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ESCHERICHIA COLI STRAINS PRODUCING *STREPTOCOCCUS MUTANS* PROTEINS RESPONSIBLE FOR COLONIZATION AND VIRULENCE*

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INTRODUCTION

Streptococcus mutans is a principal etiologic agent of dental caries and is likely one of the most ubiquitous bacterial infectious disease agents worldwide.[1-3] The ability of *S. mutans* to colonize the oral cavity is due to sucrose-independent and sucrose-dependent adherence to the pellicle-coated tooth surface with glucan facilitated aggregation between cells to result in plaque. Cariogenicity is then caused by the ability of *S. mutans* in plaque to metabolize free sugars and both extra and intracellular complex carbohydrates to yield predominantly lactic acid.[1-3] The *S. mutans* gene products that contribute to colonizing ability and thus virulence include glucosyltransferases, glucan-binding proteins, and a diversity of less well-characterized cell-surface proteins and carbohydrate antigens that may also promote adherence or aggregation.

Until recently, *S. mutans* was unable to be analyzed genetically by classical methods of mutagenesis and gene transfer for mapping and complementation. We thus chose to use gene cloning technologies to introduce *S. mutans* genes into suitable strains of *Escherichia coli* K-12. *S. mutans* plasmid[4] and chromosomal[5-7] genes are expressed very well in *E. coli*. For example, the *S. mutans* gene for aspartic acid semialdehyde dehydrogenase possesses a very unique promoter sequence region that results in 7% of the total *E. coli* protein being the product of this one *S. mutans* gene.[5,7] Furthermore, *S. mutans* gene products can substitute for *E. coli* gene products that are missing because of the presence of gene mutations or deletions in the *E. coli* recipient strain.[5,7] In that regard, Perry and Kuramitsu[8] have developed a method for transformation of *S. mutans* strain GS-5 (serotype *c*), making it possible to mutate *S. mutans* genes cloned in *E. coli*, to return them to *S. mutans* and then to examine the effect of a known mutation altering a well-characterized gene product on *S. mutans* virulence. Similarly, we have been able to use antibodies against *S. mutans* gene products made by

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recombinant *E. coli* strains to select *S. mutans* mutants lacking that gene product.

We have begun systematically to clone *S. mutans* genes encoding cell-surface proteins and then to determine the contribution of these gene products to the ability of *S. mutans* to colonize the tooth surface. An associated objective is to use some of the *S. mutans* gene products synthesized by recombinant *E. coli* clones for the analysis of the immune response to *S. mutans* and for the development of an effective anti-caries vaccine.

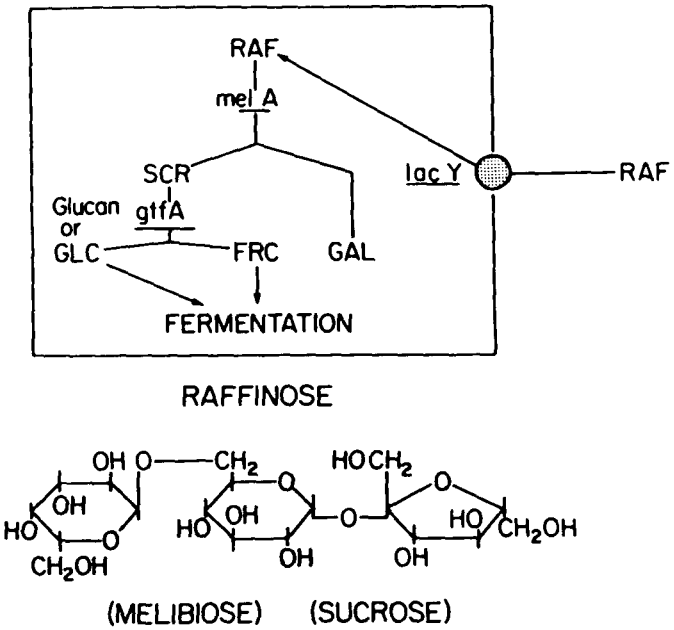

FIGURE 1. Strategy for successful cloning of *S. mutans* genes specifying enzymes for hydrolysis of sucrose into strains of *E. coli* K-12 (see text).

RESULTS

Cloning of S. mutans Genes with Sucrose Hydrolyzing Activity

E. coli is unable to metabolize sucrose because of the absence of enzymes with invertase-like activity and furthermore is unable to transport sucrose efficiently across the cytoplasmic membrane. Because we were uncertain whether *S. mutans* gene products with sucrose hydrolyzing activity would leave the cytoplasm, it was necessary to devise a means to present sucrose to such enzymes in the *E. coli* cytoplasm. FIGURE 1 depicts the strategy for accomplishing this. The trisaccharide raffinose is an α-galactoside containing galactose and glucose in an $\alpha-1\rightarrow6$ linkage; thus raffinose can be transported across the *E. coli* cytoplasmic membrane by way of the galactoside permease that is the product of the *lac*Y gene. Raffinose can then induce expression of the genes in the *mel* operon with the *melA* gene specifying an α-galactosidase that cleaves raffinose to yield galactose and sucrose. We thus shotgun cloned *S. mutans* PS14 (serotype *c*) DNA using

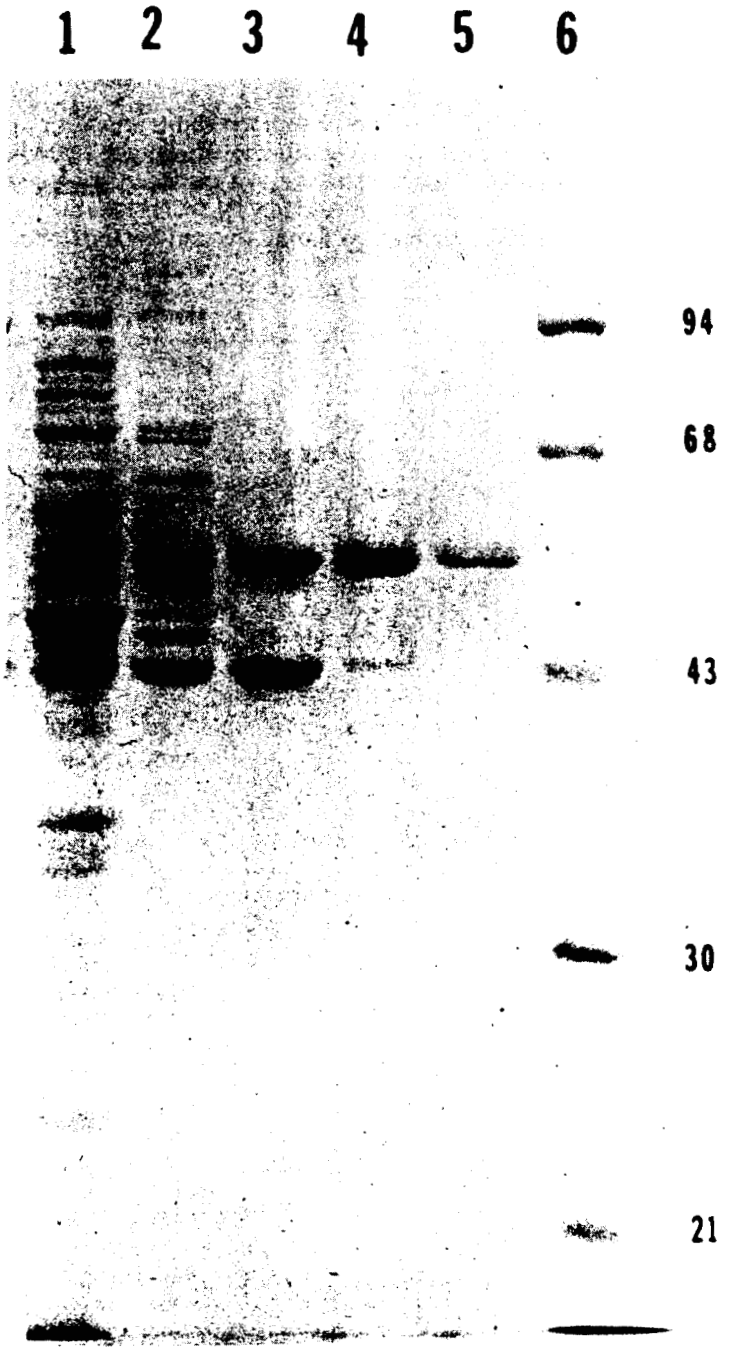

FIGURE 2. SDS-polyacrylamide gel electrophoresis of proteins in fractions containing *gtfA* enzyme activity at different stages of purification. Activity was measured by generation of reducing sugars from sucrose.[9] Lane 1: crude extract of *E. coli* K-12 containing pYA601 obtained by sonication followed by sedimentation of cell debris. Lane 2: fractions after chromatography on a DE-52 column. Lane 3: fractions after chromatography on an Ultrogel AcA54 column. Lane 4: fractions after chromatography on an Ultrogel AcA44 column. Lane 5: purified *gtfA* enzyme following precipitation with 33% ammonium sulfate. Lane 6: molecular mass markers (kdal).

the pBR322 plasmid cloning vector into an *E. coli* strain possessing a deletion of the *gal* operon and selected recombinant clones able to grow on raffinose as the sole carbon source.[6,9] Treatment of *E. coli* cells able to grow on raffinose with toluene led to a linear increase in the amount of reducing sugar generated over time when sucrose was used as a substrate. One of the recombinant *E. coli* clones with sucrose hydrolyzing activity contained a recombinant plasmid designated pYA601. Analysis of proteins specified by the recombinant plasmid, pYA601, using purified minicells containing the plasmid, revealed the presence of a 55,000 molecular weight protein encoded by 1730 base-pair fragment of *S. mutans* DNA. Based on the molecular weight of this protein, a strategy was devised for its purification to homogeneity (FIGURE 2). This purified protein could then be used

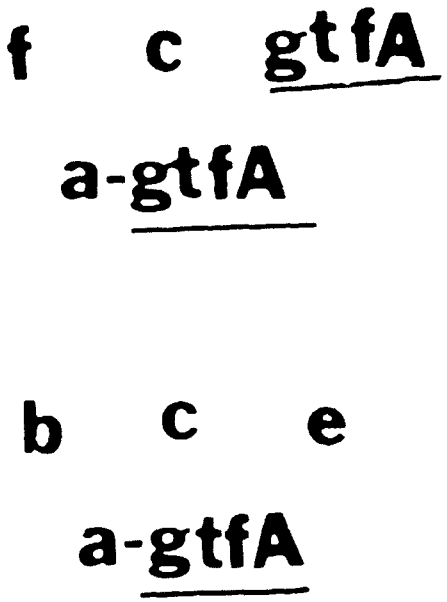

FIGURE 3. Immunodiffusion analysis employing antibodies against purified *gtfA* protein produced by *E. coli* (a-*gtfA*) and extracts of cell-surface proteins obtained from *S. mutans* strains in serotypes *b*, *c*, *e*, and *f* and the purified *gtfA* protein from the serotype *c S. mutans* strain, PS14.

to prepare monospecific and monoclonal antibodies against the protein as well as to characterize the protein for its enzyme activity.

The protein encoded by pYA601 is a glucosyltransferase[6,9] that hydrolyzes sucrose to synthesize a glucan polymer and an equivalent amount of fructose. Antibody against the product of this gene that we have designated *gtfA* reacts with immunologically identical proteins from serotypes *c*, *e*, and *f S. mutans* strains and cross-reacts with a protein from serotype *b S. mutans* (FIGURE 3). No reaction was detected with proteins produced by *S. mutans* serotypes *a*, *d*, or *g*.

The purified glucosyltransferase has a K_m of 1.2 *mM* for sucrose, a pH optimum of about 6.5 and hydrolyzes sucrose to a water-soluble glucan of low

molecular weight and fructose. This glucosyltransferase does not require a primer, and the protein is transported across the *E. coli* cytoplasmic membrane into the periplasm without processing or modification. Thus, *E. coli* containing the pYA601 plasmid can grow on sucrose because sucrose is transported across the outer membrane into the *E. coli* periplasm. *E. coli* recombinants containing pYA601 synthesize about 100,000 molecules of the *gtfA* enzyme per cell. This fact accounts for about 3 to 4% of the total *E. coli* soluble protein.

Cloning of S. mutans Genes Specifying Cell-Surface Proteins Lacking Enzyme Activity

DNA from *S. mutans* strain 6715 (serotype g) was shotgun cloned into the cosmid vector, pJC74,[10] so as to reduce the size of the clone bank that would need to be screened for expression of *S. mutans* cell-surface protein antigens. The cosmids packaged *in vitro* into infectious λ-phage particles were introduced into an *E. coli* strain lysogenic for the λ*cI857* thermo-inducible prophage and plated at 30° C. Colonies were patched onto an agarose medium containing high titer antisera raised against *S. mutans* cell-surface proteins, and after overnight incubation at 30° C, the plates were shifted to 42° C to cause thermo-induction of the λ-prophage leading to lysis of cells and release of protein antigens that they contain.[11] Precipitin rings formed around those colonies expressing a protein antigen reacting with the antibodies in the minimal agarose medium.[6,12] One of the recombinant clones produced a strong precipitin band and has been studied most extensively. This clone contained the recombinant cosmid, pYA721, that had an 8.3 kilobase insert of *S. mutans* 6715 DNA. This insert was subsequently cloned into the plasmid vector, pACYC184, to yield the recombinant plasmid, pYA726.[12] We have designated the gene specifying this surface protein antigen as *spaA* and have purified the *spaA* protein to homogeneity both from *S. mutans* and from *E. coli* recombinants. The protein purified from *S. mutans* has an apparent molecular weight of 210,000 by SDS-polyacrylamide gel electrophoresis and of

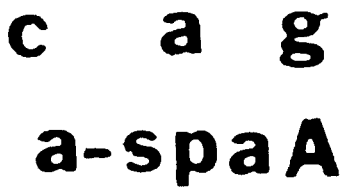

FIGURE 4. Immunodiffusion analysis of cell-surface proteins produced by *S. mutans* serotypes *a*, *c*, and g against antisera raised to purified *spaA* protein (a-*spaA*). It should be noted that proteins from serotypes *c*, *e*, and *f* are all identical in regard to response to a-*spaA* serum as are the proteins produced by serotypes *d* and g *S. mutans* strains.

TABLE 1

PRESENCE OF ANTIGENS CROSS-REACTING WITH 6715 SPAA PROTEIN AMONG THE VARIOUS SEROTYPES OF *S. MUTANS*

Strain	Serotype	Supernatant Fluid*	Cell Surface†
HS6	*a*	Yes	Yes
BHT	b	No	No
Ingbritt	c	Yes	Yes
OMZ176	*d*	Yes	Yes
LM7	e	Yes	Yes
OMZ175	*f*	Yes	Yes
6715	g	Yes	Yes

*Extracellular protein fractions of the various *S. mutans* serotypes grown in defined media[12] were prepared from cell-free culture supernatant fluids and were examined for antigens cross-reacting with the *spaA* protein of *S. mutans* 6715 by immunodiffusion analysis.

†*S. mutans* cells of the various serotypes were grown in defined media[12] and washed with 5 *M* NaCl. The presence of cell-surface antigens cross-reacting with the *spaA* protein of *S. mutans* 6715 was determined by direct immunofluoresence using rhodamine-conjugated a-*spaA* as a probe.

180,000 when produced by *E. coli*. *E. coli* cells with the *spaA* gene produce 7% of their total soluble protein as the *spaA* protein and translocate 60% of this protein to the periplasmic space.[12] The *spaA* protein reacts with antibody against the *spaA* protein as well as with antibody against the antigen I/II, purified and characterized from serotype *c S. mutans* strains by Russell, *et al.*[13] In addition, antibody purified against the *spaA* protein reacts with antigen I/II from serotype c and also with antigens produced by serotypes *a*, *d*, e, *f*, and g, but not by b strains of *S. mutans* (FIGURE 4, TABLE 1). The *spaA* protein produced by *S. mutans* contains an antigenic determinant not present on the *spaA* protein produced by *E. coli* recombinants. This phenomenon is possibly due to the absence of carbohydrate antigenic determinants on the *E. coli*-produced gene product. This hypothesis is currently under test. The *spaA* protein is found free in the supernatant fluid of *S. mutans* strains grown in FMC medium[12] (TABLE 1), but is also found to be tightly bound to the cell surface even after washing in 5 *M* NaCl. By using antibodies directed against the *spaA* protein, it has been shown that these antibodies will inhibit sucrose-induced aggregation between *S. mutans* cells. Furthermore, *S. mutans* mutants lacking the *spaA* protein as selected by using antiserum against the *spaA* protein are also defective in aggregation.

DISCUSSION

We have found that a diversity of *S. mutans* genes for cell-surface protein antigens from both serotype *c* and serotype g strains are expressed very well in strains of *E. coli* K-12. Furthermore, most of these *S. mutans* cell-surface proteins are able to be translocated across the *E. coli* cytoplasmic membrane into the *E. coli* periplasm. Because of this fact, we are continuing to identify and characterize other *S. mutans* cell-surface protein antigens produced by recombinant *E. coli* strains. Analysis of these proteins is facilitated by the fact that there are few, if any, *E. coli* proteins that cross-react with *S. mutans* proteins or vice versa. Monoclonal antibodies against the *spaA* and *gtfA* proteins have been produced

from hybridomas,[14] and these monoclonal antibodies are being used to facilitate purification of the *spaA* and *gtfA* proteins by immunoabsorbent chromatography. It should be pointed out that relatively satisfactory methods for the purification of these proteins from *E. coli*, as well as *S. mutans*, have been worked out.

Other workers have used purified glucosyltransferases[15-17] and the antigen I/II protein,[13] also termed B,[18,19] from serotype c and g *S. mutans* strains to immunize rats, hamsters, and Rhesus monkeys to evaluate induction of immunity to *S. mutans*-induced dental caries. Some,[15,19] but not all,[16,17] of these studies gave promising results.

Because of these previous studies, the purified *S. mutans* cell-surface proteins are being used in several interrelated investigations. Dr. Michalek, Dr. McGhee, and their colleagues are using these proteins, as well as lipoteichoic acid and type-specific carbohydrate antigens, to screen humans as a function of age for titers of various Ig isotypes in saliva and serum. Both enzyme-linked immunosorbent assays (ELISA) and radioimmunoassays (RIA) have been developed for most of these *S. mutans* cell-surface proteins. These individuals are also being evaluated for caries incidence, and for titers and serotypes of *S. mutans* in plaque. High titers of antibodies, especially secretory IgA, against one or more of these antigens in conjunction with low titers of *S. mutans* in plaque and/or low incidence of dental caries would be suggestive of protein antigens that should be explored for their ability to induce protective immunity against *S. mutans*-induced dental caries.

In this last regard, we are collaborating with Dr. Mestecky, Dr. Michalek, Dr. McGhee, and their colleagues to use purified protein antigens for oral (by gastric intubation) and systemic immunization of mice and gnotobiotic rats to analyze the immune response and evaluate effective immunity to *S. mutans*-induced dental caries. We have also devised a new approach for oral immunization so as to stimulate cells of the Peyer's patches to result in a strong secretory IgA response. Specifically, we are starting with enteric pathogens that are able to attach to and invade cells of the gut-associated lymphoreticular tissue (GALT) and then rendering them avirulent by genetic manipulation as used in our development of the safer *E. coli* host, χ1776, for recombinant DNA research.[20] These avirulent pathogens can then be endowed with genetic information to permit high-level production of *S. mutans* cell-surface protein antigens, and because of their ability to attach to and invade cells of the GALT, we anticipate that a secretory immune response against these *S. mutans* protein antigens will be induced. If so, a safe effective vaccine against *S. mutans*-induced dental caries should become a reality. In addition, this method would also be applicable to vaccine construction and immunization to preclude infection by any viral, bacterial, mycotic, or parasitic agent that invades a mucosal surface.

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DISCUSSION OF THE PAPER

T. LEHNER (*Guy's Hospital, London, England*): I am impressed by the cross-reactivity between serotypes, except for serotype *b*, which is a rat strain. Can you

tell us whether there is complete or partial cross reactivity, because you have not shown us all the data on this situation?

R. CURTISS (*University of Alabama in Birmingham*): Only partial cross-reactivity occurs here. Hopefully by using the cloned genes from serotypes *c*, *a*, and g, we will be able to determine what particular domains in this very large protein are shared and those that are dissimilar.

LEHNER: It also appears that this protein has the two antigen determinants that we called antigens I and II. Have you tried to digest away the antigen I component of this antigen I/II complex to leave the antigen II behind?

CURTISS: Dr. Russell and Dr. Holt in our laboratory have shown that most of the similarity between the *spa A* proteins from serotypes *c* and g seem to be in the antigen I component.

LEHNER: I was going to ask about the considerable ease with which you have identified the antigen I/II. What about the antigen III/IV? Have you found any other synthesized by *E. coli* that you could use?

CURTISS: We have quite a diversity of recombinant clones that produce at least 10 different *S. mutans* cell-surface proteins, and we just have not characterized all of these. They do not have glucosyltransferase activity. One of the problems is that we have not used the antibodies against antigen III or IV to analyze any of our clones. This method might be the simplest way, assuming that they will also be cross-reactive.

L. J. SAIF (*Ohio Agricultural Research and Development Center*): For your future studies, if you used avirulent enteric strains, do you think that they, indeed, would be able to attach and colonize?

CURTISS: Yes. We are currently using derivatives of *Salmonella* and *Salmonella-Escherichia* hybrids. Others have used a *Salmonella* derivative that lacks the ability to produce enterochelin, and although it is avirulent, this strain still invades. The real problem with their mutant is that it ends up in the spleen, where it persists for several months. We are developing strains that will not get that far, but hopefully, will just hang up in the Peyer's patches.

M. BLAKE (*Rockefeller University, New York, N.Y.*): Do you have evidence that your *E. coli* regulates the genes that you are introducing?

CURTISS: Yes.

BLAKE: Do you have any difficulty with a posttranslational modification problem? Do you think that will be a problem in the future?

CURTISS: The answer to the first question is yes. We do get genes regulated in some cases. Ordinarily, they are constitutively expressed in *E. coli*, in fact, at a very high level. But, a cluster of three or four genes for galactose metabolism are coordinately regulated in *E. coli* in the same manner as they are in *S. mutans*. Although we thought we had an operon, we did not. There are at least three transcriptional units, and one of the gene products actually is a positive regulator acting to cause increased synthesis of the other enzymes in the pathway.

In terms of posttranslation modification, we now have antibodies that are directed at the unique antigenic determinants on some of these proteins made in *S. mutans* that are not present on the proteins made in *E. coli*. These antibodies are now being used to screen for cloning of the genes that would cause the proteins in *E. coli* to be modified. So, by using sequential cloning, we assume that we will be able to demonstrate the synthesis of glycoproteins by recombinant techniques, where we have to introduce genes for several enzymes from the donor—in this case *S. mutans*. This technique should then allow one to look at the different antigens with and without the modifications, in terms of their ability to induce a protective response.