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Galactose Oxidase-Glucan Binding Domain Fusion Proteins as Targeting Inhibitors of Dental Plaque Bacteria

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In order to inhibit the growth of bacteria present in the human oral cavity, a novel system which targets antimicrobial agents to dental plaque has been developed. This system involves a hybrid protein consisting of a peptide expressing the bactericidal properties of galactose oxidase (GAO) fused to the glucan binding domain (GBD) of the *Streptococcus mutans* glucosyltransferase-S enzyme. A gene encoding GAO from the fungus *Fusarium* sp. has been inserted into an *Escherichia coli* expression vector and fused to sequences encoding the GBD, which binds to the glucans synthesized by oral streptococci. Bacterial extracts expressing the hybrid protein were tested for their ability to target the GAO activity to an in vitro plaque model consisting of streptococcal cells bound to microtiter plate wells. The binding of the hybrid protein to the streptococcal cells through its GBD and the dependence of binding on the production of glucans by bacteria were demonstrated. Furthermore, killing of three different species of oral streptococci by bound hybrid protein in conjunction with the galactose-lactoperoxidase-iodide cytotoxic system has been demonstrated. These results suggest a novel strategy for controlling dental plaque formation as well as dental caries in humans.

Several chemotherapeutic agents have been utilized to inhibit the growth of cariogenic microorganisms present in dental plaque (2). Among these agents, iodine has been shown to be effective when applied topically on tooth surfaces (2). Another potent bactericidal agent, hypoiodite (OI^-), is produced by peroxidase using hydrogen peroxide and iodide as substrates (9). One potential approach for the use of hypoiodite as an antiplaque agent would be to deliver specifically to dental plaque an enzyme whose activity would ultimately lead to the local production of this antimicrobial agent. Such an antiplaque system would require coupling the effector activity to another component capable of targeting it specifically to dental plaque. The glucan binding domain (GBD) is a protein component which constitutes the carboxyl-terminal region of glucosyltransferase-S, the extracellular enzyme from *Streptococcus mutans* that catalyzes the synthesis of water-soluble glucans (4, 5). The GBD can be expressed as a separate peptide in *Escherichia coli*, and its strong affinity for glucans has been demonstrated (5a, 7). Since some of the streptococci colonizing the tooth surface produce large amounts of glucans from dietary sucrose (3), the GBD was chosen as a potential means of delivering an effector component to plaque. Glucans represent a significant component of human dental plaque and should be amenable to targeting of antimicrobial agents.

One potential antibacterial effector is the copper-containing enzyme galactose oxidase (GAO), which is produced and secreted by the fungus *Fusarium* sp. (1). This enzyme converts D-galactose and other substrates to their aldehyde forms, concomitantly producing hydrogen peroxide. In the proposed antiplaque system, after attachment of the GAO activity to surfaces colonized by cariogenic bacteria, the addition of D-galactose would stimulate local production of hydrogen peroxide. The latter could be utilized by lactoperoxidase, an enzyme present in saliva, to oxidize added iodide to hypoiodite (8, 9).

In the present communication we describe the construction of a gene encoding a hybrid protein composed of GAO and GBD, its expression in *E. coli*, and the evaluation of its potential to target and kill cariogenic bacteria present in an in vitro plaque model. This system represents a potential novel antiplaque strategy for controlling dental plaque formation in humans.

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MATERIALS AND METHODS

Bacterial strains, growth conditions, and preparation of extracts. *E. coli* DH5α was used for DNA manipulations, and strain GI698 (F^- λ^- *lacI*^{q} *lacPL8* *ampC*::P_{trp}*c*I) (Invitrogen, San Diego, Calif.) was used for expression of proteins. *Streptococcus* strains *S. sanguis* 10556, *S. gordonii* Challis, *S. mutans* GS-5, and *S. mutans* GS-5ΔBCDF (12a) were used for GAO targeting assays. Strain DH5α was grown in Luria-Bertani medium; streptococci were cultivated in Todd-Hewitt broth or on tryptic soy broth (TSB) agar plates.

Expression of proteins and isolation of bacterial extracts were performed with the ThioFusion Expression System (Invitrogen) according to the manufacturer's instructions. Extracts were supplemented with Cu^{2+} ions by either directly adding $CuSO_4$ to a final concentration of 0.4 mM or dialyzing against 0.4 mM $CuSO_4$ in phosphate-buffered saline (PBS; 0.8% NaCl, 0.02% KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4).

Construction of plasmids. A 178-bp *Eco*RI-*Bam*HI DNA fragment (Fig. 1A) from plasmid pGAO9 (10), containing the 5′ end of the sequence coding for mature GAO, was subcloned into vector pUC118. One of the two *Dde*I sites on this fragment, located downstream from the codon adjacent to a cleavage site of the leader peptide, was chosen as a point of fusion with the 5′ end of the *lacZ* fragment. The 800-bp *Dde*I fragment consisting of the subcloned *gao* sequence and part of pUC118 was excised and, after the ends were filled in with the Klenow fragment of DNA polymerase I, inserted into the *Sma*I site of vector pUC118Nar (a derivative of pUC118 lacking the *Nar*I site). The resulting plasmid, pO5, was digested with *Nar*I and *Xba*I, and a *Nar*I-*Xba*I DNA fragment from pGAO9, containing the 3′ end of the gene, was inserted, generating plasmid pR3 (Fig. 1B).

The fusion of the GAO and GBD coding sequences was accomplished by isolating from pGAO9 a 495-bp *Dde*I fragment whose 3′ end is located within the last codon of the *gao* gene, filling in its ends, and inserting it into the *Hin*cII site of the vector pUC119. The sequences encoding the GBD were inserted as a *Xba*I-*Eco*RI fragment excised from plasmid pKmOZ′19GBD (12a). The generated construct containing the 3′ end of the *gao* gene in frame with the sequence encoding the GBD was removed by digestion with *Eag*I and *Bam*HI and inserted into plasmid pR3 which had been cut with *Eag*I and *Bcl*II. The resulting plasmid, pU4 (Fig. 1B), contains a complete gene encoding the GAO-GBD hybrid protein under the control of the *lac* promoter.

Plasmids pTrxFusGAO and pTrxFusGAOGBD (Fig. 1C) were constructed in two steps. Initially, the *Kpn*I-*Pst*I fragment containing the 5′ end of the *gao* gene was inserted into the expression vector pTrxFus. Then, one of the two *Pst*I fragments, containing either the 3′ end of the *gao* gene (from pR3) or that of the gene encoding the hybrid protein (from pU4), was inserted into the single *Pst*I site.

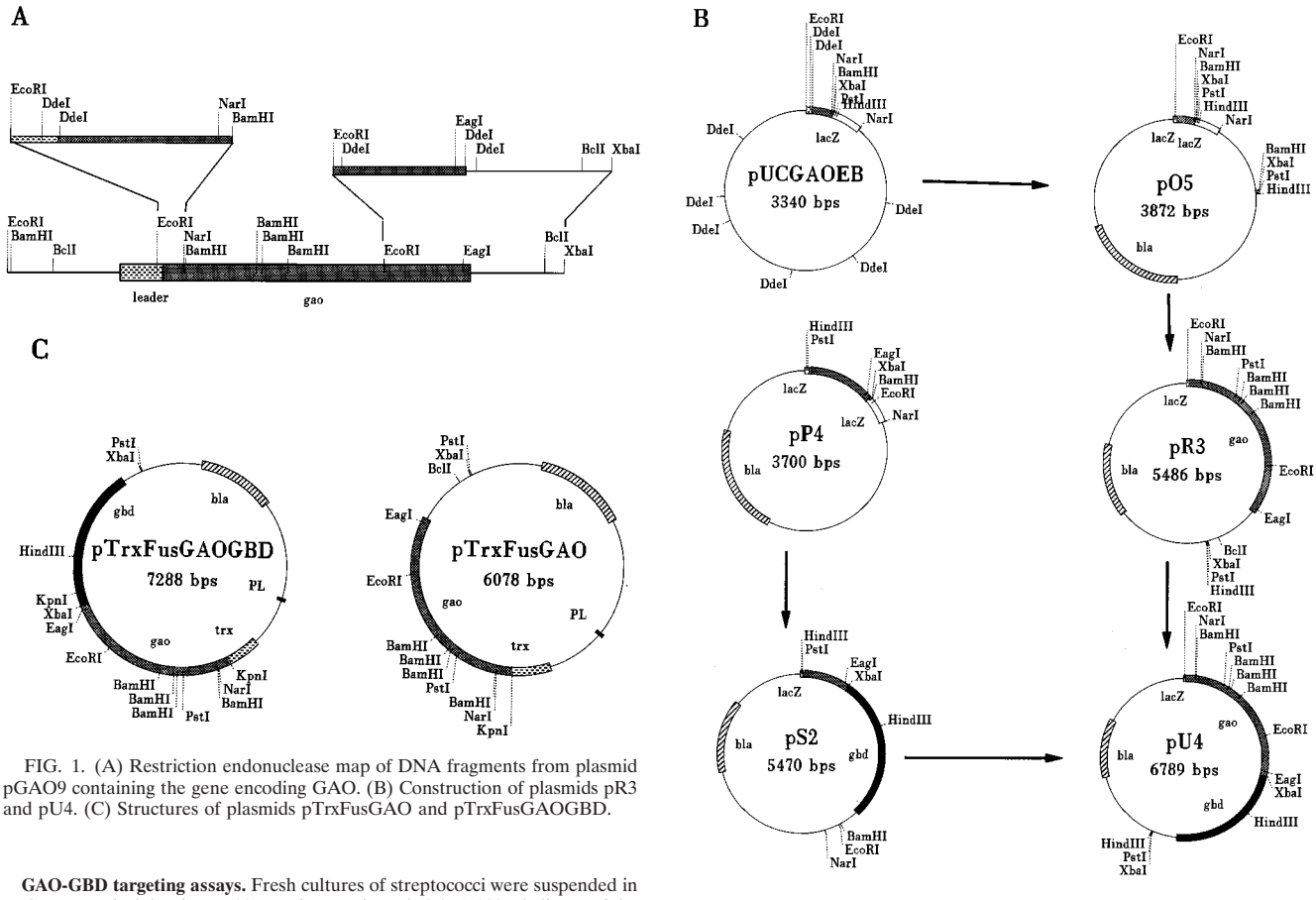

FIG. 1. (A) Restriction endonuclease map of DNA fragments from plasmid pGAO9 containing the gene encoding GAO. (B) Construction of plasmids pR3 and pU4. (C) Structures of plasmids pTrxFusGAO and pTrxFusGAOGBD.

GAO-GBD targeting assays. Fresh cultures of streptococci were suspended in PBS to an optical density at 630 nm of approximately 0.2. A 200-μl aliquot of the suspension was added to each microtiter plate well, and the plate was incubated for 18 to 24 h at room temperature. The suspension, containing unbound cells, was removed from each well by vacuum aspiration, 100 μl of 10 mM sodium acetate buffer (pH 6.0) or that buffer plus 5% sucrose (0.5% in experiments performed with *S. mutans*) was added to each well, and the plate was incubated overnight at 37°C. After removal of the buffer, each well was filled with an *E. coli* extract. All extracts were adjusted to a protein concentration of approximately 1.9 mg/ml in PBS. Following incubation of the plate for 2 to 4 h at room temperature and removal of extracts, each well was washed three times with 200 μl of PBS containing 0.05% Triton X-100.

The GAO activity bound to the bacterial cells was monitored by adding 100 μl of reaction solution (5 mg of galactose, 50 μg of horseradish peroxidase, and 50 μg of *o*-dianisidine per ml in 0.1 M sodium phosphate buffer, pH 7.0) (14) and measuring the absorbance at 490 nm in a microplate reader at various time intervals.

The bactericidal activity of bound GAO was assessed by incubating the cells for 2 to 6 h at room temperature after filling each well with 100 μl of a solution containing 5 mg of galactose per ml, 10 μg of lactoperoxidase per ml, and 5 mM KI in 0.1 M sodium phosphate buffer, pH 7.0. After the solutions were removed, each well was washed once with 200 μl of PBS. The cells bound to the bottoms of the wells were covered with Todd-Hewitt broth medium, detached by scraping with a micropipette tip, plated on TSB agar, and incubated for 1 to 2 days anaerobically at 37°C.

For the dextran competition assays, extracts containing the fusion proteins were mixed with appropriate concentrations of dextran T10 and incubated for 1 h at 4°C prior to being added to the wells containing bound streptococcal cells.

Gel electrophoresis. Bacterial extracts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% gels. Western blots were performed with antiglucosyltransferase-S (anti-GTF-S) antibody (13). For GAO activity staining, the gels were washed for 1 h in PBS containing 1% Triton X-100 and then incubated in GAO reaction solution (14), supplemented with 0.4 mM $CuSO_4$, for 1 to 18 h at room temperature.

RESULTS AND DISCUSSION

Expression of GAO and hybrid proteins in *E. coli*. In our initial attempts to express GAO in *E. coli* and to construct GAO-GBD fusions, we used derivatives of vector pUC118 (see Materials and Methods). In both constructs (named pR3 and pU4, respectively) the GAO sequence starts from the second codon of the mature protein, and in pR3 it ends at its original termination codon. In pU4 the GAO sequence is followed by an in-frame sequence encoding the GBD. When GAO alone was expressed in *E. coli* cells, its activity was readily detected, while extracts of bacteria expressing the hybrid protein exhibited relatively low (though detectable) GAO activity (data not shown).

In order to obtain a higher level of expression of the hybrid protein, we utilized the ThioFusion expression system, in which foreign proteins can be expressed from a λ P_L promoter fused to a 12-kDa thioredoxin peptide which confers solubility to heterologous proteins. The sequences encoding both GAO and the GBD fusion proteins were inserted into the vector pTrxFus as described in Materials and Methods. The extracts of bacteria containing the resultant plasmids, pTrxFusGAO and pTrxFusGAOGBD, both exhibited significant levels of GAO activity, but the activity of the former was severalfold higher than that of the latter (data not shown) (see also Fig. 3C).

In contrast to an earlier report (11), which indicated that only enzyme containing a leader peptide sequence exhibited detectable GAO activity when expressed in a bacterial host, our findings demonstrated that active GAO in its mature form can be produced by *E. coli* cells. Furthermore, this activity may be dependent upon the additional amino acid sequences present at the N terminus of the constructs as well as supplementation of the extracts with Cu^{2+} ions.

The electrophoretic analysis of bacterial extracts (Fig. 2), involving GAO activity staining of the gels as well as Western

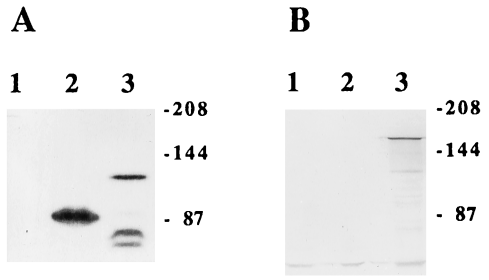

FIG. 2. GAO activity staining (A) and Western blotting with anti-GTF-S antibody (B) following SDS-PAGE of extracts of bacteria containing the following plasmids: pTrxFus (lanes 1), pTrxFusGAO (lanes 2), and pTrxFusGAOGBD (lanes 3). Molecular mass markers, in kilodaltons, are on the right.

blotting with anti-GTF-S serum (recognizing the GBD), revealed that the full-sized hybrid protein (158 kDa) did not appear to express detectable GAO activity. However, this activity was exhibited by a 122-kDa peptide visible as a distinct band on both activity-stained gels and Western blots. Two other, lower-molecular-weight bands exhibited activity following GAO staining but were not recognized by anti-GTF-S antibody. The latter peptides appear to be products of proteolytic cleavage of the fusion protein during the preparation of extracts. While it has not yet been demonstrated which part of the protein is proteolytically cleaved, a likely explanation for these results may be that the presence of an intact GBD affects the conformation of the whole protein, abolishing its enzymatic activity. In contrast, the extract expressing GAO demonstrated only a single band of approximately 90 kDa following activity staining.

The presence of the major inactive form of the hybrid protein may contribute to the marked difference in the GAO activities of the GAO and the GAO-GBD extracts. Another possible factor is the relatively low solubility of the GBD when expressed in *E. coli* from a strong promoter, which has been observed in our laboratory during previous studies of this domain.

The glucan binding capability of the 122-kDa peptide was confirmed in an experiment involving passage of the extracts through dextran T2000-Sepharose beads. The adsorbed proteins were eluted from the beads with electrophoresis loading buffer and analyzed for GAO activity following staining of SDS-PAGE gels (6). No GAO-positive protein other than the 122-kDa species was able to bind to dextran-Sepharose (data not shown).

Targeting of the GAO-GBD hybrid protein to glucan-streptococcus complexes. To initially assess the potential of the GAO-GBD hybrid protein to bind to dental plaque, we utilized microtiter plate wells covered by a layer of sucrose-grown streptococcal cells as an in vitro model plaque system. The levels of hybrid protein bound to the cellular matrix were measured as bound GAO activity in a peroxidase-coupled assay system (see Materials and Methods). Figure 3 depicts the binding of GAO and the GAO-GBD fusion protein to *S. sanguis* cells incubated with (Fig. 3A) or without (Fig. 3B) sucrose. In both cases, only negligible activity could be detected for in vitro plaque to which GAO was added, while the activity associated with the hybrid protein bound to the plaque was significant and depended on prior incubation of the cells with sucrose. The difference between the proteins is not due to the lower degree of stability of GAO, since the extract containing GAO still had severalfold higher activity than that containing the hybrid protein, as measured by direct enzyme assays following incubation at room temperature (Fig. 3C). The dependence of the binding of the hybrid protein on the presence of glucan synthesized from sucrose is consistent with our expectation that targeting of GAO activity to plaque would result from interaction between GBD and glucans. To further confirm this, we investigated the ability of dextrans (α-1,6-linked glucans) to bind to the GBD domain of the fusion protein and prevent binding of the fusion protein to the streptococcal cells. The extracts containing the hybrid protein were incubated with various concentrations of dextran T10 before cells were added. Concentrations of dextran T10 as low as 0.001% drastically reduced the targeting ability of the fusion protein (Fig. 4), while it had no effect on its galactose oxidase activity. Similar

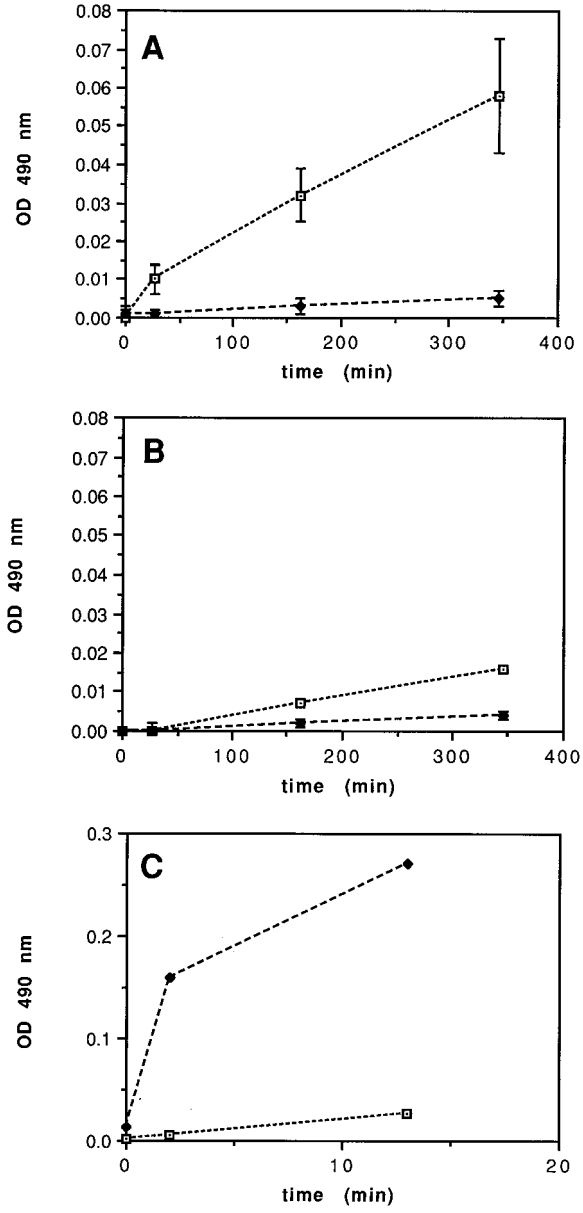

FIG. 3. GAO activities bound to *S. sanguis* cells incubated with (A) and without (B) sucrose, and direct GAO assay of extracts following incubation at room temperature (C). Symbols: ⊡, extracts containing GAO-GBD fusion; ◆, extracts containing GAO.

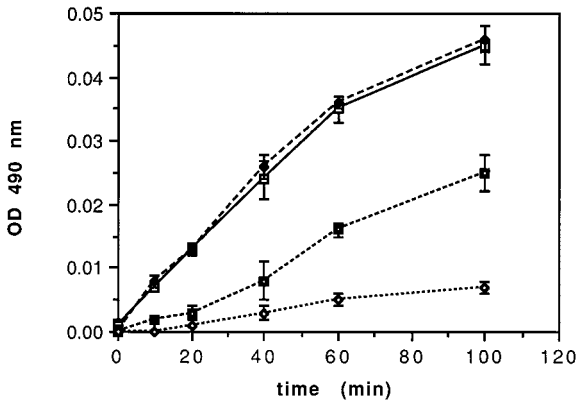

FIG. 4. Inhibition of binding of GAO activity in GAO-GBD extracts to *S. sanguis* cells by dextran T10. Concentrations of dextran are designated as follows: □, 0%; ◆, 0.0000001%; ■, 0.00001%; and ◆, 0.001%.

results were obtained when dextran T2000 was used (data not shown).

Antibacterial activity of the GAO-GBD hybrid protein. The antibacterial properties of the hybrid protein were determined as described for the targeting assays except that after the proteins were attached to streptococcal cells, the plates were incubated with a solution containing galactose, lactoperoxidase, and potassium iodide. The lactoperoxidase utilizes the hydrogen peroxide produced from galactose by GAO to convert iodide to bactericidal hypoiodite, resulting in the killing of bacterial cells (8, 9). The viability of bacteria was assessed following the plating of the plaque cells onto TSB agar plates. To *S. sanguis* cells attached to microtiter wells and incubated with (Fig. 5A) and without (Fig. 5B) sucrose were added extracts of *E. coli* carrying vector pTrxFus (bars 1 in Fig. 5) or pTrxFusGAO (bar 2 in Fig. 5A), each diluted 10-fold, or 10-, 33-, or 100-fold dilutions of extracts of bacteria containing plasmid pTrxFusGAOGBD (bars 3, 4, and 5 in Fig. 5A and 2, 3, and 4 in Fig. 5B, respectively). The viability of cells incubated with GAO alone was approximately 1 order of magnitude lower than that of the control cells. This may have resulted from nonspecific binding to the bacteria of GAO from extracts containing very high levels of the enzyme. The killing capability of extracts containing the fusion protein was dose dependent and was elevated with respect to cells previously incubated with sucrose. The killing of all bacteria in the absence of sucrose by 10-fold-diluted GAO-GBD extract (Fig. 5B, bar 2) suggests that small amounts of glucan are produced by the cells from trace amounts of sucrose in the growth medium before the cells attach to the microtiter plates. To further confirm the role of glucans in targeting the GAO-GBD fusion protein as well as to test the fusion protein on another dental plaque constituent, the targeting and killing of two strains of *S. mutans*, the wild-type strain GS-5 and its mutant, GS-5ΔBCDF, which is devoid of any GTF activity (12a), was examined (Fig. 6). Cells of both strains were incubated with sucrose prior to the addition of the extracts. While GAO alone did not affect the growth of either strain, the hybrid protein reduced the viability of only the wild-type strain, providing

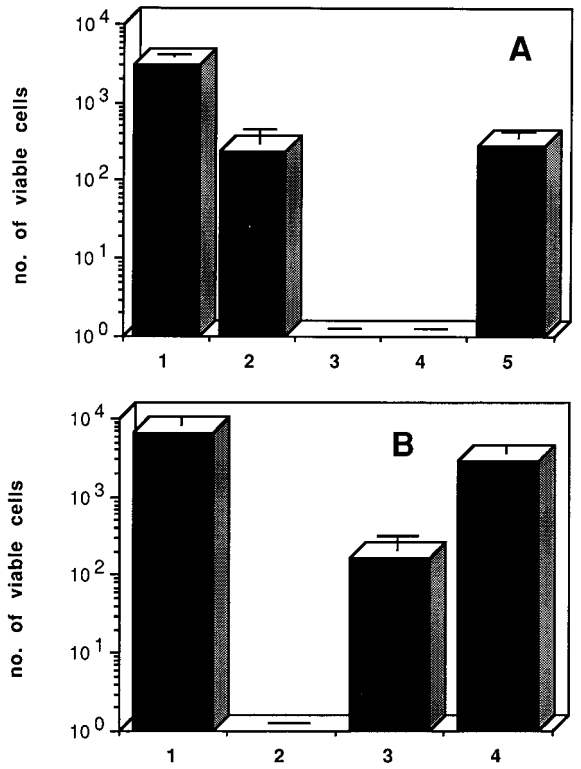

FIG. 5. Targeting and killing of *S. sanguis* cells incubated with (A) and without (B) sucrose prior to addition of extracts. (A) Cells were incubated with extracts of bacteria containing the following plasmids: pTrxFus (10-fold dilution), lane 1; pTrxFusGAO (10-fold dilution), lane 2; and pTrxFusGAOGBD (10-, 33-, and 100-fold dilutions, respectively), lanes 3 to 5. (B) Cells were incubated with extracts of bacteria continuing pTrxFus (10-fold dilution; lane 1) or pTrxFusGAOGBD (10-, 33-, and 100-fold dilutions, respectively; lanes 2 to 4).

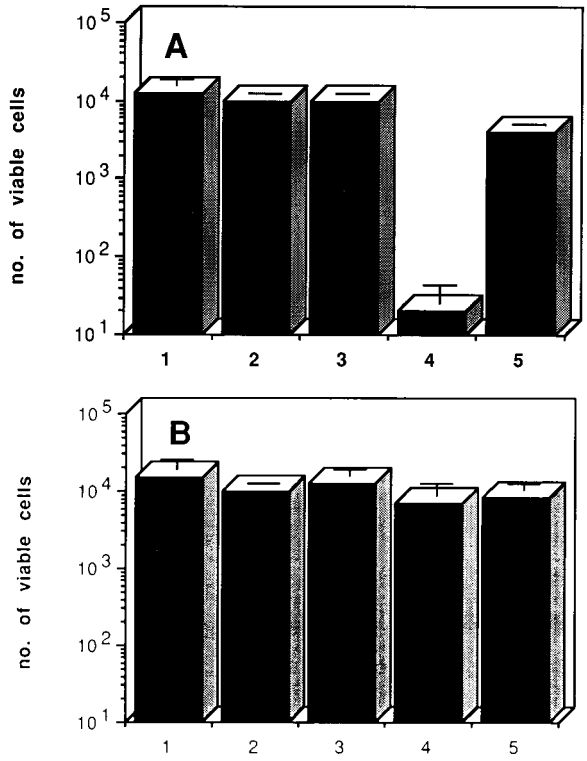

FIG. 6. Targeting and killing of two *S. mutans* strains: wild-type strain GS-5 (A) and GTF-deficient strain GS-5ΔBCDF (B). Cells were incubated with extracts of bacteria containing the following plasmids: pTrxFus (10-fold dilution), lane 1; pTrxFusGAO (10-fold dilution, lane 2); pTrxFusGAOGBD (10-fold dilution), lanes 3 and 4; and pTrxFusGAOGBD (33-fold dilution), lane 5. In lane 3, lactoperoxidase was omitted during incubation with the killing solution.

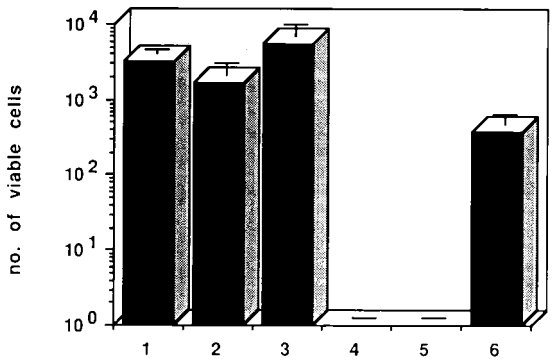

FIG. 7. Targeting and killing of *S. gordonii* cells incubated with extracts of bacteria containing the following plasmids: pTrxFus (10-fold dilution), lane 1; pTrxFusGAO (10-fold dilution), lane 2; pTrxFusGAOGBD (10-fold dilution), lanes 3 and 4; pTrxFusGAOGBD (33- and 100-fold dilution), lanes 5 and 6, respectively. In lane 3, lactoperoxidase was omitted during incubation with the killing solution.

further evidence of the critical role of glucans in targeting of the hybrid protein to streptococcal cells. This experiment also demonstrated the essential role of lactoperoxidase, whose absence in the killing solution completely abolished inhibition of bacterial growth even in the presence of bound hybrid protein (Fig. 6, bars 3). The targeting and killing of the bacterial cells by the GAO-GBD fusion protein was also confirmed with respect to another species of oral streptococcus, *S. gordonii* (Fig. 7). In this case, also, the killing of cells depended on the amount of GAO-GBD extract applied as well as on the presence of lactoperoxidase.

The GBD of GTFs from *S. mutans* has been studied extensively in several laboratories, and its ability to bind to glucans (7, 12, 15, 16) as well as to samples of human dental plaque (2a) has been well established. The data presented in this communication demonstrate the feasibility of utilizing the GBD as a means of delivering antimicrobial agents to dental plaque containing streptococcal cells producing glucans. While this antibacterial system still needs to be evaluated in in vivo animal models, the GBD, which is a component of an enzyme produced and secreted by *S. mutans* in vivo, can be expected to be stable in the oral environment. GAO, which is known for its exceptional stability, appears to be a good candidate for the second component of an antibacterial hybrid protein. Because it is not inherently bactericidal, it can be easily produced by bacterial hosts. The enzyme plays only an indirect role in antibacterial activity, leading to the production of hypoiodite, which has been demonstrated to be a highly effective bactericidal agent (8).

The present results have demonstrated targeting of a novel antibacterial system to artificial dental plaque in vitro. Improved binding and bacterial killing might be obtained by genetically engineering the GAO-GBD fusion protein gene to express a smaller GBD component, which might enhance enzyme activity without compromising targeting. In addition, such constructs need to be tested in animal model systems to examine their relative effectiveness in vivo. These results further suggest that GAO could be fused to other targeting domains for use as an antibacterial agent in other accessible regions of the body.

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