012). The co-operation of *P. gingivalis* with other oral species such as *F. nucleatum* has also been demonstrated. The ability of some *F. nucleatum* strains to tolerate higher oxygen concentrations than *P. gingivalis* means that *F. nucleatum* facilitates generation of reduced oxygen conditions that promote growth and survival of *P. gingivalis* (Bradshaw *et al.*, 1998; Diaz *et al.*, 2002). This modification of the microenvironment by *F. nucleatum* may allow growth of other strictly anaerobic oral species (Kolenbrander *et al.*, 1995). *F. nucleatum* can also elevate the pH of its environment through the generation of ammonia, thus neutralizing acid produced by fermenting microorganisms and creating a more favorable environment for *P. gingivalis* and other acid-sensitive organisms (Takahashi, 2003).

Bacterial interactions are often established by pairings of adhesin (protein) and receptor (saccharide) components found on the surfaces of the associated bacteria (Kolenbrander *et al.*, 2006). An example of such interaction involves the type 2 fimbriae on the surface of *A. oris (naeslundii)* that recognize a (GalNAcβ1→ 3Gal) linkage present within cell wall polysaccharide on *Streptococcus oralis* thus allowing the cells to co-aggregate (Palmer *et al.*, 2003; Yoshida *et al.*, 2006). In the case of interaction of *P. gingivalis* with *F. nucleatum* (Rosen & Sela, 2006), the latter expresses a lectin adhesin that specifically recognizes galactose, which is present in the capsule and lipopolysaccharide of *P. gingivalis*. Similar galactose-containing receptors are found on *Aggregatibacter actinomycetemcomitans* (Rupani *et al.*, 2008) and *T. denticola* (Rosen *et al.*, 2008), in the form of the serotype-specific O polysaccharide and outer membrane carbohydrate groups, respectively. Thus, strains of *F. nucleatum* actively bind these different organisms, both earlier and later colonizers. Also, *T. denticola* and *T. forsythia* each express leucine-rich repeat proteins that mediate mutual attachment and facilitate binding to *F. nucleatum*, further adding to the developing bacterial network (Ikegami *et al.*, 2004; Sharma *et al.*, 2005) (Fig. 1).

METABOLIC NETWORKS

Many bacteria rely on metabolic cooperation provided by close proximity of cells to grow and become incorporated within oral microbial communities. For example, *Veillonella* species are Gram-negative, anaerobic cocci, which occur in plaque in high abundance (Bik *et al.*, 2010) and are part of the pioneer oral community after birth (Cephas *et al.*, 2011). Growth of streptococci leads to the formation of lactic acid, which is a favored substrate of *Veillonella atypica*. This in turn accelerates the glycolysis rate in streptococci by removing the end-product (lactate) inhibition (Fig. 1).

When *S. gordonii* and *V. atypica* are grown in co-culture a diffusible signal produced leads to up-regulation of the *S. gordonii* amylase gene, *amyB*. Increased amylase activity on a starch substrate would produce more fermentable glucose, generating further lactic acid and more favorable conditions for *V. atypica* (Egland *et al.*, 2004). On the other hand, *S. gordonii* appears to benefit from interaction with *A. naeslundii* (Egland *et al.*, 2001). When co-cultured, a number of genes involved in arginine biosynthesis are differentially expressed in *S. gordonii* (Jakubovics *et al.*, 2008) potentially increasing the efficiency of arginine

biosynthesis in *S. gordonii*. An observation that highlights the benefits of interspecies cooperation is in the degradation of salivary mucins. Individually, *Streptococcus* species do not necessarily produce all of the required enzymes for mucin hydrolysis, while cooperatively they are able to more efficiently utilize the mucin oligosaccharides for growth (Byers *et al.*, 1999).

The examples above begin to paint a picture of a web of metabolic exchanges that occur in the oral cavity (Jenkinson, 2011). But more simply, colonization by early pioneering colonizers e.g. streptococci can enhance the growth and virulence of potentially pathogenic bacteria such as *P. gingivalis* and *T. denticola*. This has led to the mitis-group streptococci e.g. *S. gordonii*, S. oralis etc.being termed accessory pathogens in the oral cavity (Whitmore & Lamont 2011).

Antagonism

Microorganisms are not always greeted into a community with open arms. A number of bacterial species have evolved specific mechanisms to inhibit growth and attachment of competing organisms. Hydrogen peroxide produced by some of the oral streptococci provides one mechanism of bacterial antagonism (Holmberg & Hallander, 1973). However, interspecies interactions are often multi-threaded. Kreth *et al.* (2005) observed two separate mechanisms by which *S. sanguinis* and *S. mutans* are mutually antagonistic, based upon hydrogen peroxide (H_2O_2) production by *S. sanguinis* and bacteriocin production by *S. mutans*. When grown simultaneously, both species proliferate; however, prior establishment of one of the species prevents the other from occupying the same niche (Kreth *et al.*, 2005). Further work demonstrated that *S. gordonii* also inhibits growth of *S. mutans*, and that this is promoted under aerobic conditions which led to elevated H_2O_2 levels. Interestingly, H_2O_2 production by bacteria may have also co-evolved to act as a signaling molecule for the fungus *Candida albicans* to undergo filamentation (Srinivasa *et al.*, 2012). More aerobic conditions appear to stimulate production of bacteriocins by *S. mutans*, through activation of the Gram-positive competence-stimulating peptide (CSP) signaling system encoded by the *com* genes (Kreth *et al.*, 2008). As covered in more detail below, *S. mutans* expresses a eukaryotic serine/threonine type kinase known as STPK which contributes to resistance to peroxide (Zhu & Kreth, 2010). This thrust and counter-thrust, driven by co-evolution, continues through strategies to subvert the production of antagonistic elements. An example of this is a gene *sgc* in *S. gordonii* encoding a protease capable of interfering with bacteriocin production in *S. mutans* (Wang & Kuramitsu, 2005).

Certain oral streptococci have been shown to have a negative impact on biofilm formation by *P. gingivalis*. Contact with the later streptococcal colonizer *S. cristatus* has been shown to down-regulate expression of *fimA*, which encodes the major fimbrial adhesin of *P. gingivalis*, and thus prevent *P. gingivalis* accumulation on *S. cristatus* -rich substrata (Wang *et al.*, 2009). Arginine deaminase (ArcA) in *S. cristatus* provides the communication signal responsible for the down-regulation of *fimA* in *P. gingivalis*, although enzymatic function of ArcA is not essential for signaling activity (Xie *et al.*, 2007). *S. intermedius* also produces arginine deaminase that can repress the expression of both FimA and Mfa1 (minor fimbria) in *P. gingivalis* (Christopher *et al.*, 2010). Although *S. gordonii* produces ArcA, *cis* catabolite response elements function to repress expression in *S. gordonii* in comparison to *S. cristatus* (Lin *et al.*, 2008). This antagonistic interaction has been shown to have biological consequences. In a mouse model colonization of the oral cavity by ArcA-expressing *S. cristatus* followed by *P. gingivalis* infection reduces the levels of *P. gingivalis* colonization and subsequent bone loss (Xie *et al.*, 2012).

Detachment of microorganisms from the biofilm may occur as a consequence of antagonism, or exclusion. Release of cells from the biofilm is anyway an important

mechanism for dispersal, and may be passive or active. The main mechanism is shear force, such as salivary flow or external applications such as tooth-brushing. However, some microbial species are known to actively disperse from the biofilm. Following *P. aeruginosa* biofilm formation, cells on the outer layer remain as a stationary biofilm phenotype, while cells on the inside of the biofilm become motile (planktonic phenotype) and can swim out of the biofilm, leaving a hollow mound (Sauer *et al.*, 2002). The dental pathogen *A. actinomycetemcomitans* is not a motile species, but does have the ability to become released from the biofilm, utilizing dispersin B, a biofilm-releasing β-hexosaminidase (Manuel *et al.*, 2007). Salivary flow then seeds other areas of the mouth with the released *A. actinomycetemcomitans* cells (Kaplan & Fine 2002). *Candida albicans* biofilms are composed mainly of a network of hyphae (filaments) that provide the biofilm structure. A transcriptional regulator Nrg1p blocks yeast to hyphae transition and controls dispersion of yeast morphology cells from the biofilm (Uppuluri *et al.*, 2010).

INTERACTIONS OF STREPTOCOCCI WITH PORPHYROMONAS AND CANDIDA

The AgI/II adhesins of oral streptococci

The streptococcal AgI/II family of proteins are multifunctional adhesins with the ability to bind a variety of host components such as collagen, laminin, and salivary substrates, as well as other microorganisms (Brady *et al.*, 2010). Ag I/II family protein functions vary according to streptococcal species and strains in which they are expressed. For example, *S. mutans* strain NG5 expresses a single Ag I/II polypeptide, SpaP, whereas *S. gordonii* strains expresses two AgI/II family proteins (SspA and SspB) The production of these proteins is affected by environmental factors including salivary proteins, variations in pH, osmolarity and temperature. SspA and SspB expression levels increase under elevated temperature and acidic pH, while SspB expression is reduced under lower NaCl concentration (El-Sabaeny *et al.*, 2000). Also, SspA levels are generally higher than those of SspB, and the SspA polypeptide positively regulates *sspB* by binding its promoter region (El-Sabaeny *et al.*, 2001). One major receptor for AgI/II is salivary glycoprotein gp340, also found in most if not all mucosal secretions. This protein participates in innate immunity by promoting microbial cell agglutination and clearance (Prakobphol *et al.*, 2000). However, when gp340 is adsorbed onto an oral cavity surface it provides sites for streptococcal binding (Lamont *et al.*, 1991). The AgI/II proteins play a critical role in the association of *P. gingivalis* with *S. gordonii* as described in more detail below.

The SspA and SspB proteins also appear to mediate interactions of *S. gordonii* with hyphal filaments of *C. albicans*, thus promoting co-colonization by these microorganisms (Bamford *et al.*, 2009) (Fig. 1). The receptor on *C. albicans* that interacts with SspB is a protein expressed on the hyphal cell surface, designated Als3 (Silverman *et al.*, 2010). This is one of a group of proteins expressed by the agglutinin-like sequence (*ALS*) family of genes (Hoyer *et al.*, 1995). They encode surface glycoprotein adhesins involved in host-pathogen interactions and other adhesive functions (Hoyer *et al.*, 2008). There are eight Als proteins (Als1-8p), but the largest decrease in *C. albicans* adhesion is observed by deletion of both *als3* alleles (*C. albicans* is generally diploid) (Zhao *et al.*, 2004). Als3p is hypha-specific (Murciano *et al.*, 2012) and is in all probability involved in early establishment of biofilms in addition to interacting with oral streptococci (Silverman *et al.*, 2010).

P. gingivalis fimbriae

Numerous peritrichous fimbriae protrude from the cellular envelope of most strains of *P. gingivalis* (Listgarten & Lai, 1979; Handley & Tipler, 1986). Binding of *P. gingivalis* to sites in the oral cavity and to other bacteria is dependent, at least in part, upon these fimbrial

structures (Slots & Gibbons, 1978) which are of two kinds. The major fimbriae are longer, with lengths 0.3–1.6 μm and width approximately 5 nm (Yoshimura *et al.*, 1984), while the minor fimbriae are 80–120 nm in length and 3.5 – 6.5 nm in diameter (Park *et al.*, 2005). The major fimbriae bind to salivary proteins and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) found on the surface of *S. oralis* (Maeda *et al.*, 2004b) and other streptococci.. The primary unit of the major fimbria is fimbrillin (FimA). There are at least six different variants of *fimA* among strains of *P. gingivalis*, and binding differences among these fimbrillin types could impact the likelihood of periodontal disease development (Amano *et al.*, 2004). The *fimA* locus is flanked by genes that are involved in transcriptional regulation or encode proteins that contribute to the structure of the fully mature fimbriae. The two genes upstream of *fimA* are involved in regulation via a response regulator, FimR (Nishikawa *et al.*, 2004), part of the FimS/FimR two-component signal transduction system which governs transcriptional levels of *fimA* (Hayashi *et al.*, 2000). Environmental cues detected by *P. gingivalis* that influence *fimA* expression include changes in temperature and hemin concentration (Amano *et al.*, 1994; Xie *et al.*, 1997). The arginine and lysine-specific gingipains produced by *P. gingivalis* also regulate the amount of FimA on the bacterial surface, potentially affecting adhesion and colonization (Xie *et al.*, 2000). The genes downstream of *fimA* encode the products FimC - FimE, which are minor components of the mature fimbriae. FimE is responsible for assembly of FimC and FimD proteins onto the fimbrial fiber and for maintaining a stable attachment to the bacterial surface (Nishiyama *et al.*, 2007). The absence of any of these three accessory proteins manifests as a significant reduction of FimA binding to GAPDH, which in turn affects initial *P. gingivalis* binding to oral streptococci (Maeda *et al.*, 2004a).

The minor fimbriae bind to components of the streptococcal surface. The primary subunit of the minor fimbriae, Mfa1 (67 kDa) has a role in inflammatory processes and is involved in induction of several cytokines such as IL-1α, IL-1β, and TNF-α in peritoneal macrophages, in response to *P. gingivalis* (Lin et al., 2006). Additional genes encode accessory proteins that associate or co-operate with the main protein subunit. Downstream of *mfa1* is a co-transcribed gene encoding the protein Mfa2. This protein is known to have a role in the regulation of the length of the minor fimbriae and is required for their attachment to the cell envelope (Hasegawa *et al.*, 2009). *P. gingivalis* cells that do not express Mfa2, but still produce Mfa1, have abnormally lengthened minor fimbrial extensions and these fimbriae are only weakly bound to the cells. There are three additional, less characterized products encoded by the genes situated downstream of *mfa2* which are predicted to be accessory proteins that interact directly with the polymerized Mfa1 since they are co-purified with the filaments (Hasegawa *et al.*, 2009).

Interaction of SspA and SspB with Mfa1

P. gingivalis binding to *S. gordonii* SspA and SspB proteins is important for development of dual species biofilm communities (Lamont *et al.*, 2002). *P. gingivalis* does not interact with all AgI/II family members, despite conservation in primary amino acid sequences and secondary structure (Brady *et al.*, 2010). For example, *P. gingivalis* does not recognize SpaP of *S. mutans* (Brooks *et al.*, 1997). The precise region of SspA/B involved in binding *P. gingivalis* was determined by examining a series of truncated SspB polypeptides and chimeric proteins consisting of portions of SspB and SpaP. The adherence characteristics of the chimeras, composed of different sections of SspB and SpaP sequences, pointed toward a region (aa residues 1167 – 1250 in SspB) as being necessary for *P. gingivalis* binding. This region was designated BAR (SspB adherence region) and shown to have a significantly different secondary structure from the corresponding region in SpaP (Forsgren *et al.*, 2010). A critical region of 26 amino acid residues within BAR contains the necessary motifs recognized by Mfa and a synthetic BAR peptide can adhere to *P. gingivalis*, whereas the

corresponding SpaP peptide does not. It was also shown that BAR peptides with specific mutations confer conformational changes that ablate binding to *P. gingivalis* cells (Demuth *et al.*, 2001).

The NITVK motif within BAR helps define the binding specificity for AgI/II protein members as NITVK is only found in the AgI/II proteins of the oralis group of streptococci, which includes *S. gordonii, S. oralis* and *S. sanguinis*. The AgI/II proteins expressed by other streptococci have Gly in place of Asn^{1182}, Pro in place of Val^{1185}, or potentially both of these substitutions. Both Gly and Pro are known to terminate α helixes in proteins, and these particular substitutions inhibit *P. gingivalis* binding. A VQDLL motif upstream of NITVK is also conserved in the oralis group and resembles a nuclear receptor box domain of eukaryotic proteins that is involved in protein-protein interactions (Daep *et al.*, 2008). This motif is also flanked by lysine residues, and the positive charge of these residues could participate in electrostatic interactions that stabilize the complex of SspB with Mfa1. The VQDLL motif is in an α-helical region while NITVK is in a predicted β sheet, and this region extends outside of the SspB core, making it accessible to Mfa1 (Forsgren et al., 2010). The importance of the BAR region for co-colonization with *P. gingivalis* has been established in a mouse model. Peptides derived from BAR inhibited *P. gingivalis* colonization and disease in mice pre-infected with *S. gordonii* (Daep *et al.*, 2011). The efficacy and low toxicity to the host of BAR derivatives suggest that they could be developed as a therapeutic or prophylactic agent in periodontal disease (Daep *et al.*, 2008).

GENE REGULATION WITH THE COMMUNITY ENVIRONMENT

Transcriptional studies on the differences between planktonic versus the sessile (biofilm) state have highlighted the fundamental shift an organism undertakes as it becomes part of the biofilm community (O'Toole & Kolter, 1998). A dramatic response to monospecies biofilm formation is exhibited by *P. gingivalis*, with 18% of the genome differentially regulated compared to planktonic organisms. Many of the regulated genes are associated with cell envelope biogenesis, DNA replication and metabolism, supporting the concept that cells in the transition from planktonic to biofilm state exhibit a lower rate of growth and cell metabolism (Lo *et al.*, 2009). Genes involved in adhesion and early biofilm formation, *fimA* and *mfa1*, were up-regulated in early biofilms, while *fimA* was down-regulated in the later stages (Yamamoto *et al.*, 2011). In a community with *S. gordonii*, thirty-three *P. gingivalis* genes showed up- or down-regulation by microarray analysis (Simionato *et al.*, 2006), one of which was *ltp1*, a tyrosine phosphatase.

Prevailing environmental conditions, influenced by diet, can have an important role in regulating gene activity within the oral microbial community (Bradshaw *et al.*, 1989; Percival *et al.*, 1991). Different sugars or complex carbohydrates influence expression of specific metabolic pathways (Klein *et al.*, 2010) and impact on mechanisms controlling cell integrity and secretion of extracellular biofilm matrix. Carbohydrate metabolism is integrated with cell-cell signaling systems, such as the autoinducer-2 (LuxS/AI-2, see below) pathway. A LuxS deficient strain of *S. mutans* was affected in expression of genes involved in carbohydrate metabolism, DNA repair, amino acid and protein synthesis and stress tolerance (Wen *et al.*, 2011). A phenotypic outcome of loss of LuxS is a fundamental difference in biofilm architecture.

The correlation between mRNA levels and protein amounts is not always strong (Nie *et al.*, 2006) and thus it is also important to understand community adaptation at the proteome level. In *T. forsythia*, 44 proteins were found to be altered between planktonic and biofilm cultures (Pham *et al.*, 2010). Many of these proteins were associated with the outer membrane and transport systems, and effects were observed in amount of S-layer produced

by the cells. Oxidative stress response proteins were up-regulated and the resulting biofilm cells were 10- to 20-fold more resistant to oxidative stress as compared to their planktonic counterparts. This could enhance survival of *T. forsythia* in the presence of H_2O_2-producing streptococci (Pham *et al.*, 2010).

P. gingivalis displays differential abundance of 47 proteins when grown in planktonic versus biofilm conditions (Ang *et al.*, 2008). A high percentage of these changes were associated with the cell envelope. Increased presence of proteins associated with hemin transport and metabolism indicated that *P. gingivalis* cells were entering a starved state (Ang *et al.*, 2008). In a three-species community with *S. gordonii* and *F. nucleatum*, levels of *P. gingivalis* proteins involved in cell envelope structure and DNA repair were decreased (Kuboniwa *et al.*, 2009), indicating that the multi-species community environment was less stressful to *P. gingivalis*.

Signaling within a biofilm setting

Communication is an important part of any society, including bacterial communities, and intra- and inter-species communication facilitates community development. The first example of bacterial signaling was described in the marine organism *Vibrio fischeri* where a diffusible signal N-acyl homoserine lactone (AHL) was responsible for the induction of bioluminescence (Nealson & Hastings, 1979). The same AHL molecule is utilized by both *P. aeruginosa* and *Burkholderia cepacia* in co-ordination of virulence genes and biofilm formation in cystic fibrosis (Eberl & Tummler, 2004). AHL is not commonly utilized by oral bacterial species for communication (Kolenbrander *et al.*, 2002); rather oral bacteria rely on two distinct signaling systems. The first, restricted to Gram-positive organisms such as the early colonizing streptococci, utilizes short peptides termed Competence Signaling Peptides (CSP) (Suntharalingam & Cvitkovitch, 2005) or other small peptides (Son *et al.*, 2012). These have been described in a number of streptococci, including *S. mutans* and *S. gordonii*, where they play a role in genetic exchange and virulence. The second signaling system involves autoinducer-2 (AI-2), a family of signaling molecules produced by the action of the LuxS enzyme on S-ribosyl-homocysteine (SRH), generating 4,5-dihydroxyl-2,3-pentanedione or DPD, which breaks down to produce AI-2 (Sun *et al.*, 2004). AI-2 was originally described in the marine organism *Vibrio harveyi* (Bassler *et al.*, 1993) and is now recognized as a species-independent signal that is widespread in oral bacteria including *P. gingivalis, A. actinomycetemcomitans* and also oral streptococci such as *S. gordonii* and *S. mutans*.

There are a number of studies on the role of AI-2 and community development within the oral cavity. For example, AI-2 is required for biofilm growth of *A. actinomycetemcomitans* (Shao *et al.*, 2007). AI-2 is linked to the two-component system QseBC, which is induced by AI-2 through uptake of AI-2 into the cell. This uptake is reliant on two AI-2 receptors, linked to ABC transporters, termed LsrB and RbsB. Deletion of either of these elements reduces *A. actinomycetemcomitans*-induced alveolar bone resorption in animal models. Loss of QseC also diminishes biofilm formation consistent with a role for this system in colonization and virulence (Novak *et al.*, 2010)(see Fig. 3). Other oral species are also dependent on AI-2 signaling for biofilm formation. *A. oris* can utilize AI-2 produced by *S. oralis* for biofilm growth and development. Interestingly, the concentration of signal is an important factor in dual species biofilm formation, and at higher concentrations of AI-2 there are significantly lower levels of biofilm formation (Rickard *et al.*, 2006). Moreover, the AI-2 concentration in *A. oris-S oralis* biofilms decreases over time, possibly contributing to the persistence of the dual species communities (Rickard *et al.*, 2008).

S. gordonii also exhibits altered biofilm development in the absence of AI-2, and AI-2 is required for community development between *S. gordonii* and *P. gingivalis* (McNab *et al.*,

2003). Interkingdom effects of AI-2 have been seen between *C. albicans* and *S. gordonii*, where a l*uxS* knockout of *S. gordonii* is substantially affected in dual-species biofilm formation with *C. albicans* (Bamford *et al* 2009). It is suggested that *luxS* mutation affects the ability of *S. gordonii* to promote hyphal growth in *C. albicans*. This might occur by suppressing effects of the quorum-sensing molecule farnesol, which normally inhibits filamentation (Hornby *et al.*, 2001) (Fig. 4).

A range of signaling molecules have been identified produced by bacteria that affect *C. albicans* biofilm formation or morpogenesis. These include lactic acid, H_2O_2, CO_2, and bacterial peptidoglycan (Xu *et al.*, 2008) all of which appear to promote filamentation, while HSLs inhibit filamentation (Hall *et al.*, 2011). By way of return, fatty acids, carboxylic acids and glycans produced by *C. albicans* are able to promote growth of bacteria, while farnesol inhibits bacterial biofilm formation (Pammi *et al.*, 2011). Thus filamentation of *C. albicans* and mixed-species biofilm formation are regulated by recognition of a complex array of self- or non-self signaling molecules (Fig. 4).

A number of genes are regulated by LuxS in *S. gordonii* including those involved in carbohydrate synthesis (McNab *et al.*, 2003). The pathways in *P. gingivalis* involved in signal transduction, including AI-2 dependent signaling, following contact with *S. gordonii* were identified byChawla *et al.* (2010). A LuxR family orphan transcriptional regulator designated CdhR was shown to constrain development of *S. gordonii* and *P. gingivalis* communities. The community function of this regulator was attributed to two genes that are under its control. The first is *mfa1*, which as mentioned previously encodes the minor fimbrial antigen in *P. gingivalis* and is responsible for primary interactions with AgI/II polypeptides on the surface of *S. gordonii*. CdhR was also shown to regulate LuxS which directly affects the amount of AI-2 thus ultimately affecting dual species development (Chawla *et al.*, 2010). CdhR was also shown to be part of the same regulatory circuit as Ltp1, a tyrosine phosphatase.

PHOSPHORYLATION AND BIOFILM COMMUNITY DEVELOPMENT

The importance of post translational modification on serine, threonine and tyrosine residues has long been known in eukaryotic systems, and is gaining increasing significance in bacterial systems. Bacteria and some plant systems also possess another type of phosphorylation system based on histidine and aspartate. Two component signal transduction systems (TCSs) comprise a sensory kinase and response regulator and are the most common examples of bacterial regulatory systems involving phospho-transfer. The sensor kinase responds to external signals resulting in the autophosphorylation of a histidine residue. The transfer of the phosphate group to the response regulator results in the phosphorylation of an aspartate residue and a downstream effect on gene transcription (Gao & Stock, 2009). TCSs have been shown to be involved in biofilm development in a number of bacteria (Kolar *et al.*, 2011; Zhang *et al.*, 2009).

Serine/threonine protein kinases (STPKs)

A number of STPKs now described in bacteria are of the Hanks-type kinase, and show homology to eukaryotic kinases. This allows intracellular bacteria to utilize STPKs and the corresponding phosphatases to subvert host signal transduction via phosphorylation and de-phosphorylation of signaling components within the host (Kobir *et al.*, 2011). As well as having a role in virulence and host subversion, STPKs have been implicated in biofilm formation e.g. PrkC of *Bacillus subtilis* (Madec *et al.*, 2002) and Stk in *S. epidermidis* (Liu *et al.*, 2011). *S. mutans* possesses a homologue of the eukaryotic-like STPKs, PknB. A strain deficient in PknB exhibited reduced biofilm formation on hydroxyapatite discs, and an inability to tolerate acid conditions (Hussain *et al.*, 2006). Further characterization of PknB,

and the corresponding phosphatase PppL, showed that both enzymes were important for biofilm formation, cell shape, acid tolerance, genetic transformation and cariogenicity in a rat model (Banu *et al.*, 2010). PknB is also involved in controlling bacteriocin production, possibly through modulating the activity of a TCS in *S. mutans*. Additionally, PknB participates in oxidative stress tolerance, and a decreased fitness was observed in a *pknB* deficient strain of *S. mutans* when grown with *S. sanguinis* (Zhu & Kreth, 2010). Taken together, the results indicate that the STPK *pnkB* in *S. mutans* provides an important link in the establishment of a *S. mutans* community and persistence within the oral cavity.

Tyrosine kinases

Over recent years a number of tyrosine kinases and phosphatases have been described in prokaryotes leading to the realization that phosphorylation on tyrosine is not limited to occurring in eukaryotic systems, as was once believed. Significant structural differences are apparent between Gram-positive and Gram-negative bacterial tyrosine kinases (BY-kinases). In Gram-negative species, the BY-kinase comprises a single polypeptide. A short N-terminal region is usually present within the cytoplasm, followed by a transmembrane stretch and a region occupying the periplasmic space. A second transmembrane stretch brings the polypeptide back into the cytoplasm where the C-terminal region contains the enzymatically active Walker (ATP binding) domains. In Gram-positive bacteria the kinase domain and transmembrane regions are encoded by neighboring genes on the bacterial chromosome (Lee & Jia, 2009). Initially thought of as purely autophosphorylating peptides, the discovery of phosphorylation of specific substrates has lead to the appreciation that BY-kinases play a critical role in many aspects of virulence. One of the first substrates of BY-kinases to be recognized was a UDP-glucose dehydrogenase in *E. coli* (Grangeasse *et al.*, 2003). Phosphorylation of this substrate increases its activity in generating precursors for polysaccharide synthesis. BY-kinases have since been shown to play a significant role in the transport and synthesis of cellular polysaccharide (Fig. 3), and are thus likely to impact community development and biofilm formation. Tyrsoine kinase activity has been found to be important for biofim formation by *Bacillus subtilis* (Kiley & Stanley-Wall, 2010); however, the function of tyrsosine kinase in oral bacteria has yet to be investigated.

Tyrosine phosphatases

Tyrosine kinases generally have partner phosphatases, such that reversible phosphorylation of substrates allows for regulation of cellular processes. Bacterial protein tyrosine phosphatases (PTPs) possess similar structures to those typically found in eukaryotes. Bacterial PTPs fall into 3 classes: the conventional PTP; dual specificity phosphatase (DSP); and a low molecular weight (LMW) PTP class. The LMW-PTP class of phosphatases has been found to be important in virulence and other physiologically important cellular events (Grangeasse *et al.*, 2007). In Gram-positive species, LMW-PTP genes are found upstream of their corresponding kinase genes, whereas in Gram-negative species, the genes are at distinct locations on the chromosome.

In *P. aeruginosa*, deletion of *tpbA* encoding a tyrosine phosphatase resulted in >100-fold increase in biofilm formation over an 8 h period. This dramatic effect was attributed to increase in initial attachment levels, decrease in swimming activity and loss of swarming motility (Ueda & Wood, 2009). TpbA also appeared to constrain pellicle and EPS formation. Two potential mechanisms account for the observed phenotype. The first is increased transcription of the *pel* locus responsible for production of EPS. The second is regulation of cyclic di-GMP, also shown to contribute to biofilm formation in *P. aeruginosa* (Ueda & Wood, 2009). The *tpbA* gene is regulated by AHL signaling in *P. aeruginosa*, indicating cross-talk between quorum sensing and tyrosine phosphatase activity (Ueda & Wood, 2009). Another aspect of the functionality of TpbA is the ability to regulate the

amount of extracellular DNA released from *P. aeruginosa* cells (Ueda & Wood, 2010). Extracellular DNA is a major component of the biofilm matrix and can provide a structural role in biofilm development (Whitchurch *et al.*, 2002; Martins *et al.*, 2010).

A tyrosine phosphatase has also been characterized in *P. gingivalis* (Maeda *et al.*, 2008). Ltp1, a LMW-PTP, influences a number of cellular processes and is a key component of a regulatory pathway that constrains heterotypic community development between *P. gingivalis* and *S. gordonii*. Ltp1 activity restricts EPS production at the transcriptional level and also negatively regulates expression of *luxS*. By contrast, Ltp1 through modulating the activity of the transcriptional regulator CdhR (see above), positively regulates *hmu* (hemin uptake operon), thus increasing hemin/iron uptake by the organism (Fig. 3). Secretion of the RgpA/B gingipain (proteinase) is reduced in a *ltp1* mutant while increase in the cell-associated Kgp gingipain is observed (Maeda *et al.*, 2008). It is interesting to note that deletion of the genes encoding the Kgp and RgpA/B gingipains markedly alters the mono-species biofilm phenotype (Kuboniwa *et al.*, 2009a). Thus, kinase-phosphatase networks have pleiotropic effects on the formation and stability of oral microbial biofilm communities.

CONCLUSIONS

Biofilm development and maintenance is an essential factor for microbial survival and growth, both in the environmental setting and the host. Benefits of the biofilm lifestyle include reduced sensitivity to mechanical shearing and to the actions of antimicrobial agents, and enhanced nutritional flexibility. Cells undergo a multitude of changes in the transition from planktonic to biofilm mode of growth. These begin with the sensing of diffusible signals that are secreted by one set of microbial cells and recognized by others. Quorum sensing along with contact dependent sensing instigates changes in gene and protein expression. Specific cell-to-cell adherence as dictated by adhesin-receptor pairings on respective bacterial surfaces can also direct community development. Interbacterial binding helps optimize co-localization of species that can coexist in a mutually beneficial relationship. Underpinning many of these processes are the intracellular phosphorylation events that regulate a large number of bacterial cell processes relevant to community formation and development.

Acknowledgments

Research of the authors is currently funded by the National Institutes of Health (NIDCR) (DE11111, DE12505, DE16690, DE17921, and DE22867) and by the Wellcome Trust (097285). Owing to space limitations it has sometimes not been possible to cite all previous publications related to a finding or topic, and for this we apologize.

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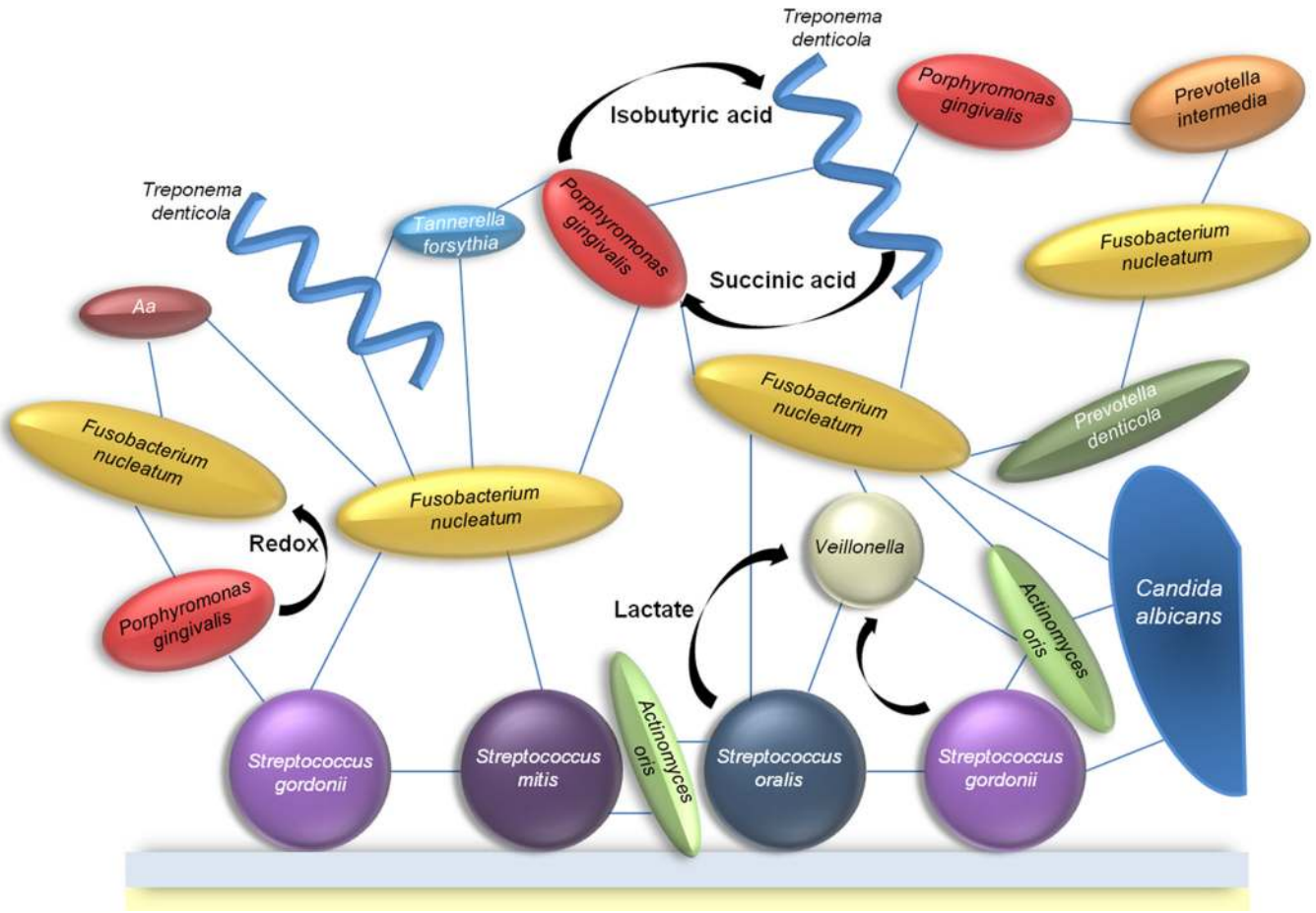

Fig. 1.

Bacterial cell-to-cell interactions in the dental plaque biofilm. One example of mutualism is displayed between *P. gingivalis* and *T. denticola*. Each releases a metabolic factor that the other can utilize, thus enhancing the growth of both species. Lines linking the microorganisms represent adhesive interactions. Aa = *Aggregatibacter actinomycetemcomitans*

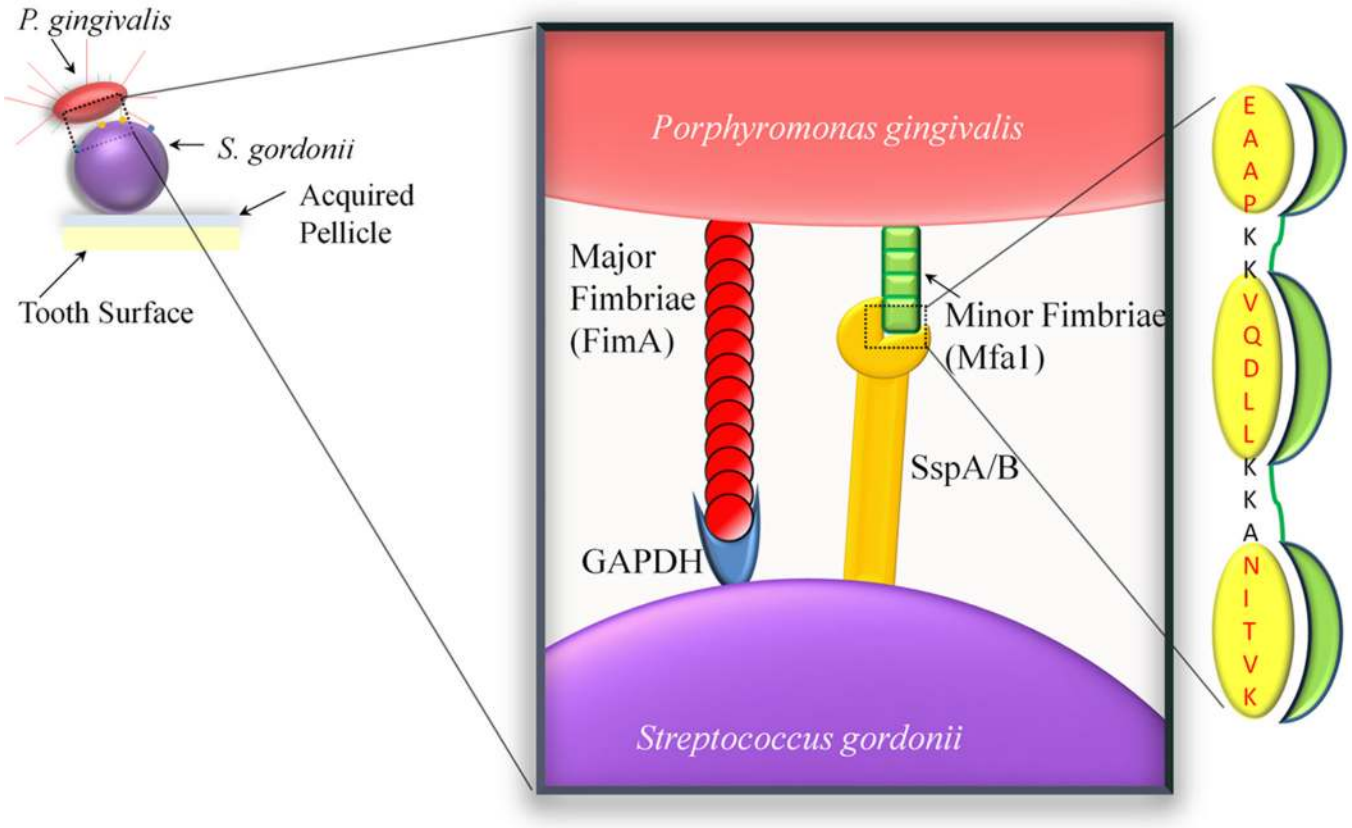

Fig. 2.

The interaction of *P. gingivalis* with *S. gordonii* depends upon two sets of adhesin-receptor pairs. The major and minor fimbriae of *P. gingivalis* bind glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and SspA/B on the surface of *S. gordonii*, respectively. The domains of SspA/B that interact with the minor fimbriae subunit, Mfa1, are highlighted in the yellow ovals. These domains reside within the SspA/B adherence region (BAR) and are required to maintain the contacts between the two species in the oral cavity.

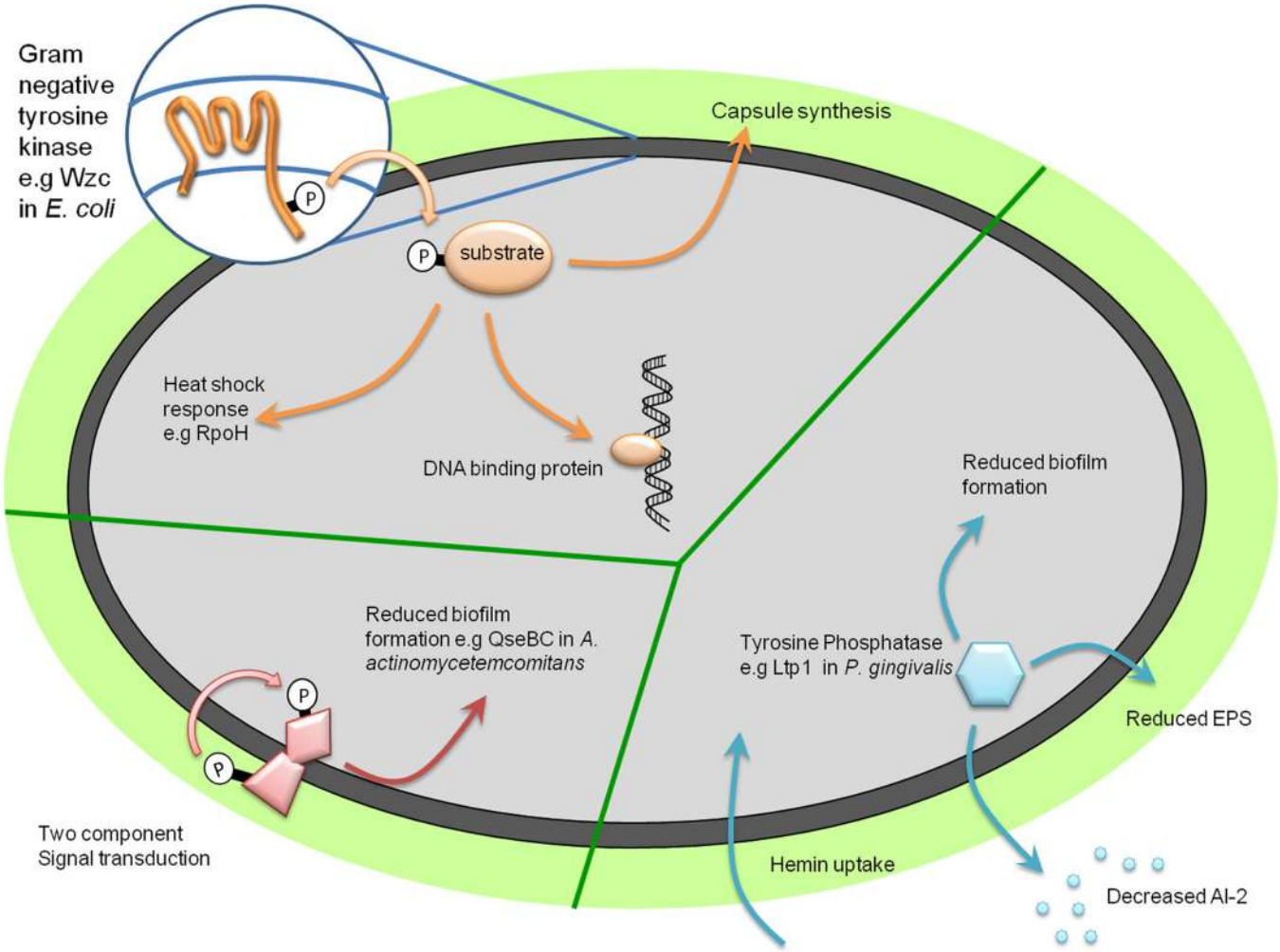

Fig. 3.

Phosphorylation events within a bacterial cell are complex and dynamic. The two-component system in *A. actinomycetemcomitans* is induced by AI-2, with the loss of this TCS resulting in diminished biofilm formation. Tyrosine kinases in Gram-negative bacteria such as Wzc in *E. coli* have been shown to regulate a variety of targets including DNA-binding proteins, capsule synthesis genes and the heat shock response. The phosphatase Ltp1 in *P. gingivalis* has been shown to affect a number of cellular activities including dual-species community formation, reduced EPS synthesis and hemin uptake.

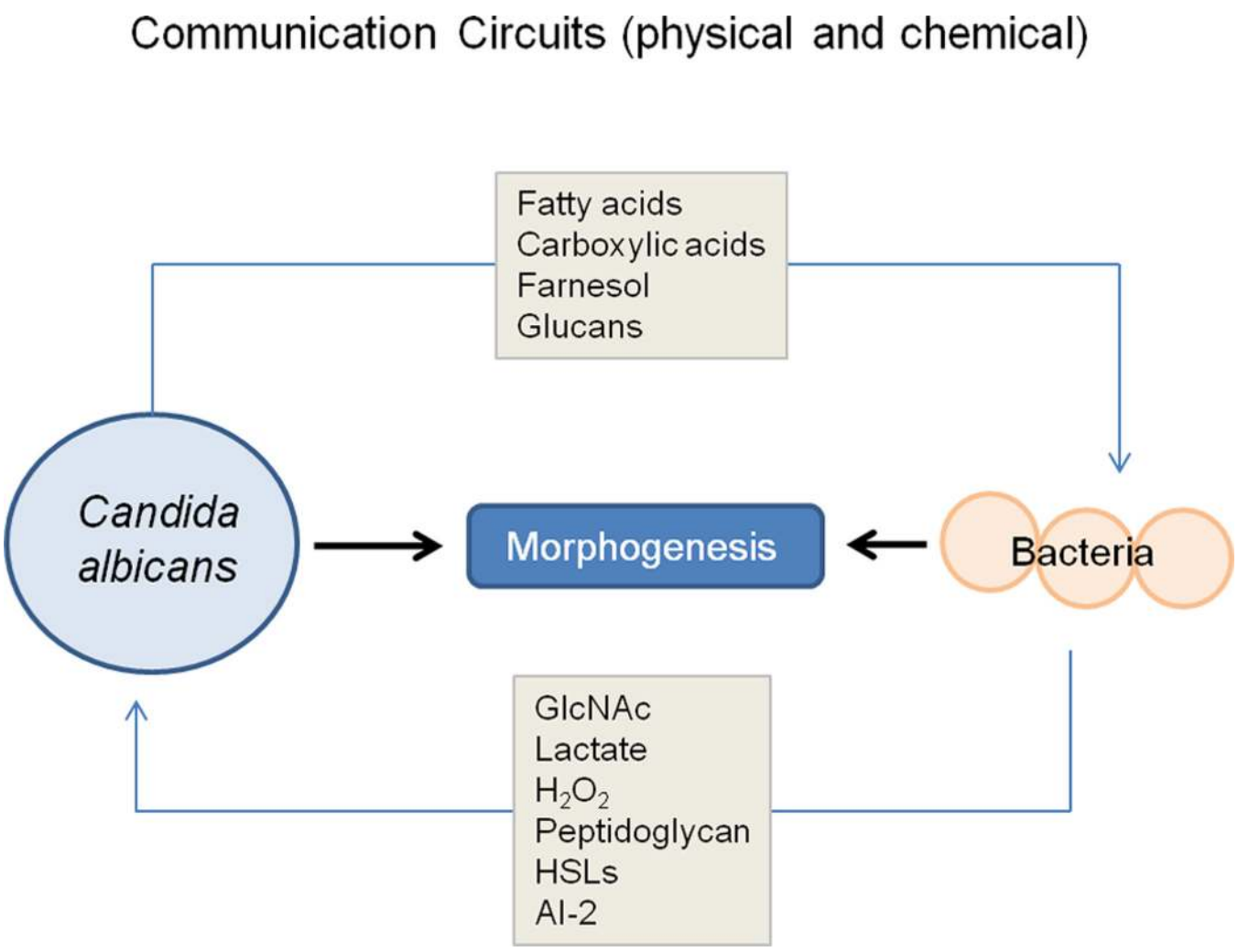

Fig. 4.

Communication circuits between *C. albicans* and oral streptococci. The diagram depicts self- or non-self control of the yeast to hypha transition, with bacterial products variously impacting on morphogenesis and biofilm formation, and *C. albicans* products positively or negatively influencing bacterial growth or biofilm formation (see text for discussion).