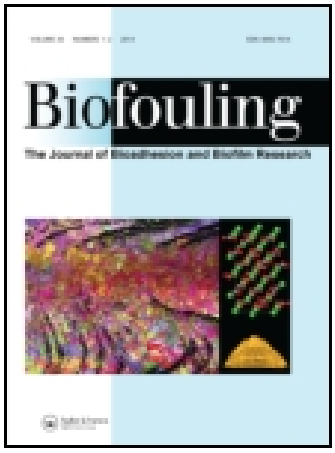

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Mini-review: Microbial coaggregation: ubiquity and implications for biofilm development

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Mini-review: Microbial coaggregation: ubiquity and implications for biofilm development

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Coaggregation is the specific recognition and adherence of genetically distinct microorganisms. Because most biofilms are polymicrobial communities, there is potential for coaggregation to play an integral role in spatiotemporal biofilm development and the moderation of biofilm community composition. However, understanding of the mechanisms contributing to coaggregation and the relevance of coaggregation to biofilm ecology is at a very early stage. The purpose of this review is to highlight recent advances in the understanding of microbial coaggregation within different environments and to describe the possible ecological ramifications of such interactions. Bacteria that coaggregate with many partner species within different environments will be highlighted, including oral streptococci and oral bridging organisms such as fusobacteria, as well as the freshwater sphingomonads and acinetobacters**.** Irrespective of environment, it is proposed that coaggregation is essential for the orchestrated development of multi-species biofilms.

Keywords: polymicrobial; biofilm; cell-cell adhesion; coaggregation; cell-cell interactions; ecology

Coaggregation and biofilm formation

Biofilms represent the dominant mode of microbial existence (Costerton et al. 1995). A biofilm is an interface-associated community which is typically composed of multiple microbial species in close proximity with one another (Stoodley et al. 2002). Species within biofilms form interdigitated cellular mosaics, facilitating metabolic cross-feeding and cell–cell signaling (Davey & O'Toole 2000; Hojo et al. 2009). Organization and cell–cell interactions enhance the persistence of both individual species and the biofilm as a whole (Tolker-Nielsen & Molin 2000; Kolenbrander et al. 2010). This review will discuss how coaggregation, defined as the specific recognition and adhesion of genetically distinct microorganisms (Rickard et al. 2003) occurs, and will demonstrate its contribution to biofilm development. There has been a resurgence of interest in interbacterial interactions, such as those mediated through cell–cell signaling and coaggregation, in order to decipher which factors contribute to competition or cooperation in biofilms. However, within typical multi-species biofilms, there are many thousands of potential cell–cell interactions. Understanding which species physically bind to one another in different environments may help to establish which are likely to be the most important interactions, and hence the ones that might be useful to target in anti-biofilm strategies. It is in this context that this review paper will also focus on the importance of coaggregation in altering the individual and collective behavior of biofilm species. As such, a holistic view of coaggregation will be taken to discuss findings relevant among different environments and highlight the potential for novel biofilm control strategies.

Coaggregation was first observed between bacteria isolated from human dental plaque in the 1970s (Gibbons & Nygaard 1970) and it has since been shown to occur between bacteria isolated from environments such as drinking water, wastewater, the human intestinal tract, and even within domestic showerheads (Elliott et al. 2006; Ledder et al. 2008; Simoes et al. 2008; Min & Rickard 2009; Vornhagen et al. 2013). In each of these environments, coaggregation has been focused on because of its potential to hinder or support the colonization and expansion of pathogenic or problematic microbial populations. Examples include the hindrance of colonization of periodontal pathogens in dental plaque biofilms and the expansion of coaggregating species in freshwater biofilms associated with biofouling (Jakubovics et al. 2008b; Simoes et al. 2008; Min & Rickard 2009).

A key role of coaggregation is in the development of multi-species biofilms. Evidence for such a role can be made by simply taking a scraping of supragingival dental plaque and visualizing the sample under a light microscope. Counter to the diagram drawn by Anton Von Leuwenhoek in 1683 (Figure 1A) (Dobell 1958; Porter 1976), dental plaque scrapings contain, in addition to single cells, morphologically complex cellular aggregates (Figure 1B) that are likely formed by coaggregation interactions (Kolenbrander et al. 2006). For example, corncob-like structures of rods and cocci are formed due

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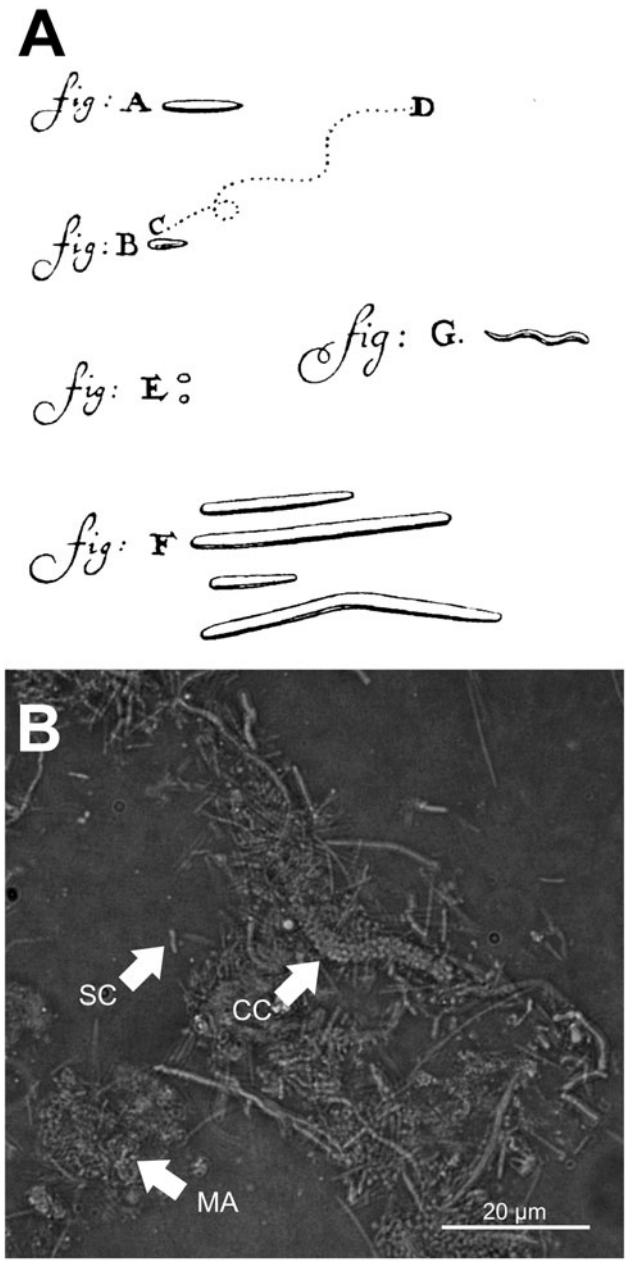

Figure 1. Past perceived simplicity *vs* recently realized complexity. (A) Drawing by Anton Von Leuwenhoek in 1683 showing single cells from a dental plaque scraping using his primitive (but groundbreaking) microscope *vs* (B) a recently taken micrograph of plaque harvested using a similar approach. SC: streptococcal chain, CC: 'corncob' structure, MA: multi-species aggregate. For further details of Figure 1A, see Porter (1976). Figure 1A is from an image originally published in 1695 and reprinted in Dobell (1932).

to coaggregation between *Fusobacterium nucleatum* and *Streptococcus* spp. (Lancy et al. 1983; Kaufman & DiRienzo 1988) (Figure 1B). Other structurally ornate co-aggregates can be formed, such as rosettes consisting of *Streptococcus sanguinis* surrounded by *Prevotella loescheii* (Kolenbrander & Andersen 1988) and interdigitated masses consisting of *Streptococcus gordonii* and *Actinomyces oris* (Jakubovics et al. 2008a). As suggested in the paper 'Adhere today, here tomorrow' (Kolenbrander & London 1993), such structured coaggregates enhance bacterial integration into oral biofilms. It has since become evident that coaggregation may also facilitate, by virtue of the specificity of adhesion, the successional development of biofilms (Hojo et al. 2009).

A first step in the successional development of dental plaque biofilms, and arguably multi-species biofilms in many other environments, is the coating of uncolonized surfaces with polysaccharides and proteins (described as an acquired pellicle or conditioning film), which enhances attachment of initial colonizing bacteria (Figure 2A) (Sutherland 2001; Huang et al. 2011). These primary colonizers grow in surface-attached micro-colonies and become enmeshed in a matrix of self-produced, extracellular polymeric substances (EPS) (Sutherland 2001) (Figure 2B). As the micro-colonies develop, additional species, so-called secondary colonizers, are recruited through coaggregation and non-specific aggregation interactions (Busscher & van der Mei 1997; Bos et al. 1999), increasing the biofilm biomass and species complexity (Figure 2C). Secondary colonizers continue to integrate, further increasing diversity and biomass (Figure 2D). This successional process allows species that cannot adhere to the acquired pellicle to become part of the biofilm (Rickard et al. 2003). An often overlooked additional role in succession, however, is that coaggregation promotes cellular juxtaposition between the colonizing species and thus, by virtue of decreased distance between the coaggregating cells, facilitates enhanced cell–cell interactions, such as the exchange of metabolites (Egland et al. 2004) and cell signaling molecules (Rickard et al. 2006). These and other coaggregation-enhanced roles will be discussed below, but first the mechanisms that bring about coaggregation need to be addressed.

Mechanisms of microbial coaggregation

There are a number of approaches to measure coaggregation between bacteria, and these are often used to characterize the mechanisms that facilitate these interactions (Bos et al. 1999). These approaches typically include the semi-quantitative visual coaggregation assay which relies upon categorizing the size of coaggregates formed in a glass tube against a categorical scoring system (0–4, where '0' denotes no coaggregation and '4' represents pairs that coaggregate to yield a clear suspension with large flocs) and more complex spectrophotometric-based assays that quantify coaggregation as a function of settling rate (ie the larger the coaggregate the more rapid the decrease in the optical density of a suspension). In addition, radiolabeling has been used to quantify

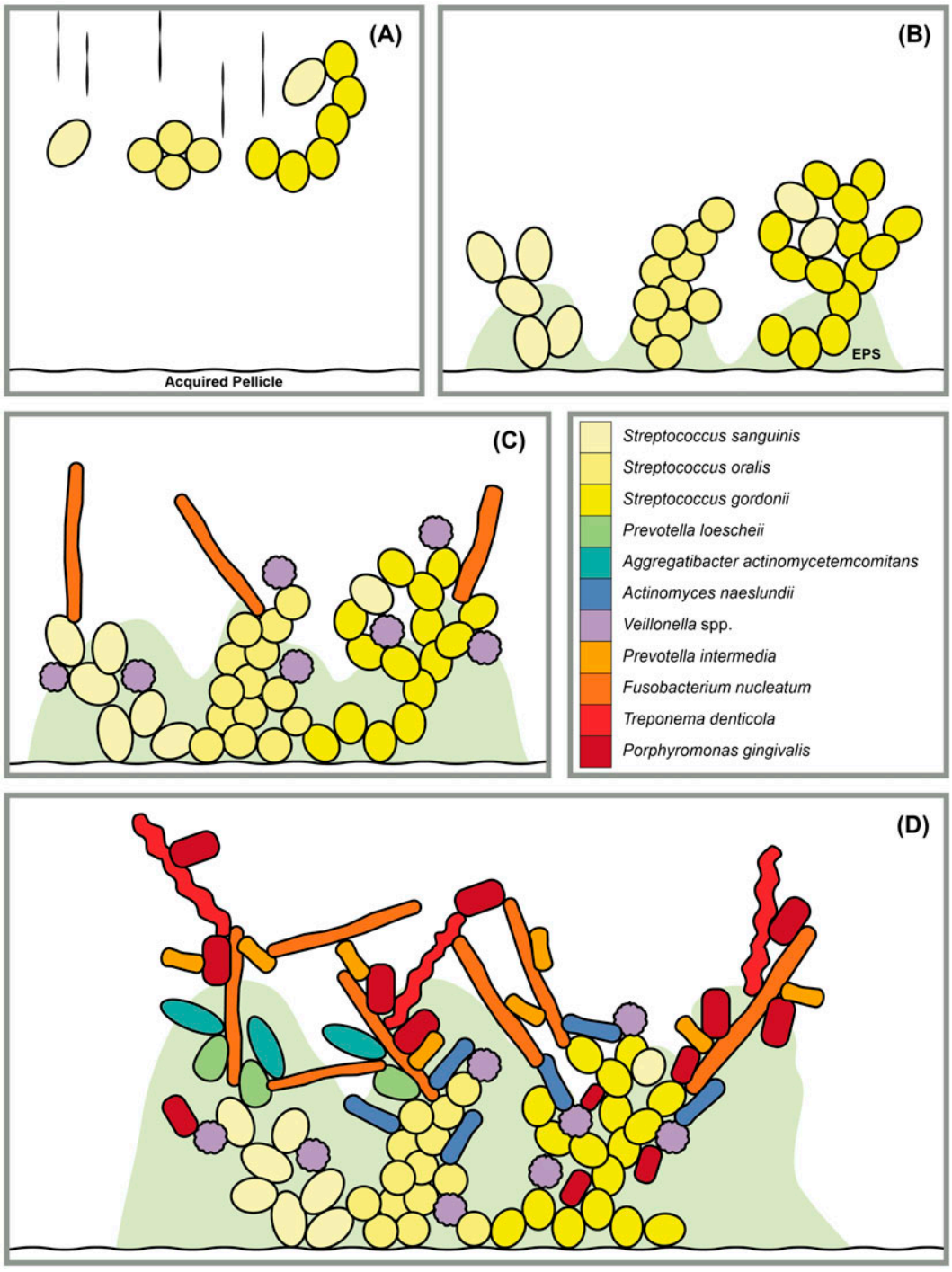

Figure 2. Diagram showing the sequential development of oral biofilms. (A) Planktonic single cells, autoaggregates, or coaggregates adhere to the acquired pellicle. (B) Growth and production of EPS by primary colonizing cells. (C) Recruitment of secondary colonizing species. (D) Additional recruitment of potentially pathogenic species, in part due to the ability of *Fusobacterium nucelatum* to coaggregate with many partners.

attachment of bacteria in suspension to immobilized cells (Wyatt et al. 1988; Jenkinson et al. 1993). A review detailing approaches to measuring coaggregation was published by Bos et al. (1999).

Coaggregation is mediated by the interaction between specific macromolecules on the cell surface of one species with cognate macromolecules expressed on the cell surface of the partner species. Microbial cells may

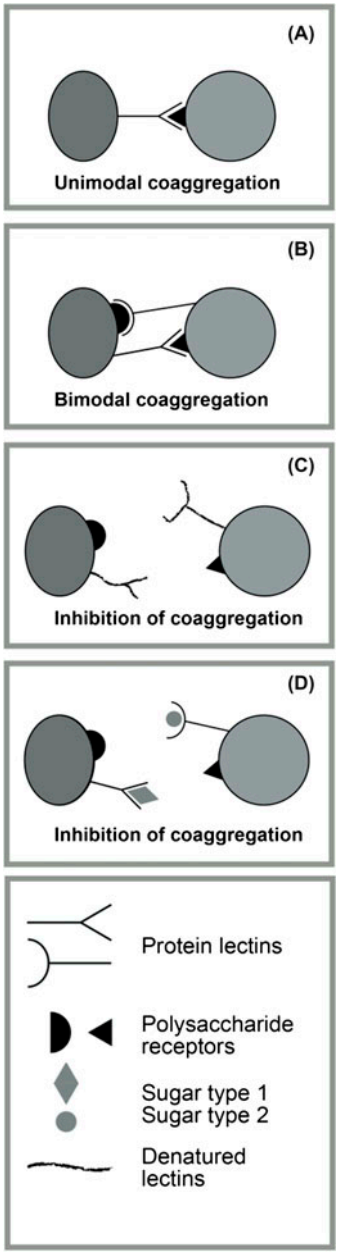

Figure 3. Typical coaggregation interactions. (A) An example of a unimodal coaggregation interaction mediated by a cell-surface-associated protein (lectin) adhesin and a complementary cell surface-associated polysaccharide receptor. (B) An example of bimodal coaggregation interaction involving a pair of adhesin–receptor interactions where each pair is unique with respect to the sugar being recognized. Note, one strain could conceivably express both adhesins while the other could express both receptor polysaccharides. (C) Adhesins can be identified by heat or through the addition of protease, both of which inactivate the adhesins but not the receptors. (D) Sugars are added to determine the type of sugar recognized by the adhesin. If bimodal coaggregation occurs, no one sugar will reverse coaggregation to yield uncoaggregated single cells. The size and shapes of the cells and appendages are not to scale.

also come into contact through hydrophobic interactions or electrostatic forces irrespective of the presence of specific interacting macromolecules, but these associations tend to be relatively weak and, for the purpose of this review, are not considered true coaggregation. Coaggregation-mediating proteins are referred to as adhesins, and polysaccharide moieties are referred to as receptors (Kolenbrander & Phucas 1984). Coaggregation may occur between lectin-like protein adhesins and polysaccharide receptors (Kolenbrander et al. 2006; Kline et al. 2009) or by protein–protein (adhesin–adhesin) interactions (Daep et al. 2008), and in some cases is bimodal, involving two different interacting pairs of macromolecules (Kolenbrander 1982; Figure 3). Whether a coaggregation partner strain bears an adhesin or a receptor can be determined by applying heat or adding protease to each partner and evaluating the effect of the treatment on coaggregation using a visual assay (Cisar et al. 1979). The mechanistic nature of adhesin–receptor interactions in coaggregation can be further tested by assessing inhibition or reversal of coaggregation by specific sugars or amino acids (McIntire et al. 1978). In the case of oral streptococci, many of the interactions are inhibited by lactose (Kolenbrander 1988; Kolenbrander et al. 2006). Using these approaches, McIntire et al. (1978) were among the first to show that coaggregation between the two primary colonizing oral bacteria *A. oris* T14 V (previously *A. viscosus*) and *Streptococcus oralis* 34 (previously *S. sanguinis*) is mediated through surface-expressed protein adhesins and polysaccharide receptors, respectively.

Beyond simple characterization, the identification of specific adhesins or receptors requires in-depth molecular genetic approaches. For the oral bacterium *S. gordonii*, gene disruption, production of recombinant polypeptides, and the expression of adhesins on the cell surface of heterologous hosts have been employed to elucidate the roles of the antigen I/II family adhesins SspA and SspB in coaggregation with *Actinomyces oris*, *Porphyromonas gingivalis* and interkingdom coaggregation with *Candida albicans* (Jenkinson et al. 1993; Holmes et al. 1996; Jakubovics et al. 2005; Daep et al. 2011). The characterization of polysaccharide receptors is more complex since several genes are involved in the biosynthesis of a single polysaccharide and there is often a great deal of heterogeneity in polysaccharide structures. Nevertheless, a ‘carbohydrate engineering’ approach, involving the transfer of genes between streptococci that produce polysaccharides of differing coaggregation specificities, has been developed to identify the key coaggregation-mediating moieties of streptococcal receptor polysaccharides (Yoshida et al. 2005). In the case of *A. oris* T14 V and *S. oralis* 34, coaggregation has been shown to occur between CafA protein, localized at the tip of *A. oris* type

2 fimbriae, and a '1Gn' type of receptor polysaccharide on *S. oralis* (Reardon-Robinson et al. 2014; Yang et al. 2014). While little is known regarding the identity of the adhesins or receptors that mediate coaggregation between bacteria outside the oral cavity, there seems to be a similar involvement of currently unidentified surface-associated coaggregation adhesins that recognize surface-associated polysaccharide receptors between bacteria isolated from aquatic and wastewater environments (Rickard et al. 2000; Adav et al. 2008).

Coaggregation between bacteria in the human oral environment

Comprising over 700 species/phylotypes, the human oral cavity constitutes a taxonomically and architecturally complex community of microorganisms (Aas et al. 2005; Hojo et al. 2009). Visualization techniques such as fluorescent *in situ* hybridization (FISH) (Zijnge et al. 2010) and the use of polyclonal antibodies (Palmer et al. 2003) have enabled qualitative and quantitative monitoring of the initiation, succession, and maturation of oral bacterial communities and provide strong evidence that dental plaque biofilms are highly structured (Kolenbrander et al. 2006). It is becoming widely accepted that bacterial coaggregation promotes a specific sequence of colonization by oral bacteria. In the case of dental plaque, for example, *Streptococcus* spp., *Veilonella* spp., and *Actinomyces* spp. are often considered primary colonizers and adhere to the tooth pellicle (Ritz 1967; Nyvad & Kilian 1987; Diaz et al. 2006; Periasamy & Kolenbrander 2010). Anaerobic Gram-negative secondary colonizers such as *Porphyromonas gingivalis* and *Fusobacterium* spp. are subsequently recruited, in part through coaggregation interactions, resulting in a number of complex cell–cell interactions within dental plaque. In an elegant demonstration that coaggregation interactions likely stabilize primary colonization, Chalmers et al. (2008) micromanipulated a community of *S. oralis*, *S. gordonii* and a *Veillonella* species that naturally developed upon human enamel chips *in vivo* and demonstrated that they coaggregated with one another *in vitro*.

Within the human oral environment, secondary colonizers only form substantial biofilms after the establishment of primary colonizers such as *Streptococcus* and *Actinomyces* (Periasamy et al. 2009; Figure 2). The primary colonizers and a select few secondary colonizers are able to strongly coaggregate with multiple partners and form multi-species bridges consisting of >3 partner species (Figure 4). For instance, *P. loescheii* and *F. nucleatum* can act as bridges across biofilm space to provide linkages for colonization between stages of succession (Kolenbrander et al. 1985, 1989). Viewed in cross-section, *F. nucleatum* is often observed in the middle echelons of dental plaque biofilms and is able to coaggregate with both early and late colonizers (Kolenbrander et al. 1985, 1989; Bradshaw et al. 1998; Zijnge et al. 2010; Nobbs et al. 2011). Considering that *F. nucleatum* coaggregates with periodontal pathogens such as *P. gingivalis* and *Aggregatibacter actinomycetemcomitans*, this organism's multi-species bridging capacity makes it of key interest when considering the development of dental plaque biofilms (Kolenbrander & Andersen 1989; Kolenbrander et al. 1989; Rosen et al. 2003). *F. nucleatum* is able to partner with at least 17 different genera, although there are strain-dependent specificities; different strains of *F. nucleatum* recognize different partner species and the strengths of coaggregation are pair specific (Kolenbrander et al. 1989, 2002). The specificity and bridging functions of *F. nucleatum* are demonstrated in its interactions with *Selenomonas flueggei*, a later secondary colonizer. *S. flueggei* cannot coaggregate with primary colonizing species such as streptococci and instead must rely on *F. nucleatum* to successfully integrate into dental plaque biofilms (Kolenbrander et al. 1989). Thus, the presence of primary colonizers is critical; without primary colonizers such as *S. gordonii*, the secondary colonizing *F. nucleatum* and *P. gingivalis* form poor biofilms (Lamont et al. 2002; Periasamy et al. 2009). Coaggregation between *P. gingivalis* and *S. gordonii* is seldom and, arguably, very weakly detected by visual coaggregation assays. However, when immobilized on a surface, *S. gordonii* interacts with *P. gingivalis* through two distinct adhesin–receptor pairings. Recognition of *P. gingivalis* major fimbriae is mediated by glyceraldehyde 3-phosphate dehydrogenase, and the *P. gingivalis* minor fimbriae are bound by antigen I/II adhesins SspA and SspB (Wright et al. 2014). *P. gingivalis* population expansion within heterotypic biofilms is further enhanced by sensing through tyrosine phosphatase Ltp1, which modulates the expression of a variety of virulence factors (Maeda et al. 2008). Such interactions are intriguing from an oral health perspective, especially when the potential for different pioneer colonizing oral streptococci to either facilitate or inhibit the colonization of periodontal pathogens is considered (Whitmore & Lamont 2011).

To date, there have been no quantitative studies of oral biofilm development using wild-type and defined coaggregation deficient mutants to compare their ability to form dual and/or multi-species biofilms under conditions representative of the human oral cavity. Technologies such as those involving confocal laser scanning microscopy, flowcells, and microfluidic systems are developing at an impressive rate. Such technologies will facilitate qualitative and quantitative studies in four dimensions (ie spatiotemporal biofilm development) (Jensen & Tolker-Nielsen 2011; Nance et al. 2013; Sanchez et al. 2013).

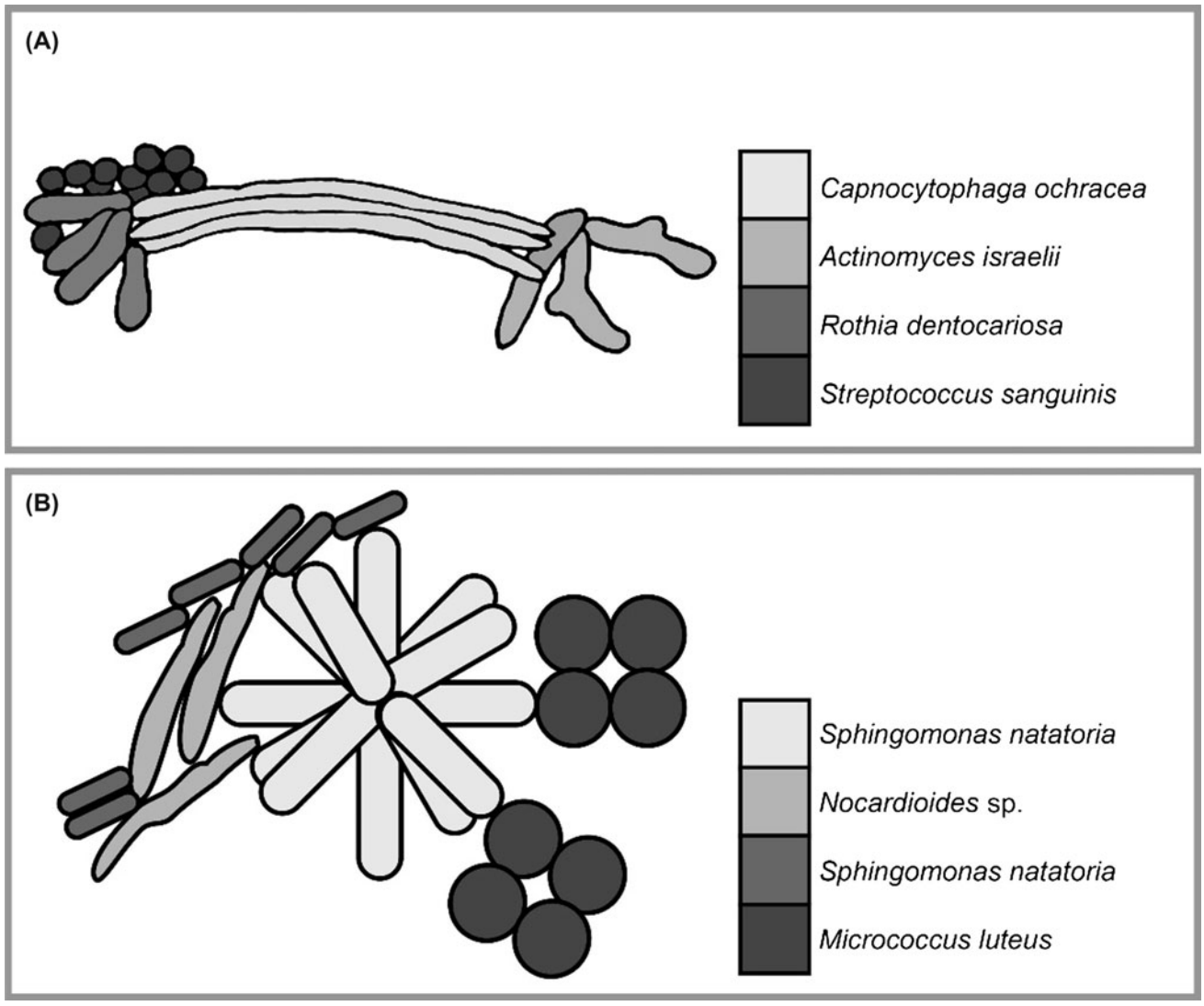

Figure 4. Diagram demonstrating possible arrangements of bridging organisms in oral (A) and freshwater (B) bacterial communities. Cell shapes and scales are approximate. *Capnocytophaga ochracea* and *Sphingomonas natatoria* are highlighted as bridging organisms for the oral and the freshwater environment, respectively. Figure 4A is derived from Kolenbrander and Andersen (1984) with permission.

Coaggregation interactions elsewhere within the human body

The human body contains numerous species of bacteria that, on a cell-per-cell basis, collectively outnumber human cells by ~10:1 (Blaser 2006). However, the composition of the human microbiome is susceptible to perturbations due to disturbances from infections or environmental changes (Ley 2010; Koren et al. 2011). Despite extensive work over the past two decades using culture-independent approaches to describe changes in community composition, the functional and spatial relationships of each species within a given community have received relatively little attention. Of this spatio-functional research, much has focused on the ability of bacteria to coordinate activities through cell–cell signaling rather than the propensity of the bacteria to coaggregate (Parsek & Greenberg 2005; Stevens et al. 2012). Apart from those within the human oral cavity, only microbial communities from the human urogenital tract have been studied in detail in the context of coaggregation (Marrie et al. 1978; Reid et al. 1988).

Pathogenic biofilms within the female urogenital tract are responsible for approximately one billion urogenital infections in women each year (MacPhee et al. 2010; McMillan et al. 2011). Consequently, particular attention has focused on the probiotic potential of *Lactobacillus* spp. to maintain health-promoting microbial communities and prevent the colonization of pathogens, such as uropathogenic *Escherichia coli* (McMillan et al. 2011). Reid et al. (1988) suggested that coaggregation may play a

significant role in stabilizing the resident microbiota of the vaginal epithelium. In particular, *Lactobacillus* spp. have been shown to coaggregate with a number of Gram-negative and Gram-positive bacteria including *Staphylococcus epidermidis* and *E. coli* under aerobic and anaerobic conditions (Reid et al. 1988, 1990; Ekmekci et al. 2009). The ability to coaggregate with numerous species conceivably has a dual role in this microbial community, as it may aid in the retention of lactobacilli within urogenital tract biofilm communities while also facilitating intimate juxtaposition with pathogenic species. This latter role is important because lactobacilli produce inhibitory compounds including bacteriocins (Reid et al. 1988, 1990), which can rapidly reduce the viability of uropathogens and disrupt the integrity and architecture of the biofilms (McMillan et al. 2011; Darouiche & Hull 2012). Thus, a probiotic approach through the introduction of coaggregating lactobacilli may offer potential advantages over standard antimicrobial therapy in that such methods would prevent re-establishment of uropathogens in a sustained manner while also being localized, impacting primarily the urogenital tract.

Although studies are still in their infancy, chronic wounds have recently been shown to harbor biofilms that contain coaggregating species. A study of 32 bacterial species isolated from chronic wounds showed that *Parvimonas micra* (formerly *Micromonas micros)* F21B and *Peptostreptococcus anaerobius* B12 strongly coaggregated with other wound isolates (Hill et al. 2010). Chronic wounds are notoriously difficult to treat and currently affect >3 million patients costing $5–10 billion per annum in the USA (Werdin et al. 2009). By better understanding the microbial interactions (including coaggregation) within chronic wound biofilms, it may be possible to design novel approaches to modulate biofilm formation either by promoting bacterial interference or by destabilizing key biofilm interactions.

Coaggregation between bacteria in the aquatic environments

Over the last decade, significant attention has focused on the propensity of aquatic bacteria to coaggregate. Most studies have investigated biofilm bacteria isolated from borehole-derived freshwater, municipal drinking water, re-circulating domestic aquarium water and wastewater (Rickard et al. 2002; Kerr et al. 2003; Malik et al. 2003; Simoes et al. 2008; Min & Rickard 2009). The rationale for understanding the role of coaggregation in aquatic biofilm development relates to concerns regarding the growth and retention of pathogens within biofilms in flowing (low and high shear) environments, microbial induced corrosion, biofouling of surfaces, and increased resistance to antimicrobials that is afforded by the biofilm mode of growth (Stewart & Costerton 2001; Kerr et al. 2003). Despite the infancy of this field of study, it has been clearly demonstrated that freshwater-based coaggregation is important for bacterial colonization and biofilm formation (Simoes et al. 2008; Min & Rickard 2009).

There are a number of nuances particular to studying coaggregation of freshwater bacteria that have hindered research. Unlike coaggregation between oral bacteria, coaggregation between freshwater bacteria is often growth-phase dependent (Rickard et al. 2000, 2002). In the study by Rickard et al. (2002), 19 freshwater strains were compared for their coaggregation ability after different periods of growth in batch culture. A total of 171 pair-wise combinations of strains were examined with every strain coaggregating with at least one partner species. Coaggregation was temporally defined with total possible pair-wise combinations after 36, 72 and 144 h occurring 22, 36.2, and 21% of the time, respectively (Rickard et al. 2002). One strain, *Sphingomonas* (*Blastomonas*) *natatoria* 2.1, coaggregated (visual score ≥2) with 16 of 18 other strains. Some coaggregation interactions were between members of the same species (intra-species coaggregation) or between members of the same genus (intra-generic coaggregation) while others were between genera (inter-generic coaggregation) (Rickard et al. 2002).

Coaggregation between aquatic bacteria can be mediated by adhesin–receptor and occasional adhesin–adhesin interactions (Rickard et al. 2000, 2003). Unlike coaggregation between bacteria from the human oral cavity, lactose or N-acetyl-galactosamine are not the only major sugars that block coaggregation. For example, coaggregation between *S. natatoria* 2.1 and *S. natatoria* 2.3 utilized lactose-inhibitable receptors, while coaggregation with *S. natatoria* 2.6 involved galactosamine-inhibitable interactions and both galactose and galactosamine inhibited coaggregation with *S. natatoria* 2.8 (Rickard et al. 2000). More recent work by Simoes et al. (2008) has shown that, in addition to galactosamine, galactose, and lactose, the sugar fucose also blocks coaggregation between specific freshwater bacterial species. Thus, it is possible that freshwater bacteria are able to use a more functionally diverse suite of adhesins/receptors for coaggregation than oral bacteria.

Similar to coaggregation between oral bacteria, freshwater bacterial coaggregation may promote bacterial succession. A recent study, which focused on the colonization of reverse osmosis (RO) membranes, demonstrated that biofilm development proceeded sequentially (Bereschenko et al. 2010). Importantly, *Sphingomonas* spp., which are known to coaggregate with many species (Rickard et al. 2002; Simoes et al. 2008; Phuong et al. 2009), were found to be critical for primary colonization and to facilitate the integration of other species into RO membrane-associated

biofilms. Secondary colonizers included members of the phyla Bacteroidetes, Proteobacteria, and Verrucomicrobium (Bereschenko et al. 2010). Akin to the growth-phase-dependent nature of coaggregation (Rickard et al. 2000), after initial colonization, *Sphingomonas* spp. appeared to exit these biofilms as single cells, presumably allowing for the colonization of distal surfaces (Bereschenko et al. 2010). Such a process may be spatiotemporally orchestrated and a common feature of many developing freshwater biofilms (Stoodley et al. 2002; Wagner-Dobler 2003; Lyautey et al. 2005).

Simoes et al. (2008) have also demonstrated coaggregation between a number of species found in freshwater (drinking-water) environments and took an intriguing approach to illustrate the importance of just one single coaggregating species in stabilizing biofilms. *Acinetobacter calcoaceticus* coaggregated strongly with *Mycobacterium mucogenicum*, *Burkholderia cepacia*, *Methylobacterium* sp., *Sphingomonas capsulata*, and *Staphylococcus* sp., all of which were isolated from the same drinking water. By mixing all the species together except one, biofilms of substantial biomass were developed in five-species mixtures except when *A. calcoaceticus* was absent (Simoes et al. 2008). This suggests that *A. calcoaceticus* may behave as a bridging organism similar to the oral bacterium *F. nucleatum* in its ability to foster multi-species bacterial biofilm development.

The importance of specificity and the role that bridge organisms play in coaggregation is evident from studies of bacteria isolated from different freshwater environments (Vornhagen et al. 2013). Specifically, Vornhagen et al. (2013) examined coaggregation between bacteria isolated from three different showerheads, finding evidence for coaggregation as well as the presence of numerous bridging organisms. While many of the partner strains did not coaggregate with one another directly, *Brevundimonas lenta* HM006, *Micrococcus luteus* AH004 and *Lysobacter gummosus* HM010 appeared to act as bridge organisms, coaggregating with 17, 17 and 14 partners respectively (Vornhagen et al. 2013). This not only suggests the broader occurrence of coaggregation in aquatic environments, but also the ability of species to specifically recognize and adhere to species from environmentally (well water *vs* metropolitan water) as well as compositionally distinct biofilms (Vornhagen et al. 2013). As such, it is conceivable that coaggregation acts as a targeting mechanism that facilitates the integration of species into biofilms even when the microbial community in the biofilm is markedly different from where the species originated. Such an ability would help microorganisms to traverse different aquatic environments.

Coaggregation in aquatic environments is not exclusive to pristine situations, and it has also been observed in activated sludge within water treatment facilities. Thus, coaggregation may have importance in maintaining biofilms of biotechnological relevance. For instance, the non-flocculating bacteria *Acinetobacter johnsonii* S35 and *Acinetobacter junii* S33 were identified and proposed to play a role in the dynamics of sewage floc formation by acting as a bridge organism and coaggregating with numerous other wastewater species (Malik et al. 2003). *Acinetobacter* spp. often possess a metabolic versatility uncommon to members of other genera (Imperi et al. 2011; Peleg et al. 2012). Together with their ability to coaggregate, this makes the genus a prospective candidate for use in bioremediation (Choi & Oh 2002; Saadoun 2002; Malik et al. 2003).

It is likely that coaggregation also occurs in marine environments, possibly contributing to the colonization of surfaces in the oceans. Only one very recent paper has examined the possibility of coaggregation interactions by marine bacteria and this is by the lactic acid bacterium, *Leuconostoc lactis*, isolated from the marine black porgy fish (*Sparus macrocephalus*) (Zhang et al. 2013). There is interest in this strain of *L. lactis* as it may use coaggregation to protect the host fish from infection. It would be interesting to expand upon this study to determine whether coaggregation occurs between taxonomically diverse marine bacteria. Further, it is possible that inter-kingdom coaggregation occurs between marine bacteria and diatoms, just as inter-kingdom coaggregations have been seen between bacteria and *Candida* spp. isolated from the human body (Shirtliff et al. 2009).

Coaggregation meditated interactions and potential outcomes for biofilm communities

The importance of coaggregation in the development of biofilms has yet to be satisfactorily explored. From an ecological perspective, the ability of a species to coaggregate will likely impart a selective advantage over non-coaggregating species. Such advantages will extend beyond improved biofilm colonization through enhanced adhesion. The impact of coaggregation on bacteria may be considered from the point of view of the whole coaggregate, ie the coaggregate level (CoL), or from the perspective of single cells within the coaggregate, the cellular level (CeL). At the CoL, in a dual-species coaggregate, the single unit that is the coaggregate will potentially impart benefits to both participating species. At the CeL, one of the partner species (each partner is considered a single unit) will benefit from being within a coaggregate while the other may also gain advantages or be at a disadvantage, as compared to planktonic cells. As a consequence of coaggregation-mediated interactions at the CeL and CoL, competition or mutualism will likely occur, which will be translated to changes in spatiotemporal biofilm development. Examples are shown in Figure 5 and each will be discussed below.

Enhanced cell–cell adhesion

The most obvious outcome of coaggregation is the rapid formation of inter-species aggregates (Figure 5). While the spatial arrangement of the cells will have meaning for functional interactions beyond adhesion (eg protection against environmental stress, see below), it is clear that loss of coaggregation ability will reduce the likelihood of a species adhering to surface-bound partner organisms and forming a dual-species biofilm (potential CeL and CoL effects). This has been documented in studies of coaggregation by oral species (Jenkinson et al. 1993; Lamont et al. 2002) and by freshwater species (Min & Rickard 2009). The physico-chemical interactions between coaggregating species have also been extensively studied and it is becoming clear that the environmental conditions required for coaggregation to occur are extremely important (Bos et al. 1999; Postollec et al. 2005; Min et al. 2010). For example, Min et al. (2010) demonstrated that coaggregation between two freshwater bacteria was dependent on the ionic strength of the solution in which the species were mixed, the types of ions present, the temperature, pH, and viscosity. Thus, it is conceivable that coaggregation is maximized under conditions conducive to growth of the coaggregating species. This is an interesting possibility as it is possible that some species may be able to coaggregate under different environmental conditions, when others cannot (a significant advantage at the CeL). The strength of coaggregation may contribute to changes in biofilm species composition and also provide a target for strategies to control biofilms.

Metabolic interactions

When considering the specificity of coaggregation and the possible conferred benefits at the CeL, an obvious potential benefit to one (or possibly both) species is the exchange of metabolites (Figure 5). Two studies have drawn attention to the possibility of coaggregation-enhanced metabolic interactions. Work by Ishii et al. (2005) showed that coaggregation facilitates hydrogen transfer between the thermophilic syntrophic bacterium *Pelotomaculum thermopropionicum* strain 'SI' and the hydrogenotrophic methanogen *Methanothermobacter thermautotrophicus* strain 'ΔH'. Members of these two genera are important in high-temperature anaerobic digestors (Sekiguchi et al. 1998; Leclerc et al. 2004). The authors demonstrated that propionate substrate oxidation and hydrogen flux between the two species was only possible over distances <2 μm and that such intimate physical contact of these two through coaggregation is indispensable for efficient syntrophic propionate oxidation. Another study by Egland et al. (2004) focused on the oral coaggregating bacteria *S. gordonii* V288 and *Veillonella* sp. PK1910. *Veillonella* spp. can only use lactate as an energy source, while *Streptococcus* spp. ferment carbohydrates to produce lactate (Distler & Kroncke 1981). Egland et al. (2004) demonstrated that cellular juxtaposition between these two species was essential for the *Veillonella* to grow within biofilms under conditions of salivary flow. Biofilm growth was expressed through the development of dual-species microcolonies or single-species microcolonies of *S. gordonii* V288. No micro-colonies of significant biomass developed that contained only *Veillonella* sp. PK1910. Such a result demonstrated that a short distance between the two species was required for metabolic interactions (in this case, the use of lactate by *Veillonella* sp.). In this study, potential benefits of cell–cell juxtaposition were identified for *S. gordonii*. Specifically, *Veillonella* sp. PK1910 promoted the upregulation of an alpha-amylase gene in *S. gordonii* V288 (as inferred by the differential expression of a GFP reporter). The authors speculated that juxtaposition not only allowed metabolic exchange but also facilitated cell–cell signaling: *Veillonella* sp. PK1910 was producing a signal molecule that was detected by *S. gordonii* DL1, resulting in the upregulation of alpha-amylase. This signal may be maltose or maltooligosaccharides that are produced by *Veillonella* spp. (Johnson et al. 2009).

Coaggregation-influenced predation and killing

Conceivably, the ability to coaggregate could aid one bacterial species to kill/inactivate another or to protect itself and others from predation. Indeed, at the CoL, a role for coaggregation in preventing microorganisms from being consumed by protozoa has recently been demonstrated by comparing the susceptibility of coaggregates of *Sphingomonas natatoria* 2.1 and *Micrococcus luteus* 2.13 against non-coaggregating suspensions of *S. natatoria* 2.8 and *M. luteus* 2.13. Under the selective pressure of grazing by a protozoan bacterivorous flagellate *Ochromonas* sp., *S. natatoria* 2.1 cells coaggregating with *M. luteus* 2.13 were able to outcompete non-coaggregating *S. natatoria* 2.8 (Thomas et al. 2011). This was likely due to the protective effects afforded by living within coaggregates (Thomas et al. 2011).

Often, coaggregation has been considered to facilitate synergistic interactions (Rickard et al. 2003; Kolenbrander et al. 2006). However, it is equally possible that coaggregation can serve as a method for one bacterium to prey upon another, and thus confer benefits for one species (but not the other coaggregating partner) at the CeL. Such a possibility was investigated by Reid et al. (1988), whose research showed that coaggregating lactobacilli produce inhibitory substances against uropathogenic *E. coli*. Furthermore, studies by Vornhagen et al. (2013) identified a coaggregating *Lysobacter gummosus*. *Lysobacter* species are well known to have highly specific and potent

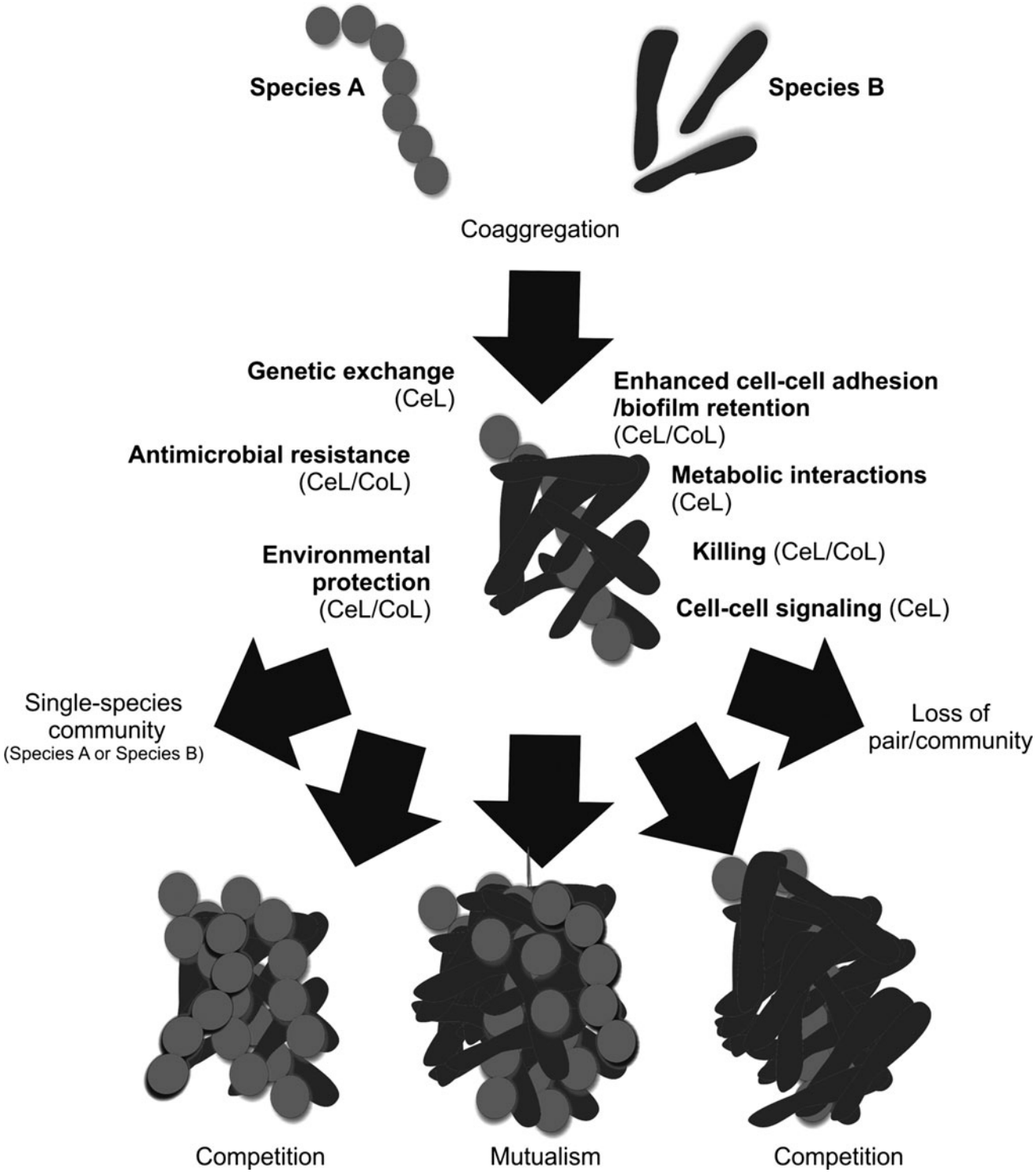

Figure 5. Diagram showing possible coaggregation-mediated interactions between a pair of coaggregating bacteria (black and gray cells). Coaggregation between different species facilitates interactions that can confer benefits at the cellular level (CeL) and the coaggregate level (CoL). Coaggregation-mediated effects at the CeL and CoL would be displayed as changes in spatiotemporal biofilm development. Image modified from Rickard et al. (2013) with permission.

anti-bacterial and anti-fungal activities (Xie et al. 2012; Pidot et al. 2014). Thus, it is conceivable that the specificity of coaggregation can act as a targeting mechanism through which a species can predate on susceptible microorganisms. From a biotechnological standpoint, the manipulation of such processes has promise as it offers an

approach by which problematic species can be controlled without the use of harsh chemicals that will affect other microorganisms and the broader environment.

Cell–cell signaling

The term sociomicrobiology was introduced by Parsek and Greenberg (2005) and refers to 'investigations of any group-behaviors of microbes.' Thus far, the main area of focus in this field has been on cell–cell signaling between bacteria (also referred to as quorum sensing) and how it mediates group behavior. However, such cell–cell signaling can only occur if bacteria are in close proximity to one another or are producing enough signal molecules to counteract loss through diffusion over larger cell–cell distances (Figure 5). Only relatively recently has coaggregation been proposed to enhance cell–cell signaling by bacteria through reducing cell–cell distance between specific species (Kolenbrander et al. 2010). Indeed, when considering a role for coaggregation and cell–cell signaling, work by Rickard et al. (2006) and Cuadra-Saenz et al. (2012) focused on a family of cell–cell signaling molecules that are produced by coaggregating pioneer colonizing dental plaque bacteria. The signal molecule that is produced by these bacteria is a collection of inter-convertible forms that are collectively called autoinducer-2 (AI-2). When certain coaggregating AI-2 producing species are in close proximity, evidence suggests that AI-2 cell–cell signaling mediates mutualism (Rickard et al. 2006) or competition (Cuadra-Saenz et al. 2012). However, the contribution of AI-2 was focused on at the expense of coaggregation: the effect of introducing coaggregation deficient mutants that are able to perform AI-2 signaling was not investigated. Similar to metabolic interactions, it is conceivable that the ability of coaggregation to reduce the distance between cells will enhance the detection of AI-2, which is especially useful when the cells are only able to produce low nanomolar quantities of AI-2 (Rickard et al. 2006; Kolenbrander et al. 2010).

Environmental protection, antimicrobial resistance, and genetic exchange

It is well known that bacteria can protect each other from potentially adverse events, especially when the cells are located within a biofilm (Mah & O'Toole 2001; Gilbert et al. 2002; Hall-Stoodley et al. 2004). Two notable examples include the protection of one species by another from oxygen and cross-species protection from antimicrobials. Protection can be afforded at the CeL and CoL and can be classified as tolerance or resistance. At the CeL, cells contained within a coaggregate can conceivably become more resistant to antimicrobials due to: altered growth rate as a consequence of nutrient depletion (Evans et al. 1990; Roberts & Stewart 2004), the localized increase in cell–cell signaling molecules which can enhance antimicrobial/acid tolerance (Li et al. 2001; Ahmed et al. 2007), and phenomena such as contact-dependent gene expression (Park & Lamont 1998; Aoki et al. 2009). Similarly, as demonstrated in oral biofilm communities, one species could protect another from potentially deleterious environmental effects such as the presence of oxygen. As described earlier, bacterial corncobs are microscopically conspicuous coaggregates containing *F. nucleatum* at the core surrounded by a number of other oral species (Lancy et al. 1983; Figure 1). Using a model biofilm system, Bradshaw et al. (1998) showed that coaggregation-mediated interactions between *F. nucleatum* and other oral species promote the survival and growth of obligate anaerobes under aerobic conditions. This is likely created by a bacterial shroud-like effect, whereby the species on the outer extremities of the corncobs use and remove molecular oxygen and protect the obligate anaerobe *F. nucleatum* at the core of the structure. It is not clear, however, if any benefit at the CeL is conferred by *F. nucleatum* to the shrouding organisms. It is interesting to note, however, that this shroud also enhances the integration of *F. nucleatum* into pre-formed oral biofilm communities, a phenomenon that is likely related to evasion of contact-dependent expression of hydrogen peroxide by other oral species (He et al. 2012). As opposed to these coaggregation-induced changes at the CeL, coaggregation-mediated interactions at the CoL relate primarily to the generation of physiological gradients throughout the coaggregated community. Much akin to biofilms, these will be expressed as changes in antimicrobial tolerance by virtue of reaction-diffusion limitation and gross changes in growth rates. While yet to be explored, it would be fascinating to examine the impact of coaggregate size and spatial organization of cells in coaggregates to discern the relative contribution of CoL and CeL to the resilience of the coaggregating cells to adverse environmental conditions such as antimicrobial challenges or oxygen deprivation/accumulation.

A topic on the minds of many microbiologists and health practitioners is the development and spread of antimicrobial resistance. With the expanded use of antimicrobials in medical, industrial, agricultural, domestic, and environmental settings, there is increased selective pressure for microorganisms to adapt through genetic exchange (Bloomfield 2002; French 2010; Davis et al. 2011). Coaggregation likely plays a role in facilitating the development and spread of antimicrobial resistance by promoting juxtaposition and thus facilitating DNA exchange within coaggregates. Specifically, juxtaposition would promote DNA exchange through transduction, conjugation, and enhanced transformation efficiency *via* elevated localized concentrations of extracellular DNA or

enhanced cell–cell signaling (Figure 5) mediated by higher levels of competence signaling peptides (Petersen et al. 2004). Inter-species DNA exchange within biofilms has been demonstrated to occur between the oral bacterium *Veillonella dispar* and four *Streptococcus* species within a laboratory-based multi-species oral biofilm model (Hannan et al. 2010). The findings suggested that this was through conjugation and transformation (Hannan et al. 2010), but the role of coaggregation has not been evaluated. It is well documented that streptococci and *Veillonella* coaggregate (Foster & Kolenbrander 2004) and it would be interesting to explore the contribution of coaggregation in DNA exchange between coaggregating and isogenic non-coaggregating mutants of these species.

Ultimately, a focused effort to examine the role of coaggregation in protecting species from transient environmental perturbations (such as an antimicrobial treatment) *via* reaction-diffusion limitation effects, changes in growth, and long-term genome modifications *via* DNA exchange has yet to be explored. From a standpoint of biofilm control, inhibiting coaggregation may well reduce tolerance to short-term perturbations and also retard the acquisition of antimicrobial resistance within biofilms. Given that coaggregation involves highly specific pair-wise interactions, it may be possible to target specific pairs in biofilms (for example, pathogens coaggregating with commensal species).

Future directions and concluding remarks

Over the last 15 years, coaggregation has become recognized to be important for the successional development of environmental, medical, and dental biofilms. While the precise nature and contribution of coaggregation to biofilm development is still being evaluated, it is clear that an ability to control coaggregation has great potential in preventing or controlling the rate of biofilm development or altering the species composition of biofilms. As a case in point, mechanistic, ecological, and epidemiological studies show that coaggregation is likely important in succession and progression towards disease in dental plaque biofilms. This can be seen when examining the findings of Socransky et al. (1998), Teles et al. (2012) and Ximenez-Fyvie et al. (2000) who used epidemiological and ecological data to classify oral species into health-associated groups (described as a yellow complex), transitional from health to periodontal disease groups (described as purple, green, and blue complexes), and groups increasingly associated with periodontal disease (orange and red complexes) (Figure 6A). Taking these data and applying the complex colors to the coaggregation maps constructed by Kolenbrander et al. (2002) and colleagues (Figure 6B), a similar arrangement of colors can be seen. In particular, early colonizers are predominantly associated with health while the later colonizers are associated with disease. Following the co-aggregation interactions mediated by adhesins and complementary receptors from the acquired pellicle (conditioning film), there are multiple routes to proceed from the primary colonizing yellow-colored streptococci to the periodontal pathogens displayed as orange and red colored cell types. Thus, mapping coaggregation interactions in biofilms may shed light on the response of communities to certain pair-specific coaggregation inhibitors and alternative outcomes with respect to the rate of biofilm development and community membership.

From a perspective of crafting communities by manipulating coaggregation interactions, it is becoming apparent that coaggregation may be used to enhance cell–cell interactions so that biofilm communities can be augmented to perform specific tasks. One example involving coaggregation-based augmentation of biofilms centers on approaches to enhance environmental bioremediation. Adav et al. (2008) demonstrated that coaggregation can increase phenol degradation by pairing *Acinetobacter calcoaceticus* I6 with *Bacillus thuringiensis* I2 and *A. calcoaceticus* I6 with *Candida tropicalis* I9 in co-culture as opposed to individual inoculations. Similarly, in medically relevant scenarios involving the human urogenital tract, increasing evidence suggests that coaggregating lactobacilli can be used to treat bacterial and fungal infections (Saling & Schreiber 2005; McMillan et al. 2011; Verdenelli et al. 2014). Thus, there is an increasing recognition by the scientific community that coaggregation interactions can be manipulated to alter microbial communities to a favorable state.

When considering approaches to target specific coaggregation interactions within a biofilm, work focused on bacterial arginine deiminase (*arcA*) has shown that *Streptococcus cristatus* downregulates *fimA* gene expression in *P. gingivalis* by upregulating ArcA expression (Xie et al. 2000, 2007). Reduced expression of *fimA* results in the abrogation of *P. gingivalis* fimbriae expression that is required for coaggregation. Approaches to upregulate ArcA expression may thus be explored as a mechanism for reducing coaggregation between species in the oral cavity. More broadly, the protein lectins and polysaccharide receptors that facilitate coaggregation may themselves become targets for inhibition. This could conceivably require the use of enzyme technology or simple sugars.

In conclusion, studies of coaggregation are still in their infancy due to technological insufficiencies and, arguably, limited knowledge exchanged between different research fields. With recent advances in multidisciplinary approaches to study biofilms and a resurgent interest in the role of multi-species communities in health, disease, and broad biofilm homeostasis, many functional and ecological studies of biofilms that focus on the role of coaggregation are now commencing. With the burgeoning

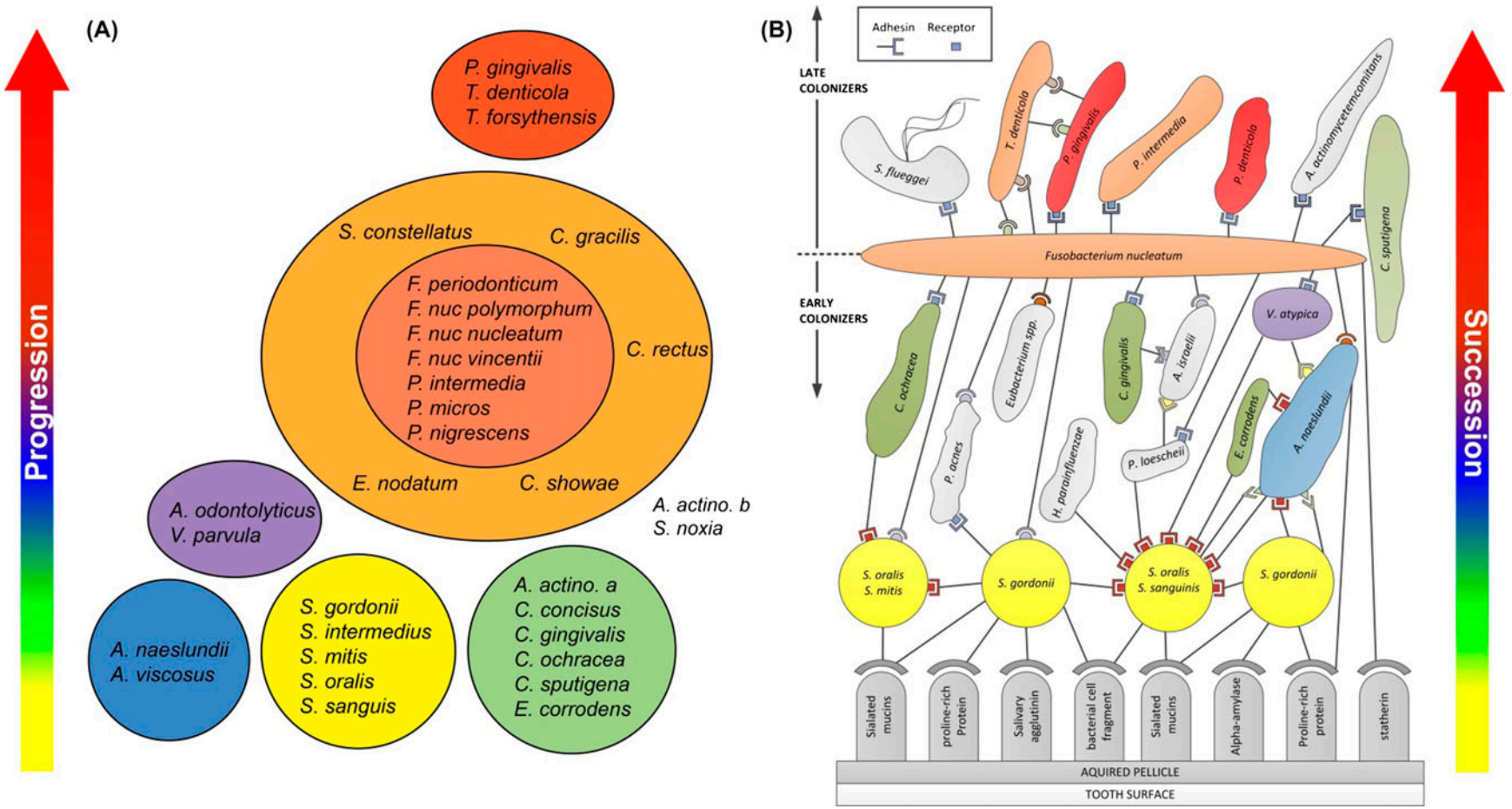

Figure 6. A proposed role of coaggregation in oral biofilm succession and progression from health to periodontal disease. Diagram shows the relationships of dental plaque biofilm species within and between 'microbial complexes' (A), and their coaggregation patterns (based upon visual assays) in relation to health and disease (B). Complexes are color coded according to their presence in dental plaque biofilms isolated from healthy individuals or from those displaying periodontal disease. Red complex bacteria are associated with severe forms of periodontal disease and were rarely isolated in the absence of members of the orange complex. Conversely, yellow, blue, green and purple complexes were often isolated from dental plaque biofilms that were not causing periodontal disease. *Aggregatibacter actinomycetemcomitans* serotype b (*A. acino. b*) and *Selenomonas noxia* (*S. noxia*) represent outlier species not assigned to a complex. Figure 6A is modified from Socransky et al. (1998) with permission and Figure 6B is modified from Kolenbrander et al. (2002) and Rickard et al. (2008) with permission. Note that Figure 6B is not intended to be comprehensive; other different coaggregation partnerships have been indicated using a variety of other laboratory techniques.

issues associated with the over-use and abuse of antimicrobials, especially when trying to treat highly recalcitrant multi-species biofilm communities, novel approaches to prevent biofilm development or destabilize mature communities have become a major research focus. Acknowledging that coaggregation plays an integral role in the development of many environmentally distinct multi-species biofilms is the first step in a research direction that may yield new technologies to treat such communities or prevent them from being problematic.

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