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Genetic Rearrangement Associated with In Vivo Mucoid Conversion of *Pseudomonas aeruginosa* PAO Is Due to Insertion Elements

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The conversion of *Pseudomonas aeruginosa* PAO to the mucoid phenotype has been reported for a chronic pulmonary infection model in rats (D. E. Woods, P. A. Sokol, L. E. Bryan, D. G. Storey, S. J. Mattingly, H. J. Vogel, and H. Ceri, J. Infect. Dis. 163:143–149, 1991). This conversion was associated with a genetic rearrangement upstream of the exotoxin A gene. To characterize the genetic rearrangement, the region upstream of the *toxA* gene was cloned from PAO, PAO-muc (a mucoid strain), and PAO-rev (a nonmucoid revertant strain). The nucleotide sequence of a 4.8-kb fragment from PAO-muc was determined. A+T-rich regions of approximately 2 kb (IS-PA-4) and 0.4 kb (IS-PA-5) were identified in this fragment. DNA probes constructed internal to these regions hybridized to PAO-muc but not to PAO or PAO-rev, suggesting that PAO-muc contains an insertion element. Sequence analysis of the nonmucoid clones indicated that a 2,561-bp fragment corresponding to IS-PA-4 and a 992-bp fragment corresponding to IS-PA-5 were not present in PAO or PAO-rev. Both nonmucoid clones, however, contained in the same location as IS-PA-4, a 1,313-bp region which was not present in PAO-muc. DNA probes complementary to this sequence, designated IS-PA-6, did not hybridize with PAO-muc, indicating that this sequence had been replaced upon conversion to the mucoid phenotype. Between IS-PA-4 and IS-PA-5 there was a 500-bp sequence which was 94% identical to the 500-bp sequence downstream of IS-PA-6. These insertion elements had some DNA sequence similarity to plasmid and transposon sequences, suggesting that they may be of plasmid origin. IS-PA-4 and IS-PA-5 were shown also to be present in two mucoid isolates from cystic fibrosis patients. The insertions occurred in the same location upstream of the *toxA* gene, suggesting that this type of genetic recombination may also be associated with mucoid conversion in some *P. aeruginosa* clinical isolates.

One of the most notable features of strains of *Pseudomonas aeruginosa* colonizing the airways of cystic fibrosis patients is the predominance of colonies with the mucoid phenotype because of the production of excessive amounts of alginate (10). Evidence suggests that the respiratory tracts of cystic fibrosis patients are initially colonized by nonmucoid *P. aeruginosa* strains, which are converted to the mucoid phenotype (21, 36). Reversion to the nonmucoid phenotype readily occurs when mucoid isolates are cultured on artificial media in the laboratory.

The synthesis of alginate is extensively regulated, presumably because the production of this material is energetically expensive for the organism (35). A number of biosynthetic and regulatory genes required for the production of alginate have been identified, and their role in alginate synthesis was recently reviewed (34). Flynn and Ohman have identified two closely linked genes, *algS* and *algT*, which are involved in the switch between the mucoid and the nonmucoid phenotypes (12). The *algB* gene appears to be involved in the production of high levels of alginate, although it is not required for alginate synthesis (17). This gene has been shown to have sequence similarity with genes encoding the response regulatory class of two-component regulatory proteins. The central domain of *algB* has significant homology to the NtrC subfamily of transcriptional activators, and *algB* has been shown to be required for the transcriptional activation of *algD* (53). Three other genes, *algR1*, *algR2*, and *algR3* (alternatively designated *algR*, *algQ*, and *algP*, respectively), have been shown to be required for *algD* transcription (8, 9, 17, 25, 27, 53). The *algR1* gene product also appears to belong to a regulatory class of two-component sensory transduction systems but is distinct from the *algB* gene product (8, 9). *algP*, or *algR3*, is a histone-like element which is involved in the regulation of *algD* yet is independent of the signal transduction regulators *algB* and *algR* (25, 27). *algU*, a gene which regulates *algD* transcription and has sequence similarity to the gene encoding an alternative sigma factor, was recently described (32). The production of alginate requires, therefore, a complex network of regulatory elements involving many different loci on the chromosome and several levels of control, including bacterial signal transduction systems and histone-like elements.

A number of environmental factors have been shown to influence mucoid conversion in vitro. The growth of nonmucoid strains in static cultures with acetamide as the sole carbon source resulted in mucoid isolates for 19% of the strains examined (43). Mucoid conversion was demonstrated in a chemostat system under culture conditions which resulted in a slow growth rate or nutrient deprivation (47, 48). Energy-poor carbon or nitrogen sources consistently produced mucoid subpopulations, whereas energy-rich carbon and nitrogen sources, such as glutamate or gluconate, failed to induce mucoid conversion (47). Phosphate or iron limitation also resulted in the conversion to mucoid populations in continuous cultures (47, 48). Additionally, mucoid colonies were reported to have a growth advantage over nonmucoid colonies under conditions of severe nutrient deprivation (48). These studies (43, 47, 48) suggested that the energy state of the organism

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has a major role in mediating conversion to the mucoid phenotype.

We have reported the conversion of *P. aeruginosa* to the mucoid phenotype for a chronic pulmonary infection model in rats (52). At 6 months after the initial inoculation, organisms isolated from the lungs demonstrated the mucoid phenotype. These studies indicated that in vivo conversion to the mucoid phenotype by *P. aeruginosa* is not unique to the cystic fibrosis lung environment but may be associated with chronic *P. aeruginosa* infection states. The trigger which results in mucoid conversion may be the nutrient deprivation and low energy status associated with chronic infections.

The mucoid strains recovered from the rat lungs produced significantly lower levels of exotoxin A, exoenzyme S, phospholipase C, and pyochelin than the parent strain. The production of these enzymes returned to parental levels in the nonmucoid revertant strains. Digestion of chromosomal DNA from the parent, mucoid, and nonmucoid revertant strains and hybridization with virulence factor-specific probes demonstrated that conversion to the mucoid phenotype was associated with a rearrangement of chromosomal DNA upstream of the exotoxin A gene (52). This rearrangement could have occurred by deletion, inversion, duplicative transposition, or insertion, although the mapping data indicated that the gene rearrangement likely occurred via an insertion or an inversion. The objective of the present study was to determine the type of recombinational event which occurred upstream of the exotoxin A gene and which was associated with mucoid conversion in these strains and to determine whether the same event occurred in *P. aeruginosa* isolates from cystic fibrosis patients. To further investigate this mechanism of gene rearrangement, the region of DNA upstream of the exotoxin A gene was cloned from the parent, mucoid, and nonmucoid revertant strains and comparatively analyzed.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *P. aeruginosa* PAO is the nonmucoid parent strain used in this study. PAO-muc is the mucoid derivative strain previously isolated from rats chronically infected with PAO for 6 months (52). PAO-rev is the nonmucoid revertant strain isolated from PAO-muc after passage on artificial medium (52). *P. aeruginosa* mucoid and nonmucoid isolates from adult cystic fibrosis patients were generously provided by H. R. Rabin, University of Calgary. Strains 3144 and 3162, which have been described in detail, are both mucoid isolates from this group of patients. Genetic manipulations were performed with *Escherichia coli* DH5α F′.

Cultures were routinely grown in Luria broth (GIBCO) or on Luria agar containing 100 μg of ampicillin per ml for plasmid maintenance. *P. aeruginosa* strains were cultured on modified Mian's medium (28) to maintain the mucoid phenotype.

Genetic manipulations. Chromosomal DNA was isolated by the method of Goldberg and Ohman (16). Plasmid DNA was isolated either by the alkaline sodium dodecyl sulfate (SDS) method of Birnboim and Doly (2), followed by cesium chloride gradient ultracentrifugation, or the method of Takahashi and Nagano (46) for small-scale preparations.

Cloning of DNA fragments containing the region upstream of *toxA* was performed with pUC19 (54). Chromosomal DNA was digested with *Eco*RI and fractionated on a 10 to 40% sucrose gradient. Fragments of 10 to 15 kb were ligated into pUC19 and transformed into *E. coli* DH5αF′ by electroporation with a Bio-Rad Gene Pulser apparatus under the recommended conditions. Clones with DNA inserts were transferred to nylon membranes (GeneScreen Plus; Dupont) and screened by colony hybridization (51) with the 0.7-kb *Pst*I-*Nru*I fragment upstream of *toxA* as a probe (49). DNA fragments used as probes were isolated from agarose gels with Gene-Clean (Bio-Can Scientific, Mississauga, Ontario, Canada) and labeled with [^{32}P]dCTP by use of an oligonucleotide labeling kit (Pharmacia). For hybridization experiments with isolated DNA, restriction endonuclease digests of chromosomal or plasmid DNA were transferred to GeneScreen Plus by use of a Pharmacia vacuum blotter. Hybridization was performed at 65°C with 10 ml of 1% SDS–10% dextran sulfate. The membranes were washed twice for 5 min each time in an aqueous solution containing 0.3 M sodium chloride and 0.03 M sodium citrate at room temperature. This step was followed by two washes at 65°C for 30 min each time in the same buffer containing 1.0% SDS. Two final washes were done at room temperature for 30 min each time in 0.015 M sodium chloride–0.0015 sodium citrate. After hybridization, the blots were dried and subjected to autoradiography overnight at −70°C. Oligonucleotide probes used in hybridization experiments were end labeled with [γ-^{32}P]ATP by use of T4 polynucleotide kinase (GIBCO-BRL, Bethesda, Md.). Hybridization with oligonucleotide probes was performed at 55°C.

Northern (RNA) blot analysis of *toxA* mRNA was performed as described by Frank et al. (13) with the internal 1.5-kb *Bam*HI fragment as a probe to measure *toxA* transcript accumulation.

Sequence analysis. Appropriate fragments of plasmid DNA were subcloned into M13mp18 and M13mp19 vectors (54). Nested deletions were constructed in M13mp19 single-stranded DNA by the method of Dale et al. (7) with a Cyclone Biosystem kit (International Biotechnologies, Inc., New Haven, Conn.). Sequencing was performed by the dideoxynucleotide chain termination method (40) with T7 DNA polymerase (Sequenase; U.S. Biochemical Corp.) according to the manufacturer's recommended procedure. In some cases, terminal deoxynucleotidyltransferase was added after termination of the reactions to resolve ambiguities in G+C-rich templates (30). Sequencing was completed with synthetic oligonucleotide primers (University of Calgary Regional DNA Synthesis Laboratory). Both strands of DNA were sequenced. DNA sequences were analyzed with the IBI Pustell and PC/Gene (Intelligenetics) analysis systems.

PCR. The PCR was performed with a Perkin-Elmer Cetus Thermocycler. Reaction mixtures contained 100 pmol of each primer, approximately 1 ng of target DNA, 4 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate and 2.5 U of *Taq* polymerase. The DNA was initially denatured at 97°C for 10 min immediately prior to cycling reactions. The DNA was subsequently denatured at 90°C for 1 min, primers were annealed at 55°C for 1 min, and products were extended at 72°C for 1 min. Thirty cycles were performed.

RESULTS

Cloning of the region upstream of the *toxA* gene in mucoid and nonmucoid PAO. In a previous study (43), Southern hybridization analysis of chromosomal DNA isolated from the PAO, PAO-muc, and PAO-rev strains suggested that a chromosomal rearrangement had occurred upstream of the exotoxin A gene in the mucoid strain. The 0.7-kb *Pst*I-*Nru*I toxin A upstream probe hybridized to a 9.7-kb *Bgl*II fragment in PAO and PAO-rev, whereas it hybridized to an 8.0-kb *Bgl*II fragment in PAO-muc. A 1.5-kb *Bam*HI-*Bam*HI fragment internal to the toxin A structural gene also hybridized to the

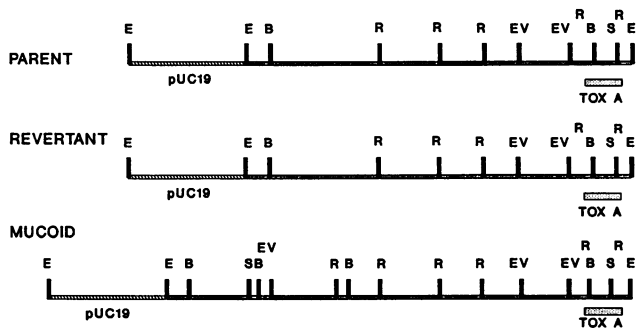

FIG. 1. Restriction endonuclease maps of clones isolated from the PAO, PAO-muc, and PAO-rev strains. The clones from the parent (pSL-NM) and the revertant (pSL-REV) contain a 10-kb *Eco*RI insert. The clone from PAO-muc (pSL-MUC) contains a 12-kb *Eco*RI insert. The location of the exotoxin A gene is indicated by the bar below each map. The restriction enzymes shown are as follows: E, *Eco*RI; B, *Bgl*II; S, *Sal*I; EV, *Eco*RV; R, *Bam*HI.

same fragments as well as to a 6.9-kb *Bgl*II fragment, since there is a *Bgl*II site internal to the toxin A gene. The 6.9-kb *Bgl*II fragment was identical in the DNAs from the nonmucoid and mucoid strains. These studies, along with hybridization experiments involving digestion with different restriction endonucleases, suggested that a chromosomal rearrangement had occurred between 5.0 and 8.0 kb upstream of the exotoxin A gene in PAO-muc.

To determine the mechanism of chromosomal rearrangement which had occurred in the mucoid strain, the region upstream of the toxin A structural gene was cloned from PAO, PAO-muc, and PAO-rev. *Eco*RI fragments were cloned to allow the cloning of a single DNA fragment which included the toxin A gene and approximately 9.0 kb upstream of this gene. The upstream *Pst*I-*Nru*I fragment was used as a probe to identify clones containing this fragment of DNA in pUC19, transformed into *E. coli* DH5αF′. The three clones, pSL-NM (from PAO), pSL-MUC (from PAO-muc), and pSL-REV (from PAO-rev), contained *Eco*RI inserts of approximately 10.0, 12.0, and 10.0 kb, respectively.

The restriction maps of these clones were determined by restriction endonuclease digestion and comparison with previously published maps of the exotoxin A region (31, 49). The results are shown in Fig. 1. The restriction sites mapped on pSL-NM and pSL-REV were in all cases identical, suggesting that chromosomal changes which had occurred in PAO-muc reverted to the parental genotype in PAO-rev. As had been suggested by the hybridization experiments, a 6.5-kb fragment extending from the first *Bam*HI site on the left side of pSL-NM to the *Eco*RI site on the right side of *toxA* appears to be conserved in all three plasmids. Similarly, the 0.7-kb *Eco*RI-*Bam*HI fragment on the left side of the insert is present in the three clones. pSL-MUC contains at least five restriction sites not present in pSL-NM or pSL-REV. These include a *Sal*I site 1.3 kb from the *Bgl*II site on the left side, two *Bgl*II sites, an *Eco*RV site, and a *Bam*HI site, as shown in Fig. 1. The presence of the additional *Bgl*II site about 4.7 kb from the left side suggests that a rearrangement has occurred in a region of at least 2.5 kb.

Sequence analysis of the PAO-muc clone and evidence for insertion elements. To further examine the gene rearrangement in PAO-muc, a 4.8-kb fragment from pSL-MUC was sequenced. This fragment extends from the *Bgl*II site 0.7 kb downstream of the left *Eco*RI site to the *Bam*HI site 5.5 kb downstream. The nucleotide sequence and location of pertinent restriction endonuclease sites are shown in Fig. 2.

The base composition of the sequenced region is shown in Fig. 3. Conserved coding and noncoding regions of *P. aeruginosa* DNA have an average G+C content of approximately 67%. Two distinct regions of the sequenced fragment are significantly A+T rich. One is approximately 2,000 bp, with an average G+C content of less than 40%, and the second is approximately 800 bp, with an average G+C content of about 47%. Between these two A+T-rich regions is a region of about 600 bp which is very G+C rich and is more typical of *P. aeruginosa* DNA.

Pritchard and Vasil (38) have described insertion elements located within 3.0 kb upstream of the exotoxin A gene in *P. aeruginosa*. These putative insertion sequences, designated IS-PA-1 and IS-PA-2, are about 1,000 and 785 bp, respectively, have 5-bp direct repeats at their boundaries, and have an average G+C content of 50% or less (38). Since the regions that we have sequenced share some properties with the elements described by Pritchard and Vasil (38) and are in the same area of the chromosome, we have designated the 2-kb region and the 1-kb region IS-PA-4 and IS-PA-5, respectively.

To determine whether the A+T-rich regions are due to insertion elements, DNA probes constructed internal to these regions were used in hybridization experiments with chromosomal DNAs isolated from PAO, PAO-muc, and PAO-rev, as well as plasmids pSL-NM, pSL-MUC, and pSL-REV. The 0.6-kb *Sal*I-*Bgl*II fragment was used as an internal probe for the IS-PA-4 sequence. This probe hybridized to a 1.9-kb *Bgl*II fragment and to a 9.0-kb *Sal*I fragment from PAO-muc chromosomal DNA (Fig. 4A) and pSL-MUC (data not shown) but did not hybridize to chromosomal DNA from either PAO or PAO-rev (Fig. 4A). This probe also did not hybridize to the clones from the nonmucoid strains (data not shown). In some experiments, faint bands approximately 10 kb in size were observed in both *Bgl*II and *Sal*I digests upon extended exposure of the X-ray film. To determine whether the IS-PA-4 probe was hybridizing specifically to these fragments, PCR amplification was also used to determine whether this sequence was present in the nonmucoid strains. The oligonucleotide primers 5′-TTGATGCTATGCTCATTGAC-3′ and 5′-AAACTGAAAGAACGAGAAAGTTTCA-3′ were used to amplify a 734-bp fragment containing the *Sal*I site. The results, shown in Fig. 4B, confirm that this DNA sequence is only present in strain PAO-muc and pSL-MUC and is not present in the chromosomal DNAs of the nonmucoid strains. Oligonucleotide primers complementary to the IS-PA-5 sequence were hybridized to chromosomal DNAs from PAO, PAO-muc, and PAO-rev and to plasmids pSL-NM, pSL-MUC, and pSL-REV digested with *Bgl*II. As was the case with the IS-PA-4 probe, the IS-PA-5 probe only hybridized to *Bgl*II fragments from pSL-MUC and PAO-muc chromosomal DNA (Fig. 4C). These results indicate that the genetic rearrangement in PAO-muc is due to the presence of inserted DNA and not to an inversion in this region. The putative insertion elements are present in a single copy in PAO-muc and do not appear to be present in chromosomal DNAs isolated from PAO and PAO-rev.

There are no inverted repeat sequences or duplicated target sequences flanking IS-PA-4. IS-PA-5 has direct repeats of 54 bp, with only four mismatches flanking the A+T-rich region. These direct repeats extend from bp 3726 to 3780 and from bases 4766 to 4820.

Sequence analysis of the nonmucoid clones. To determine the precise locations of the IS-PA-4 and IS-PA-5 insertions, the corresponding 2.5-kb *Bgl*II-*Bam*HI fragment was se-

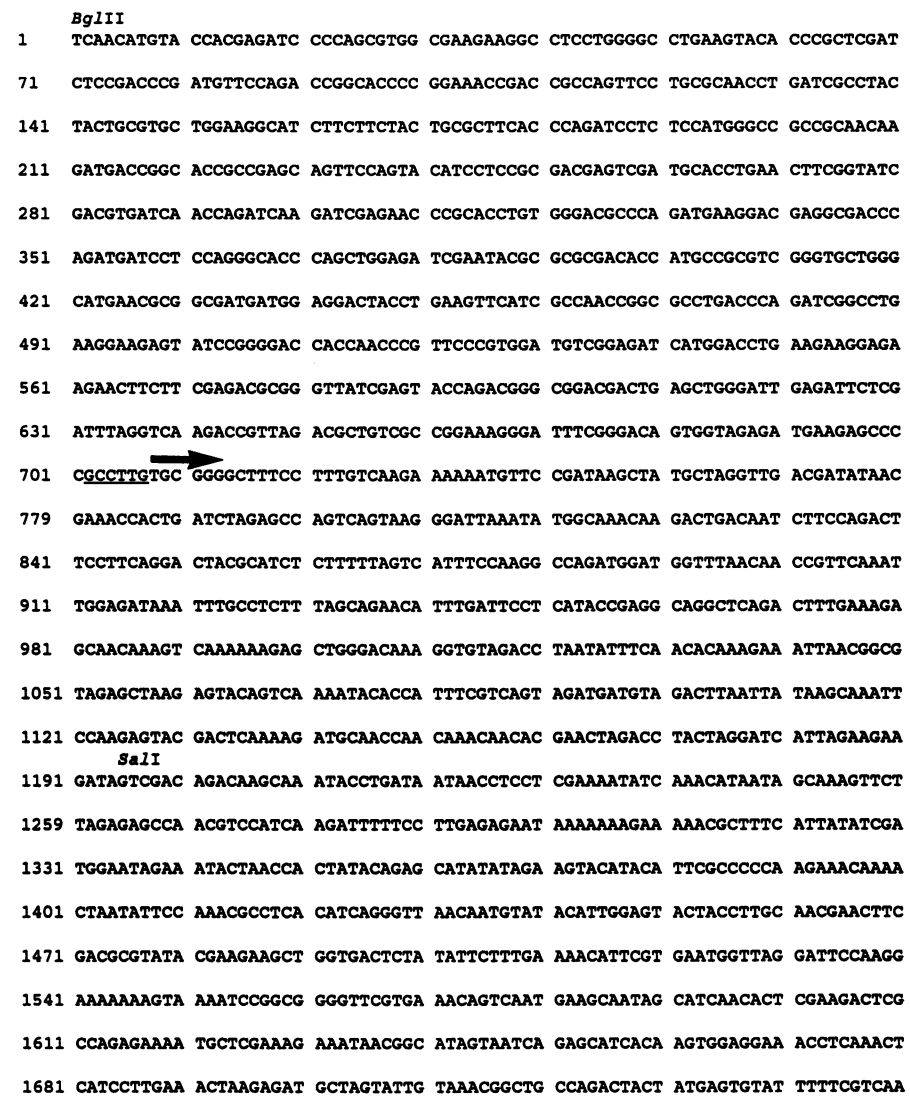

FIG. 2. Nucleotide sequence of the 4.8-kb *Bgl*II-*Bam*HI fragment from pSL-MUC. Both strands of DNA were sequenced completely. Selected restriction endonuclease sites are indicated above the sequence. The beginnings and ends of the IS-PA-4 and IS-PA-5 sequences are indicated by the arrows above the sequence. The potential target sequence duplications are underlined.

quenced from pSL-NM. The nucleotide sequence is shown in Fig. 5. Portions of pSL-REV were also sequenced, and in all cases the sequences obtained from PAO and PAO-rev were identical. A diagram depicting the comparison of the mucoid and nonmucoid sequences is shown in Fig. 6. The first 709 bases downstream of the *Bgl*II site were identical in the clones from PAO and PAO-muc. PAO-muc contained a 2,561-bp region from bp 710 to 3271 which corresponded to IS-PA-4 and which was not present in PAO or PAO-rev. PAO and PAO-rev, however, contained a 1,313-bp region which was not present in PAO-muc. Both PAO and PAO-muc contained an approximately 500-bp region downstream of their unique sequences which was 94.6% identical. IS-PA-5 was located just downstream of this region in PAO-muc, and the repeats at the end of IS-PA-5 were almost identical to the sequences flanking the region upstream of the *Bam*HI site in PAO or PAO-rev.

To determine whether the 1,313-bp region found in PAO and PAO-rev was present in more than one copy in the nonmucoid strains and to determine whether this sequence was present elsewhere in the mucoid strain, an oligonucleotide probe complementary to this sequence was used in hybridization experiments with these strains. This probe hybridized to PAO, PAO-rev, pSL-NM, and pSL-REV but not to PAO-muc and pSL-MUC, indicating that this sequence was present in the nonmucoid PAO strains in a single copy and was not present in the chromosome of PAO-muc (Fig. 7). The 1,313-bp fragment from the nonmucoid strains, designated IS-PA-6, has a G+C content typical of *P. aeruginosa* DNA; however, there are A+T-rich regions at both ends of this fragment. At the beginning of IS-PA-6 there is a 225-bp region with a G+C content of less than 50%. There is a 75-bp region at the end of IS-PA-6 with a G+C content of 50%. There are no inverted repeats or target sequence duplications at the ends of IS-PA-6.

Similarity of IS-PA-4, IS-PA-5, and IS-PA-6 to known sequences. To determine the possible source or function of the elements contained in the mucoid and nonmucoid clones, the

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1751 TCCTGCCGAT GGACATGTGC TAGCAAAAAT TGTAGAGACA GAAGATTCAT GGGAGATAGC TAAGGCGGCC
                                                 EcoRV                    BglII
1821 AATTTCCAGA CAGAAATAGA ACGAATAGAA CTTGAACTAG CCAGATATCT AAGACCACAA CTTGCCAGAT

1891 CTGTTGGAGC AGAGAATAAT TTTAAATTCG ACCAAAAAGA AGTAACAAAA GGCAAATCAG CCTTTGCTCT

1961 ACTCGACCAA ATATATAAAG ATGAAATTTG CTATGATGAA CTAAAATCGA TATTTATCGG ACTATTTTCA
                    EcoRV
2031 CGCTCTGCAA ACAATGCGAT ATCGCCAAAC TATACCGAGC TTAGAATTGA TGTACTTCAA AACTTCGAAA

2101 GAGACTCTGA AAAATCTAAA TTCCTAGAGG CTCTTTTCGT GCTCCACAGC AAATCATCTA CGGCGATGGA

2171 GTCTTTGAAA GATGGTTTGT TAAAACCTGA AATTATGGAT CTATTTAAAA GATTTTGGAA AGAAGACAAA

2241 CCATCATATA GGGCATTTGT CACACTCTTA GCCATATTCA GCGCCCTTGA CAAAAAAAAT CGCAGGTTTG

2311 AAGACTACAA CGACATAAAG AGCGGAATAA TAAAACTAGC AGGACAAATA GAAATCGACC CCAGCGAATA

2381 CATAGAAACA TATATAAAAG CCTTTAAAAC TATAGCGCTA GACGTCCTCA AAGGCAGCGA GGACAAGGAT

2451 AAAATGCTGC AAAGCATGTA TCATCACATT GGCTCAATGA ATTTTGAAAA TGCTTTACTA TCCATGTCAC

2521 TATTGTAAAT AACTCCCTAA GGAGCTATTC CTCAAATTTT TATTTTCTGA CACTCCCTTT CGCCTCCGGA

2591 GCGTCACTTT TGCCAGTCGC GGCAAAAGTA ACCAAAAACG CTTGCCCCTG CATCCGGCCC CAGTCGCTTC

2661 GCGACTGGGG TTCCCTCCAT CGCCGTTCCG GGGGCACGCC GCCACGGTCC TTCGTGGACC GAGGCGGCTT

2731 TCGCGGCATC CTGCCGCTCA ACCCCCTACA CGACGATTCC GCTCGGCCTC CTGATGGGGC GGACCGGAGC

2801 GTCGGATATT TCTCTGGAAG AGGTAGGGCA AATCACGCAC AGCGTGATTC GCAAGATGGG GCAGAGTTGC

2871 CAGCGCCGGG CGGCGCAGGT TTGGTAAAAA GCCTTGTTTC ATGTGGTTTC TTTGATAGAG AGAGTCTTTC

2941 AGCTTCGCTC ATTCCGCTCG GTTGTTTCGT TCCTCTCGTC TCAATACTCT GCCACCGGCG CCTAAGAAAC

3001 GCTAAACCAC GCAGCGGCCA GACCGCTCCC GACAGCCAAG CGGCTTTTTT GAGTCTGCGG TATTCTCTCG

3081 CCCTCAAAAT CTCTGCAACG CCGGGAGTGG ACGAATACAA GACCCGAAAG GAAATATGTC CGGCCCTCTG

3159 CGTGGGGTTT TCTTAACTCC CGGCACCCCA GCCCCAAGAA AGCTGGTCAC ATCACGCAGA GGTAACGGAA

3221 ATGCCTATCA CCAATCCCAT ATCTCCATCC ACACCGCTTC ATCCCACTAC CTCCCCGGAG GTGCGCCATG

3291 TCTGACGCCC CCCTCCACTA CAGCCGTTTC ACCCACCACC ATCGGGTCCT GCGCGCCGTG CTGCTGGATG

3361 AGGAAGGCTG GTTCGTGCTC TCCGACCTGG TACGGCTGCT GGGCCGTTAC CTGGGCTGTC GGGCGCCGGC

3431 GGCGTTGTGT GACGAGGCCG TGGCCGCTGG CGACGGCGGA ACAGCGCGAG CGCTTGTTCG CCCTCTGTCA

3501 TGCGTTGGAG CGGCATCTGG ACACCGATCA GTGGCGGCTC GCCTGGCTCC ATGACGAACG CCACGGGCCA

3571 CGCCAGGATT GCCTGGTCAG CGAGTCCGGG CTCTATGCCT TGCTCTGGCT GCGCTGCCAG GCGCGGCCGG
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FIG. 2—*Continued.*

nucleotide sequence of each element as well as the 500-bp similar region was used to search the data base of prokaryotic DNA sequences. IS-PA-4 had approximately 50% sequence homology with sequences from *Chlamydia trachomatis* plasmids (6, 44), *Streptococcus faecalis* plasmid pAD1 (15), *Bacillus* plasmid pTB913 (37), *Lactobacillus hilgardii* plasmid pLAB1000 (24), *Staphylococcus aureus* plasmid pE194 (22), transposon Tn*917* (42), and *Clostridium butryicum* plasmid pCB101 (accession number X62684).

The first 98 bp of IS-PA-5 were 92% identical to an interstrain *toxA* homologous region from *P. aeruginosa* WR5 (38). IS-PA-5 had the highest homology (50 to 52%) with plasmid sequences, including those of plasmid RSF1010 (41), the 28-kDa VirA protein from the *Salmonella typhimurium* virulence plasmid (19), the 29-kDa protein from the *S. choleraesuis* virulence plasmid pKDSC50 (33), integron IN0 from *P. aeruginosa* plasmid pVS1 (3), Tn*1696* from plasmid R1033 (50), and the *E. coli* plasmid-associated trimethoprim-resistant dihydrofolate reductase gene (55).

IS-PA-6 had 52.6% homology with *Mycobacterium smegmatis* transposase insertion sequence IS*6120* (18). The conserved region between IS-PA-4 and IS-PA-5 had 57% homology with the *P. aeruginosa* TrpI transcriptional activator of the *trpAB* genes (5). It also had homology to the putative integrase/transposase gene from *Streptomyces clavuligerus* IS*116* (29). The corresponding sequence from the nonmucoid strains also had homology with the IS*116* gene as well as the *tnpA* gene for the transposase from *P. aeruginosa* Tn*501* (4). The nonmucoid sequence also had 57% homology with the PilR transcriptional activator of the *P. aeruginosa pilA* gene (23).

A number of potential open reading frames have been identified in the sequenced regions from the mucoid and nonmucoid clones. The locations and sizes of these open reading frames are indicated in Fig. 6. When the SwissProt data base was searched with the translated sequence of these open reading frames, no significantly similar sequences were identified, despite the extent of the DNA homology observed.

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3641 GGGTTTGCGT CGGGTCAGCG GCTCGGTACT GCCACGCCTG CGCAGCCAGG CCCACCCCAA CGCCACGCCC
                                                                      BamH1
3711 CAGCGCGCCG TGCTGCACTG GAAAACCGCC GAGATCGACA CGCTGCACTG GCAAGGCAAG ACCTGGATCC
3781 CCCTCTCCGA CTGCCCCCAA CTCCTCGACA GCCCACGCCC GCTGATCCGG GCCTGACCCC CGTCATACGC
3851 CCTGAAAGGA GTCAGGGCTT GTAACAACAG CACGACCTTT GGCAACCTTT CCCCAATGCC CCGGCACGCT
3921 CGTCTGTACA GATCCTAGGA CTGGATTTCC AAGCCCTGTT GCTACCCAGG GCCCCGCAGA AATACCAAAG
3991 CCAAGGTCCT TCCAAAGCCC CTCGTTCGAA TCGTCTGGTA ACCAGACAAA CGGCCCGGAG CACTTACTGA
                         BglII
4061 AACTTATGCC ACCACCAATA GAGATCTAGA TCACATTTTA TTGTAAGCAG TGATGGCAAA GTTCCCGCAG
4131 AGCGACACAA AGACCACTGG AAGTCCACTT GAATGGCTCA CCAGAAACGC TATTCGACTA ATGAGAGGAA
4201 CATGGTGAGC GACAAGAAGT GGTGCGTTAT CGCTGTAGCA GCGCTTTATC TGCTCAGTGG AATACTCTTG
4271 ATAAGGTCTC CGATCAGCCT GCCCCCCTTC GTCGAAAGCG TATGGAGGTT CTACAGCAGC ATTGGTTTCG
4341 ATATGAGTCG AGACATTGCG CGAGCTGTCA TACTCTCTCT GAGTCTTTCA TGCTTGGCCC ACGCATTAAT
4411 AATACTCACG ACTTATATCA CCAATGGAGG GCTACCAAAG GTTCTCAGGG GGCATGAGCA GTATCTCTTC
4481 CTCAGAGCGC TGTTTTACCT TGGCCTTTGC ATTCTGGGTA TTGGGGCGAT GGGAGACAAT ATATCCGCAA
4551 GGAGAACCTC TTCATTCAGC AAGGAACTAT CGATTCTGCT GGGAGCTTCT ATCTGGGAGG AGTGGTTTGG
4621 ACAGTGAGTG ATTTGCTTGC GTCAATCATC CTGAAGTTTA AGAGGTGAGT ACGACAGCCA ACTCGCCTGG
4691 GGGCAATTCT CCAACGTTCT CACCGCACCT CCACCCTAGT CGTTCACCCA CCACCGGGCC TTGCGCGCCG
4761 CGCTGGCAGA CCGCCGAGAT CGACACCCTG CACCGGCAAG GCAAGACCTG GAT
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FIG. 2—*Continued.*

Therefore, it was not possible to infer the function of any of these potential coding sequences.

Evidence for the presence of IS-PA-4 and IS-PA-5 in *P. aeruginosa* isolates from cystic fibrosis patients. Since IS-PA-4 and IS-PA-5 were identified in *P. aeruginosa* strains which were converted to the mucoid phenotype during chronic infections in rats, it was important to determine whether this phenomenon occurs in respiratory isolates from cystic fibrosis patients. Chromosomal DNAs from 10 mucoid and 7 nonmucoid strains were hybridized with the IS-PA-4 and IS-PA-5 probes (data not shown). Both probes hybridized to DNA fragments from two mucoid strains, 3144 and 3162. The hybridizing fragments in these strains were identical in size to the fragments which hybridized in PAO-muc DNA. These probes did not hybridize to any DNA fragments in the nonmucoid strains or in the other eight mucoid strains examined. These data indicate that these insertion elements are present in at least some respiratory isolates from cystic fibrosis patients.

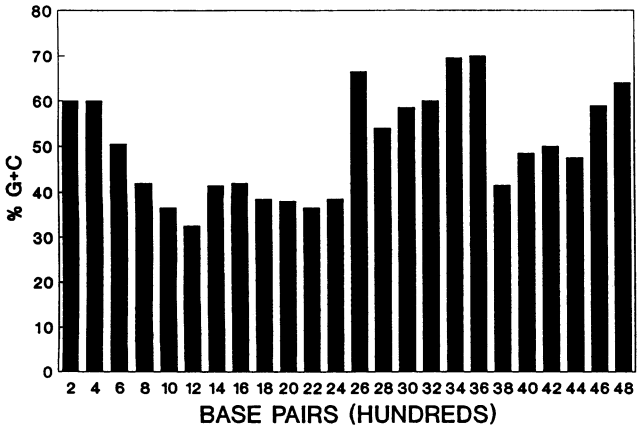

FIG. 3. Base composition of the 4.8-kb *Bgl*II-*Bam*HI fragment from pSL-MUC. Each bar on the graph represents the average G+C content of each 200 bp of this fragment. Segments from 8 to 24 and 38 to 44 represent areas of higher A+T content than is typically observed in *Pseudomonas* DNA.

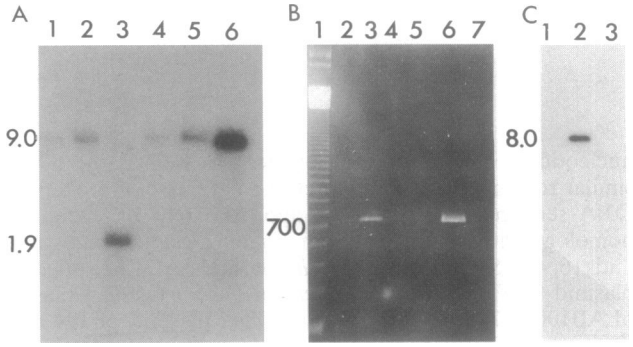

FIG. 4. (A) Southern hybridization with the IS-PA-4 DNA probe. A 0.6-kb *Sal*I-*Bgl*II fragment internal to IS-PA-4 was hybridized to *Bgl*II (lanes 1 to 3) and *Sal*I (lanes 4 to 6) digests of genomic DNAs from PAO-rev (lanes 1 and 4), PAO (lanes 2 and 5), and PAO-muc (lanes 3 and 6). (B) PCR amplification of the IS-PA-4 sequence. Oligonucleotide primers TTGATGCTATGCTCATTGAC and ACTT TGAAAGAGCAAGAAAGTCAAA amplified a 734-bp fragment containing the *Sal*I site. Lanes: 1, molecular weight standards; 2, PAO genomic DNA; 3, PAO-muc genomic DNA; 4, PAO-rev genomic DNA; 5, pSL-NM; 6, pSL-MUC; 7, pSL-REV. (C) Southern hybridization with the IS-PA-5 DNA probe. Lanes: 1, PAO; 2, PAO-muc; 3, PAO-rev. Numbers at left of panels A and C are in kilobases.

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675  GGGACAGTGG TAGAGATAAA GAGCCCCGCC TTGTGGTGCT CTTCATTAAA GAGAAATAAA AACCCAAGGT
745  GTTCAGCAAG TTAATTCACC AAATCAATAG AAGATATGTG CCAATATGTG CCAAGCTCAC TATCCCCAAG
815  ACACCATCGA CAGACTATTT GAGTCAAACA GCCCTAGAAA ACAAGAAATA AAAACATGCT CAACCATTCG
885  CTCAATCTGA AATCTCCGTG ACGGTAGAAT AACGCGCAGC GTTATCCGCC AGCTGGCCAA GGTACGCTCC
955  GAAATCCTTC TGGATACAGG ACGATCCCCA ATGCCAAGCT ACCGACGAAC TGGGTCCCCG GCGGCACCTA
1025 TTTCTTCACC GTCACCCTGC ATGACCGCCG CTCCAACCTG CTGACCCGCG AAATCGACCT GCTGCGCCGC
1095 GTGGTCGCCC AGACCAGGCG CCGGCATCCC TTCCGCATCG ATGCCTGGGT CGTGCTGCCG GAGCACATGC
1165 ATTGCCTCTG GACCCTGCCG CCCGACGACG CCGATTTCGC CACCCGCTGG AAGGTCATCA AGTCCGGCTT
1235 CGCACGGCGC ATCCCCTGCC ACGCATCACG TACCTTCGCG CAACGGCGGC GAGGACAACG CCGCATCTGG
1305 CAGCAGCGCT ACTGGGAACA CCTGATTCGC AATGACACGG ACTACCGGCG CCATTTCGAC TACATCCATC
1375 TCAACCCTGT GAAACACGGG CTGGTTACGG CGGTCAAGGA TTGGCCCTTC TCCACCTTCC ACCGGGCCGT
1445 GGCTGATGGG CTTTATCCCG AGGATTGGGC CGGTGATCCT TCCTTGGAGG TCCGGGCGGC CGAGCGGGTT
1515 TGAGTCTGCT GCGGGATAGG ACTGTGCCTT ATTCGCCCTA CCCCGGCTAC CAAGTCTTTT TCTCGGCCTC
1590 GCAGGATGGC CGGCCCCGAG AAGTAACCAA AGCGCCTTGC CCCTGCATTC GGCCTCCTAC ACGACGATTC
1660 CGCTCGGCCT CCTGATAGGG CGGACCGGAG CATCGGATGT TTCTCTGGAA GAGGTAGGCG AATAGACGAA
1730 GCGTTATCCG CAGCTACACA GGATTTTCCT GTAACGCCGA GCATCGGGGG ATTGAAAGAA CTTCGTCAAG
1800 TCGATGTACA AGCAATGGCT TCAGAGAATC GGCGGATAAC TGCTTTGCAG TTATTCGCCC TACCGTTGAC
1870 CGCTATCGGT GCGCTCCATA ATCATTCTTC GGCGCTAAGA ACGCATACCA CCCAGCGGCA AACCGTTCCC
1940 GAAAGTAGCG GTGTATTCCA TTGAGGAAAT GCGTATGCCA TTCGTTTTTC AACCCACCCC ATTCATCCCT
2010 CGCGGCACGA CACCAACACC ACCTCCCCGG AGGTGCGCCA TGTCTGACAC CCTCCTCCAA CCCAGCCGTT
2080 TCACCCACCA CCATCGGGTC CTGCGCGCCG TGCTGCTGGA TGAGGAAGGC TGGTTCGTGC TCTCCGACCT
2150 GGTACGGCTG CTGGGCCGTT ACCTGGGCGG TCGGCGGCCG GCGGCGTTGT GTGACGAGGC CGTGGCCGCT
2220 GGCGACGGCG GAACAGCGCG AGCGCTTGTT CGCCCTCTGT CATGCGTTGG AGCGGCATCT GGACACCGAT
2290 CAGTGGCGGC TCGCCTGGCT CCATGACGAA CGCCACGGGC CACGCCAGGA CTTGCCTGGT CAGCGAGTCC
2360 GGGCTCTACG CTCTGCTCTG GCTCGCACGC CTGCGCGGCA CGGGGGTTTG CGCCGTTGGG TCAGCGGCTC
2430 GGTGCTGCCA CGCCTGCGCA GCCAGTCCCG CCCCAACGCC ACGCCCCAGC GCGCCGTGCT GCACTGGAAA
2500 ACCGCCGAGA TCGACACCCT GCACTGGCAA GGCAAGACCT GGATCCTCT
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FIG. 5. Nucleotide sequence of the nonmucoid 2.5-kb *Bgl*II-*Bam*HI fragment. The first 675 bp, which are identical to the mucoid sequence, are not shown. The beginning and end of the IS-PA-6 sequence (see the text) are indicated by the arrows above the sequence.

To determine whether these insertion elements are inserted randomly in the chromosome or whether there is a target site upstream of the toxin A gene, *Bgl*II digests of DNAs isolated from strains 3144 and 3162 were hybridized to the *Pst*I-*Nru*I upstream sequence probe and the IS-PA-4 probe. The IS-PA-4 probe hybridized to a 1.9-kb *Bgl*II fragment in PAO-muc and strains 3144 and 3162 (Fig. 8A). The *Pst*I-*Nru*I probe hybridized to an 8.0-kb *Bgl*II fragment in PAO-muc, 3144, and 3162 and to a 9.7-kb *Bgl*II fragment in PAO (Fig. 8B). These experiments indicated that the insertion elements are located in the same site upstream of the toxin A gene in the patient isolates and suggested that there is a specific insertion target site for these elements.

Effect of IS-PA-4 or IS-PA-5 on *toxA* transcription. In a previous study, the mucoid strains isolated from rats produced less toxin A than the parent or revertant strains (52). To determine whether the presence of IS-PA-4 or IS-PA-5 affects *toxA* transcription, *toxA* transcript accumulation was examined for cultures of PAO, PAO-muc, and PAO-rev grown in low-iron medium. There were no significant differences in growth rates between the mucoid and nonmucoid strains. No appreciable difference in transcript accumulation was observed for PAO, PAO-muc, or PAO-rev (data not shown). It is possible that *toxA* transcription is not directly influenced by the presence of IS-PA-4 or IS-PA-5 upstream. However, it is also likely that the Northern (RNA) hybridization assay is not sufficiently sensitive to detect a decrease of 20%, as was observed for the ADP-ribosyltransferase assays of the mucoid strains (52).

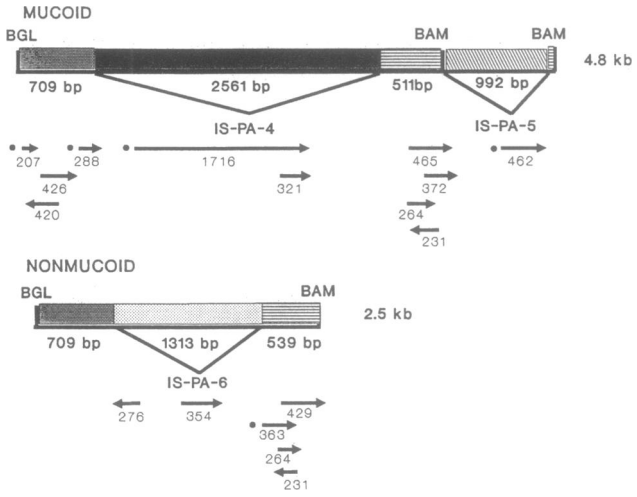

FIG. 6. Comparison of the mucoid and nonmucoid sequences. Bars indicate the locations of homologous or unique sequences in corresponding fragments of the mucoid and nonmucoid clones. Arrows indicate the locations of potential open reading frames longer than 200 bp, as defined by the method of Fickett (11). Circles at the ends of arrows indicate potential prokaryotic coding sequences determined by the method of Kolaskar and Reddy (26).

DISCUSSION

Evidence has been presented that the genetic rearrangement associated with the in vivo conversion of *P. aeruginosa* PAO to the mucoid phenotype is due to the insertion and deletion of insertion-like sequences located 5 kb upstream of the *toxA* gene. Two A+T-rich regions, designated IS-PA-4 and IS-PA-5, were shown to be present only in chromosomal DNA isolated from PAO-muc and not in the parent or revertant strains. A 1.3-kb fragment, designated IS-PA-6, was present in the corresponding region of PAO and PAO-rev but was not present in PAO-muc. Each of these elements was present in a single copy in the chromosome. A 0.5-kb conserved but not identical region was located between IS-PA-4 and IS-PA-5 and immediately downstream of IS-PA-6. The upstream and downstream regions flanking these putative insertion sequences appear to be conserved in both the mucoid and the nonmucoid strains. These data suggest that the rearrangement is not due to an inversion or duplication.

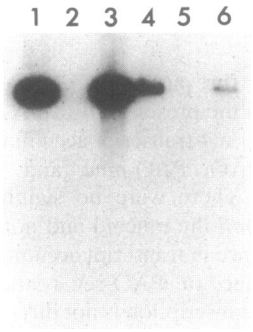

FIG. 7. Southern hybridization analysis with the IS-PA-6 probe. An oligonucleotide probe corresponding to the IS-PA-6 region was hybridized to *Bgl*II digests of plasmid (lanes 1 to 3) or genomic (lanes 4 to 6) DNA from PAO, PAO-muc, and PAO-rev. Lanes: 1, pSL-NM; 2, pSL-MUC; 3, pSL-REV; 4, PAO; 5, PAO-muc; 6, PAO-rev.

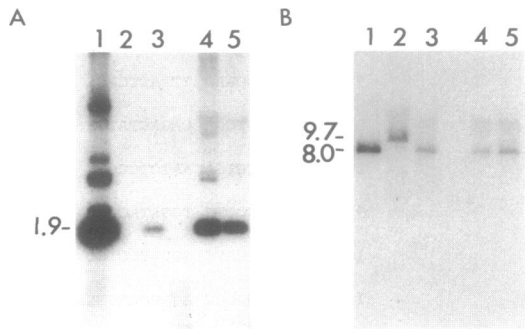

FIG. 8. (A) Southern hybridization of the IS-PA-4 probe to two mucoid cystic fibrosis patient isolates. The *Sal*I-*Bgl*II probe was hybridized to genomic DNAs digested with *Bgl*II. Lanes: 1, pSL-MUC; 2, PAO, 3, PAO-muc; 4, strain 3144; 5, strain 3162. (B) Southern hybridization of the toxin A gene upstream sequence probe to the same isolates. The *Pst*I-*Nru*I probe was hybridized to *Bgl*II digests of the same strains as those in panel A. Numbers at left of panels are in kilobases.

Although we have defined IS-PA-4 and IS-PA-5 as two entities, the data suggest that these elements are transposed as a single unit (IS-PA-4/5). There are no inverted repeats or duplicated potential target sequences flanking either IS-PA-4 or IS-PA-5. However, there is a potential target sequence duplication at the beginning of IS-PA-4 and at the end of IS-PA-5. There is a duplication of a 6-bp sequence, GCCTTG, at bases 702 to 707 and bases 4748 to 4753 (Fig. 2). At the same sites, it is possible that there are actually 10-bp repeats with one mismatch, as the sequences are GCCTTGTGCG and GCCTTGCGCG (mismatch in boldface type), respectively. The 0.5-kb conserved region between IS-PA-4 and IS-PA-5 is only 94% identical in the mucoid and nonmucoid clones. This result would also suggest that these elements are transposed as a unit which contains this conserved region. In hybridization experiments with other *P. aeruginosa* strains, IS-PA-4 and IS-PA-5 have always been found together. We have not yet identified any strains containing only one of these elements. It is possible that IS-PA-4 and IS-PA-5 form a compound transposon. Compound transposons consist of two insertion sequences containing a central nontransposable element (14). The conserved region between IS-PA-4 and IS-PA-5 could be analogous to this central element. Studies are in progress to examine additional mucoid and nonmucoid isolates from other infected animals to determine whether IS-PA-4 and IS-PA-5 are always present together.

IS-PA-4 and IS-PA-5 resemble the IS-PA-1 and IS-PA-2 elements described by Pritchard and Vasil (38) in that they are A+T rich and lack direct or inverted repeats. IS*492*, an insertion sequence from *P. atlantica*, is also A+T rich (1). IS*492* and IS*801* from *P. syringae* pathovar phaseolicola do not have terminal inverted or direct repeats (1, 39). This fact suggests that insertion elements from *Pseudomonas* spp. lack the typical terminal repeat sequences common to other insertion elements. IS*492*, IS*801*, IS-PA-1, and IS-PA-2 all appear to duplicate a 5-bp target sequence, whereas the composite IS-PA-4/5 duplicates at least a 6-bp target sequence. IS*492* was shown to be precisely excised from the chromosome of *P. atlantica* (1). The excision of IS-PA-4/5 is also precise, as the sequences of PAO and PAO-rev are identical in this region. It has not been determined whether IS*801*, IS-PA-1, and IS-PA-2 are excised precisely, since the sequences of chromosomal fragments which have lost these elements have not been reported.

Although the 1.3-kb unique insert in PAO and PAO-rev has been designated IS-PA-6, it does not resemble the other *Pseudomonas* insertion sequences. Its G+C content is more typical of *Pseudomonas* DNA than is the G+C content of IS-PA-4 or IS-PA-5, although it is flanked by A+T-rich regions. Insertion sequences are usually inserted in A+T-rich regions (14). IS-PA-6 does not have any duplicated target sequences. GCCTTGTG is present in the nonmucoid sequence at the beginning of IS-PA-6 but is not duplicated at the end of the sequenced region.

Translated open reading frames from these insertion elements show no significant similarity to known protein sequences in the SwissProt data base. Searches of the EMBL DNA sequence data base suggested that IS-PA-4, IS-PA-5, and IS-PA-6 have sequence similarity to several plasmid and transposon sequences. Homology searches of the DNA and protein data bases with IS-PA-1, IS-PA-2, IS*492*, and IS*801* revealed no significant similarity to other known sequences (1, 38, 39). Therefore, it is not surprising that significant similarity was not found between IS-PA-4, IS-PA-5, and IS-PA-6 and other sequences in the data base, since insertion elements do not appear to have highly conserved sequences. It is also possible that these insertion elements belong to another class of site-specific elements, such as integrons or Tn*7* (20, 45). Integrons, however, have been shown to have highly conserved segments flanking inserted antibiotic resistance genes (45). Although some homology was detected with integron IN0, the degree of homology was not as extensive as that between other integrons sequenced to date.

These insertion elements showed sequence similarity to plasmid DNA. IS-PA-4 and IS-PA-5, because of their G+C contents, certainly do not resemble *P. aeruginosa* sequences. This result suggests that these sequences may have originated from a plasmid or a bacteriophage. Insertion sequences have been shown to occur more frequently in plasmids per length of DNA than in bacterial chromosomes (14). PAO may have acquired a plasmid in vivo from another organism present in the lung environment. Plasmids could have originated from normal flora or some other organism infecting the lung during the 6-month study period. Studies are under way to determine the prevalence of these elements in normal flora from rats as well as other bacterial species which could colonize the lungs of cystic fibrosis patients as well as animals in an animal model. These studies will be useful in determining the reservoir of the insertion elements.

It is likely that the source of these elements is extrachromosomal DNA. The method used to isolate chromosomal DNA would remove some plasmids. Also, large low-copy-number plasmids might not be isolated by the procedures used in this study. It is also possible that the elements are phage encoded. Although the mechanism of insertion has not yet been determined, the data suggest that the acquisition of IS-PA-4/5 and the loss of IS-PA-6 may be due to a double-crossover event with another genetic element. When PAO-muc reverts to the nonmucoid form, there is a second allelic exchange with the same genetic element, resulting in the reacquisition of IS-PA-6 and the loss of IS-PA-4/5. Further studies are needed to determine whether there is a phage or plasmid associated with this process.

All conversions of *P. aeruginosa* to the mucoid phenotype are obviously not due to this exchange of insertion sequences. However, the identification of IS-PA-4/5 in at least two mucoid *P. aeruginosa* clinical isolates suggests that this recombination mechanism may occur in some cystic fibrosis patients. Further studies are in progress to determine how this genetic rearrangement is involved in mucoid conversion. It may be due to a regulatory gene present on the insertion elements, or insertion may affect the expression of a gene downstream of the insertion location.

ACKNOWLEDGMENTS

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