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Genomic and phenotypic diversity of *Streptococcus mutans*

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ABSTRACT

Background: *Streptococcus mutans* (*S. mutans*) is a commensal microorganism found in the human oral cavity. However, due to environmental changes, selective pressures, and the presence of a variable genome, it adapts and may acquire new physiological and metabolic properties that alter dental biofilm homeostasis, promoting the development of dental caries. Although the plasticity and heterogeneity of *S. mutans* is widely recognized, very little is known about the mechanisms for the expression of pathogenic properties in specific genotypes.

Highlight: The implementation of molecular biology techniques in the study of *S. mutans* has provided information on the genomic diversity of this species. This variability is generated by genome rearrangements, natural genetic transformation, and horizontal gene transfer, and continues to grow due to an open pan-genome. The main virulence factors associated with the cariogenic potential of *S. mutans* include adhesion, acid production (acidogenicity), and acid tolerance (aciduricity), and also show variability. These factors coordinate the modification of the physicochemical properties of the biofilm, which results in the accumulation of *S. mutans* and other acidogenic and aciduric species in the oral cavity.

Conclusion: We review the current literature on the main processes that generate *S. mutans* genomic diversity, as well as the phenotypic variability of its main virulence factors. *S. mutans* achieves its pathogenesis by sensing the intra- and extracellular environments and regulating gene transcription according to perceived environmental modifications. Consequently, this regulation gives rise to differential synthesis of proteins, allowing this species to potentially express virulence factors.

Contents

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1. Introduction

Oral human microbiota are comprised of a great diversity of microorganisms that are part of a multispecies complex. The dynamic environment of the oral cavity allows for the selection of certain dental biofilm cells to not only adapt metabolically but also to develop its potential and genomic content [1]. Although microorganisms belonging to the same genus and species present a common gene set denoted *core-genome*, which is essential for cellular functioning and the survival of the species, they can differ in their physiological and virulent properties because of their *strain specific genes* [2–5]. Comparative genomic analysis between multiple genomes of individual species has revealed an extensive intra-species genomic diversity. The rest of the genome, referred to the accessory functions, is generally known as the *not essential or dispensable genome* and is not shared by all strains. Therefore, the disposable genome contributes to the diversity of the species and probably provides functions that are not essential for survival. It also confers selective advantages such as survival and adaptation to different ecological niches, antibiotic resistance, and the capacity to colonize new hosts [4]. This diversity is generated by a variety of processes which include genome rearrangements, horizontal gene transfer (HGT) by natural genetic transformation, and exposition to genetic mobile elements such as bacteriophages, plasmids, insertion elements (IS), transposons (Tn), and genomic islands (GI) [6,7]. Although the impact of *S. mutans* specific accessory genes on oral health maintenance, or in the development of pathological processes, is not yet completely clear, there is no doubt that they constitute an important reservoir of variability [4,8] which requires further investigation.

In order to broaden our understanding of the physiopathological behavior of *S. mutans*, this review aims to examine the mechanisms which explain its genomic variability, as well as its phenotypic variability, associated mainly with adhesion processes, acidogenicity, and aciduricity, which allow *S. mutans* to colonize the oral cavity, form biofilms, produce organic acids, and survive in acidic environments. Moreover, in addition to considering that specific genetic constitution for each strain can be associated with the ability of *S. mutans* to acquire and express pathogenic properties, we review how, under specific environmental conditions, gene regulation plays an important role in the control of the expression of these virulence factors (Fig. 1).

2. Genomic diversity

The implementation of diverse molecular biology techniques in the study of *S. mutans* has provided information on the genomic diversity of this species which appears to continue to grow due to an open pan-genome [9], as will be discussed in this section. Three main natural strategies can be distinguished in the generation of these genetic variations: (1) small local changes in the nucleotide sequence of the genome that explain, for example, the presence of four serotypes; (2) the intragenomic reorganization of segments of genomic sequences; and (3) the acquisition of DNA sequences from another organism.

2.1. S. mutans serotypes

A recent molecular systematic study based on the DNA sequences of the 16S rRNA of *rpoB, sodA, tuf, rnpB, gyrB, dnaJ*, and *recN* gave evidence to propose an alternative phylogeny for the genus *Streptococcus* [10] which differed from previous taxonomic

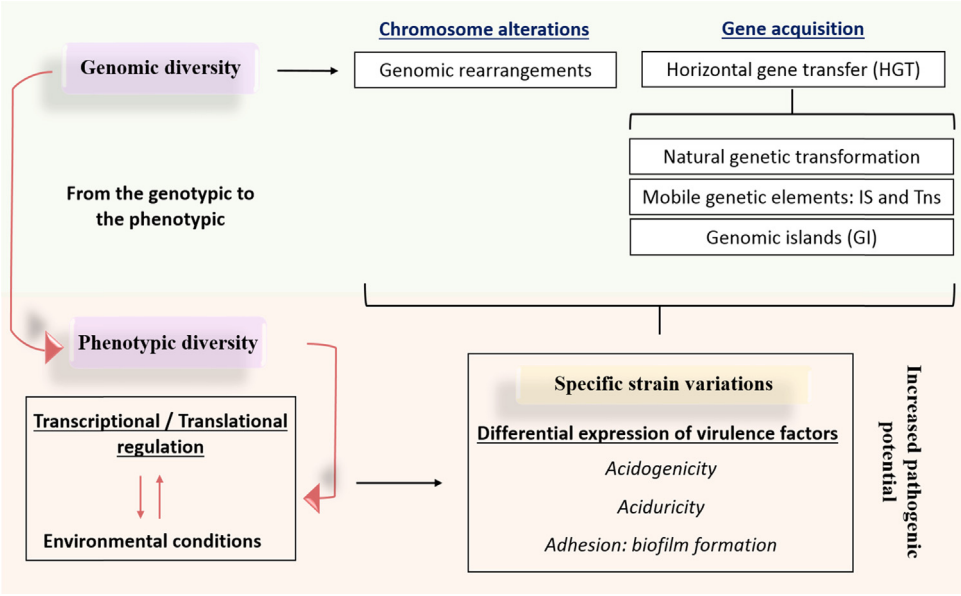

Fig. 1. Overview of the genomic and phenotypic diversity related to the acquisition and expression of virulence factors in *S. mutans*. The genomic diversity of *S. mutans* is generated by genomic rearrangements that involve alterations of the chromosome by deletions, insertions, duplications, inversions, or translocations. In addition, through HGT, *S. mutans* can acquire new genes by natural genetic transformation or by exposure to mobile genetic elements, such as IS, Tns, and GI. These processes can cause phenotypic variations that are specific to each strain. However, the expression of phenotypes is conditioned by transcriptional and translational regulation and depends on the recognition of chemical signals and environmental stimuli. This regulation causes the differential expression of virulence factors in *S. mutans*, which could explain the differences in the pathogenic potential between isolates.

studies [11–14]. This study proposed to subdivide the genus into 8 phylogenetic groups and confirmed that *S. mutans* is a member of the mutans group along with other 19 species [10]. Additionally, it has been reported that *S. mutans*, along with *S. sobrinus, S. ratti, S. criceti, S. downei, S. macacae*, and *S. ferus*, can be classified into specific serotypes. This serological specificity is defined by cell wall antigenic polysaccharides known as rhamnose-glucose polymers (RGPs) [15,16]. Unlike other species present in humans, *S. mutans* has the highest genetic variation and is classified into four serotypes (c, e, f, and k) since the strains express RGPs with different chemical compositions [17]. The use of polymerase chain reaction (PCR) with primers designed based on the sequence differences of the *rgp* gene [17,18], has allowed to determine the serotype frequencies and distribution among clinical isolates. While serotype c is the most prevalent in the oral cavity and corresponds to approximately 70–80% of the isolates, serotype e is the next most common (20%), and serotypes f and k correspond to less than 5% and 2%, respectively [18-21]. This distribution also highlights different pathogenic patterns in *S. mutans*. Although all four serotypes are found in the oral cavity, they have the ability to migrate into the bloodstream by evading phagocytosis due to the hydrophilic nature of RGPs, and thereafter reach the endothelial cells of coronary arteries in humans, where serotype k is the most common [22]. A common biological feature of this serotype is its low cariogenicity due to the lack of important antigens related to the onset and development of dental caries. In contrast, it survives in blood for a longer duration due to its low antigenicity, which enables virulence over a longer period of time and explains its association with the pathogenesis of certain cardiovascular diseases [16,20,22,23].

2.2. Genomic rearrangements

Chromosome rearrangements can be classified as deletions, duplications, insertions, inversions, and translocations. These events are caused by a break in long stretches of DNA involving at least two different locations, followed by a re-ligation of the broken ends to produce a new chromosomal arrangement [24]. In some cases, the rearrangement could encompass genes, and even operons, or a large number of genes depending on the size of the rearranged fragment [25]. Rearrangements can therefore influence the structure of the chromosome causing important effects on the phenotype and generating variability through the disruption of an existing gene, the creation of a new gene, or by changing the distance of a gene from the origin of chromosome replication (oriC), thereby modifying the number of copies of the gene and affecting its expression [24].

The complete genome sequencing of the strain UA159 in 2002 has helped elucidate the complexity and genetic specificity of *S. mutans* [26]. As a consequence, UA159 is frequently used as a reference strain for variability studies [3]. To date, a large number of *S. mutans* genomes have been sequenced and 188 genomic sequences of this species are available in the NCBI database (https://www.ncbi.nlm.nih.gov/). Genomic sequences are frequently used in comparative genomics studies to identify virulence factors. This method involves comparing genomes from different strains and detecting the differences and similarities, thereby revealing common molecular and pathogenic mechanisms which can be related to specific phenotypic characteristics [9]. One of the pioneering studies on *S. mutans* comparative genomic analysis, carried out by Maruyama et al., provided extensive information on the species-specific genetic content of *S. mutans,* when comparing strain UA159 with NN2025. These strains are classified within the same serological group (serotype c) and show the same biochemical (fermentation of various sugars), adhesive, and cariogenic properties. Approximately 90% of the genes present in NN2025 are also present in the UA159 genome. However, despite the fact that the order and disposition of the shared genes are conserved to a great extent, there are more than 30 genome rearrangements. Unique strain-specific regions were identified. The NN2025 genome contains eight strain-specific regions containing 65 ORFs that were unique in comparison with UA159. In contrast, the UA159 genome contains nine strain-specific regions containing 70 unique ORFs. In addition, eight regions exist in both strains, but the content of the ORFs is highly diverse. These regions are designated as “variable regions”. For example, region 1 contains genes of the purine nucleotide biosynthesis pathway (*pur* genes) and is thought to be important for bacterial growth, but genetic variation is found within this region between the two genomes [27]. Song et al. sequenced the genomes of six *S. mutans* clinical isolates and cross-compared them to the genome contents of the reference strains *S. mutans* UA159 and NN2025, focusing on characteristics related to pathogenicity. Genome alignments revealed the existence of chromosomal rearrangements. It was also observed that virulence-related genes had high variations between strains. In addition to these studies, genomic regions required for *S. mutans* survival in different environments have also been discovered [28]. In their study, Shields et al. used transposon mutagenesis coupled with next-generation DNA sequencing, a technique known as transposon sequencing (Tn-seq). They determined that only 11% of the *S. mutans* UA159 genome is essential, with the presence of genes encoding products required for replication, translation, cell wall biogenesis, and the lipid metabolism, as well as genes necessary for survival, growth, and persistence colonization in both in vitro (sustained growth in rich or defined medium) and in vivo (mouse model for dental caries) conditions [29]. These findings suggest that *S. mutans* strains evolve through chromosomal shuffling, which plays an important role in the genomic diversity of this species.

2.3. Horizontal gene transfer

Specific strain variations can be generated due to HGT, loss, duplication, or modification of existing genes [2]. HGT occurs among a wide range of the bacteria which inhabit the human oral cavity [1], explaining why HGT is considered the most important mechanism for the acquisition of new phenotypic traits, thereby providing a selective advantage to microorganisms [27]. Therefore, due to the genomic diversity present among different isolates, the genome content of a single strain does not necessarily represent the genomic potential of certain species. With the rapid development of DNA sequencing technologies, a large amount of information has been provided on the evolution, pathogenesis, diversity, metabolic activities, and virulence properties of microorganisms [30]. Using these techniques, it was possible to demonstrate that some genes associated with virulence in *S. mutans* were acquired by HGT. Hoshino et al. and Argimón et al. performed a phylogenetic analysis to determine the origin of glycosyltransferases (GTFs) in the *Streptococcus* genus. These enzymes are encoded by *gtf* and catalyze the synthesis of glucans from sucrose. The authors proposed that the genus *Streptococcus* acquired the genes *gtf* by HGT when they found in the oral cavity with lactic acid bacteria, thanks to the implementation of fermentable foods in the human diet. *S. mutans* became a microorganism capable of forming cariogenic biofilms through the acquisition of GTFs [31,32].

Cornejo et al. sequenced 57 *S. mutans* isolates in order to determine the general structure and the potential adaptive characteristics of both the core and dispensable genomes. The genomes of the studied *S. mutans* strains are highly variable and their global genetic compositions differ markedly from one isolate to another due to the high HGT rate in this species [5]. Meng et al.

performed a pan-genome analysis of 183 *S. mutans* strains and determined that this species presents an open pan-genome, indicating that new genes can be found as more genomes are sequenced. This finding is relevant since an open pan-genome has been associated with the adaptation of the bacterial species [9]. Moreover, this is a characteristic of species which colonize diverse habitats and coexist with other microorganisms in large communities, and is typical of species that have the ability to exchange genetic material and have a high rate of HGT [33]. Therefore, the aforementioned findings suggest that the genome of *S. mutans* may be expanding gradually over time [9].

2.3.1. Mobile genetic elements

Insertion elements (IS) and transposons (Tns) are present in all bacterial genomes. However, the genes associated with these elements can influence the metabolism or pathogenic potential of some microorganisms [26]. ISs are short sequences that can move from one chromosomal position to another, and usually code only for functions required for their own mobility. It is important to highlight that ISs can inactivate gene expression by the mechanisms of insertion, deletion, or modulation [34]. Some ISs and conjugative transposons are prominent in the *S. mutans* genome. The IS3 family of insertion elements is widely distributed in the Eubacteria domain and has been reported in over 40 g-positive and -negative species. Using bioinformatics analysis, it was possible to identify seven complete elements in the UA159 strain, as well as 15 fragments of the IS3 family. Six of the members of the IS3 family are closely related to the IS861 of *Streptococcus agalactiae* and are almost identical at the nucleotide level, suggesting a clonal origin. The seventh member of the family is a variant of the IS1193 element of *Streptococcus thermophilus*. Other intact ISs were found in *S. mutans* UA159 intergenic regions and may generate a neutral effect on the function of genes where they are integrated. The conjugative transposon TnSmu1, which is related to Tn916 of *Enterococcus faecalis* and contains tetracycline resistance genes, was also identified in this strain [26].

2.3.2. Genomic islands

Bioinformatics studies have shown that GIs contains more novel genes (i.e., those that do not have orthologues in other species) than the rest of the genome [35]. This suggests that GIs have become strongly selected for adaptive and auxiliary functions [7]. Therefore, they have a significant impact on the plasticity and evolution of the genome and often play an important role in the dissemination of antibiotic resistance, virulence genes, and in the formation of new catabolic pathways [7,36]. GIs present a wide spectrum of varieties in terms of organization and genetic functionality and are considered a family of discrete DNA elements, which include mobile DNA elements, such as integrative and conjugative elements, conjugative transposons, and some prophages, integrated into the bacterial chromosome and can drive strain differentiation [7].

GIs are relatively large chromosomal regions, usually between 10 and 200 kb, which are part of the dispensable genome and represent DNA segments that have been transferred by other mobile genetic elements, contributing to the diversification and adaptation of microorganisms. They are characterized by the presence of ISs, direct repeats sequences (16–20 bp) at each of their ends, and G + C content that differs from the genome average. They also contain mobile genes that codify for integrases or transposases required for chromosomal integration and cleavage [36,37].

Ajdic et al. identified a broad region in the *S. mutans* UA159 genome denoted as TnSmu2 and classified it as a GI of approximately 50 kb (representing 2.76% of the total genome size), with a G + C content of 28.9%. This GI is flanked by multiple remnants of transposases from the ISL3 family, and contains several gene fragments from other IS families, homologous genes similar to gramicidin and bacitracin synthetases, as well as accessory proteins and regulators involved in the biosynthesis of non-ribosomal peptides [26]. Interestingly, the sequences of these genes, their organization, and their genomic location are very divergent between *S. mutans* strains [38]. Waterhouse et al. investigated the occurrence of TnSmu2 in 40 strains of *S. mutans* and found that only 6 contained the same GI, TnSmu2, with a similar size and most likely in the same location as that of strain UA159. The remaining isolates also presented the TnSmu2 region but with variable lengths with respect to the reference strain. Differences between the strains were detected possibly due to the action of different insertion/deletion events [3].

2.3.3. Natural genetic transformation

This process provides the recipient microorganism with the ability to acquire new phenotypic traits [28,39] by facilitating the integration of dispensable genes. In doing so, it generates a wide range of genome heterogeneity [3,40] by promoting the emergence of resistance, genetic variation, and the rapid evolution of virulence factors [28,39]. This process is carried out as long as the cells have the ability to enter a physiological state known as competence which allows them to take up exogenous DNA from their environment and incorporate it into their genome [41]. Natural competition seems to have evolved simultaneously with the ability to lyse neighboring bacterial cells Which reside within the same microenvironment [42]. This is genetically programmed and requires complex changes which only occur at certain growth stages [41]. Therefore, natural genetic transformation constitutes a mechanism of ecological importance by which *S. mutans* manages to adapt to changing environments, originating from different selective pressures [28,29].

2.4. S. mutans gene regulation

Although *S. mutans* has been studied in some detail at the genetic level, an understanding of its gene regulation has only started to emerge in recent years [43]. *S. mutans* has evolved from a network of regulators to integrate its cellular response to environmental change [44]. Herein, we describe the latest insights into global gene regulation in *S. mutans*, including mechanisms of competence, signal transduction, quorum sensing, and small RNA (sRNA) regulation.

2.4.1. Competence regulation

The acquisition of natural competence is transient and, in most cases, the genes encoding products required for DNA uptake are not expressed [45]. The activation of the transcription of these genes occurs in response to specific signals, and only when environmental conditions allow it. In streptococcal species, competence is regulated by secreted signal peptides, and improve the synthesis of ComX, also known as the alternative sigma factor (SigX), the master regulator of natural transformation in these species, which associates with RNA polymerase and allows for the recognition of competence gene promoters [46]. In *S. mutans,* a network of genes and at least two peptide signaling molecules, competence-stimulating peptide (CSP) and ComX-inducing peptide (XIP), control the development of its genetic competence [39,45,47]. Most strains of *S. mutans* encode an apparently functional ComCDE, as well as the ComRS pathways, and either of the two signal peptides (CSP or XIP) can trigger the positive regulation of *comX*. However, specific conditions, such as the growth phase of the cells, and environmental parameters, such as the composition of the medium and pH, influence the effectiveness of each pathway [48,49]. In addition, natural genetic transformation varies

among clinical isolates. Palmer et al. verified this variation when they showed that 15 isolates from geographically different patients with caries presented a disparity in the genetic content and phenotypic characteristics associated with virulence. The development of competence of the evaluated strains could be correlated with the presence or absence of *comCDE*, *comX*, and the genes for bacteriocins synthesis [50]. Strain UA159 is naturally competent, is transformable, and has the operon *comCDE* dedicated to competence and quorum sensing, which is regulated by the signal peptide CSP. This operon is composed of genes that code for a peptide pheromone precursor and a two-component signal transduction system, responsible for the regulation of pheromone production [26]. Additionally, these systems coordinate the development of antagonistic interactions and the production of antimicrobial bacteriocins as a means to acquire transforming DNA, either by eliminating closely related streptococcal species or through an altruistic suicide mechanism among a subpopulation of competent cells within the community of *S. mutans*, increasing the availability of genetic material for homologous recombination [50–52]. *S. mutans* can also trigger a competence cascade by detecting the peptide XIP, a *comX* inducer, which interacts with the ComRS quorum sensing system. *S. mutans* synthesizes ComS and exports it to the extracellular space, but in its split form, XIP. Other cells of the population internalize XIP through the oligopeptide permease transporter, Opp. Once inside the cell, it binds to the transcriptional regulator ComR, forming the ComR-XIP complex, which activates the transcription of *comX* and *comS*, promoting the formation of competence phenotypes in almost all cells of the population (Fig. 2) [48,49].

Using Tn-seq in *S. mutans* UA159, Shields et al. detected and characterized 20 novel genes that have a substantial impact on competition regulation and competence-related phenotypes, in addition to the identification of genes previously known for their role in the expression of *comX* (*comR*, *comS*, *comD*, *comE*, *cipB*, *clpX*, *rcrR*, and *ciaH*) to activate the competent state. Because many of the discovered genes have not been studied in the context of genetic competence, these findings contribute to the general understanding of cell-cell communication in *S. mutans* [45].

In addition to the existing association between natural genetic competence and stress response pathways, multiple environmental factors of the oral cavity generate a direct impact on the competence cascade of *S. mutans* [53]. This is evident in experiments carried out in cells exposed to low pH conditions where the acidic environment directly affects *comX* transcription and reduce its expression. Extracellular pH determines the sensitivity of the competition peptides, CSP and XIP [54,55]. Likewise, oxygen is considered a key factor and significantly alters *S. mutans* transcriptional regulation, given the availability of this element to strongly alter the competition by decreasing bacteriocins expression [56]. Moye et al. observed a significant increase in *comX* activity after exposing *S. mutans* cells grown on media with various carbohydrates to CSP. This finding indicates that the carbohydrate source has a considerable influence on the progression of the competent state of cells [53].

2.4.2. Two-component signal transduction systems (TCSs) regulation

To survive in the continuously changing oral cavity, microorganisms use regulatory systems to detect and respond rapidly to the stimuli generated [57]. Some studies have documented the existence of several regulatory genes involved in biofilm formation, suggesting a coordinated process that depends on internal and external signals [58]. For example, TCSs are important for cellular adaptation processes in diverse stress environments. In *S. mutans*, multiple genes that are part of the TCSs, in addition to modulating some physiological properties of this species, are involved in biofilm formation. One of these genes is *vicX*, which is

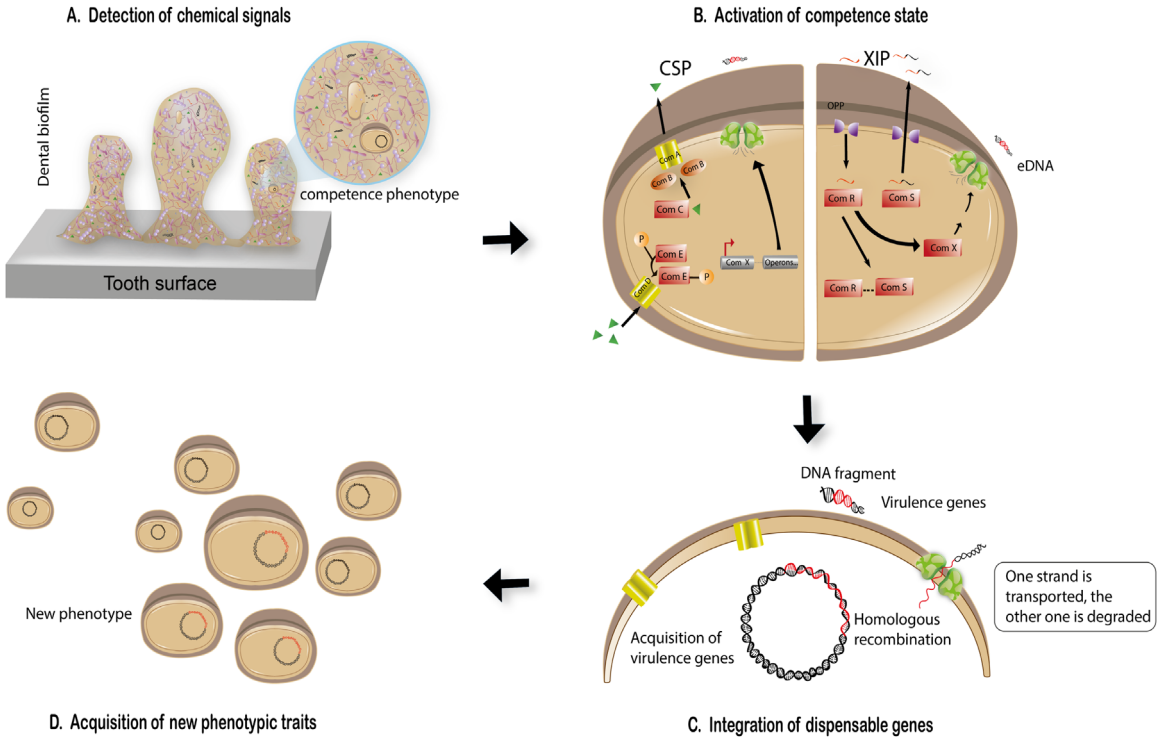

Fig. 2. Natural transformation in *S. mutans*. A) The dental biofilm harbors extracellular DNA (eDNA) that provides a source of genetic material for competent cells. *S. mutans* detects chemical signals from its environment and recognizes signal peptides once they reach a critical concentration. **B)** *S. mutans* presents two signaling pathways that activate genetic competence: a system mediated by the signal peptide CSP and another mediated by XIP. Both peptides activate the transcription of the alternative sigma factor, ComX. **C)** ComX induces the transition to the competence state by the activation of operons that code gene products for the uptake and processing of eDNA. Through homologous recombination, dispensable genes that can be virulence genes are integrated. **D)** A new phenotype with specific virulence characteristics is generated, and if it adapts within the biofilm, a new strain of *S. mutans* with pathogenic potential is also established.

part of the VicRK, one of the TCSs in *S. mutans*. Besides regulating the expression of *gtfB* and *gtfC* genes, VicRK participates in the genetic competence of S. *mutans*, increasing its transformation rate [59]. The *rnc* gene also regulates the expression of virulence in bacteria. *rnc* deletion could decrease *S. mutans* cariogenicity by repressing its downstream VicRKX expression at the post-transcriptional level [60]. It was further demonstrated that the *rnc* gene participates in biofilm formation by evaluating the expression of biofilm formation-associated genes by qRT-PCR in *S. mutans*. It was found that *rnc* positively modulates the expression of *gtfB/C/D* genes, as well as *dexA* and *gbpB.* Therefore, it was determined that *rnc* regulates the formation of stable biofilms through the positive control of exopolysaccharide synthesis and bacterial adhesion [61]. It has also been shown that the LuxS/autoinducer-2 (AI-2) quorum sensing system plays an important role in the metabolic regulation and the interaction between bacterial species. A recent genome-wide transcriptome analysis found that the expression of 30% of all *S. mutans* genes was affected by the mutation of the *luxS* gene, and the expression of these genes could not be completely restored by the AI-2 system [62]. Additionally, an alteration in the formation, architecture, and composition of the biofilm was observed after inducing a mutation of the *luxS* gene in a dual-species biofilm formed by *S. gordonii* and *S. mutans* [63].

2.4.3. sRNAs regulation

Besides transcriptional and translational gene regulation through direct interaction with mRNAs, bacterial regulatory pathways involving sRNAs are now being elucidated and their functions are coming to light, particularly with regards to bacterial physiology, adaptation, and pathogenesis [64–66]. In *S. mutans*, sRNAs were first described after high-throughput experiments and a kingdom-wide prediction of bacterial sRNA-encoding genes using the SIPHT (sRNA identification protocol using high-throughput technologies) method. As a result, 18 trans-encoded RNA (treRNA) genes of *S. mutans* were predicted. However, their functions, as those of most *S. mutans* treRNAs, remain elusive [67]. Biocomputational studies predicted that three treRNAs were putatively regulated by cia-dependent small RNAs (csRNA23-1, csRNA23-2, and csRNA24) [68]. Xia et al. described for the first time a pH-dependent sRNA in *S. mutans*. They predicted a total number of 334 sRNAs by combining different bioinformatics approaches. However, only the treRNA, L10-leader, was verified by qRT-PCR and northern blot analyses due to its high abundance. The expression level of the L10-leader was growth phase-dependent and was highly affected by pH. In clinical strains, its expression was higher in most adhering and acidic strains, and could be linked to virulence [69].

Recently, a variety of sRNAs have been also identified in *S. mutans* using planktonic cells. Lee et al. reported over 900 potential miRNA-size small RNAs (msRNAs) in *S. mutans* [70]. Additionally, specific sRNAs of a small size (18–50 nucleotides) are induced under acid stress conditions, as revealed in previous studies [71]. A total of 736 differentially expressed candidate sRNAs were predicted, including 352 sRNAs located on the antisense to mRNA (AM) and 384 sRNAs in intergenic regions (IGRs). The top 7 differentially expressed sRNAs were successfully validated by qRT-PCR in UA159, 2 of which were further confirmed in 100 clinical isolates [43].

Wenderska et al. utilized high-throughput RNA sequencing (RNAseq) to provide a greater understanding of how global gene expression patterns change in response to competence stimulating peptide XIP. Their study demonstrated that, in *S. mutans,* the XIP peptide not only controls DNA transformation and bacteriocin production but also induces a response that resembles the stringent response to amino acid starvation. They further reported on the discovery of 11 intergenic regions that expressed sRNAs and provided the first evidence for a heat shock-regulated intergenic region that negatively regulates genetic competence [72]. RNAseq and bioinformatics analyses have also confirmed previously reported patterns of *S. mutans* gene expression changes in response to aerobic [56] and heat stress [73] and have identified for the first time a vancomycin stress transcriptome characterized by primarily upregulated expression of genes involved in the pyruvate metabolism and ABC transporters [74]. The importance of sRNAs-mediated regulatory mechanisms depends on a fine-tuning of the expression of an armament of virulence factor genes [75,76]. Deciphering the role of sRNAs in *S. mutans* regulatory networks may provide some clues for the design of novel therapeutic strategies for dental caries [77].

3. From the genomic diversity to the phenotypic diversity of the main *S. mutans* virulence factors

The remarkable variability of *S. mutans* has been demonstrated by several studies using molecular techniques such as multilocus sequence typing (MLST) [78,79], pulsed field gel electrophoresis (PFGE) [80,81], PCR with arbitrary primer (AP-PCR) [82–84], PCR amplification of repetitive extragenic palindromic elements (Rep-PCR) [85–87], and the comparison of sequenced genomes [26,27]. Around 52 different *S. mutans* genotypes have been reported in saliva and dental biofilms [88,89], with the presence of 1–5 different *S. mutans* genotypes in the same individual [86]. In addition, the variability of individual *S. mutans* genes has been identified from these studies [3,90,91]. As a result of the differences in genetic content and gene regulation, *S. mutans* strains also exhibit phenotypic variability [17,92,93]. It has been suggested that not all strains have the same virulence capacity to promote the formation of dental caries [93,94]. Therefore, it seems reasonable to expect that at least some of the genes related to virulence are not distributed in the same way in the strains according to their caries status [95].

In particular, acidogenicity, aciduricity, and adhesion, considered as the key virulence factors of *S. mutans* [96–98], have been compared extensively among a large number of clinical isolates with genotypic differences, demonstrating variability at the same time [50]. The study of *S. mutans* virulence factors and their correlations with other species present in the biofilm is fundamental in order to understand the role of colonization of multiple genotypes in the same individual [88]. In addition, the ability of *S. mutans* to respond to environmental stresses, that is salivary flow, acid pH, oxidative stress, and changes in carbohydrate source and availability, is essential for *S. mutans* survival and predominance in caries lesions. Importantly, *S. mutans* has evolved a network of regulators to integrate its cellular response to environmental changes [99,100]. Under these conditions, *S. mutans* gains a competitive growth advantage due to its acidogenic and aciduric potential ability to transport and metabolize dietary carbohydrate over a broad range of external concentrations and pH, and adherence capabilities [97,101], as will be reviewed in the following sections.

3.1. Acidogenicity

The expression of genes related to carbohydrate absorption and metabolism seem to have contributed to the successful adaptation of *S. mutans* in the oral cavity. This species has the capacity to transport and degrade a large amount of carbohydrates, which in turn contributes to its pathogenic potential. The internalization of these carbohydrates is mainly carried out by the activity of two main transporters: ABC-type transporters for multiple sugar

metabolism (msm) and *malXFGK* transport systems. However, the predominant route for the uptake of mono- and disaccharides by *S. mutans* is the phosphotransferase system (PTS) [102]. To show their importance, *S. mutans* UA159 contains more than 280 genes associated with these transport systems, which represent almost 15% of the total ORFs found in its genome [26].

Acidogenicity refers to the ability of some microorganisms to produce organic acids from the carbohydrate metabolism under anaerobic conditions, which results in a pH reduction to values below 4.0 [96,103]. Carbohydrate fermentation carried out by *S. mutans* is a key strategy for its survival. *S. mutans* have all the genes needed for a complete glycolytic pathway for the production of lactic acid, formate, acetate, and ethanol fermentation products [26]. However, lactic acid is the main final product of *S. mutans* fermentation when the sugar concentration is high,which leads to the possible appearance of dental caries [104,105]. The streptococci of the mutans group are capable of producing large amounts of acid, even though the pH of the oral cavity is relatively low. In addition, pH decreases can generate a highly acidic biofilm that can favor the enamel demineralization process [106,107]. There is evidence that *S. mutans* is one of the most acidogenic species inhabiting the dental biofilm. de Soet et al. analyzed 47 strains of the genus Streptococcus for the ability to produce acid at pH values between 5.0 and 7.0, with nine species isolated from dental biofilm. Differences were observed in acid production when strains were maintained at a pH of 7.0. However, at lower pH values, the percentage of variation was lower. In addition, the average speed of acid production at different pH values was significantly higher in *S. mutans* and *S. sobrinus* compared to the other evaluated species, reaffirming their participation in carious processes [107].

S. mutans can also lower the dental biofilm pH due to the intracellular polysaccharide (IPC) metabolism. These are amylopectin-glycogen molecules, stored in intracellular granules, which contain mainly α1,4 and α 1,6 glycosidic bonds. IPCs are an endogenous source of carbohydrates and can be metabolized to produce acid upon during periods of nutrient depletion in the oral cavity. Therefore, IPCs are of great importance in the absence of fermentable carbohydrates from the diet and can contribute to the cariogenicity of this microorganism [108,109]. Harris et al. generated an IPC-deficient strain (SMS203) from the cariogenic strain, *S. mutans* UA130, classified as serotype c. The acidogenicity of the mutant strain was lower than the wild-type progenitor strain, especially when cultured in the absence of exogenous glucose, and showed a significantly reduced cariogenic potential in germfree rats [110]. These findings help to elucidate the role of IPCs in the virulence of *S. mutans*, and how it can be influenced by conditioned environmental conditions.

3.2. Aciduricity

Biofilm sustained acidification can lead to a significant increase in the proportions of acidogenic and acid-tolerant species, increasing caries risk. Therefore, the ability to survive rapid and extreme changes in pH is one of the most important attributes of cariogenic bacteria, especially after carbohydrate intake, when the sugar level increases and the pH decreases to values around 4.0 in the dental biofilm. In general, *S. mutans* can grow and carry out glycolysis at low pH values, acquiring a selective advantage over less aciduric species [111,112]. In order to do this, *S. mutans* has an adaptive acid tolerance response (ATR) mechanism and is able to modify its physiology and survive in these environments. This aciduricity or acid tolerance has been largely attributed to the presence of proton translocator F_0F_1-ATPase pumps expressed at higher levels compared to many other oral microorganisms [111,113]. F_0F_1-ATPase is a multimeric enzyme capable of transporting protons out of the cell, allowing the maintenance of a more alkaline cytoplasmic pH compared to the extracellular environment (a difference referred to as ΔpH), to carry out different bioenergetic processes, and conferring protection to acid-sensitive glycolytic enzymes [111,114,115].

In contrast to *S. mutans*, commensal streptococci can catabolize urea using urease enzymes and/or arginine via the arginine deiminase system (ADS), liberating ammonia that alkalinizes the cytoplasm of the commensal and, in turn, increasing the pH of biofilm communities. In addition to moderating environmental acidification, the hydrolysis of urea by ureases, and in particular, of arginine by the ADS, can promote the stability of health-associated biofilms by providing bioenergetic advantages to the commensal [116]. However, *S mutans* has evolved to catabolize agmatine via the agmatine deiminase system (AgDS) for alkali production. Although agmatine is a decarboxylated derivative of arginine and the AgDS is highly similar to the ADS, with end products being putrescine, ammonia, CO2 and ATP, the agmatine catabolism may have a significant effect on oral biofilm ecology, but not in the way that arginine or urea metabolism does [117]. AgDS may increase the competitive fitness of *S. mutans*, contributing in major ways to the pathogenesis of this organism [118]. In fact, it has been postulated that the AgDS, unlike the ADS or urease, does not produce sufficient quantities of alkali to impact the pH of oral biofilm. Moreover, the AgDS may actually enhance the acid tolerance of cariogenic organisms, such as *S. mutans,* through ATP generation and increasing of the cytoplasmic pH, which could enhance the ability of the bacteria to continue to catabolize carbohydrates at low pH values [118].

It is clear that *S. mutans* has evolved numerous strategies to become established in oral biofilms, to antagonize the growth of commensal organisms, to produce organic acids from dietary carbohydrates, and to grow and metabolize in acidic conditions that are favorable for the initiation and progression of dental caries [116]. Consequently, acidogenicity and aciduricity can be considered as the main factors contributing to *S. mutans* cariogenicity [97,98], such that variations in these characteristics could help explain the differences in virulence among clinical isolates [93].

3.3. Adhesion

Colonization and survival of *S. mutans* is facilitated by adherence to the tooth surface [111]. This adhesion, within the dental biofilm, can be mediated by two mechanisms: sucrose-independent and sucrose-dependent mechanisms [97,119]. The first mechanism, also known as the antigen I/II or Pac, is facilitated by the surface-associated protein P1 (SpaP). This molecule is an adhesin that promotes bacterial adhesion to the components of the acquired enamel pellicle. However, it is not relevant for *S. mutans* virulence [98,111]. In contrast, the sucrose-dependent mechanism is considered responsible for dental surface colonization [97]. This is generated by the action of GTFs, enzymes that participate in the synthesis of extracellular polysaccharides called glucans. The GTFs have enzymatic activity that hydrolyses sucrose into glucose and fructose, and binds glucose residues together by glycosidic bonds in order to form a glucan polymer. In addition to promoting bacterial adhesion to tooth surfaces, these glucans promote the aggregation and co-aggregation of microorganisms. Due to this process, microcolonies are formed and favor biofilm formation [97,98,119]. *S. mutans* possesses three GTFs encoded by the *gtfB*, *gtfC*, and *gtfD* genes. Collectively, GTFs synthesize soluble and insoluble glucans. Soluble glucans have predominantly linear polymers linked by α 1,6-bonds (dextrans), which are easily degraded and therefore can serve as an extracellular energy reserve in times of nutrient deprivation. On the other hand, insoluble glucans (mutans) have a higher degree of branching and predominantly

α1,3 bonds, and cannot be degraded by bacterial enzymes, so their participation as a potential source of extracellular energy is doubtful [97,120].

The GtfB enzyme synthesizes insoluble glucans responsible for the adsorption to surfaces. GtfC catalyzes the synthesis of a mixture of insoluble and soluble glucans, responsible for providing binding sites for *S. mutans* establishment in solid surfaces as well as facilitating the aggregation with other bacterial cells, while GtfD synthesizes only soluble glucans [121–123]. The genes encoding GtfB and GtfC are close to each other, have 95% sequence homology, and are subject to the same regulatory processes. These genes are expressed in response to glucose or sucrose excess, or acidification of the environment [98]. The loss of any of these genes is associated with a reduction in *S. mutans* virulence. Yamashita et al. demonstrated that the inactivation of *gtfB* and *gtfC* genes causes a reduction in the synthesis of extracellular polysaccharides and, consequently, less biofilm production by *S. mutans*, as well as a decrease in smooth-surfaces caries incidence in a murine model [124].

In addition to GTFs, *S. mutans* produces fructosyltransferase (FTF), which participate in extracellular fructans synthesis from sucrose. The function of these fructans is to serve as short-term nutrient storage reservoirs. In addition, they play an important role in bacterial colonization and adhesion to tooth surfaces, which adds to the dental biofilm pathogenicity [108,111,125]. Studies have shown the importance of fructans in the virulence of *S. mutans* by insertional inactivation of the gene *ftf*, responsible for fructan production in the *S. mutans* V403 strain [126]. In addition, by generating a FTF-deficient strain in fructan synthesis (V1741), it was possible to suggest that FTF activity contributes to the pathogenicity of the V403 strain, possibly by generating extracellular fructans which serve as storage compounds [127]. Considering that *S. mutans* virulence is related to its ability to colonize dental surfaces, it is important to highlight that a large number of clinical isolates exhibiting different genotypes presented a significant variability in their capacity to form biofilms, suggesting that these variations may be associated with the differential expression of proteins involved in the development and growth of the dental biofilm [128].

Overall, these findings indicate that the acquisition of virulence genes is only a first step on the path towards the *S. mutans* pathogenic lifestyle. What makes *S. mutans* a pathogenic microorganism is not limited to genes acquired to activate virulence, it also requires mild genetic changes mediated by regulatory genes to adapt the expression of phenotypes with pathogenic potential according to environmental pressures [61].

4. Conclusions

The intra-species variations as well as the gene expression modulation orchestrated by the environmental conditions of the oral cavity, revealed in the reviewed investigations, highlight the complexity and dynamic changes present in the genetic components of *S. mutans*. These changes may possibly explain the existence of specific molecular and/or metabolic mechanisms that could be related to the inherent phenotypic characteristics of each *S. mutans* strain present in the oral cavity. Although the association between number, genotypic diversity, and caries status of an individual is still controversial, it is possible that the simultaneous action of different genotypes, with different phenotypic potentials, results in different virulence attributes which can increase the risk of developing caries. Therefore, improving our knowledge of this diversity will be essential in future studies in order to be able to explore, through a combination of genotypic and phenotypic analyses, the mechanisms of selection, adaptation, and competence that contribute to *S. mutans* cariogenicity.

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Conflict of interest

The authors declare no conflict of interests.

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