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Direct Selection of IS*903* Transposon Insertions by Use of a Broad-Host-Range Vector: Isolation of Catalase-Deficient Mutants of *Actinobacillus actinomycetemcomitans*

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Transposon mutagenesis in bacteria generally requires efficient delivery of a transposon suicide vector to allow the selection of relatively infrequent transposition events. We have developed an IS*903*-based transposon mutagenesis system for diverse gram-negative bacteria that is not limited by transfer efficiency. The transposon, IS*903*φ*kan*, carries a cryptic *kan* gene, which can be expressed only after successful transposition. This allows the stable introduction of the transposon delivery vector into the host. Generation of insertion mutants is then limited only by the frequency of transposition. IS*903*φ*kan* was placed on an IncQ plasmid vector with the transposase gene located outside the transposon and expressed from isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoters. After transposase induction, IS*903*φ*kan* insertion mutants were readily selected in *Escherichia coli* by their resistance to kanamycin. We used IS*903*φ*kan* to isolate three catalase-deficient mutants of the periodontal pathogen *Actinobacillus actinomycetemcomitans* from a library of random insertions. The mutants display increased sensitivity to hydrogen peroxide, and all have IS*903*φ*kan* insertions within an open reading frame whose predicted product is closely related to other bacterial catalases. Nucleotide sequence analysis of the catalase gene (designated *katA*) and flanking intergenic regions also revealed several occurrences of an 11-bp sequence that is closely related to the core DNA uptake signal sequence for natural transformation of *Haemophilus influenzae*. Our results demonstrate the utility of the IS*903*φ*kan* mutagenesis system for the study of *A. actinomycetemcomitans*. Because IS*903*φ*kan* is carried on a mobilizable, broad-host-range IncQ plasmid, this system is potentially useful in a variety of bacterial species.

Transposons are remarkably effective tools for the genetic characterization and manipulation of bacterial genomes. They provide a means to interrupt, mark, identify, characterize, clone, and insert genes of interest (2, 28). Several different transposon mutagenesis systems are available to generate essentially random insertions into host chromosomes (3, 28). In general, these systems require that the transposon be introduced into a target bacterium by a plasmid or phage suicide vector that is unable to replicate or integrate into the bacterial chromosome. Cells that have undergone rare transposition events are then selected by stable expression of a selective marker within the transposon. One disadvantage of such systems is that transfer of the suicide vector into the target host must occur at a high enough efficiency to allow the detection of rare transposition events (1 event per 10^4 to 10^6 cells).

IS*903* is an insertion sequence of 1,057 bp that contains 18-bp inverted repeats at its ends and a single gene for transposase (*tnp*). IS*903* transposes predominantly by a simple insertion pathway and generates 9-bp target duplications on insertion (18). IS*903* transposition has two requirements: (i) the inverted repeat sequences must be present at the ends, and (ii) the transposase gene must be present in *cis* to the transposon for efficient transposition (9). This simple system is easily manipulated to construct insertion mutagenesis vectors that facilitate stable insertions in host genomes. For example, an IS*903* derivative has been used for insertion mutagenesis in *Legionella pneumophila*, the causative agent of Legionnaires' disease (7, 49). Here we describe a new derivative, IS*903*φ*kan*, which allows direct selection of insertion mutations into actively expressed genes. The system does not require efficient delivery of the suicide vector to the host to detect large numbers of transposon insertion mutants, a property particularly important for the mutagenesis of bacterial species that lack efficient systems for the introduction of transposon vectors.

One such bacterium is *Actinobacillus actinomycetemcomitans*, a gram-negative facultative anaerobe that is believed to cause severe localized juvenile periodontitis, as well as other human infections, including endocarditis, meningitis, pneumonia, septicemia, urinary tract infections, vertebral osteomyelitis, and abscesses (13, 48). Very little is known about the genetics of *A. actinomycetemcomitans* colonization and virulence factors, in part because it has been difficult to generate transposon insertion mutations in this organism; transposition frequencies are extremely low (10^{-7}), and no efficient delivery system has been developed (29, 40). In this work, we demon-

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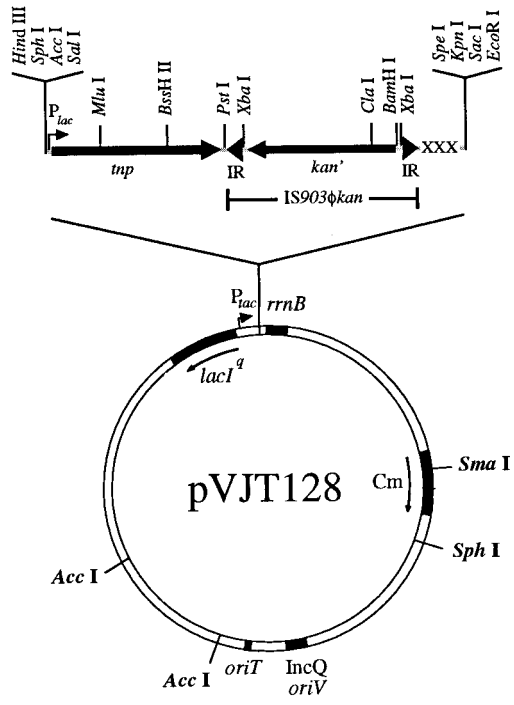


FIG. 1. Map of the pVJT128 plasmid. *tnp* is the IS*903* transposase gene from pKD368, and *kan′* is the kanamycin resistance gene from Tn*903* beginning with the second codon. IR indicates the 18-bp inverted-repeat sequence of IS*903*. The three amber stop codons in different reading frames are indicated by XXX. The boundaries of the transposable element IS*903φkan* are noted. The *Mlu*I and *Bss*HII sites used to create the transposase-minus derivative, pVJT131, are shown.

strate the utility of IS*903φkan* for efficient, random mutagenesis of *A. actinomycetemcomitans*. Expression of catalase activity is a defining characteristic of *A. actinomycetemcomitans*, and it may be an important defense mechanism against oxidative killing by phagocytic cells. From a library of 4,000 IS*903φkan* insertion mutants of *A. actinomycetemcomitans*, we isolated mutants defective in catalase activity and identified the gene for catalase (*katA*). Examination of the intergenic regions flanking *katA* revealed multiple copies of a potential DNA uptake sequence for natural transformation.

MATERIALS AND METHODS

Cell growth and storage. *Escherichia coli* strains were grown on Luria-Bertani (LB) plates and broth as previously described (39). Where appropriate, media were supplemented with 20 μg of chloramphenicol per ml, 50 μg of kanamycin per ml, and 0.01, 0.1, or 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The *E. coli* strains used included DH1 (F$^-$ *supE44 hsdR17 recA1 gyrA96 relA1 endA1 thi-1* λ) (20) and a spontaneous rifampin-resistant mutant of JA221 (F$^-$ *lacY leuB6 trpE5 hsdR recA1* λ$^-$) (received from C. Yanofsky). *A. actinomycetemcomitans* strains were grown in AAGM broth (17) containing 30 g of Trypticase soy broth (BBL), 6 g of yeast extract (BBL), 0.75% glucose, and 0.4% $NaHCO_3$ per liter. The glucose and $NaHCO_3$ were added to the medium after autoclaving. AAGM plates were made similarly, except that 40 g of Trypticase soy agar was substituted for the Trypticase soy broth. Where appropriate, media for *A. actinomycetemcomitans* were supplemented with 20 μg of kanamycin per ml, 2 μg of chloramphenicol per ml, 20 μg of nalidixic acid per ml, and 1 or 10 mM IPTG. The plates were incubated at 37°C in a CO_2-enriched environment in a sealed Gaspak container (BBL) for 48 to 72 h. Broth cultures of *A. actinomycetemcomitans* were grown in screw-cap plastic tubes at 37°C for 15 to 20 h. *A. actinomycetemcomitans* strains can be stored by concentrating overnight AAGM broth cultures 10-fold and placing them at −70°C in AAGM broth containing 10% dimethyl sulfoxide. *A. actinomycetemcomitans* Y4Nal was isolated as a spontaneous Nal^r mutant of strain Y4 (ATCC 43718) after plating of dense suspensions of cells on medium containing nalidixic acid. To determine the effect of H_2O_2 on *A. actinomycetemcomitans*, strains were grown overnight in AAGM broth. Each culture was diluted to a final absorbance at 600 nm (A_{600}) of 0.030 in 10 ml of fresh medium with or without 0.1 mM H_2O_2 in screw-cap plastic tubes and grown at 37°C. During growth, 1-ml aliquots were removed and the A_{600} was monitored.

Plasmid construction. pVJT128 (Fig. 1) is based on a derivative of the mobilizable IncQ expression plasmid pMMB67HE (16), into which a Cm^r marker was cloned to generate pJAK17 (30). pVJT128 contains the IS*903* transposase gene and inverted repeat sequences from pKD368 (7); located between the inverted repeat sequences is the kanamycin resistance gene (*kan*) beginning with the second codon. An 838-bp PCR product containing the *kan* gene flanked by *Xba*I was generated with primers OKD110 and OKD115 (Table 1) and the template pKD100 (9). OKD115 anneals to the 5′ end of the Tn*903 kan* gene beginning with the second codon and contains both *Xba*I and *Bam*HI sites at the 5′ end. OKD110 anneals to the 3′ end of the Tn*903* kanamycin gene and contains the stop codon and an *Xba*I site. The construct was further modified by replacing an *Spe*I-*Cla*I fragment, including the 5′ end of the *kan* gene and the region immediately upstream, with a sequence which introduced three out-of-frame stop codons and eliminated any translational start sites between the stop codons and codon 2 of the *kan* gene. Primers OKD121 and OKD122 (Table 1) were used to generate the replacement fragment, a 193-bp PCR product flanked by *Spe*I and *Cla*I sites, which incorporates the three out-of-frame stop codons and a TAG stop codon instead of an in-frame ATG codon. OKD121 anneals upstream of the *kan* gene and the inverted repeat sequence. OKD122 anneals within the *kan* gene.

A *tnp* deletion derivative of pVJT128 was constructed by removing 443 bp between the *Mlu*I and *Bss*HII sites within the transposase coding sequence. The

TABLE 1. Properties of primers used in the construction of pVJT128 and pVJT131 plasmids

Primer	Sequence and salient features	Annealing position
OKD110	5′-GGCTCTAGATGATTAGAAAAACTCATCGAGCAT-3′ (*Xba*I; *kan* gene stop codon)	3′ end of the Tn*903* kanamycin gene
OKD115	5′-CCGCTCTAGAGGATCCCAGCCATATTCAACGGGAAACGT-3′ (*Xba*I; *Bam*HI; *kan* gene codon #2)	5′ end of the Tn*903* kanamycin gene beginning with the second codon
OKD121	5′-CCAACTAGTTAGATAGGCATAGTAACCCGGC-3′ (*Spe*I; Stop; Stop; Stop; Stop)	Upstream of the *kan′* gene and inverted repeat sequence
OKD122	5′-CAATCGATAGATTGTCGCAC-3′ (*Cla*I)	Internal to the *kan′* gene

resulting construct, pVJT131, is otherwise isostructural to pVJT128 and is used as a transposase-minus control.

Transposition in *E. coli*. $CaCl_2$-competent DH1 was transformed with pVJT128 and pVJT131 (39). Transformants were selected on LB containing chloramphenicol and IPTG (1 mM). After overnight growth, two transformants from each plasmid were picked and resuspended in 1 ml of LB and plated on LB containing kanamycin and LB containing chloramphenicol. To determine the frequency of transposition within the colony, the number of kanamycin-resistant colonies was divided by the number of chloramphenicol-resistant colonies.

Transposition in *A. actinomycetemcomitans*. The IncP *oriT*-minus RK2 derivative, pRK21761 (42), was used to mobilize the IncQ plasmids pJAK17, pVJT128, and pVJT131 from *E. coli* to *A. actinomycetemcomitans* (17). The *E. coli* donor strains were constructed by $CaCl_2$ transformation of JA221Rif (pRK21671) with the three IncQ plasmids. Donor strains and the recipient strain, Y4Nal, were grown to stationary phase, mixed in a ratio of 1:10, spotted on AAGM plates, and incubated at 37°C for 16 h. The mating mixtures were scraped from the plates with a cell scraper (Fisher Scientific), resuspended in 1 ml of AAGM, and plated on AAGM plates supplemented with nalidixic acid and chloramphenicol to select for transconjugants. The presence of the transferred plasmids in the transconjugants was confirmed by DNA extraction (Qiagen Midi column), restriction endonuclease digestion, and electrophoresis in a 0.8% agarose gel. Y4Nal containing the plasmid pJAK17, pVJT128, or pVJT131 was grown in AAGM-chloramphenicol broth for 15 h. A 10^{-6} dilution was plated on AAGM-chloramphenicol plates containing 0, 1, or 10 mM IPTG and incubated at 37°C for 48 h to allow transposition. Four colonies from each of the three strains were picked, resuspended in 1 ml of AAGM broth, and plated on AAGM and AAGM-kanamycin. To determine the frequency of transposition within the colony, the number of kanamycin-resistant cells per colony was divided by the total number of cells per colony.

Curing *A. actinomycetemcomitans* strains of the IncQ plasmids. To cure strains of the transposon donor plasmid, kanamycin-resistant potential transposon mutants were picked and streaked onto AAGM-kanamycin. Kanamycin-resistant colonies were picked and grown in 5 ml of AAGM-kanamycin broth for 15 h. Cultures were then streaked on AAGM-kanamycin plates. Individual colonies were then picked and restreaked on both AAGM-kanamycin and AAGM-chloramphenicol. More than half of the colonies were Km^r and Cm^s, indicating curing of the transposon donor plasmid.

Southern blot analysis. Genomic DNA was isolated from *A. actinomycetemcomitans* by following the large-scale protocol of Ausubel et al. (1). A 1.5-kb *Hae*II fragment containing the Tn*903 kan* gene of pMK20 (25) was electroeluted (39) from a Tris-acetate-EDTA (TAE)–0.8% agarose gel to use as a probe (*kan* probe) for the presence of the transposon. Based on a partial sequence from the *A. actinomycetemcomitans* Genome-Sequencing Project database (35), primers 5′-GTGGATAATGACAACACCATG-3′ (positions 558 to 578 in Fig. 4) and 5′-GCCGTTGTGATCTACGCGCAT-3′ (positions 1640 to 1620 in Fig. 4) were designed to amplify a putative catalase gene to use as a probe (*cat* probe). Both the *kan* and *cat* probes were labeled with digoxigenin by using the digoxigenin labeling kit (Boehringer-Mannheim) as specified by the manufacturer. Southern blotting was done by standard procedures (39).

Catalase activity. To screen for catalase activity, individual colonies were picked with a toothpick and dipped into a beaker of 3% H_2O_2 or smeared on a slide and tested with one drop of 3% H_2O_2. Catalase-positive colonies gave visible oxygen bubbles. Loss of catalase activity was confirmed by a quantitative assay. A 1-ml volume of an overnight broth culture was centrifuged at $2,000 \times g$ in an Eppendorf microcentrifuge for 5 min. The pellet was washed twice with 0.85% phosphate-buffered saline (PBS) and then resuspended in 0.1 ml of PBS. A 0.9-ml volume of 18 mM H_2O_2 in PBS was added, and the suspension was incubated at 25°C for 5 min. Cells were centrifuged at $16,000 \times g$ for 1 min, and the A_{240} of the supernatant was measured to determine the concentration of remaining H_2O_2. The molar concentration of H_2O_2 was based on an extinction coefficient of 81 cm^{-1} M^{-1} (34).

Inverse PCR. Inverse PCR was done by published methods (47). A 1-μg portion of *Hin*dIII-digested genomic DNA was ligated with 10 U of DNA ligase (New England Biolabs) in a 50-μl reaction mixture at 14°C for 16 h. The large reaction volume was used to facilitate intramolecular ligation. The *A. actinomycetemcomitans* genomic DNA adjacent to the transposon was amplified by PCR with primers which hybridize within the transposon and are oriented outward: *kan*Start (5′-GTTTCCCGTTGAATATGGCTGGG-3′) and *kan*Stop (5′-GCAGTTTCATTTGATGCTCGA-3′).

Sequencing. PCR products were cut out of ethidium bromide-stained 1% Tris-acetate gels, and DNA was electroeluted (39). The purified DNA was sequenced at the Columbia University DNA Sequencing Facility by using Perkin-Elmer Applied Biosystems automated DNA sequencer 373A. Three overlapping PCR products were made to sequence the catalase region of Y4Nal. Both strands were sequenced by using the same primers that were used to produce each PCR product. The primer pairs used for PCR and for sequencing were 5′-ATCGCCGGTTTCGTGAAAGTC-3′ (starting 51 bases and ending 30 bases upstream of the catalase region [see Fig. 4]) and 5′-CAGCGCAGCTTTGGTGTATTG-3′ (positions 749 to 729 in Fig. 4), yielding an 800-bp PCR product; 5′-GTGGTGCACGCCAAGGGTTCC-3′ (positions 669 to 689) and 5′-GTGTGTGCCGCCGTTGTGATC-3′ (positions 1649 to 1629), yielding an 981-bp PCR product; and 5′-TGCCCGTATCACACCACCCAC-3′ (positions 1587 to 1607) and 5′-TGACCTGCTGCAAGTGTCTGA-3′ (starting 23 bases and ending 2 bases downstream of the catalase region), yielding a 1,013-bp PCR product. To further confirm some of the sequences in the 1,013-bp PCR product, the internal primer 5′-ACTCAAGAACCGACCGCACT-3′ (positions 1957 to 1976) was also used for sequencing. To look for frameshift mutations in the kanamycin gene in one of the catalase mutants, Aa1394, primers 5′-CAGCGATCGTGGTATTCCGG-3′ (positions 1052 to 1071) and 5′-TAACCAACGCAGAAGCGGCG-3′ (positions 1186 to 1205), hybridizing upstream and downstream of the insertion site, respectively, were used for PCR and were used for sequencing.

RESULTS

Construction of IS*903*ϕ*kan* for selection of insertions by expression of *kan* gene fusions. We constructed an IS*903*-based transposon with a cryptic *kan* gene (Fig. 1). The 5′-end of the *kan* open reading frame in this element, named IS*903*ϕ*kan*, extends through one of the IS*903* inverted-repeat sequences flanking the transposon. *kan* is not expressed, because it lacks a start codon, ribosome binding site, and promoter. In addition, the IncQ plasmid carrying IS*903*ϕ*kan* (pVJT128) has three tandem stop codons in different reading frames as well as the *rrnB* transcriptional terminator, all located immediately upstream of *kan* and outside IS*903*ϕ*kan* to further reduce any residual expression from the vector. Activation of the cryptic *kan* gene would occur by transposition of IS*903*ϕ*kan* into an expressed gene in the appropriate reading frame to generate a gene fusion with *kan*.

Transposition of IS*903*ϕ*kan* from pVJT128 was designed to be inducible by IPTG. Transcription of IS*903 tnp* is initiated from the Lac repressor-regulated promoters *lacUV5* and *tac* (Fig. 1). To further increase the expression of transposase, we replaced the GTG start codon of the *tnp* gene with ATG and altered a strong transcriptional terminator that occurs within the gene, as described previously (7). The *tnp* gene is located outside the transposon and immediately adjacent to IS*903*ϕ*kan*; thus, it becomes physically separated from IS*903*ϕ*kan* following transposition. Subsequent transposition events should be less likely because IS*903* transposase is predominantly *cis* acting (10).

We expected that expression of Km^r by pVJT128-containing cells would be dependent upon transposition of IS*903*ϕ*kan*. To test this, the *E. coli recA* strain DH1 was transformed with pVJT128 and Cm^r transformants were selected in the presence of 1 mM IPTG to induce transposase expression. Individual colonies were picked and resuspended in broth, and cells were plated on kanamycin-containing medium to select for cells that may have undergone a transposition event. The colonies each contained an average of 5.4×10^3 Km^r cells in a total of 2.3×10^7 cells, yielding a frequency of Km^r mutants of 2.3×10^{-4}. As a control, the experiment was repeated with cells carrying plasmid pVJT131, which is identical to pVJT128 except for a 443-bp *Mlu*I-*Bss*HII deletion in *tnp*, which prevents the expression of functional transposase. With pVJT131, Km^r cells occurred at a frequency of 10^{-7} or lower. Thus, the 10^3-fold-higher-frequency appearance of Km^r mutants in the pVJT128-containing colonies was dependent on expression of the IS*903* transposase, as expected if transposition of IS*903*ϕ*kan* had occurred. The apparent transposition frequency of 2.3×10^{-4}, as reflected by the appearance of Km^r mutants, is consistent with transposition frequencies of other IS*903* derivatives assayed by conjugation (8).

Transposition of IS*903*ϕ*kan* in *A. actinomycetemcomitans*. The IncQ plasmid replicon of the IS*903*ϕ*kan*-containing plasmid pVJT128 is functional in a wide variety of gram-negative bacteria, including the periodontal pathogen *A. actinomycetemcomitans* (17). To determine if IS*903*ϕ*kan* could be used for the isolation of insertion mutants of this bacterial species, plasmids pVJT128 (IS*903*ϕ*kan tnp*$^+$), pVJT131 (IS*903*ϕ*kan*

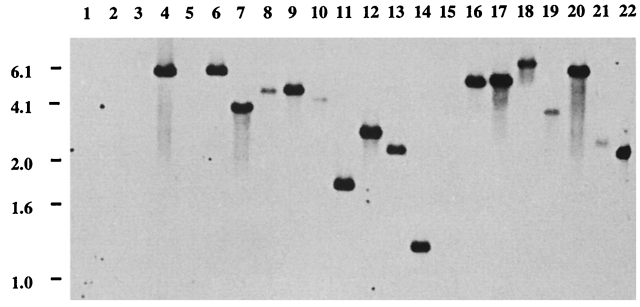


FIG. 2. Southern blot of IS*903φkan* insertions. Shown is the Southern blot of *Eco*RI-digested genomic DNA. Digoxigenin-labeled DNA containing the *kan* gene was used as a probe. The sizes of DNA markers are shown on the left in kilobases. The control lanes contain DNA from Y4Nal (lane 1), Y4Nal(pJAK17) (lane 2), Y4Nal(pVJT131) (lane 4), and Y4Nal(pVJT128) (lane 6). The bands observed in lanes 4 and 6 are due to hybridization with the pVJT parent plasmids. Kanamycin-resistant mutants were derived from Y4Nal(pJAK17) (lane 3), Y4Nal(pVJT131) (lane 5), and Y4Nal(pVJT128) (lanes 7 to 22). All mutants were cured of the parent plasmid.

Δ*tnp*), and pJAK17 (vector) were introduced into *A. actinomycetemcomitans* Y4Nal by conjugation. We used *E. coli* donor strains containing an *oriT* mutant of RK2, which is able to mobilize IncQ plasmids but is defective in self-transfer (42). Transconjugants were selected on medium containing chloramphenicol and nalidixic acid. Individual transconjugant colonies for each plasmid were purified and tested for sensitivity to kanamycin to confirm that RK2 was not present and that the *kan* gene of IS*903φkan* was not expressed. Southern blot analysis of DNA from the transconjugants with a *kan*-specific probe confirmed that transposition of IS*903φkan* had not occurred (Fig. 2).

To test for transposition of IS*903φkan* in *A. actinomycetemcomitans*, the strains were plated for individual colonies on AAGM containing chloramphenicol and 0, 1, or 10 mM IPTG. After 48 h, individual colonies were picked, resuspended in broth, and then titrated on nonselective medium for total cells and on selective medium for Km^r cells. Colonies from the pVJT128-containing strain showed a significant increase in the fraction of Km^r cells with increasing concentration of IPTG (Table 2). In contrast, the low frequency ($<10^{-6}$) of Km^r cells arising from the plasmidless and pJAK17-containing control strains reflected the spontaneous rate of chromosomal mutation to resistance. For the control strain carrying pVJT131 (Δ*tnp*), Km^r cells occurred at a frequency approximately equivalent to that of the uninduced pVJT128 cells. This frequency is higher than the background due to spontaneous chromosomal mutation, owing to plasmid DNA rearrangements that activate the cryptic *kan* gene on the plasmid copy of IS*903φkan*. This conclusion is based on the following evidence: (i) nearly all of the Km^r pVJT131-containing cells became Km^s upon loss of the plasmid and (ii) plasmids from these isolates were able to confer Km^r in *E. coli* (data not shown). In contrast, curing of plasmids from the Km^r mutants isolated after IPTG induction of pVJT128-containing cells rarely resulted in the loss of Km^r, as expected if IS*903φkan* transposed to the chromosome in these strains (see below).

TABLE 2. Transposition of IS*903φkan* in *A. actinomycetemcomitans*

Plasmid	Description	No. of Km^r CFU/total CFU (10^6) in presence of IPTG at:		
		0 mM	1 mM	10 mM
None		<1	3.0 ± 2.1[a]	<1
pJAK17	Vector	<1	<1	<1
pVJT128	IS*903φkan tnpA*$^+$	4.4 ± 2.0	74 ± 49	351 ± 146
pVJT131	IS*903φkan tnpA*	9.6 ± 1.3	11.6 ± 1.9	1.4 ± 0.1

[a] Represents one colony.

To confirm that transposition of IS*903φkan* had occurred in *A. actinomycetemcomitans*, Southern blot hybridization analysis was done with genomic DNA of various Km^r mutants after they were cured of pVJT128, pVJT131, or pJAK17 as described in Materials and Methods (Fig. 2). No hybridization was detectable with DNA of Km^r mutants derived from pVJT131-containing and pJAK17-containing control strains (Fig. 2 and data not shown). This confirms that these mutants arose by spontaneous chromosomal mutation rather than by transposition of IS*903φkan* to the bacterial chromosome.

In contrast, genomic DNA from 15 of 16 Km^r mutants isolated from the IPTG-induced pVJT128-containing strain hybridized to the *kan* probe (Fig. 2). Each of these isolates showed a single hybridizing band, indicating (i) that multiple transposition events within a single cell are infrequent under the conditions used here and (ii) that none of the mutants contains an insertion of the complete pVJT128 plasmid, as expected from their Cm^s phenotype. The variable sizes of the hybridizing fragments are consistent with the target specificity expected for IS*903* (22). One of the 16 Km^r mutants arising from the pVJT128-containing strain did not hybridize to the *kan* gene probe (Fig. 2, lane 15). This mutant is likely to have occurred by chromosomal mutation.

Isolation of catalase-deficient mutants of *A. actinomycetemcomitans*. A distinguishing feature of *A. actinomycetemcomitans* is the expression of catalase (43). To identify the catalase gene, we used IS*903φkan* to obtain insertion mutants defective in catalase activity. Strain Y4Nal(pVJT128) was grown on medium containing chloramphenicol and 10 mM IPTG to induce the transposition of IS*903φkan*. Seventy Cm^r colonies were pooled and plated on kanamycin-containing medium to select a library of transposon insertion mutants. A total of 4×10^3 Km^r colonies were screened individually by a rapid toothpick assay for the ability to hydrolyze H_2O_2, as described in Materials and Methods. We identified and confirmed three mutants (Aa1393, Aa1394, and Aa1395) with no apparent catalase activity. The frequency of catalase-defective mutants (3/4,000) is consistent with an estimated frequency (1/1,000) based on the assumptions that (i) the size of the genome is 2×10^6 bp, of which half codes for essential genes; (ii) the size of the catalase gene is 1,000 bp; and (iii) IS*903φkan* inserts randomly. All three mutants grew more slowly than the wild type in broth containing 0.1 mM H_2O_2, and the MICs of H_2O_2 are 1 mM for wild-type Y4Nal and 0.3 mM for each of the mutants (data not shown).

Southern blot hybridization analysis with the *kan* probe showed that all three mutants displayed a hybridizing *Hin*dIII fragment of about 2.8 kb whereas the wild-type genomic DNA did not hybridize (Fig. 3A). By searching the raw data from the *A. actinomycetemcomitans* Genome Sequencing Project database (35) for similarities to catalase sequences from other bacteria, we identified two contigs with sequences predicted to encode catalase-related proteins. Using one primer specific for each contig, we showed by PCR that the two contigs contained nonoverlapping segments of the same putative catalase-encoding gene. The PCR product was then used as a hybridization probe for this gene (*kat* probe). For all three catalase-deficient mutants, the *kat* probe hybridized to a *Hin*dIII fragment of about 2.8 kb (Fig. 3B), the same size as the *kan*-hybridizing fragment (Fig. 3A). In wild-type Y4Nal, the fragment that hybridized to the catalase probe was smaller by approximately

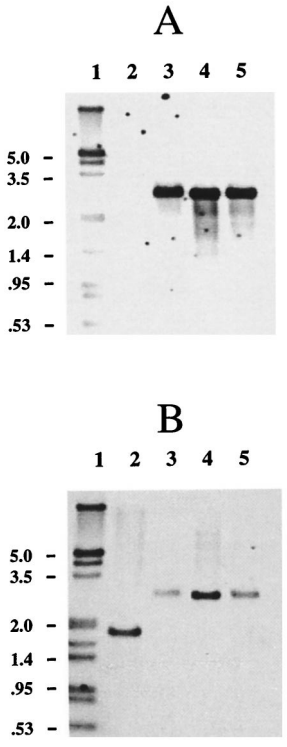


FIG. 3. Southern blots of catalase-defective mutants. Shown are the Southern blots of *Hin*dIII-digested genomic DNA from the wild type, Y4Nal (lane 2), and catalase-negative mutants, Aa1393 (lane 3), Aa1394 (lane 4), and Aa1395 (lane 5). Lane 1 contains digoxigenin-labeled DNA molecular weight markers (shown in kilobases) (Boehringer Mannheim). Probes were digoxigenin-labeled DNA containing the *kan* gene (A) or the *katA* gene (B).

1 kb (Fig. 3B), which is the size of IS*903*ϕ*kan*. The insertion sites were identified precisely by sequencing the products of inverse PCR with primers pointing outward from the ends of IS*903*ϕ*kan* (see Materials and Methods).

The *A. actinomycetemcomitans* Genome Sequencing Project used a different strain from that described here. We have therefore confirmed the sequence of a 2,577-bp region containing the IS*903*ϕ*kan* insetion mutations and the putative catalase gene of strain Y4Nal used in our studies (Fig. 4). Apart from a few individual nucleotide differences, our sequence is identical to that of the Genome Sequencing Project (Fig. 4). The region contains only one open reading frame able to encode a polypeptide larger than 60 amino acids. This large open reading frame was interrupted by IS*903*ϕ*kan* in all three catalase-deficient mutants. The predicted polypeptide product of the open reading frame has 484 amino acids and shows more than 50% identity to several bacterial catalases (Fig. 5). Upstream of the open reading frame are a reasonable Shine-Dalgarno sequence for ribosome binding and a predicted strong σ^{70} promoter. We conclude that this open reading frame is the structural gene for catalase in *A. actinomycetemcomitans*, and we have designated it *katA*.

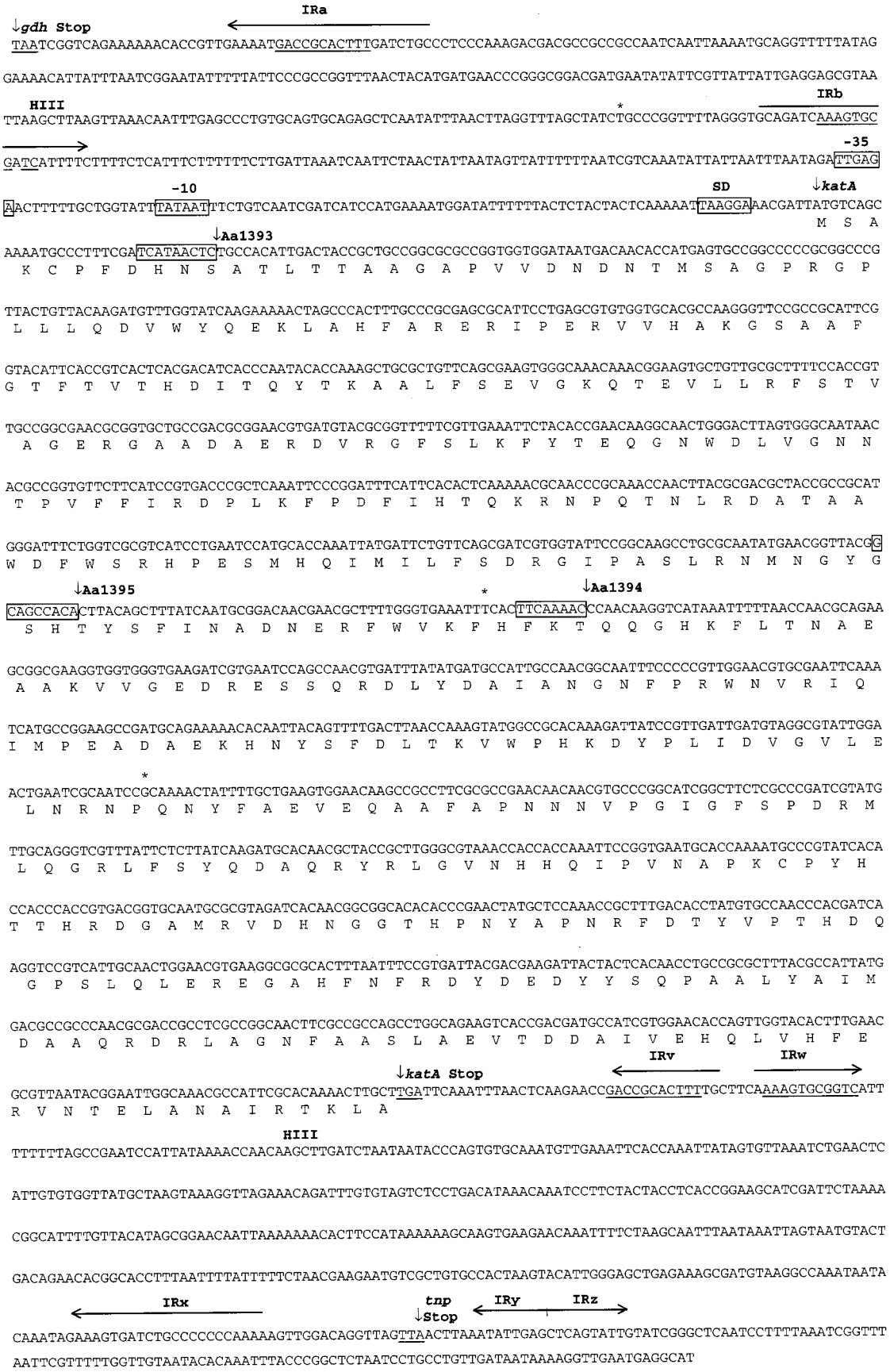
Multiple copies of a potential DNA uptake sequence in the *katA* region. Several significant inverted- and direct-repeat sequences of potential regulatory significance were found in the upstream and downstream noncoding regions (Fig. 4). Two sets of related inverted repeats are particularly interesting. The upstream region contains two nearly perfect 24-bp repeats in inverted orientation that are separated by 237 bp (IRa and IRb in Fig. 4). Downstream of *katA* is an inverted repeat (IRv and IRw) with 13-bp arms separated by a 3-bp spacer. The inverted repeat arms IRa, IRv, and IRw share an 11-bp sequence (Fig. 6), and IRb differs from this sequence by only a single base. This 11-bp sequence contains the 9-bp core DNA uptake signal sequence (USS) for natural transformation in *Haemophilus influenzae*, which occurs 1,465 times in the *H. influenzae* genome (44).

DISCUSSION

We constructed a transposon, IS*903*ϕ*kan*, that allows direct selection of insertions without the need for either a suicide vector or an efficient DNA transfer system. Because the *kan* gene on IS*903*ϕ*kan* is cryptic, the transposon can be maintained in a bacterial population without expressing Km^r. Following induction of IS*903* transposase expression by IPTG, Km^r insertion mutants are readily detected within the population whenever IS*903*ϕ*kan* transposition results in the fusion of *kan* to an expressed open reading frame in the target genome. To demonstrate the utility of IS*903*ϕ*kan*, we generated a library of random insertion mutants of the periodontal pathogen *A. actinomycetemcomitans* and screened for mutants defective in catalase activity. All three mutants isolated contained an IS*903*ϕ*kan* insertion in an open reading frame whose predicted product displays greater than 50% identity to several known catalases from other bacterial species. We conclude that this open reading frame is the structural gene for catalase (*katA*) in *A. actinomycetemcomitans*.

The genetic analysis of the human pathogen *A. actinomycetemcomitans* has been hindered by the absence of a suitable transposon mutagenesis system. Tn*5* (29) and Tn*916* (40) transpose in *A. actinomycetemcomitans*, but their utility has been limited by the efficiency of DNA transfer in this bacterial species. The IncQ plasmid-based system used here to carry IS*903*ϕ*kan* can replicate in a broad range of gram-negative bacteria and can be mobilized to an even wider range of bacterial species by the conjugative transfer systems of promiscuous IncP plasmids (19). Furthermore, the simple genetic requirements of IS*903* transposition (9) (Fig. 1), combined with its ability to transpose randomly in a variety of bacteria (*L. pneumophila*, *E. coli*, and *A. actinomycetemcomitans*), suggest that IS*903*ϕ*kan* may prove a potentially useful tool for mutagenesis of diverse bacterial species. If delivery of the IS*903*ϕ*kan* donor plasmid to the target host is efficient by either conjugation or transformation, Km^r insertion mutants can be selected immediately, as was done in the *E. coli* transposition assay described in above. However, the greatest advantage of the IS*903*ϕ*kan* system will be conferred on hosts that do not transform well and conjugate poorly with the known plasmid transfer systems. For these hosts, it will be

FIG. 4. Nucleotide sequence of *katA* and flanking regions. Shown is the 5′→3′ sequence of a 2,577-nucleotide segment of Y4Nal genomic DNA starting from the stop codon of a predicted glutamate dehydrogenase gene (GenBank accession no. AF162654). The predicted amino acid sequence of the *katA* product is also shown. The −35 and −10 promoter sequences and the Shine-Dalgarno sequence (SD) are boxed. The insertion sites for IS*903*ϕ*kan* are shown by vertical arrows above the sequence. The nucleotides duplicated at the sites of IS*903*ϕ*kan* insertions are also boxed. The 11-bp putative DNA uptake sequences are underlined. Inverted repeats are indicated by horizontal arrows above the sequence. Asterisks indicate nucleotide differences with respect to the Genome Sequence Project database. None of the differences result in a change of amino acid. The two *Hin*dIII sites present in the region are indicated above the sequence.


IRa
↓gdh Stop
HIII
IRb
-35
-10
SD
↓katA
↓Aa1393
↓Aa1395
↓Aa1394
↓katA Stop
IRv
IRw
HIII
IRx
tnp
↓Stop
IRy
IRz

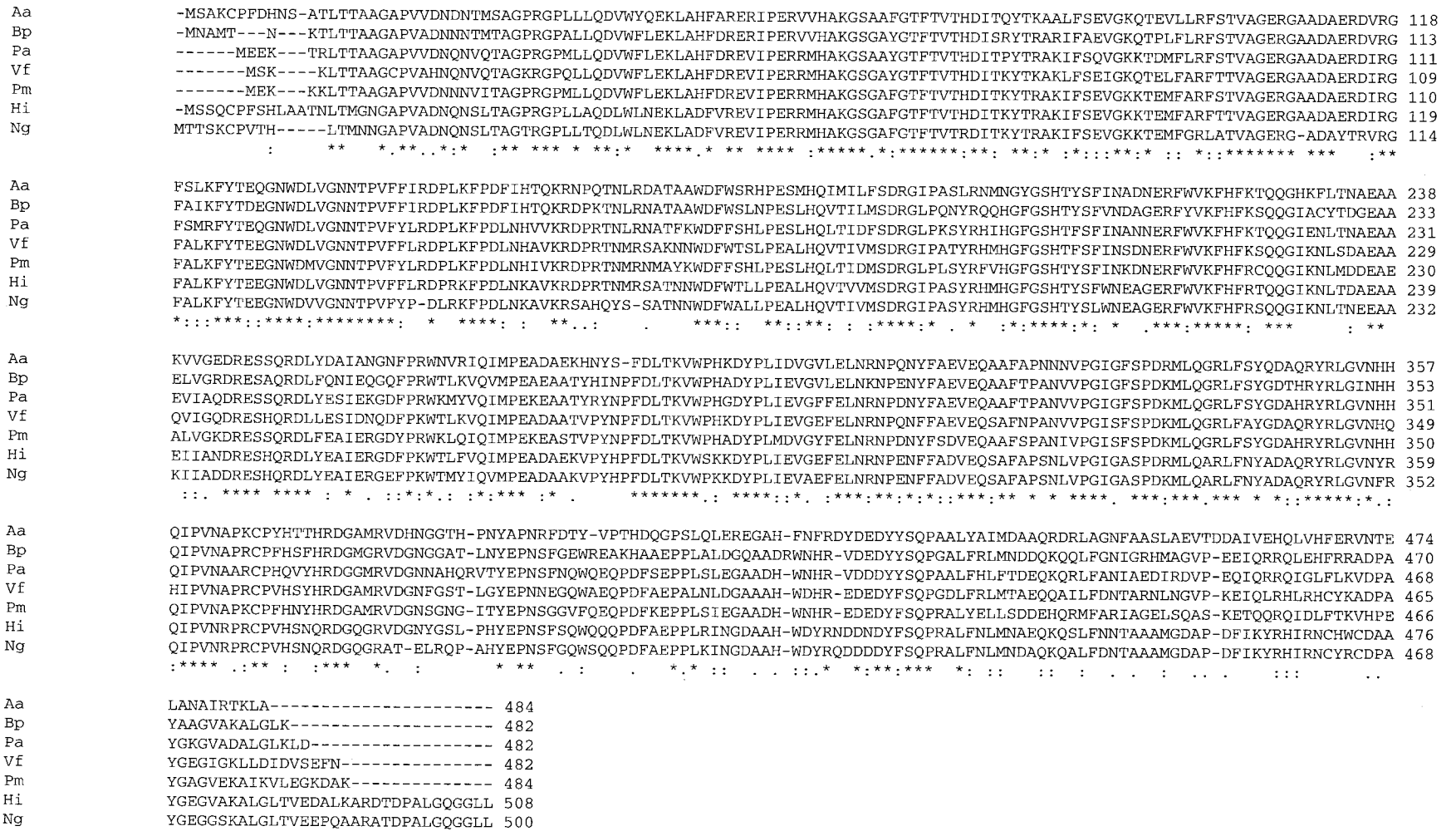


FIG. 5. Alignment of related bacterial catalases. The predicted amino acid sequence of catalase from *A. actinomycetemcomitans* is aligned with known bacterial catalases that have the highest identity to it: *A. actinomycetemcomitans* (Aa), *Bordetella pertussis* (Bp) (accession no. 1345688), *Pseudomonas aeruginosa* (Pa) (accession no. 2896139), *Vibrio fischeri* (Vf) (accession no. 3064126), *Proteus mirabilis* (Pm) (accession no. 1345690 [6]), *H. influenzae* (Hi) (accession no. 1168784 [4]), and *Neisseria gonorrhoeae* (Ng) (Accession no. 1016016 [23]).

```
IRa   G C A G A T C A A A G T G C G G T C A T T T T C
IRb   G C A G A T C A A A G T G C G A T C A T T T T C
IRv             C A A A A G T G C G G T C
IRw             C A A A A G T G C G G T C

A. actinomycetemcomitans      A A A G T G C G G T C
H. influenzae                   A A G T G C G G T
```

FIG. 6. Alignment of putative DNA uptake sequences. The four arms of inverted repeats that contain the 11-bp consensus sequence (see Fig. 4) were aligned. Shown below are the 11-bp sequence that is repeated 848 times in the incomplete *A. actinomycetemcomitans* genome sequence and the 9-bp DNA uptake sequence (USS) that is repeated 1,465 times in the *H. influenzae* genome.

necessary only to establish the IS*903φkan*-containing plasmid in a single transformant or transconjugant. After growth of the clone, induction of transposase should yield large numbers of IS*903φkan* transposon insertion mutants. We have been particularly interested in "rough," adherent, clinical isolates of *A. actinomycetemcomitans* (12, 37), which are exceedingly poor recipients in conjugation and are even more difficult to transform than "smooth," nonadherent, laboratory variants such as the Y4Nal strain used here. As a consequence, rough *A. actinomycetemcomitans* have been refractory to genetic analysis. Recently, we successfully used the IS*903φkan* system to isolate mutants of rough *A. actinomycetemcomitans* without difficulty (24).

A limitation of the IS*903φkan* mutagenesis system is that only expressed, nonessential genes can be targeted. In addition, the expression of some *kan* fusions may not be sufficient to confer kanamycin resistance. Transcription or translation of the gene fusion may be too low, or the fusion protein may be unstable. We also expected that only one in six insertions should be in the proper reading frame to make a functional *kan* fusion. In practice, the frequency appears to be higher. The fusions in Aa1393, Aa1394, and Aa1395 are out of frame with *katA*. No secondary frameshifts were found in *katA* or the 5′ end of *kan* in these mutants suggesting that *kan* translation is initiated at an alternative start site. In another study, six of nine Km^r IS*903φkan* insertion mutants of *A. actinomycetemcomitans* were out of frame (24). Thus, the selection for Km^r may select for compensatory mutations to allow *kan* to be expressed from initially out-of-frame insertions and/or the *kan* fusions may be expressed from alternative translational start sites.

A. actinomycetemcomitans is a facultative anaerobe that resides in the subgingival plaque (43), where it is exposed to oxygen and by-products of oxygen metabolism generated by host cells such as phagocytic leukocytes (33, 36, 43). Catalase is likely to be part of an important defense mechanism for *A. actinomycetemcomitans* against oxidative killing by phagocytic cells. H_2O_2 is the major bactericidal agent that affects *A. actinomycetemcomitans* in periodontal pockets (11, 34). The toxicity of H_2O_2 is indirect and results from the intracellular generation of hydroxy radicals, which can cause DNA strand scission (15). Therefore, protective enzymes such as catalases and peroxidases should be important for the resistance of *A. actinomycetemcomitans* to H_2O_2. Indeed, the catalase-defective mutants isolated in this study are more sensitive to hydrogen peroxide than the wild type. However, they remain able to grow in the presence of 0.3 mM hydrogen peroxide, suggesting that there are other mechanisms of resistance, consistent with previous observations (11). Perhaps this residual resistance to H_2O_2 is encoded by catalase genes that are normally not expressed. Their activity would therefore not be detected in the initial screening assay, but their expression would be induced by the presence of H_2O_2 in the growth media.

The *A. actinomycetemcomitans katA* gene is predicted to code for a protein of 484 amino acids with a molecular weight of 54,961. The predicted polypeptide product is closely related to several other heme-binding bacterial catalases in molecular weight, pI, and amino acid sequence (Fig. 5 and data not shown). The *katA* structural gene is 52% G+C, which is close to the value of 48% G+C calculated from a sample of *A. actinomycetemcomitans* genes involved in basic cellular functions (26). The upstream and downstream intergenic regions have a significantly lower G+C content (34 and 36%, respectively). Overall, the *katA* region is 44% G+C, which is consistent with the reported overall value of 43% G+C for the genome (27). *katA* is likely to be expressed from a monocistronic mRNA. No other open reading frames of any significance occur immediately upstream or downstream of *katA* (Fig. 4). On the basis of sequences available in the Genome Sequencing Project database, the closest putative upstream gene is predicted to encode a glutamate dehydrogenase. The closest downstream gene, which is predicted to encode an ATP-binding cassette transporter, is oriented opposite to *katA* and terminates 1,021 bp from the *katA* stop codon (results not shown). A predicted strong σ^{70}-type promoter in the upstream region of *katA* is consistent with expression of catalase during logarithmic growth of *A. actinomycetemcomitans*. Catalase expression in some bacteria is inducible (4, 5, 31, 45) or growth-phase regulated (21, 32, 38). We have not yet investigated the possible regulation of catalase expression in *A. actinomycetemcomitans*, although the upstream region contains inverted and direct repeats that could act as targets for regulatory factors.

The remnant of a possible IS element is found 460 bp after the termination codon of *katA* (Fig. 4). The region contains a small open reading frame whose predicted product is related to a C-terminal portion of the IS*150* transposase of *E. coli*. A complete copy of this putative transposase gene is present on another contig in the *A. actinomycetemcomitans* genome database. The predicted complete transposase has 38% identity to the IS*150* transposase of *E. coli* (41) and 47% identity to a putative transposase from *H. influenzae* (14). The complete transposase gene is contained within a putative IS element of 1,262 bp with 22-bp inverted repeats at the termini. A copy of the terminal sequence with 21 matching nucleotides (IRx) is present near the transposase gene remnant in the region downstream of *katA* (Fig. 4). For the next 100 nucleotides further downstream of *katA*, including a portion of the putative transposase gene, this remnant is 90% identical to the analogous portion of the predicted IS element.

The intergenic upstream and downstream regions of *katA* also revealed three copies of an 11-bp sequence (5′-AAAGT GCGGTC-3′) and a fourth copy with one mismatch (Fig. 6). We note that this 11-bp sequence contains the 9-bp core USS of *H. influenzae* used to facilitate specific uptake of DNA in natural transformation (Fig. 6) (44). There are 1,465 copies of the USS in the *H. influenzae* genome. The *H. influenzae* 9-bp core USS is part of a larger 29-bp consensus sequence, which includes an AT-rich region adjacent to one side of the core. Nearly all closely spaced USS in *H. influenzae* are in inverted orientation relative to each other, and they occur predominantly in intergenic regions. Downstream inverted repeats are thought to function additionally in termination of transcription or stability of mRNA. Like *H. influenzae*, *A. actinomycetemcomitans* is capable of natural transformation (40, 46). The 11-bp repeats found in the *A. actinomycetemcomitans katA* region share the properties of the *H. influenzae* USS, including an adjacent AT-rich region (Fig. 4). These findings strongly

suggest that the 11-bp sequence may function as a DNA uptake signal for transformation in *A. actinomycetemcomitans*. The *A. actinomycetemcomitans* Genome Sequencing Project database contains 848 exact occurrences of the 11-bp sequence in 1.9×10^6 bases of DNA sequence. This 11-bp sequence is expected to occur randomly only once in every 4.1×10^6 bp. We predict that the 11-bp sequence constitutes the core USS for DNA uptake in *A. actinomycetemcomitans*. The close relationship of this sequence to the DNA uptake sequence of *H. influenzae* and the close relatedness of *A. actinomycetemcomitans* to the genus *Haemophilus* raise the possibility that these organisms readily exchange DNA by natural transformation.

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