

## *Streptococcus mutans*: a new Gram-positive paradigm?

José A. Lemos, Robert G. Quivey, Jr, Hyun Koo and Jacqueline Abranches

### Correspondence

José A. Lemos

jose\_lemos@urmc.rochester.edu

Center for Oral Biology and Department of Microbiology and Immunology, University of Rochester Medical Center, Rochester, NY 14642, USA

Despite the enormous contributions of the bacterial paradigms *Escherichia coli* and *Bacillus subtilis* to basic and applied research, it is well known that no single organism can be a perfect representative of all other species. However, given that some bacteria are difficult, or virtually impossible, to cultivate in the laboratory, that some are recalcitrant to genetic and molecular manipulation, and that others can be extremely dangerous to manipulate, the use of model organisms will continue to play an important role in the development of basic research. In particular, model organisms are very useful for providing a better understanding of the biology of closely related species. Here, we discuss how the lifestyle, the availability of suitable *in vitro* and *in vivo* systems, and a thorough understanding of the genetics, biochemistry and physiology of the dental pathogen *Streptococcus mutans* have greatly advanced our understanding of important areas in the field of bacteriology such as interspecies biofilms, competence development and stress responses. In this article, we provide an argument that places *S. mutans*, an organism that evolved in close association with the human host, as a novel Gram-positive model organism.

Advances in the field of bacteriology have relied strongly on studies involving the bacterial paradigms *Escherichia coli* and *Bacillus subtilis*. In both cases, a long history of laboratory research, ease of cultivation and genetic manipulation encouraged and provided the rationale for the emergence of these bacteria as model organisms. *E. coli* is a Gram-negative, non-sporulating bacterium that can be found free-living, in water or soil, as well as associated with plants, insects, birds and mammals. It is the most studied prokaryotic organism and comprises a very heterogeneous group containing both pathogenic and non-pathogenic strains. In addition to serving as the Gram-negative model organism, laboratory strains of *E. coli* are extremely versatile and are the quintessential lab workhorses. *Bacillus subtilis* is a Gram-positive sporulating organism commonly found in soil, plants and, transiently, on the surface of animals. Strains of *B. subtilis* are not associated with humans and are not pathogenic, although some closely related *Bacillus* species such as *Bacillus anthracis* and *Bacillus cereus* are implicated in human disease (anthrax) and in food poisoning, respectively. Because the sporulation process occurs in simple well-defined stages, *B. subtilis* sporulation has served as a paradigm for bacterial development and differentiation studies. Like *E. coli*, *B. subtilis* is also easy to cultivate and highly amenable to genetic manipulation. The wealth of information derived from investigations of the biochemistry, physiology, genetics and developmental processes of *E. coli* and *B. subtilis* laid the foundation for, and at the same time provided guidance for, studies with other bacterial species. In addition to *E. coli* and *B. subtilis*, there are numerous other organisms that have served, in a

more limited scope, as bacterial models. For example, the environmental species *Caulobacter crescentus*, *Myxococcus xanthus* and *Streptomyces* spp. are important model organisms for studying cellular differentiation and developmental processes, and the dairy bacterium *Lactococcus lactis*, due to its relatively simple fermentative metabolism, is a widely accepted model for studying growth physiology and membrane transport of lactic acid bacteria.

In contrast to *E. coli* and *B. subtilis*, the dental pathogen *Streptococcus mutans* does not have a free-living lifestyle. The natural habitat of *S. mutans* is the human mouth, more specifically dental plaque, where the bacterium resides in multispecies biofilms that form on the surfaces of teeth. While a normal inhabitant of the oral cavity, *S. mutans* is mostly known for its importance in the aetiology of dental caries and occasional association with subacute infective endocarditis. Decades of research have conclusively demonstrated that *S. mutans* is a major cariogenic organism by virtue of its contribution to the formation of the dental biofilm matrix, its capacity to produce large quantities of organic acids, and its ability to outcompete non-cariogenic commensal species at low pH conditions (Banas & Vickerman, 2003; Bowen & Koo, 2011; Gross *et al.*, 2012; Lemos *et al.*, 2005; Quivey *et al.*, 2001).

The oral cavity is a dynamic environment that undergoes large and rapid fluctuations in pH, nutrient availability and source, oxygen tension, temperature and osmolality (Lemos *et al.*, 2005). Although the intermittent ingestion of food by the human host has been suggested to expose bacteria in oral biofilms to a 'feast or famine' lifestyle

(Carlsson, 1983), the 'famine' cycle of the oral environment cannot be fully compared to the severely oligotrophic conditions encountered by bacteria with a free-living lifestyle. As a result of its obligate host-associated lifestyle, studies with *S. mutans* have identified important differences in the mechanisms by which this organism copes with fluctuations in pH, oxygen tension and carbohydrate availability. Rather than providing a comprehensive overview of the research on *S. mutans*, which is the subject of recent reviews (Bowen & Koo, 2011; Lemos & Burne, 2008; Matsui & Cvitkovitch, 2010; Nicolas & Lavoie, 2011; Smith & Spatafora, 2012), we will focus on contributions that illustrate how studies with *S. mutans* have greatly advanced our understanding of key areas in the field of microbiology (Fig. 1). Specifically, we will highlight studies that have changed existing bacterial dogmas, or that have broadly enhanced our knowledge of the biology of prokaryotes, particularly with regard to low-GC Gram-positive bacteria. The advantages of using the oral cavity for biofilm-related studies and to explore bacterial interactions will also be discussed. The goal of this article is to portray *S. mutans*, a genetically amenable and relatively safe organism, as a new Gram-positive model organism.

### History of *S. mutans* research

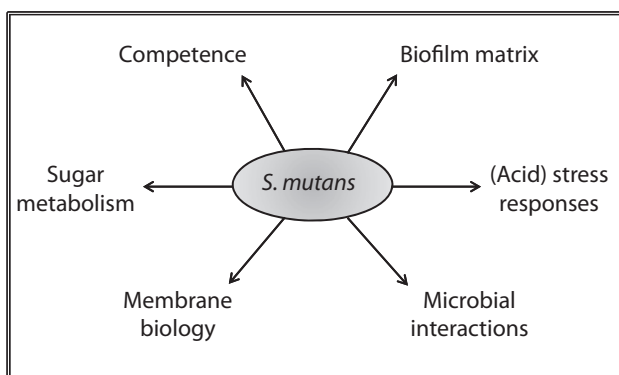
In 1924, J. Clarke isolated an organism from carious lesions and called it *S. mutans*, because he thought the oval-shaped cells observed were mutant forms of streptococci (Clarke, 1924). However, it was only in the late 1950s that *S. mutans* received greater attention from the scientific community and, by the mid 1960s, it was recognized as a major aetiological agent in dental caries (Loesche, 1986). In the subsequent two decades, researchers began to uncover the pathophysiology of *S. mutans*. During this period, the first tools for studying *S. mutans*, both *in vitro* and *in vivo*, were developed. As a result of the efforts of these pioneer researchers, the major virulence traits of *S. mutans* were established: (i) the ability to produce large quantities of organic acids (acidogenicity) from metabolized carbohydrates; (ii) the ability to survive at low

pH (aciduricity); and (iii) the ability to synthesize extracellular glucan-homopolymers from sucrose, which play a critical role in initial attachment, colonization and accumulation of biofilms on tooth surfaces (Banas & Vickerman, 2003; Bowen & Koo, 2011; Burne, 1998; Loesche, 1986). With the advances in molecular genetic techniques in the 1980s and 90s, scientists began to more rapidly understand how metabolic pathways enabled *S. mutans* to evolve into a specialized dental pathogen. Finally, with the release of the first complete *S. mutans* genome in 2002 (Ajdić *et al.*, 2002), the scientific community took full advantage of the technologies that emerged in the genomic era, applying functional genomic, transcriptomic and proteomic approaches to better dissect the physiology, genetics and virulence mechanisms of *S. mutans*.

### *S. mutans* as a model organism of pathogenic Gram-positive bacteria

Contrary to *B. subtilis*, which does not infect humans and has a free-living lifestyle, *S. mutans* is an obligate human pathogen. Thus, the knowledge gained from studies with *S. mutans* may be highly relevant to other pathogenic non-sporulating Gram-positive bacteria. With a relatively small genome of about 2 Mb with over 1900 genes (Ajdić *et al.*, 2002), the *S. mutans* UA159 genome is much closer in size to those of closely related streptococcal and staphylococcal species (ranging from about 1.8 Mb in *Streptococcus pyogenes* to about 2.8 Mb in *Staphylococcus aureus*) (Table 1). More recently, next-generation sequence technology was used to obtain genome sequences of 57 clinical isolates of *S. mutans* (Cornejo *et al.*, 2012). The *S. mutans* core genome was estimated to contain close to 1500 genes, with each genome containing, on average, 1636 genes. With a genome about half the size of *E. coli* (~4.7 Mb) or *B. subtilis* (~4.2 Mb) and with a GC content less than 40 %, it is, therefore, not surprising that a greater overlap in the mechanisms of gene regulation and metabolic pathways occurs between *S. mutans* and related Gram-positive pathogens, which also have compact genomes, low GC content and, often, a host-associated lifestyle.

Like *E. coli* and *B. subtilis*, *S. mutans* is highly amenable to genetic manipulation, is naturally competent, and is relatively safe to manipulate in the laboratory setting. Today, a large number of fast and reliable genetic tools are available to study the biology of *S. mutans*, including PCR-ligation mutagenesis (Lau *et al.*, 2002), markerless in-frame deletion systems (Atlagic *et al.*, 2006; Banerjee & Biswas, 2008; Merritt *et al.*, 2007), gene knockdown strategies (Lemos *et al.*, 2007b; Wang & Kuramitsu, 2005), integration vectors for genetic complementation or expression of foreign genes (Jung *et al.*, 2009; Wen & Burne, 2001; Zeng & Burne, 2009), and a variety of *E. coli*-streptococcal shuttle vectors and reporter systems for gene expression analysis. In addition to sophisticated genetic tools, there are also many simple and efficient *in vitro* models to study *S. mutans* biofilms, which include batch and continuous-feeding models, as well as flow systems attempting to



**Fig. 1.** Areas in the microbiology field that strongly benefited from studies conducted with *S. mutans*.

**Table 1.** Average genome size, approximate number of genes, and GC content of the bacterial paradigms *E. coli* and *B. subtilis* and selected pathogenic Gram-positive bacteria

Species	Approximate genome size (Mb)	Approximate no. of genes	Approximate GC content	Lifestyle
<i>E. coli</i> *	4.7*	4500	50 %	Free-living and host-associated
<i>B. subtilis</i>	4.2	4100	43 %	Free-living
<i>S. mutans</i>	1.6	1950	37 %	Host-associated
<i>S. pyogenes</i>	1.8	1800	39 %	Host-associated
<i>S. agalactiae</i>	2.2	2200	35 %	Host-associated
<i>S. pneumoniae</i>	2.1	2100	40 %	Host-associated
<i>Staphylococcus aureus</i>	2.8	2850	33 %	Host-associated
<i>Listeria monocytogenes</i>	2.9	2800	38 %	Free-living and host-associated

\*Sequenced genomes range in size from 4.6 to 6.2 Mb.

mimic salivary flow (Lemos *et al.*, 2010). Notably, most of these systems are suitable for the development of more complex multispecies biofilms (Bradshaw & Marsh, 1999; McDermid *et al.*, 1986; Shu *et al.*, 2003). These model systems can be easily adapted for studies with non-oral and non-streptococcal species. Some of the most recent studies using *S. mutans* biofilm models are briefly discussed below.

Despite the advantages of having powerful analytical tools to study the genetics and biology of pathogenic bacteria, the translation of *in vitro* findings to animal models is critical for a complete understanding of bacterial pathogenesis. In this regard, there are well-established *in vivo* models for *S. mutans*, including rat (Burne *et al.*, 1996; Koo *et al.*, 2005; Yamashita *et al.*, 1993) and transgenic mouse caries models (Burne *et al.*, 1996; Catalán *et al.*, 2011; Culp *et al.*, 2005; Yamashita *et al.*, 1993), as well as rabbit and mouse models of infective endocarditis (Bahn *et al.*, 1978; Paik *et al.*, 2003). In fact, it was the pioneering work conducted in the 1950s and 60s using hamsters and gnotobiotic rats that established *S. mutans* as the principal aetiological agent of dental caries (Fitzgerald & Keyes, 1960; Orland *et al.*, 1955; Zinner *et al.*, 1965). More recently, several studies have demonstrated the usefulness of the infection of the larvae of *Galleria mellonella* to study the pathogenic potential of *S. mutans* strains in systemic infections (Abranches *et al.*, 2011; Gonzalez *et al.*, 2012; Kajfasz *et al.*, 2010). Despite its obvious limitations, this increasingly popular model may be viewed as a powerful and inexpensive screening tool for studying bacterial fitness and virulence, in particular for traits that are associated with resistance to host-derived stress factors during systemic diseases.

### The oral biofilm: a battlefield of microbial interactions

Microbial biofilm communities are found on mucosal surfaces, including the gut and in the vaginal, oral and nasal cavities. It is widely recognized that the different interactions, synergistic or antagonistic, between members of these complex communities can shift the bacterial population from health to disease. This is particularly true

for the oral cavity, where *S. mutans*, among other less well-characterized lactic acid-producing bacteria such as lactobacilli and bifidobacteria, tends to dominate the oral flora as caries progresses (Becker *et al.*, 2002; Gross *et al.*, 2010; Tanner *et al.*, 2011a, b). As proposed by Marsh (1994) in the ecological plaque hypothesis, dental caries is a disease that results from a shift in the proportions of the resident flora as a consequence of changes in the environment (e.g. high sugar concentrations/low pH).

The easy access to oral samples from human volunteers and the ability to identify the major contributors in health and disease allowed researchers to probe some key bacterial interactions that disturb the homeostasis of oral biofilms. For example, *Streptococcus gordonii* and *Streptococcus sanguinis*, early colonizers of the tooth surface normally associated with health, were shown to antagonize the growth of *S. mutans* by producing H<sub>2</sub>O<sub>2</sub> (Kreth *et al.*, 2008). Likewise, *Streptococcus oligofermentans*, whose abundance appears to be inversely related to that of *S. mutans* and the presence of carious lesions, was shown to produce inhibitory quantities of H<sub>2</sub>O<sub>2</sub> using lactic acid as a substrate (Tong *et al.*, 2007). In this particular case, *S. oligofermentans* gains a competitive advantage over *S. mutans* by directly inhibiting its growth (via H<sub>2</sub>O<sub>2</sub> production) and, at the same time, by facilitating the survival of an acid-sensitive flora. Conversely, *S. mutans* can produce bacteriocins (known as mutacins) that are inhibitory against early colonizers (Merritt & Qi, 2012). By analysing the transcriptome of *S. mutans* in mixed-species cultures containing *S. mutans*, *S. gordonii* and the early colonizer *Veillonella parvula*, Liu and colleagues suggested that part of the deleterious effects of the H<sub>2</sub>O<sub>2</sub> producer *S. gordonii* against *S. mutans* was mitigated by the presence of *V. parvula* (Liu *et al.*, 2011). Many other studies, which were not restricted to *S. mutans*, have shown how microbial cell-cell interactions influence the oral microbiota composition and homeostasis (Kuramitsu *et al.*, 2007; Whitmore & Lamont, 2011). Therefore, due to the relatively well-defined microbial composition of healthy and diseased sites and easy access for sampling, the oral biofilm microflora

have served, and will likely continue to serve, as an excellent model system for studying bacterial interactions.

### **S. *mutans* as a model biofilm organism**

It is well established that *S. mutans* has a biofilm-dependent lifestyle (Bowen & Koo, 2011; Burne, 1998; Marsh, 1994). In the oral cavity, a highly diverse microbial community is constantly interacting with a proteinaceous film (containing salivary proteins and bacterial exoproteins) present on the tooth surface, known as the pellicle. Specific groups of organisms (mostly streptococci and *Actinomyces* spp.) can adhere to the pellicle in low numbers, and subsequently these organisms co-adhere with other oral microbial species (Nobbs *et al.*, 2009). *S. mutans* expresses adhesin-like surface-associated proteins (e.g. AgI/II family) that are capable of binding to receptors in the pellicle (Gibbons, 1989). Recently, the adhesin P1 (AgI/II) was shown to be an amyloid-forming protein that contributes to biofilm development by *S. mutans* (Oli *et al.*, 2012). *S. mutans* also interacts with specific salivary proteins, such as common salivary protein-1 (CSP-1), which, in turn, helps the bacterium to bind saliva-coated apatitic surfaces (Ambatipudi *et al.*, 2010). Thus, *S. mutans* can be present in this initial colonizing community on the tooth pellicle, albeit not as one of the most abundant organisms. However, when dietary sugars, particularly sucrose, become available, *S. mutans* can become the numerically dominant organism, thereby orchestrating the formation of cariogenic biofilms.

*S. mutans*-derived extracellular glucosyltransferases (Gtfs) are constituents of the pellicle and are capable of synthesizing glucans *in situ* from sucrose (Schilling & Bowen, 1992). The surface-formed glucans provide additional bacterial binding sites that favour local colonization by *S. mutans*, as it binds avidly and in large numbers to these polymers (Schilling & Bowen, 1992) through several envelope-associated glucan-binding proteins (Banas & Vickerman, 2003). Furthermore, *in situ*-formed glucans can mask host-derived bacterial binding sites in the pellicle (Schilling & Bowen, 1992), which may negatively impact adherence of other commensal species that bind more favourably to the pellicle, such as *Streptococcus oralis*, *S. sanguinis* and *S. gordonii* (Jenkinson, 2011; Nobbs *et al.*, 2009). Gtfs also bind to many oral microbes, even those that do not naturally express Gtfs, thereby converting them into *de facto* glucan producers (Gregoire *et al.*, 2011; McCabe & Donkersloot, 1977; Vacca-Smith & Bowen, 1998). The exopolysaccharides (EPS), mostly in the form of glucans, formed on the pellicle and on microbial surfaces promote local accumulation of microbial cells while forming a diffusion-limiting polymeric matrix that protects embedded bacteria (Koo *et al.*, 2010; Xiao *et al.*, 2012). In parallel, sucrose and other sugars are rapidly fermented by *S. mutans* within the EPS-rich matrix, creating highly acidic environments.

As discussed above, complex interspecies competition and synergism take place between *S. mutans* and other species during the process of biofilm development. The low-pH

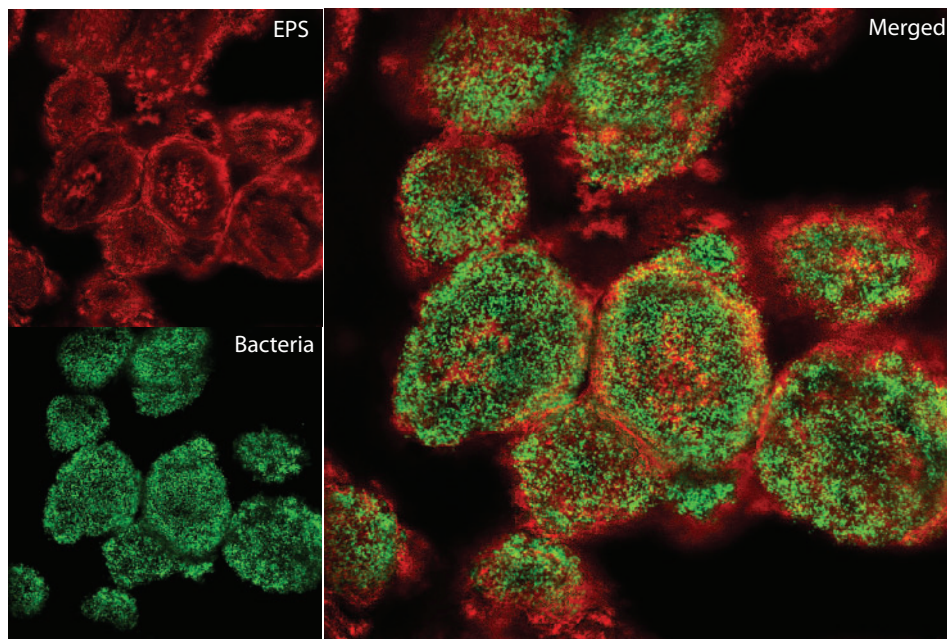
environment further induces EPS production by *S. mutans* (Li & Burne, 2001), and under these conditions the organism prospers, leading to biofilm accretion and further acidification. As the environmental stress increases (Lemos & Burne, 2008), the microbial diversity is dramatically reduced in favour of a highly aciduric and acidogenic flora (Gross *et al.*, 2012, 2010; Palmer, 2010).

Recently, attention has been devoted to understanding how *S. mutans* takes advantage of these environmental cues to optimize its metabolism in the presence of other oral bacteria within a dynamically changing biofilm milieu. A combination of *in situ* EPS imaging and 3D pH mapping approaches with high-throughput quantitative proteomics (MudPIT) has been used to dissect the mechanisms by which *S. mutans* assembles the matrix and survives within intact mixed-species biofilms. The sequential assembly of a structurally complex and spatially heterogeneous 3D matrix helps to create a highly compartmentalized architecture within intact biofilms (Fig. 2). In turn, highly localized and structured acidic microniches are dynamically formed throughout intact biofilms. The spatiotemporal assembly of a 3D biofilm matrix and the regulation of metabolic and stress survival pathways by *S. mutans* are highly intertwined, and also are dependent on the presence of other oral species (Klein *et al.*, 2012; Xiao *et al.*, 2012). More specifically, co-culture with the early oral colonizers *S. oralis* and *Actinomyces naeslundii* induces the expression of proteins associated with EPS synthesis (GtfB and GtfC) and remodelling (DexA), of the glucan-binding GbpB protein and of acid stress-related proteins such as AtpD, FabM and GroES (see below for more details) (Xiao *et al.*, 2012). While some of these interactions are unique to the oral environment, the knowledge generated from these studies has relevance beyond the mouth, as extracellular matrix and mixed-species communities are common features of many biofilms found in the body and in the environment.

Clearly, *S. mutans* represents a model that may be useful to further elucidate matrix assembly principles, responses to environmental cues and polymicrobial interactions. These studies can advance current understanding about how bacterial interactions with the matrix environment modulate the dynamics of biofilm development and how these interactions govern the shifts in microbial composition and creation of biological niches within mixed-species biofilms. Furthermore, the novel approaches to study *S. mutans* biofilms may have broader applicability. These models could be adapted for studies of other biofilm systems in which polymeric matrices are actively formed during biofilm development and varied microenvironments are created, such as those associated with cystic fibrosis and medical device-related infections.

### **S. *mutans* is the bacterial paradigm of lactic acid bacteria (LAB) stress responses**

When compared to other species of LAB, *S. mutans* is, by far, the best-studied organism in terms of the genetics and



**Fig. 2.** EPS matrix (red) creates compartmentalized bacterial (green) islets and heterogeneous microenvironments in oral biofilms. Bacterial islets enmeshed and surrounded by an intricate EPS matrix (as seen in the cross-sectional image) were identified across the 3D architecture of a mature mixed-species oral biofilm grown in sucrose. The assembly of a complex network of compartmentalized microenvironments created a range of acidic pockets and localized antimicrobial resistance within the greater biofilm architecture, which illustrates the spatial heterogeneities in the microstructure of biofilm.

physiology of stress responses. This is not entirely surprising, given that dental caries is directly linked to the ability of certain oral bacteria such as *S. mutans* to thrive under highly stressful conditions. Studies on *S. mutans* stress responses have served to reveal critical differences in the way that host-associated bacteria cope with environmental stresses when compared with bacteria that have both a free-living and host-associated lifestyle. In many cases, the stress regulon was shown to control a broader set of biological functions, including energy metabolism, biofilm formation and genetic competence (Lemos *et al.*, 2005; Lemos & Burne, 2008; Matsui & Cvitkovitch, 2010). In the following two sections we will describe the key mechanisms of stress tolerance utilized by *S. mutans* and highlight studies with broader implications for bacterial stress responses that have served to challenge long-established dogmas.

### Key contributions linked to studies on acid stress responses by *S. mutans*

The ability of *S. mutans* to cause disease depends on its abilities to irreversibly bind to teeth via the formation of extracellular glucan polymers, and to survive in the acidic environment created by its own metabolism of the sugars present in the human diet. Of note, elegant studies from the Burne group on carbon metabolism and carbon catabolism repression revealed a highly complex, hierarchical and

efficient control of energy generation by *S. mutans*, and also indicated that carbon metabolism is intertwined with the expression of virulence-related traits (Abranches *et al.*, 2003, 2008; Zeng & Burne, 2008, 2010; Zeng *et al.*, 2006).

The pressure of life at low pH values has led to the evolution of interconnected stress response pathways that allow its persistence in the oral cavity. The acid stress response not only facilitates the survival of *S. mutans*, but also enables the organism to outcompete less aciduric species of oral bacteria. Results from experiments conducted with cultures of mixed oral bacterial species have shown that *S. mutans* dominates these microbial communities during low-pH growth, whereas less aciduric species, such as *S. sanguinis*, predominate during growth at neutral pH values (Bradshaw & Marsh, 1998; McDermid *et al.*, 1986). These observations ignited a search for the mechanisms that control acid stress responses in *S. mutans*, and also generated considerable interest in understanding the interrelationships of the oral microbial flora, which has led to recent metagenomic sequencing projects.

The association between the aciduricity of *S. mutans* and its ability to outcompete other oral bacteria in mixed cultures can be explained by how the organism maintains intracellular pH homeostasis. *S. mutans* produces elevated levels of a membrane-bound  $F_1F_0$ -ATPase during growth in acidic conditions (Belli & Marquis, 1991). Moreover, counter to the well-known function of the membrane-

bound  $F_1F_0$ -ATPase in the formation of ATP in *E. coli* and in mitochondria, the principal function of the *S. mutans* F-ATPase is to operate in reverse of the established paradigm, by pumping protons out of cells at the cost of ATP hydrolysis. This maintains the intracellular pH close to neutrality, which protects acid-sensitive enzymes in the glycolytic pathway (Bender *et al.*, 1986). It was also shown that *S. mutans* produces higher levels of F-ATPase, with greater enzymic turnover at low pH, than other oral bacteria, with the exception of the highly aciduric lactobacilli (Bender *et al.*, 1986; Sturr & Marquis, 1992). Thus, *S. mutans* not only produces prodigious amounts of organic acids, which act to inhibit the growth of acid-sensitive organisms and contribute to enamel demineralization, but also expresses comparatively high levels of F-ATPase to maintain intracellular pH homeostasis and preserve its own metabolic activity (Sutton & Marquis, 1987).

The observations concerning the acid-resistant F-ATPase prompted a subsequent investigation to determine whether changes in the membrane might contribute to F-ATPase activity. This study showed that membrane composition undergoes rapid remodelling during growth at low pH (Quivey *et al.*, 2000). In fact, there is a near reversal of the proportions of saturated and unsaturated membrane fatty acids, with unsaturated fatty acids occupying nearly 60% of the membrane composition during acidic growth (Fozo & Quivey, 2004; Quivey *et al.*, 2000). Around this time, the *fabM* gene, encoding an enzyme with *trans-2-cis-3*-decenoyl isomerase activity, was identified and characterized in *Streptococcus pneumoniae* (Marrakchi *et al.*, 2002) and shown to be responsible for the formation of unsaturated fatty acids. Parallel studies, conducted with *S. mutans*, showed that following mutation of the *fabM* gene, the organism could no longer produce unsaturated fatty acids (Fozo & Quivey, 2004). Moreover, loss of the unsaturated fatty acids resulted in an extremely acid-sensitive phenotype that could be rescued by the inclusion of long-chain, unsaturated fatty acids in the growth medium (Fozo & Quivey, 2004). In addition, inactivation of *fabM* resulted in a substantial reduction in the ability of *S. mutans* to cause caries in rats (Fozo *et al.*, 2007). This was a departure from the *E. coli* paradigm of fatty acid synthesis, in which unsaturated fatty acids arise by the combined action of two enzymes, FabA, a dehydratase that forms unsaturated fatty acids from  $C_{10}$ :hydroxy-fatty acid intermediates and FabB, the condensing enzyme that is capable of elongating unsaturated fatty acids (Marrakchi *et al.*, 2002).

### Shifting long-standing paradigms in protein translocation and the stringent response

The signal recognition particle (SRP) is conserved in all three domains of life and is involved in the translocation of membrane proteins (Keenan *et al.*, 2001). The bacterial SRP translocation pathway was previously studied in *E. coli* and *B. subtilis* and in both cases was found to be essential for cell viability (Keenan *et al.*, 2001; Phillips & Silhavy,

1992). However, proteins of the SRP pathways were unexpectedly shown to be dispensable in *S. mutans* (Hasona *et al.*, 2005). Further studies revealed that overlapping and compensatory pathways exist in *S. mutans* and allow the organism to adapt and maintain membrane function in the absence of the SRP (Funes *et al.*, 2009). Specifically, a gene duplication event enabled the specialization of a protein from the YidC/Oxa1/Alb3 family, involved in the insertion of proteins into membranes of bacteria, mitochondria and chloroplasts, to overlap functionally with the SRP (Funes *et al.*, 2009). Although the SRP mutants showed impaired growth under a variety of stress conditions, this finding suggests that protein biogenesis and translocation pathways in *S. mutans*, and likely in other closely related organisms, may have fundamentally important differences when compared to the more widely studied organisms.

Another investigation that challenged a long-standing bacterial dogma came during the initial characterization of the *S. mutans* stringent response. The stringent response is a highly conserved global stress response mechanism that coordinates broad metabolic alterations necessary for adaptation under nutrient-limiting conditions (Potrykus & Cashel, 2008). This response is mediated by the accumulation of two modified guanine nucleotides, pyrophosphorylated GDP or GTP, collectively known as (p)ppGpp. For several decades, a single bifunctional enzyme known as Rsh (or RelA) was considered to be the sole enzyme controlling (p)ppGpp production and degradation in Gram-positive bacteria. However, the serendipitous finding that the *S. mutans*  $\Delta$ relA mutant was still able to produce (p)ppGpp (Lemos *et al.*, 2004) led to the discovery of two small monofunctional (p)ppGpp-synthetase enzymes, named RelP and RelQ, in *S. mutans* (Lemos *et al.*, 2007a). BLAST search analysis indicated that at least one of these enzymes was present in virtually every Gram-positive organism (Lemos *et al.*, 2007a), and subsequent studies confirmed the functionality of these enzymes in other Gram-positive bacteria such as *B. subtilis*, *S. pneumoniae* and *Enterococcus faecalis* (Abranches *et al.*, 2009; Battesti & Bouveret, 2009; Nanamiya *et al.*, 2008). The identification of these small (p)ppGpp synthetase enzymes in Gram-positive bacteria reinvigorated the scientific interest in this fundamental stress response pathway. As a result, recent bioinformatic and genetic analysis indicated that single-domain (p)ppGpp-synthesizing and -hydrolysing enzymes can be found, alone or in combination, in a number of organisms, including archaea, bacteria, plants and animals (Atkinson *et al.*, 2011; Das *et al.*, 2009; Sun *et al.*, 2010).

### *S. mutans* competence: lessons for making genetic transformation possible for all streptococci?

Natural transformation (competence) is a phenomenon observed in many bacterial species and has been linked to

increased genetic diversity, increased survival, biofilm formation, bacteriocin production and general stress responses (Kreth *et al.*, 2005; Martin *et al.*, 2006; Perry *et al.*, 2009; Suntharalingam & Cvitkovitch, 2005). The molecular mechanisms underlying competence development in *S. pneumoniae* have been thoroughly dissected (Claverys & Havarstein, 2002). In this organism, as well as in all other competent streptococci, an alternative sigma factor (ComX) functions as a master regulator of competence. ComX is activated by the product of *comC*, a secreted competence-stimulating peptide (CSP), in a density-dependent (quorum-sensing) manner. Although *S. pneumoniae* laid the foundation for understanding competence in streptococci, work with *S. mutans* indicated that specific pathways associated with competence might vary among the different streptococci (Martin *et al.*, 2006; Suntharalingam & Cvitkovitch, 2005). Interestingly, genomic analysis revealed that a complete ComX regulatory circuit and a number of the late competence genes are present in streptococcal species deemed 'non-transformable', including members of the pyogenic group (e.g. *S. pyogenes* and *Streptococcus agalactiae*). This observation raises the possibility that all streptococci may, under appropriate conditions, be capable of developing competence. However, there was no evidence that these bacteria encode a ComC-like peptide. More recently, a novel double-tryptophan peptide known as sigX-inducing peptide (XIP, encoded by *comS*) and an Rgg-type regulator (designated *comR*) responsible for inducing *comX* expression were identified in members of the salivarius group (e.g. *Streptococcus salivarius*, *Streptococcus thermophilus*), which led to the development of protocols for *in vitro* transformation of *S. salivarius* and *S. thermophilus* (Fontaine *et al.*, 2010). Remarkably, 'non-transformable' members of the pyogenic and bovis groups of streptococci also possess a regulatory system homologous to ComR/ComS (Mashburn-Warren *et al.*, 2010).

In contrast to *S. pneumoniae*, which relies on CSP to stimulate competence, *S. mutans* appears to be unique, as it carries both CSP- and XIP-dependent pathways. Despite an overlap with competence-related gene expression, the CSP regulatory circuit was later found to be dispensable for *S. mutans* competence and was implicated in non-lantibiotic bacteriocin production (Ahn *et al.*, 2006; Kreth *et al.*, 2005; Mashburn-Warren *et al.*, 2010). Using a microfluidic, single-cell method, Son *et al.* (2012) provided novel clues on how *S. mutans* responds to extracellular signals to activate ComX. More specifically, the authors showed that in a chemically defined medium, exogenous CSP does not induce *comX*, whereas exogenous XIP uniformly activates *comX* expression. On the other hand, in complex medium, XIP does not induce *comX*, while CSP elicits a bimodal *comX* response from the population (Son *et al.*, 2012). These recent findings indicate that our understanding of the mechanisms associated with bacterial competence is far from complete. The ease of genetic manipulation and the presence of the ComR/ComS regulatory pathway place *S. mutans* in a

unique position to further advance our understanding of competence in streptococci and, possibly, help to overcome the barriers that have hampered *in vitro* genetic transformation of pyogenic streptococci and other streptococcal species.

## Concluding remarks

In this article, we have presented evidence that the development of novel technologies and the rapid advances in dissecting the genetics and physiology of *S. mutans* have resulted in the emergence of this bacterium as a new Gram-positive model organism. The availability of multiple model systems to investigate the role of specific mutations in the pathophysiology of *S. mutans*, and the ability to recover living bacteria from each of the established models, strengthen the case for the use of this organism as a comparative tool for other Gram-positive pathogens. Because of the enormous amount of knowledge gathered from *E. coli* and *B. subtilis*, their contributions to present and future discoveries will continue to inform the development of additional bacterial systems. However, with the annotation of hundreds of complete bacterial genomes, functional genomics and other 'omic' technologies, it is now much easier to study other bacterial species. Thus, the progress made in recent decades places *S. mutans* in an interesting position to further advance basic microbiology research, especially in low-GC Gram-positive organisms.

## Acknowledgements

We are grateful to Steven Gill and Roberta Faustoferri for critical reading of the manuscript. This work was supported in part by grants from the National Institute of Dental and Craniofacial Research [DE019783 (J.A.L.), DE017425 (R.G.Q.), DE010174 (R.G.Q.), DE16139 (H.K.) and DE18023 (H.K.)] and a grant from the American Heart Association [10GRNT4210049 (J.A.)].

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