Directed Genomic Integration in *Actinobacillus actinomycetemcomitans*: Generation of Defined Leukotoxin-Negative Mutants

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To develop targeted gene integration in the periodontal pathogen *Actinobacillus actinomycetemcomitans*, a ColE1-based, spectinomycin-resistant plasmid containing a segment of the leukotoxin gene was electroporated into strain JP2. In all of the stable spectinomycin-resistant transformants that arose, the plasmid had recombined into the genomic leukotoxin locus since ColE1-based vectors cannot replicate extrachromosomally in *A. actinomycetemcomitans*. Directed genomic integration was then used to construct a leukotoxin-negative strain by transforming the leukotoxin-producing strain JP2 with a ColE1-based plasmid containing an internal fragment of the leukotoxin gene. Cytotoxicity assays proved that these transformants had <0.1% of the leukotoxin activity of the parental strain. These results demonstrate that integration-based approaches can be used for generating isogenic mutants in specific virulence genes in *A. actinomycetemcomitans*.

Actinobacillus actinomycetemcomitans is a gram-negative bacterium which has been implicated as a pathogen in various periodontal diseases, especially localized juvenile periodontitis (30, 37). The best-characterized potential virulence factor in *A. actinomycetemcomitans* is the 116-kDa leukotoxin protein that specifically lyses human and primate polymorphonuclear lymphocytes (33, 38). It is presumed to be involved in helping the bacterium evade host defense cells (34). The gene for the *A. actinomycetemcomitans* leukotoxin has been cloned and characterized (14, 17, 18); the leukotoxin is a member of the RTX toxin family of proteins (5, 12, 22). The structural gene for leukotoxin (*lktA*) is part of an operon that includes genes required for the activation (*lktC*) and localization (*lktBD*) of the LktA protein (6, 8, 31, 35).

In order to elucidate the functions of the leukotoxin and other putative *A. actinomycetemcomitans* virulence factors in pathogenesis, methods to generate defined mutations in their genes must be developed. Transposon mutagenesis, which was recently extended to *A. actinomycetemcomitans* (15), is a powerful method for generating such mutations if a screen or selection for the desired mutation is available. However, having a cloned virulence gene, such as the leukotoxin gene, allows more direct approaches to be taken to construct mutant strains. For example, gene replacement mutagenesis (13, 27) and mutagenesis by insertional duplication (24, 29) have been used successfully to alter specific virulence factor genes in many bacteria (2, 4, 36, 39). However, neither approach has been described for *A. actinomycetemcomitans*. To develop such protocols, we first needed to demonstrate that DNA transformed into *A. actinomycetemcomitans* could be targeted to a specific chromosomal locus.

A nonreplicating, *lkt*-containing plasmid recombines into the *A. actinomycetemcomitans* chromosome at the homologous locus. Plasmid pMC107 was constructed to test the ability of exogenously introduced DNA to recombine into a specific

chromosomal region in *A. actinomycetemcomitans*. The region chosen for these experiments contained the leukotoxin (*lkt*) operon since it has been cloned and well characterized (17, 31). In plasmid pMC107, part of the *lktC* gene has been replaced by a spectinomycin resistance gene which is now flanked by 1 kb of DNA that is normally upstream of *lktC* and 1 kb of DNA that is normally downstream of *lktC* (Fig. 1). Importantly, the plasmid has a ColE1 origin of replication which does not appear to support extrachromosomal DNA replication in *A. actinomycetemcomitans* (32). To construct plasmid pMC107, the unique *Pst*I site was first removed from the multiple cloning site of plasmid pDL269 (19). A 1.1-kb *Sph*I-*Nde*I fragment, which contains a spectinomycin resistance gene (19), was isolated from this intermediate plasmid and cloned (1, 28) into the 5.0-kb *Sph*I-*Nde*I DNA fragment from plasmid pTD24. Plasmid pTD24 has a 2.4-kb *Pst*I fragment from pTD17 (14) cloned into the *Pst*I site of the Bluescript SK phagemid vector (Stratagene Cloning Systems, La Jolla, Calif.).

In a typical experiment, 500 ng of pMC107 DNA was electroporated into *A. actinomycetemcomitans* JP2 (34) and one-fourth of the sample was plated onto TSBYE (32) with spectinomycin (50 µg/ml). After 3 to 4 days of incubation in a Coy anaerobic chamber (5% CO₂, 10% H₂, 85% N₂), 10 to 20 medium to large colonies were visible above a background of approximately 10 times as many tiny and small colonies. If tiny or small transformants were picked, streaked for isolation on TSBYE plates, and then tested for spectinomycin resistance, none were stable for the resistance phenotype. These unstable transformants are presumed to arise from nonintegrated plasmid DNA that had managed to replicate poorly in *A. actinomycetemcomitans*. It might be possible to reduce the background of abortive transformants by using a plasmid vector with a more stringent origin of replication like the R6K origin (26).

On the other hand, when the medium and large colonies were picked and streaked for isolation, about half of them proved to be stable for the spectinomycin resistance trait after one passage under nonselective conditions. The stable spectinomycin-resistant transformants were presumed to arise from recombination of the pMC107 DNA into the *A. actinomyce-*

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FIG. 1. Restriction endonuclease maps of expected recombinants at the leukotoxin locus. The physical maps of key restriction endonuclease sites in plasmid pMC107 and at the leukotoxin locus of *A. actinomycetemcomitans* JP2 are shown at the top. The expected configurations for the three possible recombinants between pMC107 and JP2 at the leukotoxin locus are shown below. The DNA segments are not drawn to scale. H, *Hind*III; N, *Nde*I; P, *Pst*I; S, *Sph*I; spec, spectinomycin resistance gene (19).

temcomitans genome. In five independent transformations, stable transformants were found at a frequency of $75 \pm 20/\mu\text{g}$ of input DNA. This is in the range of values reported for similar experiments with *Lactococcus lactis* (20) and *Streptococcus thermophilus* (23) and is about 10-fold lower than the frequencies for *Lactobacillus plantarum* (21) and *Haemophilus ducreyi* (11). These differences in frequency of transformation and recombination probably reflect the relative efficiencies of electroporation among the organisms since there is a correlation between the efficiency of transformation with replicating plasmids and the measured frequency of integration of nonreplicating DNA.

To prove that the stable transformants had recombined pMC107 into their chromosomes and to determine where that recombination had occurred, Southern blot analyses (10, 15) were performed on genomic DNA from 47 stable isolates derived from five independent transformations. The restriction endonuclease *Hind*III was used to digest the DNA because it would allow us to distinguish among the three possible recombination events: (i) a single crossover (Campbell) at the *lkt* locus (Fig. 1, types A and B), (ii) a reciprocal double crossover (gene conversion or replacement) at *lkt* (Fig. 1, type C), and (iii) an insertion at a non-*lkt* locus. All of the stable transformants had undergone a recombination event at the genomic *lkt* locus (Fig. 2 and data not shown). In 46 of the 47 transformants, the plasmid had integrated into the chromosome via a single crossover event. The restriction endonuclease chosen for use in the Southern blots allowed us to determine which plasmid segment was involved in the recombination event, the

FIG. 2. Southern blot analysis of stable transformants of *A. actinomycetemcomitans* JP2 with uncut plasmid pMC107. Approximately 5 μg of DNA from *A. actinomycetemcomitans* JP2 or one of 17 randomly selected transformants was digested with *Hind*III and analyzed by Southern blot hybridization (10, 15). In addition, 10 ng of pMC107 DNA was digested and analyzed. The blots were probed with the isolated, ^{32}P -labeled 2.9-kb *Pst*I DNA fragment from pMC107. The positions of a subset of the DNA size standards (Stds) are indicated on the right, and the positions of the bands for wild type (wt), recombinant type A, and recombinant type B are indicated on the left. The sample in lane 3 was lost during sample preparation.

1.0-kb *lkt* segment 5' of the spectinomycin resistance gene or the 1.0-kb 3' *lkt* segment (Fig. 1). The data indicated that the recombination occurs equally well with either flanking DNA segment (Table 1, uncut plasmid). Only one transformant appeared to have generated a recombinant by a reciprocal double crossover or gene conversion event between the introduced plasmid and the *A. actinomycetemcomitans* genome (data not shown). This proportion of single-crossover versus double-crossover recombinants is similar to the proportions that have been reported for other organisms (11, 20, 21, 23). Finally, seven of the transformants had a plasmid size band visible on the Southern blot (Fig. 2, sample 8, for example). Since such bands were found relatively infrequently and since ColE1-based plasmids do not appear to be able to replicate stably in *A. actinomycetemcomitans* (32), the transformants with plasmid size bands are presumed to have arisen either from recombination of a plasmid dimer into the genome or from recombination of a second plasmid with a plasmid that had already recombined into the genome. All of these results indicate that DNA introduced into *A. actinomycetemcomitans* recombines efficiently into the host chromosome at regions of homology.

Linearized DNA transforms *A. actinomycetemcomitans* less efficiently. In some organisms (23, 25), exogenously introduced linearized plasmid DNA recombines into the host chromosome with a higher frequency than supercoiled or nicked plasmid DNA. In addition, linearization within a particular DNA segment has been shown to help target the recombination to

TABLE 1. Transformation frequencies and distribution of integration sites

No. of transformants/ μg of DNA	Linearization site in pMC107	No. of stable transformants tested	No. of recombinants found by Southern blot analysis	
			Type A	Type B
75	None ^a	47 ^b	21	25
5.5	<i>Nhe</i> I	13	2	11
6.5	<i>Mlu</i> I	10	5	5
4.5	<i>Bsm</i> I	9	6	3
5	<i>Hind</i> III	5	2	3
4	<i>Xmn</i> I	4	2	2

^a Uncut plasmid.

^b A single type C (replacement) recombinant was found with uncut pMC107.

the homologous chromosomal segment (25). To test these possibilities with *A. actinomycetemcomitans*, plasmid pMC107 was linearized with one of five restriction endonucleases. One (*NheI*) linearized the DNA in the *lktA* segment, two (*MluI* and *BsmI*) cut the DNA in the 5' *lkt* segment, and two (*HindIII* and *XmnI*) cut the DNA in the vector. Each linearized DNA preparation was electroporated into *A. actinomycetemcomitans*, and spectinomycin-resistant transformants were selected as before. The frequency of stable spectinomycin-resistant transformants averaged $5 \pm 4/\mu\text{g}$ (range, 1 to 12/ μg) for the linearized DNAs. This is significantly (15-fold) lower than the frequencies found with uncut pMC107 DNA. A similar decrease in transformation efficiency by plasmid linearization has been reported for *L. lactis* (20) and *Bordetella pertussis* (39) and may be a consequence of the presence of particular exonucleases in these organisms.

The stable transformants from the linearized DNAs were then subjected to Southern blot analysis to determine which DNA segment had been involved in the recombination between the plasmid and the *lkt* locus on the chromosome. Most of the linearized DNAs recombined equally well with either the 5' *lkt* segment or the 3' segment (Table 1). Only *NheI*-linearized pMC107 DNA showed a significant ($P = 0.048$ by the Pearson chi-square test) preference for recombination with a particular segment; 11 of the 13 recombination events occurred in the 3' *lkt* segment, which is the one cut by *NheI*. However, this appears to be either a unique property of an *NheI* site or a statistical aberrancy since a similar result was not obtained with two other restriction endonucleases that cut within the insert. The apparent ability of *NheI* to target recombination would not appear to be a property of the types of ends it generates when it cuts DNA since one of the other enzymes tested (*MluI*) also leaves 4-bp single-stranded 5' ends. Overall, these results indicate that linearization, with the possible exception of linearization by *NheI*, does not target the recombination to a particular DNA segment.

Although Campbell-like recombinations between pMC107 and the chromosome occurred readily in *A. actinomycetemcomitans*, replacement recombination (Fig. 1, type C) via a double-crossover or gene conversion event occurred infrequently. We found only one replacement recombinant out of a total of 99 stable transformants screened by Southern blot analysis. The proportion of such replacement mutants can be increased in some microorganisms by transforming with a linear DNA fragment that contains no vector sequences (11, 25). To test the possibility that this might occur in *A. actinomycetemcomitans*, pMC107 DNA was digested with *PstI* and the 3.1-kb insert fragment, containing the 5' *lkt* region, the spectinomycin resistance gene, and the 3' *lkt* segment, was electroporated into strain JP2. There was only one stable transformant per microgram of input DNA, and none of the five stable transformants tested by Southern blot analysis had a pattern consistent with a replacement event. Instead, the stable transformants had a complete copy of pMC107 inserted into the 3' *lkt* segment of the *A. actinomycetemcomitans* chromosome (data not shown). These transformants are presumed to have arisen from a small amount of uncut plasmid DNA that carried through the purification protocol. Similar experiments with a *PstI*-*BglII* insert fragment isolated from pMC107 did not generate any stable transformants. These results suggest that it will be difficult to generate replacement mutations in *A. actinomycetemcomitans* with currently available techniques.

Constructing an isogenic leukotoxin-negative mutant via a Campbell insertion. In order to determine the *in vivo* function of a particular bacterial protein, it is important to be able to construct defined isogenic mutants. As a test of the ability to do

FIG. 3. Restriction endonuclease maps of wild-type and mutant leukotoxin loci. The physical maps of key restriction endonuclease sites in plasmid pDK378 and at the leukotoxin locus of *A. actinomycetemcomitans* JP2 are shown at the top. The expected configuration for a single crossover between the JP2 chromosome and pDK378 at the leukotoxin locus is shown below. The DNA segments are not drawn to scale. B, *BamHI*; H, *HindIII*; P, *PvuII*; S, *SspI*; spec, spectinomycin resistance gene (19); *lktA'*, incomplete segment of the gene encoding leukotoxin.

this for *A. actinomycetemcomitans*, we decided to construct leukotoxin-negative mutants of leukotoxin-producing *A. actinomycetemcomitans* JP2. Because replacement recombination had not worked efficiently in *A. actinomycetemcomitans*, a single-crossover insertion-duplication mutagenesis approach was used (24, 29). This approach has been employed for genes in several other organisms (2, 4, 24, 29). An internal *SspI*-*PvuII* fragment (17) encoding amino acids 27 to 243 (of 1,055 amino acids) of the LktA protein was cloned (1, 28) into the *SmaI* site of pUS19 (3), a ColE1-based vector carrying a spectinomycin resistance gene, to generate plasmid pDK378 (Fig. 3). The insert is in the reverse orientation in plasmid pDK386 (not shown). Each of these plasmids was electroporated into *A. actinomycetemcomitans* JP2, and spectinomycin-resistant transformants were selected. Five stable transformants were isolated and tested by Southern blot analysis; the results demonstrated that the plasmids had recombined into the host chromosome at the *lktA* locus as expected (Fig. 3 and 4). The mutant strains generated by integration of pDK378 were named AAM9, -10, and -11. The other mutant strains, AAM12 and -13, arose from integration of pDK386. All of these integrants appeared to be relatively stable; >90% of the transformants tested retained the integrated plasmid after more than 20 generations of growth under nonselective conditions.

The integration of plasmids pDK378 and pDK386 generates a partial duplication of the *lktA* gene such that one segment lacks the last three-fourths of the *lktA* gene and the other segment is missing both the *lkt* promoter and the translation initiation codon (Fig. 3). Thus, strains AAM9 through AAM13 should no longer produce leukotoxin. To prove that this was the case, Western blot (immunoblot) analyses were done on JP2, AAM9 (pDK378 integrant), and AAM12 (pDK386 integrant). Polyclonal antileukotoxin antibody reacted with a 110-kDa protein in the lane containing 64 μg of total protein from

FIG. 4. Southern blot analysis of transformants of *A. actinomycetemcomitans* JP2 with plasmids pDK378 and pDK386. Approximately 5 µg of DNA from *A. actinomycetemcomitans* JP2 or one of three transformants (AAM9 to AAM11) generated with plasmid pDK378 and two transformants (AAM12 and AAM13) generated with plasmid pDK386 was digested with *Hind*III. A second set of DNAs was digested with *Bam*HI, and both sets were analyzed by Southern blot hybridization (10, 15). The blots were probed with ³²P-labeled pTD24 DNA which has a 2.4-kb *Pst*I fragment from pTD17 (14) cloned into the *Pst*I site of the Bluescript SK phagemid vector (Stratagene Cloning Systems). The positions of a subset of the DNA size standards (Std) are indicated on the left. For clarity, several lanes between the JP2 samples and the AAM samples were removed from the photographs.

parental JP2 cells, but no protein bands were detected in lanes with 64 µg of total protein from the mutant strains (data not shown). However, the sensitivity of the Western blot analysis was such that leukotoxin present at 10% of the level in JP2 would not be detectable. Therefore, a more sensitive cytotoxicity assay (14) was used to look for leukotoxin in mutant extracts (31). The cytotoxicity assay of protein from wild-type and mutant cells showed that the mutants have <0.1% of the leukotoxin activity of JP2 (Table 2). These results demonstrate that the expected leukotoxin-negative mutants have been generated.

Our results indicate that homologous recombination between exogenously introduced plasmid DNA and the *A. acti-*

TABLE 2. Cytotoxicity of extracts from wild-type and mutant cells

Extract (µg)	% Lysis of THP-1 cells ^a
JP2	
53.....	73
18.....	75
6.....	69
2.....	48
0.6.....	32
0.2.....	12
AAM9	
53.....	2
18.....	1
6.....	1
2.....	1
AAM12	
53.....	1
18.....	1
6.....	<1
2.....	<1

^a Calculated as [activity (counts per minute) in the sample - background activity]/(maximum releasable activity - background activity). The background (phosphate-buffered saline alone) activity was 6,500 cpm, and the maximum releasable counts (1% Triton X-100) averaged 77,956 cpm. The data are averages for duplicate assays.

nomycetemcomitans chromosome can occur efficiently with as little as 730 bp of DNA and prove the utility of the integration-based mutagenesis approach for the generation of isogenic mutations in *A. actinomycetemcomitans*. Guthmiller et al. (7) have found that a segment of homologous DNA as short as 544 bp can direct recombination into the *A. actinomycetemcomitans* chromosome. This important genetic tool has also been used in a second strain of *A. actinomycetemcomitans*, Y4 (16). Thus, by using the techniques described here for *A. actinomycetemcomitans* and variations described for other bacteria (9, 23, 39), it should be possible to introduce a defined, specific mutation into any gene in the *A. actinomycetemcomitans* genome, providing that the gene of interest has been cloned. This will allow the comparison of wild-type and isogenic variants of *A. actinomycetemcomitans* in analyzing the role of putative virulence factors in the pathogenesis of the organism.

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