Oral Microbiology Immunology 2009: 24: 79–82
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ORAL MICROBIOLOGY
AND IMMUNOLOGY

Short communication

Distribution, regulation and role of the agmatine deiminase system in mutans streptococci

A. R. Griswold[1,3], M. M. Nascimento[2], R. A. Burne[1]
[1]Department of Oral Biology, [2]Department of Operative Dentistry, University of Florida, Gainesville, FL, USA

Griswold AR, Nascimento MM, Burne RA. Distribution, regulation and role of the agmatine deiminase system in mutans streptococci.
Oral Microbiol Immunol 2009: 24: 79–82. © 2009 The Authors. Journal compilation. © 2009 Blackwell Munksgaard.

The agmatine deiminase system (AgDS) was identified in seven strains of mutans streptococci. Genes encoding the AgDS of *Streptococcus rattus* FA-1 were sequenced and found to share homology with the *agu* genes of *Streptococcus mutans* UA159. With the exception of *Streptococcus sobrinus*, the AgDS of mutans streptococci appear to be sensitive to carbohydrate catabolite repression. Agmatine inhibited bacterial growth, suggesting that the AgDS degrades a deleterious substance into useful compounds.

Key words: biofilm; dental caries; ecology

Robert A. Burne, Department of Oral Biology, University of Florida, PO Box 100424, 1600 SW Archer Road, Gainesville, FL 32610-0424, USA
Tel.: + 1 352 392 4370;
fax: + 1 352 392 7357;
e-mail: rburne@dental.ufl.edu

Accepted for publication April 10, 2008

[3]Present address: J. H. Miller Health Science Center, University of Florida, Gainesville, FL, USA

To persist in the oral cavity, bacteria must possess sophisticated strategies for coping with frequent changes in oxygen tension, pH, and nutrient source and availability (8). During the development of dental caries, repeated and sustained acidification of oral biofilms results in increases in the proportions of organisms with a potent capacity to lower the pH through glycolysis and to grow and continue to metabolize carbohydrates at extreme pH values (9). As a counterbalance to the forces of acidification in oral biofilms, a variety of species have retained the capacity to produce alkali from salivary substrates, which appears to have a major impact on pH homeostasis and microbial ecology (2). The two primary sources of plaque ammonia production are through the hydrolysis of urea by urease enzymes or of arginine via the arginine deiminase system (ADS); and both of these systems are thought to play an integral role in caries prevention (1, 4, 6, 10, 13, 14).

We recently described an ammonia-generating system in the cariogenic organism, *Streptococcus mutans* (7). The agmatine deiminase system (AgDS) is similar to the ADS and catabolizes agmatine to produce putrescine, ammonia, and carbon dioxide, with the concomitant production of adenosine triphosphate (ATP; Fig. 1). As with the ADS of other plaque streptococci, the AgDS of *S. mutans* augments ΔpH and provides ATP, thereby contributing to acid tolerance. However, activity in the AgDS is generally lower than that in the ADS (6). Consequently, ammonia production from agmatine in the oral cavity probably does not result in significant alkalinization of the environment, unlike arginine catabolism via the ADS. While possession of the AgDS might benefit *S. mutans*, it is unlikely to have a positive effect on the persistence of acid-sensitive species in the oral cavity, as does ammonia production from arginine or urea (1). Interestingly, agmatine is inhibitory to the growth of *S. mutans* and a functional AgDS is required for the optimal growth in the presence of agmatine, which is readily measurable in dental plaque samples (7). In addition to providing bioenergetic benefits, the AgDS of *S. mutans* detoxifies a growth-inhibitory substance that can be produced by some plaque bacteria (7). The AgDS is a potentially attractive target for anti-caries strategies, so additional information about the distribution and physiological role of the system in cariogenic bacteria is needed.

Identification of streptococcal *aguA* homologues

To assess the sequence similarity of the agmatine deiminase-encoding gene, *aguA*, among the streptococci, *S. mutans aguA* (i.e. SMU.264) was used in BLAST searches of the available streptococcal genomic sequences in the NCBI, TIGR and Sanger databases. Homologues were identified in the genomes of *Streptococcus sobrinus* (72% ID), *Streptococcus uberis* (46% ID), *Streptococcus pneumoniae* (45% ID), and *Streptococcus mitis* (44% ID), *Lactobacillus salivarius* subsp. *salivarius* (strain UCC118; 24% ID), and *Lactobacillus brevis* (strain ATCC 367; 54% ID). An *aguA* homologue was not found in the partial genome of *S. gordonii*, nor in the genomes of *Streptococcus suis*, *Streptococcus thermophilus*, *Streptococcus pyogenes*, *Streptococcus agalactiae*,

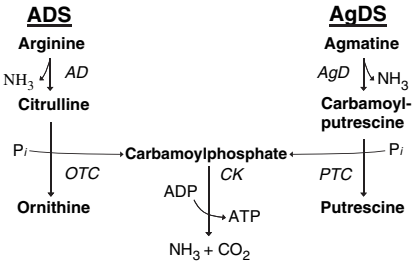

Fig. 1. The arginine deiminase system (ADS) and agmatine deiminase system (AgDS) pathways are highly analogous. Other abbreviations: AD, arginine deiminase; AgD, agmatine deiminase; OTC, ornithine transcarbamoylase; CK, carbamate kinase; and PTC, putrescine transcarbamoylase.

Streptococcus sanguinis, *Streptococcus equi*, or *Streptococcus zooepidemicus*. The genomic sequences of *Streptococcus rattus*, *Streptococcus oralis*, and *Streptococcus salivarius* are not currently available.

Measurement of AgD enzyme activity in oral streptococci

To determine if the AgDS was widely distributed among the oral streptococci, production of *N*-carbamoylputrescine from agmatine was assayed in *S. mutans* UA159, six related strains of mutans streptococci, and five strains of non-mutans streptococci, including *S. salivarius*, *S. oralis*, *S. sanguinis* genospecies 1 and 2, and *S. gordonii*. Bacterial strains were grown in tryptone-vitamin (TV) medium (3) containing 25 mM glucose, galactose or sorbitol, with or without 10 mM agmatine, at 37°C in 5% CO_2 and 95% air. AgD activity was measured by colorimetric determination of *N*-carbamoylputrescine production from agmatine as previously described (6). Briefly, bacteria were grown to an optical density at 600 nm (OD_{600}) of 0.6. Cells were harvested by centrifugation, washed once with 10 mM Tris-maleate buffer, pH 6.0, and resuspended in 1/10 of the original culture volume in the same buffer. The cells were permeabilized using 1/20 volume of toluene and two, 1-min freeze–thaw cycles. The cell suspension was centrifuged and the pellet was resuspended in 500 µl of 10 mM Tris-maleate, pH 6.0. A 50-µl aliquot of the cell suspension was used in a 500-µl reaction mixture containing 10 mM agmatine in 10 mM Tris-maleate buffer, pH 6.0. After 30 min, reactions were terminated by the addition of an equal volume of 10% trichloroacetic acid. The *N*-carbamoylputrescine was measured in a 1-ml reaction containing an appropriate aliquot of the reaction mixture, 700 µl phosphoric acid : sulfuric acid (3 : 1) and 250 µl diacetyl monoxime. The sample was heated at 100°C for 10 min, immediately cooled, and the OD_{490} was recorded and compared to a standard curve. To determine the protein concentration of the sample, a known volume of the original cell suspension was mixed with an equal volume of glass beads (0.1 mm) and completely homogenized using a Bead Beater. The samples were centrifuged for 10 min in a refrigerated microcentrifuge and the protein concentration of the lysate was measured (Bio-Rad, Hercules, CA) with bovine albumin serum as the standard. AgD activity was expressed as nmol *N*-carbamoylputrescine produced per min per mg protein. Chemical reagents were obtained from Sigma (St Louis, MO). Interestingly, AgD enzyme activity was detected in the non-mutans strains *S. salivarius*, *S. oralis*, and *S. sanguinis* genospecies 2 but not in *S. gordonii* or in *S. sanguinis* genospecies 1, even in the presence of non-repressing sugars (data not shown). The remainder of the study focused on AgD activity in mutans streptococci, many of which are associated directly with human dental caries.

AgD activity was detected in each of the mutans streptococci, with the highest levels observed in *S. mutans* strains UA159 and GS-5, and *S. rattus* strains FA-1 and BHT (Fig. 2). As previously demonstrated with *S. mutans*, the AgDS in *S. rattus* was induced by agmatine (Fig. 3A) (6). In many AT-rich gram-positive bacteria, the expression of catabolic genes and operons is regulated by carbohydrate catabolite repression in the presence of a preferred carbohydrate source such as glucose (12). The AgDS

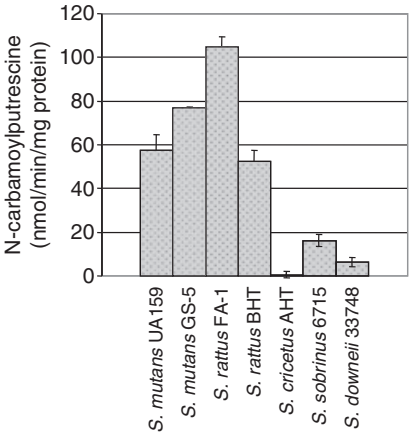

Fig. 2. Agmatine deiminase system (AgDS) enzyme activity in *mutans* streptococci grown in TV media containing 25 mM glucose and 10 mM agmatine. Results shown are the average and standard deviations (error bars) of a minimum of nine separate cultures for each strain and condition.

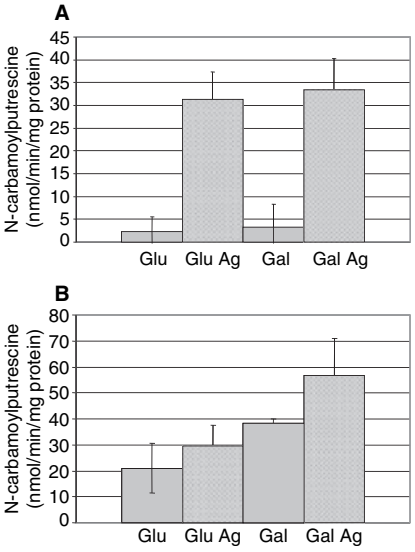

Fig. 3. (A) Agmatine deiminase system (AgDS) enzyme activity of *Streptococcus rattus* FA-1 grown in TV medium containing 25 mM glucose (Glu) or galactose (Gal), with or without the addition of 10 mM agmatine (Ag). Results shown are the average and standard deviations (error bars) of a minimum of nine separate cultures for each strain and condition. (B) AgD enzyme activity of *S. rattus* FA-1 grown in TV medium containing 250 mM glucose or galactose, with or without 10 mM agmatine. Results shown are the average and standard deviations (error bars) of a minimum of nine separate cultures for each strain and condition.

Table 1. Agmatine deiminase (AgD) enzyme activity (expressed as nmol/min/mg protein) in selected oral streptococci grown in TV media containing 25 mM galactose or 25 mM sorbitol and 10 mM agmatine (Ag)

Strain	TV sorbitol + Ag	TV galactose + Ag
S. mutans UA159	476.2 ± 120.3	170.0 ± 11.4
S. sobrinus 6715	9.7 ± 2.0	ND
S. cricetus AHT	10.9 ± 3.7	ND

Results shown are the average and standard deviations of a minimum of nine separate cultures for each strain and condition.
ND, not determined.

of *S. rattus* was relatively insensitive to carbohydrate catabolite repression compared with that of *S. mutans* (6), as determined by monitoring activity in cells grown in 25 mM of the repressing sugar glucose vs. the poorly-repressing sugar galactose. However, when *S. rattus* was grown in much higher concentrations of glucose (250 mM), the effects of catabolite repression were apparent and began to mask the effects of agmatine induction (FIG. 3B), consistent with previous studies on the arginine deiminase system of *S. rattus* that showed that a much higher concentration of glucose was needed to observe carbohydrate catabolite repression compared with what was needed to induce carbohydrate catabolite repression of the ADS in *S. gordonii* (2, 5).

The AgDS of *S. cricetus* appeared to be tightly regulated by carbon catabolite repression, because enzyme activity was only measurable when the cells were grown in a non-repressing sugar, such as sorbitol (Table 1). *S. sobrinus* displayed low, albeit consistently detectable, AgD activity regardless of whether repressing (i.e. glucose) or non-repressing (i.e. sorbitol and galactose) carbohydrates were included in the growth medium (Fig. 2 and Table 1). Thus, the AgDS has been conserved among the mutans streptococci and, with the exception of *S. sobrinus*, appears to be sensitive to carbon catabolite repression.

Sequencing of the *S. rattus agu* operon

Streptococcus rattus is currently the closest genetic relative of *S. mutans*, yet this organism is poorly cariogenic in experimental animals. To examine the genetic relationships of the *agu* genes of mutans streptococci and to dissect the basis for differential sensitivity of the operons to carbohydrate catabolite repression, the *agu* operon of *S. rattus* FA-1 was sequenced using the primers in Table 2. The *S. rattus agu* genes are organized in an *aguRBDAC* cluster and the predicted amino acid sequences are nearly identical to their counterparts in *S. mutans* (Fig. 4).

Similar to ADS enzymes, AguA shares the least similarity (79%) between the two bacteria of enzymes in the AgDS, which may contribute in part to measured differences in the AgD activities in these organisms. The functional domains of AgD have not yet been identified, but the conserved [GGGNIHCITQQ] sequence (11) present in all known AgD enzymes was identified at the C-terminus of *S. rattus* AguA, as well as in the AguA sequences of *S. sobrinus*, *S. uberis*, *S. pneumoniae*, and *S. mitis*.

Table 2. Primers used to sequence the *Streptococcus rattus agu* operon

Primer[1]	Sequence	Locus
5-S	5′-TATTTCCAATTTACGGGTGTTCT-3′	*aguR*
100-S	5′-GTGAATGTGAGTTTTTACTGTGC-3′	*aguR*
560-S	5′-TTGATTTGGTAGGTAATAGAGGT-3′	*aguR*
1050-S	5′-GGCTTTGTAAAAAAGGCATAAAC-3′	*aguR-aguB*
1760-S	5′-GAATGGAATTTGTTCACTTTGGA-3′	*aguB*
2300-S	5′-CGCAATCATGTCTGTCCTAAAC-3′	*aguB-aguD*
3080-S	5′-GCTTTTGGTATTGGCGTCTCA-3′	*aguD*
3540-S	5′-CGTCCTTTTAAGGTTAGTGGC-3′	*aguD-aguA*
4260-S	5′-GTGTCTGTTACATCTTAGTCGG-3′	*aguA*
4840-S	5′-CAGTGAAAATGGAGAAAATGTATG-3′	*aguA-aguC*
5340-S	5′-GAGGTTGGCGAAAAGTAGTTG-3′	*aguC*
560-AS	5′-TACCTCTATTACCTACCAAATCAA-3′	*aguR*
1050-AS	5′-GTTTATGCCTTTTTTACAAAGCC-3′	*aguR*
1760-AS	5′-GTCCAAAGTGAACAAATTCCA-3′	*aguR-aguB*
2300-AS	5′-GTTTAGGACAGACATGATTGCG-3′	*aguB*
3080-AS	5′-GAGACGCCAATACCAAAAGCA-3′	*aguB-aguD*
3540-AS	5′-GCCACTAACCTTAAAAGGACG-3′	*aguD*
4260-AS	5′-CCGACTAAGATGTAACAGACAC-3′	*aguD-aguA*
4840-AS	5′-CATACATTTTCTCCATTTTCACTG-3′	*aguA*
5340-AS	5′-CAACTACTTTTCGCCACCTC-3′	*aguA-aguC*
5780-AS	5′-CCGTAATTTGTGTGCCACTTC-3′	*aguC*

[1]The position of the primer relative to the 5′ end of the *agu* operon.

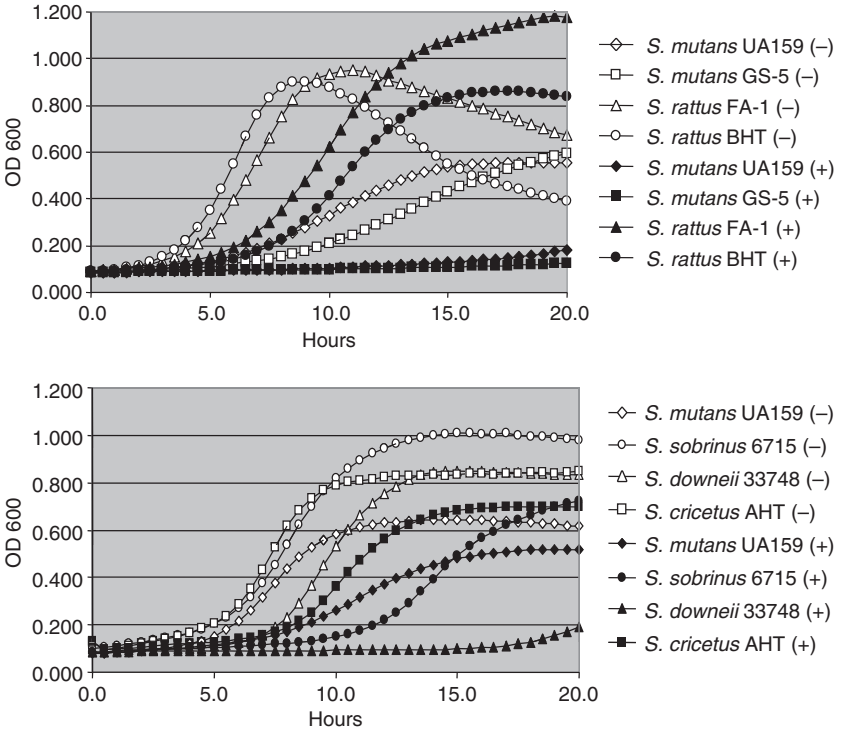

Fig. 4. The *agu* operon of *Streptococcus rattus* FA-1 and homology of each open reading frame to the corresponding gene in *Streptococcus mutans* UA159. The molecular weight and pI of each predicted agmatine deiminase (AgD) protein is shown for *S. rattus* (black) and *S. mutans* (parentheses, grey). AguR is a transcriptional activator of the agmatine deiminase operon in the presence of agmatine. The nucleotide sequences bear the GenBank accession number EF104920

Growth inhibition by agmatine

Consistent with data previously obtained with *S. mutans* UA159, the presence of 20 mM agmatine increased the doubling times of all strains tested (FIG. 5A,B) with the exception of the non-mutans streptococci (data not shown). The mechanisms of agmatine inhibition remain unclear; however, previous studies have postulated that growth inhibition results from

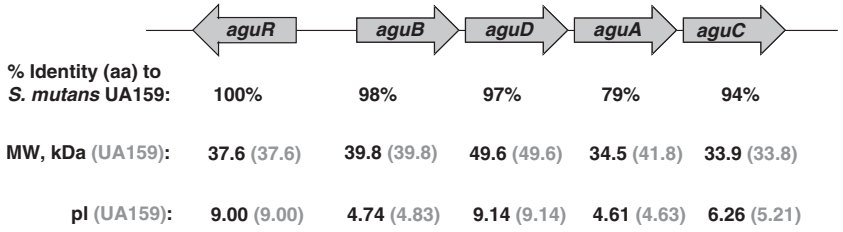

Fig. 5. (A) Growth of *Streptococcus mutans* and *S. rattus* strains in TV medium containing 25 mM galactose and 0 mM (open symbols) or 20 mM (closed symbols) agmatine. Growth curves were generated using a Bioscreen C (Oy Growth Curves AB Ltd., Helsinki, Finland). Optical density at 600 nm was recorded every 30 min with shaking for 10 s before each reading. Standard deviations for data points shown were < 0.02 for cultures grown in 0 mM agmatine and < 0.06 for cultures grown in 20 mM agmatine. (B) Growth of selected *mutans* streptococci in TV medium containing 25 mM glucose and 0 mM (open symbols) or 20 mM (closed symbols) agmatine. Standard deviations for data points shown were < 0.02 for cultures grown in 0 mM agmatine and < 0.06 for cultures grown in 20 mM agmatine.

competitive inhibition of amino acid transport or interference with translation (7). Enhanced yields, as measured by final OD, in the presence of agmatine and the non-preferred sugar, galactose, likely resulted from neutralization of the cytoplasm and environment by ammonia, coupled with increased ATP generation through the AgDS. Interestingly, *S. rattus* is the only mutans streptococcus capable of generating ammonia via the ADS, in addition to the AgDS (1). We previously demonstrated that agmatine is present in biologically relevant amounts in dental plaque and saliva (0.75 and 0.2 μmol of agmatine per mg of protein, respectively), which would be predicted to be in the range of concentrations used in this study (7). Thus, in addition to enhancing ammonia and energy production, the AgDS may function primarily to break down a growth-inhibitory component present in saliva and dental plaque.

Summary

In dental plaque biofilms, where the source and amount of nutrients are highly variable, organisms benefit from the ability to metabolize a wide variety of substrates. Here, we show that a system for agmatine utilization has been conserved among a genetically and physiologically diverse group of oral streptococci with distinct niches in oral biofilms. As the genomic sequences of much of the oral microbiome remain unavailable, it is difficult to determine the full extent to which the AgDS is distributed among oral species. However, our study reveals that the system may be absent from many non-mutans streptococci. Furthermore, agmatine inhibits bacterial growth, suggesting that the AgDS degrades a deleterious substance into useful compounds. Future studies should attempt to determine the extent to which ATP and ammonia generation via agmatine hydrolysis confers a selective advantage to bacterial species residing in the oral cavity.

Acknowledgments

This study was supported by DE10362.

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