**Streptococcus gordonii** Strains Resistant to Fluorodeoxyuridine Contain Mutations in the Thymidine Kinase Gene and Are Deficient in Thymidine Kinase Activity


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Mutants of *Streptococcus gordonii* resistant to 5-fluorodeoxyuridine (FUDR) were isolated. Each strain contained a point mutation resulting in the premature termination of the thymidine kinase (TK) open reading frame (*tdk*). In vitro translation of the mutant *tdk* coding regions resulted in synthesis of truncated TK polypeptides deficient in TK activity.

Gram-positive commensal bacteria, such as *Streptococcus gordonii*, are currently being developed as live vaccine vectors able to colonize mucosal surfaces and stimulate a secretory immunoglobulin A, as well as a systemic, immune response to a recombinant antigen displayed on the surface of these organisms (4, 7). In order to test these organisms as live vaccine vectors in humans, nonantibiotic selectable markers will be needed for the manipulation of these recombinant organisms in research and clinical laboratories. Previous studies of gram-negative bacteria have suggested it is possible to select for spontaneous resistance to the pyrimidine analog fluorodeoxyuridine (FUDR) (1, 3). All FUDR-resistant mutations on the basis of resistance to the pyrimidine analog have suggested it is possible to select for spontaneous mutations that map to the *tdk* locus and are deficient in thymidine kinase activity (*tk*) (9). Here, we demonstrate that FUDR-resistant strains of *S. gordonii* are readily selected, identify the nature of the mutations in the *tdk* locus, and assays for the loss of TK enzyme activity, and test the in vivo consequences of the mutation.

*S. gordonii* strain GP204, a spontaneous streptomycin-resistant mutant of *S. gordonii* strain V288 (ATCC 35105) (8), is the parental strain of the FUDR-resistant *S. gordonii* strains. Strain GP204 was plated on brain heart infusion agar base containing between 1 and 10 µg of FUDR per ml in the presence of uridine (12.5 µg/ml) and thymidine (2 µg/ml). Two FUDR-resistant colonies were obtained on the plates containing 1 µg of FUDR and were designated SP204(1-1) and SP204(1-2). One FUDR-resistant colony grew on the plates containing 10 µg of FUDR per ml and was designated SP204(10-1). Quantitative analyses revealed that the spontaneous mutation rate of *S. gordonii* GP204 FUDR-resistant mutants was $10^{-6}$ (data not shown).

PCR was employed to amplify the TK open reading frame (ORF) from chromosomal DNA preparations of *S. gordonii* strains SP204(1-1), SP204(1-2), SP204(10-1), and GP204. PCR primers were based on the previously published sequence of the *S. gordonii* tdk gene from strain DL-1 (Challis) (6) and were designed to amplify a 579-bp DNA product encompassing the entire *tdk* locus. The PCR products were cloned into the plasmid vector pCR2.1 (Invitrogen) and transformed into INVαF cells. The DNA from representative clones was sequenced with the M13 reverse and T7 sequencing primers.

Analysis of the DNA sequence from the FUDR-resistant *S. gordonii* mutants revealed that the *tdk* ORFs each contained a single base pair substitution which resulted in the introduction of a translational termination codon in the *tdk* ORF at a unique position in each strain, as follows: codon 86 of SP204(1-1), codon 155 of SP204(1-2), and codon 88 of SP204(10-1). The derived nucleotide sequence of *S. gordonii* GP204 differed from that published for DL-1 (Challis) (6) at a single residue, containing a silent A to G mutation at nucleotide 54 of the *tdk* ORF, as did SP204(1-1), SP204(1-2), SP204(10-1), verifying that *S. gordonii* GP204 was the parent of these strains.

The predicted molecular mass of the full-length *S. gordonii* TK polypeptide is 21,843 Da. The predicted molecular mass of the prematurely terminated TK polypeptides encoded by the SP204(1-1), SP204(1-2), and SP204(10-1) FUDR-resistant mutants are 9,800, 17,800, and 10,100 Da, respectively. To verify these phenotypes, the FUDR-resistant *tdk* loci cloned in the pCR2.1 plasmid vector were transcribed using T7 RNA polymerase, the derived transcripts were translated in rabbit reticulocyte lysates in the presence of $[^{35}S]$methionine, and the radiolabeled translation products were analyzed by gel electrophoresis. The data in Fig. 1A indicate that the apparent molecular masses of the labeled in vitro translation products observed agree with the predicted sizes of the TK truncation products of the *tdk* loci of SP204(1-1), SP204(1-2), and SP204(10-1).

Previously, Black and Hruby (2) identified seven functional domains (I to VII) that are highly conserved in both eukaryotic and prokaryotic TK enzymes. The truncated TK enzymes encoded by SP204(1-1), SP204(1-2), and SP204(10-1) would lack the essential domain VII, a four-amino-acid sequence near the carboxyl terminus. To test this prediction, unlabeled translation reactions programmed with FUDR-resistant *tdk*-derived in vitro transcription products were tested for TK activity, as measured by the ability of the extracts to convert $[^{3}H]$thymidine to $[^{3}H]$TMP (5). As is evident in Fig. 1B, no $[^{3}H]$thymidine-phosphorylation activity was present above background level in the SP204(1-1), SP204(1-2), and SP204(10-1) TK translations, whereas a high level of TK activity was evident in the parental GP204 TK translation reaction.

The TK-deficient phenotype of the FUDR-resistant *S. gordonii* mutants had no effect on the growth of *S. gordonii* cells in culture or in recipient animals. Compared to GP204 in brain heart infusion broth, SP204(1-1) grew at a similar rate and to a similar cell density (data not shown). Likewise, there was no significant reduction in the number of mice colonized or the average duration of colonization for inoculated BALB/c mice (data not shown).
The results presented here have confirmed an apparent commonality in nucleotide metabolism between gram-negative bacteria, such as *E. coli*, and the gram-positive commensal bacterium *S. gordonii*. When grown in the presence of inhibitory concentrations of FUDR, *S. gordonii* produces FUDR-resistant mutants with a frequency of about $10^{-6}$. Identification of the genomic locus responsible for the acquisition of FUDR revealed that three independent mutations [SP204(1-1), SP204(1-2), and SP204(10-1)] all mapped to the same gene, *tdk*, which encodes the nucleoside salvage enzyme TK. Each of the three FUDR mutants acquired a TK-deficient phenotype by virtue of the introduction of a nonsense mutation within the *tdk* ORF to produce truncated proteins lacking one or more motifs known to be essential for other TK enzymes. Enzyme assays confirmed that none of the truncated enzymes encoded by the SP204(1-1), SP204(1-2), and SP204(10-1) *tdk* genes retained any enzymatic activity.

The original impetus for attempting to isolate FUDR-resistant *S. gordonii* mutants was to enable this marker to be used for the in vitro selection for recombinant candidate vaccine strains and to facilitate the detection of implanted organisms in recipient animals in vivo. Selective conditions could be established (1 μg of FUDR per ml, 12.5 μg of uridine per ml, and 2 μg of thymidine per ml) which allowed the growth of FUDR-resistant mutants.

![Figure 1](http://aac.asm.org/)

**FIG. 1.** Structural and functional analysis of FUDR-resistant *tdk* genes. Transcripts of the *tdk* genes of wild-type *S. gordonii* and the FUDR-resistant mutants were prepared and then translated in rabbit reticulocyte lysates in the presence or absence of $[^{35}S]$methionine. (A) The radiolabeled translation products were resolved by electrophoresis on 12% polyacrylamide gels containing sodium dodecyl sulfate and visualized by autoradiography. Lane M, molecular weight markers (46, 30, 21.5, 14.3, 6.5, and 3.4 kDa, respectively); lane 1, no RNA added; lane 2, GP204; lane 3, SP204(1-1); lane 4, SP204(1-2); lane 5, SP204(10-1). (B) The unlabeled translation products were tested for TK enzyme activity using a previously described protocol (5) which measures the conversion of $[^3H]$thymidine to $[^3H]$thymidine monophosphate. The incorporation of the radiolabel is shown for each of the *tdk* gene products. The bar labeled “H2O” represents the incorporation directed by a reticulocyte lysate to which no exogenous RNA had been added. Results are means ± standard deviations (error bars).
while inhibiting the growth of wild-type *S. gordonii*. Furthermore, the growth of FUdR* S. gordonii* was not compromised in rich media, indicating that recombinants derived in this manner can be easily grown to high density for use as vaccine inocula. In vivo implantation experiments suggested that the ability of *S. gordonii* to establish colonization and persist in the oral cavity of the mouse is not compromised by the presence of the FUdR* mutation.

Taken together, the results obtained here indicate that FUdR* should have utility as a selection scheme and phenotypic marker in *S. gordonii*-based recombinant vaccines. This approach has two advantages. First, since FUDR is not routinely used to treat human disease there should not be a significant reservoir of FUdR* oral bacteria to complicate detection of implanted vaccines. Second, FUDR is not an antibiotic and FUdR* is not plasmid-borne. If live bacterial strains are to be used as vaccine vectors, they should not contain any engineered, or selected, markers of resistance to drugs of clinical relevance in a configuration (such as a plasmid) whereby they could be passed from the implanted commensal to an indigenous pathogen. The FUdR* genomic marker should satisfy both of the above-mentioned criteria.

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REFERENCES


