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Evidence that Accumulation of Mutants in a Biofilm Reflects Natural Selection Rather than Stress-Induced Adaptive Mutation▽

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The accumulation of mutant genotypes within a biofilm evokes the controversy over whether the biofilm environment induces adaptive mutation or whether the accumulation can be explained by natural selection. A comparison of the virulence of two strains of the dental pathogen *Streptococcus mutans* showed that rats infected with one of the strains accumulated a high proportion (average, 22%) of organisms that had undergone a deletion between two contiguous and highly homologous genes. To determine if the accumulation of deletion mutants was due to selection or to an increased mutation rate, accumulations of deletion mutants within in vitro planktonic and biofilm cultures and within rats inoculated with various proportions of deletion organisms were quantified. We report here that natural selection was the primary force behind the accumulation of the deletion mutants.

There is a 150-year-old controversy over whether selective stress can influence mutation rate (13). It has led to the concept of adaptive mutation, a process whereby environmental stress can induce the cellular mechanisms that lead to mutations. Experiments by Cairns et al. (2, 3) figured prominently in fueling the controversy as they demonstrated the reversion of a Lac$^-$ phenotype in resting *Escherichia coli* plated on lactose-containing medium. However, the controversy lingers, primarily because it is difficult to isolate the effects of mutations on growth rates or the growth of subpopulations under conditions that would ordinarily suppress growth (13).

One source of stress that has been proposed to increase the mutation rate is the environment within a biofilm (8), though a biofilm is in reality a collection of environments. In a trial to compare the virulence of the wild-type dental pathogen *Streptococcus mutans* and that of a genetically engineered mutant that no longer expressed a glucan-binding protein (Gbp) designated GbpA, it was noted that rats infected with the *gbpA* mutant accumulated high proportions (average, 22%) of organisms that had undergone a deletion (7). Recovery of deletion mutants from wild-type-infected rats was rare. The basis for the deletion was a recombination between two contiguous and highly homologous glucosyltransferase (Gtf) genes (Fig. 1). This system offered the opportunity to investigate the basis for the accumulation of the deletion mutants. We do not propose to end the controversy over adaptive mutation. However, we present evidence that natural selection was the primary basis for the accumulation of deletion mutants in this system.

The choice of *S. mutans* was based on ongoing studies of the virulence mechanisms of this species. Already, it was known that the most prominent virulence attributes of *S. mutans* were its ability to produce copious amounts of acid from the fermentation of dietary carbohydrates, its acid tolerance, and its ability to utilize sucrose to synthesize polymers of glucose, called glucans, that promote adhesion and accumulation on tooth surfaces (1). Due to the central role of glucans, *S. mutans* proteins possessing an affinity for glucan have been proposed to contribute to the establishment of the dental plaque biofilm and have been designated Gbps.

The Gbps of *Streptococcus mutans* include the Gtfs, responsible for catalyzing the synthesis of extracellular glucan, and non-Gtf Gbps that piggyback onto glucan while making a variety of contributions to the biology of the organism. In particular, GbpA has been reported to affect the depth and distribution of microcolonies (aggregates of organisms that extend outward from the substratum surface) within in vitro biofilms formed by *S. mutans* (6). It is likely that the change in biofilm architecture extends to the in vivo environment, as rats monoinfected with a *gbpA* strain of *S. mutans* displayed a higher caries rate than the wild type and accumulated deletion mutants that had undergone a recombination between two *gtf* genes (7). The accumulation of deletion mutants, designated *gtfBC* mutants, averaged 22% and mitigated the increase in virulence and resulted in a partial restoration of the biofilm phenotype associated with the loss of GbpA.

In vitro, the RecA-dependent recombination of the nearly identical *gtfB* and *gtfC* genes occurs at a frequency of 10^{-3} and results in the formation of a single Gtf that catalyzes the synthesis of mainly water-soluble glucans (14). It is specu-

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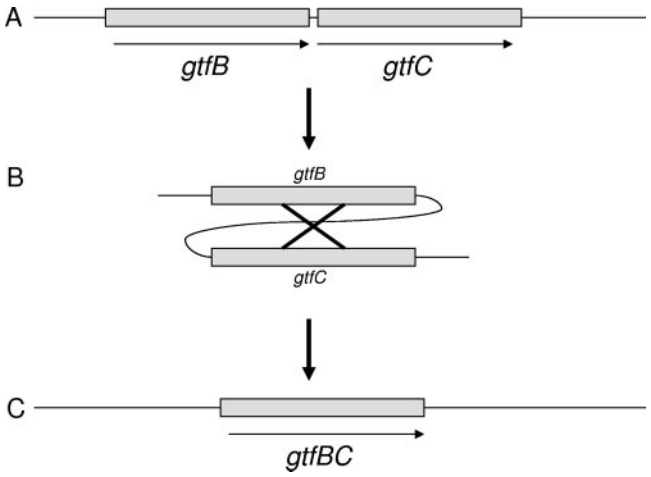

FIG. 1. (A) The *S. mutans* chromosomal *gtfB* (4.4-kb) and *gtfC* (4.2-kb) genes share 98% identity over a 2,000-bp stretch (15) between the regions encoding the signal peptide and the glucan-binding domain. (B) Homologous recombination occurs at random sites within the highly homologous regions in the two genes. (C) Due to the high level of homology, the precise sites of the deletion are difficult to determine. However, the single hybrid gene that results is thought to encode the *gtfB* active site and the *gtfC* glucan-binding domain. The resulting hybrid *gtfBC* gene encodes a Gtf that retains enzymatic activity, though the absolute amount of activity of each clone may vary. The glucan product is more similar to that catalyzed by the *gtfC* product than the *gtfB* product.

lated that *gtfBC* mutants may be more likely to dissociate from a biofilm, allowing them to colonize other sites. In rats colonized with *gbpA* mutants, the accumulation of *gtfBC* mutants may be a response to increased stress within the altered biofilm. The accumulation of *gtfBC* mutants may be explained by natural selection, an increased frequency of recombination, or a combination of both factors. The importance of distinguishing between these possibilities is fundamental to understanding the mechanism behind the contributions of GbpA and to understanding the evolutionary forces that have an impact on biofilm ecology. An increase in recombination frequency would indicate a direct or indirect influence on gene expression or enzyme activity related to basic cellular functions, like recombination. Confirmation of natural selection would suggest that a major contribution of GbpA is to the development of an optimal plaque biofilm that minimizes the stress to the bacterial population as a whole. Accordingly, experiments were undertaken to distinguish between these possibilities.

In an animal study designed to investigate the virulence of a *gbpA* strain of *S. mutans*, the accumulation of *gtfBC* mutants in *gbpA* mutant-colonized rats averaged 22% when the starting inoculum harbored approximately 0.001% *gtfBC* mutants (7). This accumulation of deletion mutants occurred over a thirty-day period of colonization. To begin to understand the forces behind the ascendancy of *gtfBC* mutants, another rat infection study was carried out; in this study, the inocula were varied with respect to the ratio of *gtfBC* deletion to nondeletion *gbpA* strains of *S. mutans*. The infection protocol was as described before (7) and involved inoculating germfree rats by swabbing their teeth on two consecutive days, allowing the rats to feed on a 5% sucrose diet ad libitum for thirty days, and then collecting plaque samples to enumerate the proportion of deletion mutants. The *gbpA* strain was engineered to harbor a kanamycin resistance cassette so that new deletion mutants could be distinguished from *gtfBC* mutants that were part of the original inoculum. Deletion mutants were recognized by their smooth-colony morphology on mitis salivarius agar plates containing sucrose (7). As shown in Table 1, when the *gtfBC* mutants were in the majority in the inoculum, their percentages decreased by the end of thirty days, whereas if the starting percentage was only 25%, their proportion increased. Reversion of the *gtfBC* deletion to the wild type was not possible, but there was a finite possibility that intact *gtfB* and *gtfC* genes could be reintroduced via natural transformation. When the inoculum was 100% *gtfBC* deletion mutants, no rough colonies (indicative of wild-type *gtf* genes) were detected after thirty days. New *gtfBC* mutants were minimal, indicating that changes in the proportions of *gtfBC* mutants were strongly influenced by natural selection. The observation that proportions of *gtfBC* mutants moved higher or lower depending on their starting percentage indicates that neither strain enjoyed a clear growth advantage in vivo and that natural selection could work to reduce the population of deletion mutants as well as increase their representation.

Next, the in vitro accumulation of *gtfBC* mutants was examined. It was reasoned that, if the loss of GbpA affected recombination frequency, the change in frequency might be detected in both planktonic and biofilm cultures. Alternatively, if natural selection was the primary force behind the accumulation of *gtfBC* mutants, then greater recovery of deletion mutants should take place only from biofilm cultures. For these experiments, two-day biofilm or planktonic cultures were grown with a change of medium after 24 h. (For planktonic cultures, the bacteria were pelleted by centrifugation prior to the addition of new prewarmed medium). The cultures were then sonicated to break up bacterial aggregates, diluted, and plated to obtain isolated colonies (30 to 300 per plate) on brain heart infusion medium with 1% sucrose. Smooth and rough colonies were then enumerated and are presented in Table 2. The highest frequency for recovering *gtfBC* mutants was from biofilm cultures of the *gbpA* strain. This frequency was significantly higher than that from planktonic cultures of the *gbpA* strain as determined by a chi-square analysis. Conversely, there was no statistical difference between the frequencies of recovering *gtfBC* mutants from planktonic and biofilm cultures of wild-type *S.*

TABLE 1. Changes in the percentages of *gtfBC* deletion mutants over time in rats

% *gtfBC* mutants		Frequency of new *gtfBC* mutants[a]
Initial inoculum	After 30 days	
25	34	Not detected
64	48	0.004
81	51	Not detected
96	69	0.020
100	100	

[a] New *gtfBC* mutants are those arising from organisms in the initial inoculum, which started with intact *gtfB* and *gtfC* genes. The new *gtfBC* mutants could be distinguished from the initial *gtfBC* mutant population by selection on medium containing kanamycin.

TABLE 2. Frequency of recovery of *gtfBC* deletion mutants in vitro

Strain and growth condition (culture)	No. of *gtfBC* mutants	Total no. of colonies	Frequency of recovery of *gtfBC* mutants
Wild type, planktonic	43	6,630	0.006
Wild type, biofilm	48	5,232	0.009
gbpA mutant, planktonic	37	6,151	0.006
gbpA mutant, biofilm	66	5,266	0.013

mutans. No deletion mutants were recovered from planktonic or biofilm cultures of a *recA* strain.

One possible basis for the observed natural selection was that pH differences existed between cultures of the two strains. To be sure that the loss of GbpA did not result in a greater reduction in pH, the terminal pH was measured in the planktonic phase above biofilms formed by the *gbpA* and wild-type strains of *S. mutans*. No difference was detected (4.3 for the wild-type versus 4.4 for the *gbpA* strain; $n = 4$). The rate of pH decline also did not differ between the two strains. If pH is a factor in the selection for *gtfBC* mutants, it must be so within localized areas of the biofilm.

Localized differences in pH are a possibility, since the loss of GbpA altered biofilm architecture (6), and rats colonized with the *gbpA* strain of *S. mutans* experienced a higher caries rate than rats colonized with the wild type (7). The accumulation of *gtfBC* deletion mutants (averaging 22%) mitigated both of these phenotypes (6, 7). It could be speculated that the accumulation of deletion mutants was due to different selection forces that accompany the change in biofilm architecture or a difference in the acid response of the *gbpA* strain.

The latter possibility was investigated by utilizing two-dimensional gel electrophoresis to compare proteins recovered from biofilms formed by the *gbpA* and wild-type strains. Several spots differed between the strains (Fig. 2). An exhaustive proteomic comparison was not an objective; however, several spots corresponding to the greatest differences in magnitude between strains were sent out for identification (Steven H. Seeholzer, Fox Chase Cancer Institute, Philadelphia, PA). Interestingly, unique or more intense spots from the *gbpA* mutant biofilms included two isoforms of RecA. These results provided the first evidence that the frequency of recombination, in addition to natural selection, could be a factor contributing to the accumulation of *gtfBC* mutants.

TABLE 3. Gene expression ratios for wild-type and *gbpA* strains of *S. mutans*[a]

Trial no.	Gene expression ratio for:	
	recA[b]	*gyrA*[c]
1	−1.39	1
2	−1.93	1
3	−1.40	1
Avg	−1.44	1
SD	0.25	

[a] RNA isolation, purification, and real-time PCR were performed as previously described (10). The expression ratios were calculated using the Pfaffl method (12). For this experiment, the ratios of gene expression were calculated from the critical-threshold values for the 400-pg cDNA dilutions.
[b] Primers (113-bp product) for *recA* were 5′-TGATGTCCGTGGCAATAC T-3′ and 5′-CCTTAAATGGTGGAGCAACTT-3′.
[c] Primers (123-bp product) for *gyrA* were 5′-AACGCGTGACCTAATGGAA GT-3′ and 5′-CGAGCACGCAAAACAATAGA-3′.

To determine if the protein spots coincided with an increased transcription of the *recA* gene, the relative expression of *recA* within *gbpA* or wild-type biofilms was measured via real-time reverse transcription-PCR. Conditions for biofilm growth matched those employed for the two-dimensional gel analysis. Instead of an increase, a slight decrease in transcription of *recA* was detected when normalized to the expression of the *gyrA* gene (Table 3). This decrease was not statistically

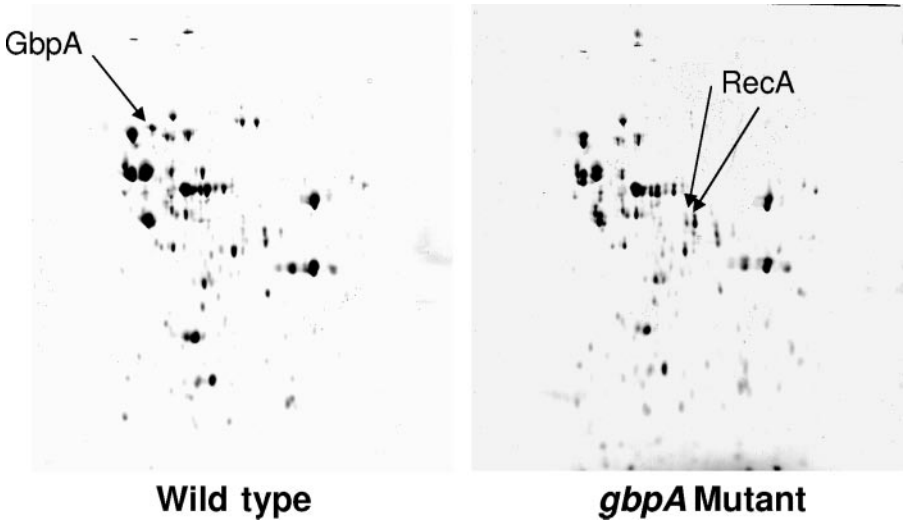

FIG. 2. Two-dimensional gel electrophoresis of proteins recovered from 48-h biofilms formed by the wild-type (left) and *gbpA* mutant (right) strains of *Streptococcus mutans*. The first-dimension isoelectric focusing employed a pH range of 4 to 8. The second-dimension sodium dodecyl sulfate-polyacrylamide gel electrophoresis was on an 8% polyacrylamide gel that was run until the dye front migrated off the gel. The gels were stained with Coomassie brilliant blue. The GbpA protein in the gel of wild-type proteins and the unique RecA isoforms in the gel of mutant proteins are indicated by arrows. Protein spots of interest were directly excised from the gel and sent to the Fox Chase Institute (Philadelphia, PA) for identification using mass spectrometry.

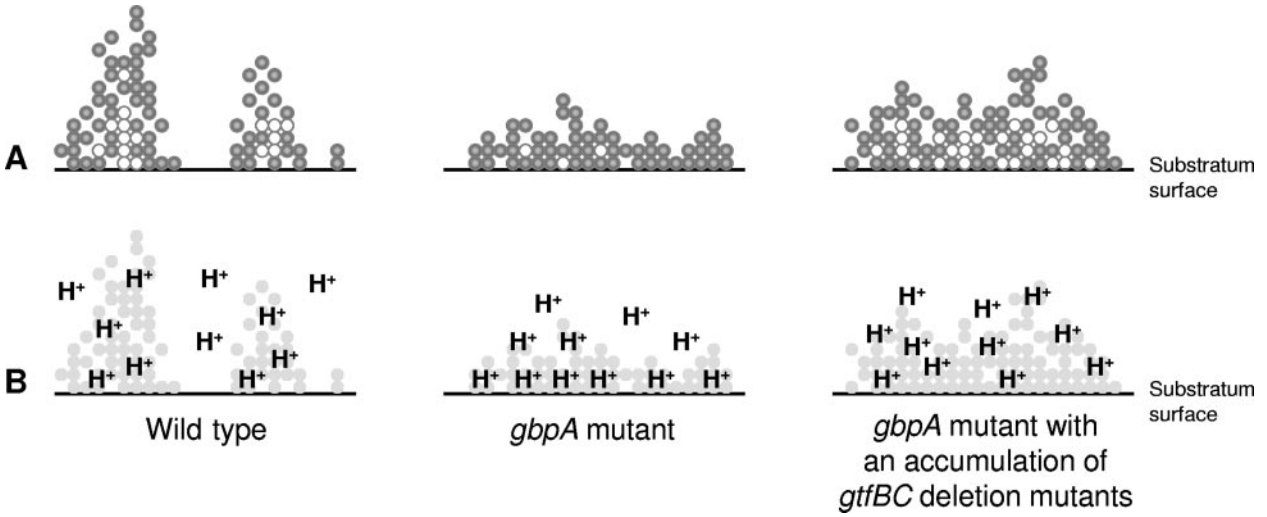

FIG. 3. A model of biofilm architecture (A) and acid distribution (B) to explain the natural selection of *gtfBC* deletion mutants. In part A, the filled circles represent metabolically active bacteria and the unfilled circles represent metabolically less active organisms. In biofilms formed by the wild-type strain, microcolonies build outward from the substratum surface (left panel). Metabolically less active organisms are confined to the inner portions of microcolonies. There are portions of the substratum that are only sparsely coated with bacteria. When GbpA is missing, *S. mutans* loses the ability to build tall microcolonies and the substratum is more evenly coated (middle panel). Consequently, there are higher numbers of metabolically active bacteria in contact with the substratum surface and a greater acid burden that could explain the higher caries rates of the *gbpA* mutant in rats. But this biofilm is more stressful for the bacteria, and so there is a selection for *gtfBC* mutants that can partially restore the biofilm architecture (right panel). The restoration of architecture adds depth to the biofilm that, in turn, sequesters some bacteria in contact with the substratum, rendering them less metabolically active and leading to a more benign distribution of acid molecules.

significant. Several groups have catalogued changes in gene or protein expression in response to pH in *S. mutans* (4, 9, 16, 17) or in other species (5, 11). One of these (9) reports an increase in RecA in response to acidic pH. This may have occurred in our system, but we compared expression levels only between strains grown under identical conditions, not between pH levels. We cannot rule out the possibility that the unique RecA isoforms in the *gbpA* mutant biofilms catalyzed recombination more efficiently or that there was a transient increase in *recA* expression in the *gbpA* biofilm at an earlier time point.

Cumulatively, the results support the conclusion that natural selection is the predominant force behind the accumulation of *gtfBC* mutants in biofilms formed by a *gbpA* strain of *S. mutans*. The in vivo fluctuations in *gtfBC* mutant proportions were almost exclusively independent of new recombination events. In vitro, *gtfBC* mutants were recovered at the highest frequency from biofilms formed by the *gbpA* strain, and this frequency was statistically higher than the recovery from *gbpA* planktonic cultures. We believe that the change in biofilm architecture that accompanies the loss of GbpA yields the conditions that drive the natural selection of *gtfBC* mutants. A model is proposed in Fig. 3. A consequence of the change in biofilm structure may be local pH drops that overwhelm the ability of the organism to adapt to an acidic environment. Although unique RecA isoforms were identified in *gbpA* biofilms, the preponderance of data make it doubtful that recombination frequency plays more than an ancillary role in the accumulation of *gtfBC* mutants. The fact that the forces of natural selection change within the context of *gbpA* biofilms highlights the contribution GbpA provides to the formation of an optimal biofilm environment. The presence of the contiguous, highly homologous *gtfB* and *gtfC* genes allows *S. mutans* to respond to biofilm stress without a need for adaptive mutation.

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