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Gene Amplification Induces Mucoid Phenotype in *rec-2 Pseudomonas aeruginosa* Exposed to Kanamycin

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Gene amplification in the chromosome of *rec-2 Pseudomonas aeruginosa* PAO2003 upon growth on kanamycin-supplemented media led to a stable mucoid phenotype. The chromosomal region controlling alginate biosynthesis was shown to be amplified four to six times as a direct tandem repeat of at least 16.8 kilobase pairs. This amplification was deduced from Southern DNA-DNA hybridization patterns of the chromosomal DNA digested with restriction endonucleases *Bgl*II and *Eco*RI and probed with a cloned DNA segment complementing the *alg-22* mutation. The part of the amplified unit carrying the novel DNA joint was cloned. The *Eco*RI junction fragment was further subcloned and used to probe chromosomes of parental strain PAO2003 and mucoid variant VD2003M. As predicted, the *Eco*RI junction fragment hybridized to the two chromosomal fragments required to produce the novel junction. Though the mucoid phenotype caused by gene amplification was stable, nonmucoid revertants were obtained at a low frequency on tetracycline-containing media. Southern hybridization of chromosomal DNA from a nonmucoid revertant revealed a reduction in the copy number of amplified DNA. These results suggest a direct relationship between amplification of this chromosomal segment and the induction of mucoidy.

Pseudomonas aeruginosa is an opportunistic pathogen with highest incidence of pulmonary infection in children with cystic fibrosis (CF) (22). In the course of antibiotic therapy for such patients, *P. aeruginosa* replaces the initial bacterial pathogens, i.e., *Staphylococcus aureus* and *Haemophilus influenzae* (24), and characteristically shifts (10) from commonly nonmucoid to a mucoid, alginate-producing (13, 26) phenotype. During the terminal stages of disease, the predominant mucoid phenotype is associated with clinical deterioration (9). However, nothing is known about the genetic basis of emergence of alginate-producing strains in CF lungs.

Most *P. aeruginosa* strains are intrinsically resistant to certain aminoglycoside and β-lactam antibiotics having chromosomally coded aminoglycoside 3′-phosphotransferase II (32) and β-lactamase (30). It has been shown that mucoid strains of *P. aeruginosa* are generally slightly more resistant to certain antibiotics (19, 20). This fact served as the basis for the isolation of mucoid variants in vitro (16, 20). Such evidence supports the suggestion that prolonged antibiotic therapy could be a causative factor in the emergence of mucoid *P. aeruginosa* in CF patients (22, 28).

Here we report the occurrence of a stable mucoid phenotype in *P. aeruginosa* PAO *rec-2* upon treatment with kanamycin leading to gene amplification. Using as a hybridization probe a cloned DNA segment that complements the *alg-22* mutation (7), we show here that the corresponding region in the chromosome of this stable mucoid isolate has been amplified four to six times as a tandem repeat in direct orientation.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are given in Table 1.

Media and bacterial growth. *Escherichia coli* strains were grown in LB medium (GIBCO Laboratories) supplemented with 1.5% agar for growth on plates. Antibiotics (Sigma Chemical Co.) used were ampicillin, 40 μg/ml; kanamycin, 50 μg/ml; and tetracycline, 25 μg/ml. *P. aeruginosa* was grown in LB liquid medium. Minimal medium for *P. aeruginosa* was as described elsewhere (2), with trace elements omitted. These media were supplemented, when appropriate, with 1.5% agar, 50 μg of tetracycline per ml, and 1 mM amino acids. *Pseudomonas* Isolation Agar (Difco Laboratories) was used for the selection and maintenance of *P. aeruginosa* strains. When required, tetracycline and kanamycin were added to 300 and 1,000 μg/ml, respectively, in *Pseudomonas* isolation agar medium. All incubations were at 37°C.

***P. aeruginosa* strain isolation.** To isolate mucoid *P. aeruginosa* by exposure to kanamycin, overnight cultures were washed, and 10^8 CFU were plated on *Pseudomonas* isolation agar plates containing 1 mg of kanamycin per ml. Growing colonies were screened for mucoid phenotype, and this selection was verified by assaying alginate (25). Exposure to ethyl methanesulfonate (EMS; Sigma) was performed as described elsewhere (7).

DNA isolation and biochemistry. Plasmid DNA was isolated by a modification (6) of the procedure described by Casse et al. (3). Total *P. aeruginosa* DNA was isolated as described previously (7). All enzymatic reactions, quantitation, and gel electrophoresis of DNA were done according to Maniatis et al. (27). Enzymes were purchased from New England BioLabs, Inc. DNA fragments were extracted from agarose gels by electrophoresing DNA bands onto DEAE-cellulose membrane (NA-45; Schleicher & Schuell, Inc.) as described elsewhere (11).

Construction of genomic libraries and DNA subcloning. A genomic library of *P. aeruginosa* 8822 was constructed in plasmid pCP13 as described previously (7). For the construction of the *P. aeruginosa* VD2003M genomic library, total chromosomal DNA was partially digested with *Bam*HI and ligated to *Bam*HI-digested pHC79 DNA. In vitro packaging into λ particles was done with packaging extracts from

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TABLE 1. Bacterial strains and plasmids

Species and strain or plasmid	Pertinent properties	Reference
E. coli		
AC80	*hsdR hsdM thr leu met*	4
JM83	*ara* Δ(*lac-pro*)*rpsL* (Str^r) *thi* φ80 d*lacZ*Δ*M15*	36
DH1	*hsdR17* (*hsdR hsdM*$^+$) *recA gyrA96 supE*	27
HB101	*recA hsdS20* (*hsdR hsdM*) *recA pro rpsL20* (Str^r)	27
P. aeruginosa		
8822	*his-1 alg-1*	7
8852	*his-1 alg-22*	7
PAO2003	*argH rec-2 alg*	5
VD2003M	*argH rec-2* Alg$^+$	This study
VD1/64	*argH rec-2 alg* (pLAFR1)	This study
Plasmid		
pHC79	6.43 kb, ColE1 Tc^r Ap^r *cos*$^\lambda$	21
pCP13	23 kb, IncP Tc^r Km^r *mob*$^+$ *tra cos*$^\lambda$	7
pLAFR1	21.6 kb, IncP Tc^r *mob*$^+$ *tra cos*$^\lambda$	15
pRK2013	48 kb, ColE1 *tra*$^+$ (RK2) Km^r	14
ps1EMBL	4.2 kb, R6K Km^r *lacZ'*	33
pVD11	43 kb, Tc^r *alg-22*$^+$ *argH*$^+$; recombinant plasmid from *Hin*dIII genomic library of *P. aeruginosa* 8822 in pCP13	This study
pVD8	45 kb, Km^r Ap^r Tc^s; recombinant plasmid from *Bam*HI genomic library of *P. aeruginosa* PAO2003 in pHC79	This study

Amersham Corp. Packaged cosmids were adsorbed to *E. coli* DH1, and the genomic library was amplified by growth in LB medium supplemented with ampicillin. Amplified genomic libraries were preserved in 50% glycerol at −20°C.

The subcloning of DNA fragments was performed by published procedures (32, 36), with plasmid ps1EMBL as vector and *E. coli* JM83 as host for screening recombinant molecules.

Conjugal plasmid transfer and genetic complementation. Plasmid transfers from *E. coli* to *P. aeruginosa* were performed by triparental filter matings (34). Stationary-phase cultures of *E. coli* carrying the plasmid to be transferred, helper strain *E. coli* HB101(pRK2013), and the *P. aeruginosa* recipient were washed and suspended to their original volumes with LB medium. Equal amounts (0.4 ml) of each culture were mixed in a syringe and filtered through nitrocellulose filters (HA, 0.45-μm pore size; Millipore Corp.). Filters were placed on LB medium plates. After overnight incubation at 37°C, bacterial growth was suspended in saline and plated on selective media.

Genetic complementation of the *alg-22* mutation was done by conjugal plasmid transfer into *P. aeruginosa* 8852. Complementation of the *argH* mutation was done by scoring transconjugants of PAO2003 for growth on antibiotic-supplemented minimal medium.

Southern blot, DNA dot blot, and colony hybridization analyses. In all procedures, nitrocellulose membranes BA85 (Schleicher & Schuell) were used for DNA immobilization. DNA hybridization probes were prepared by nick translation with [^{32}P]dCTP (3,000 Ci/mmol; Amersham), as described elsewhere (27).

Southern blots were performed as described elsewhere (27), with partial depurination of DNA in the gel after the electrophoresis. For this purpose gels were soaked in 0.25 M HCl for 30 min and subsequently rinsed with distilled water before the denaturation step.

Dot blot analysis (23) was performed as follows. DNA samples (up to 5 μg) were denatured in 300 μl of 30 mM Tris hydrochloride (pH 7.5)–0.2 M NaOH–6× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate) by incubation for 10 min at 80°C. Samples were cooled on ice and neutralized with 40 μl of 2 M Tris hydrochloride (pH 7.0) immediately before being filtered through nitrocellulose filters mounted in a Minifold apparatus (Schleicher & Schuell). After being baked for 2 h at 80°C in a vacuum oven, filters were processed exactly as for the Southern blot hybridization. After autoradiography, filters were cut into squares corresponding to the filtered areas, and bound radioactivity was determined by liquid scintillation counting.

Colony hybridization analysis was done as described elsewhere (27). Square-cut nitrocellulose membrane and petri dishes (Lab-Tek Products, Div. Miles Laboratories Inc.) were used for bacterial growth. Before prehybridization, filters were vigorously shaken in 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA) and 1% sodium dodecyl sulfate at room temperature to remove most of the cell debris. This treatment was essential to reduce false-positive hybridization signals.

RESULTS

Isolation of mucoid derivatives of *P. aeruginosa rec-2* PAO2003. After the publication of observations (16, 20) that *P. aeruginosa* could be induced to produce alginate on media containing aminoglycoside and β-lactam antibiotics, we attempted to obtain mucoid strains from *P. aeruginosa* PAO2003, which has a *recA*-like mutation, *rec-2* (5). This strain became mucoid at a fairly high frequency (approximately 10^{-3} to 10^{-4}) on exposure to EMS. However, in contrast to some other PAO strains, it was difficult to obtain any highly mucoid derivatives of this strain on kanamycin-containing media. A strain was isolated from several apparently mucoid colonies among approximately 5×10^4 screened clones selected on the *Pseudomonas* isolation agar plates supplemented with 1 mg of kanamycin per ml. This strain, designated VD2003M, produced a moderate amount of alginate, i.e., 7.3 to 15.5 μg per mg of wet cell weight. The level of alginate produced by parental strain PAO2003 was below the detection limit (1.5 μg/ml).

Detection of DNA amplification in the chromosome of strain VD2003M. Recently, a chromosomal sequence isolated from stable mucoid strain 8830 has been cloned in the broad-host-range cosmid vector pCP13 (7). The resulting recombinant plasmid, pAD1, complements the *alg-22* and *argH* mutations mapping at 19 min on the PAO chromosome. This plasmid, when introduced into either nonmucoid *P. aeruginosa* cells such as those of strain PAO or nonmucoid derivatives of mucoid CF isolates, slowly induces detectable levels of alginate (7). In this study a corresponding DNA sequence from nonmucoid strain 8822, a spontaneous derivative of the mucoid CF isolate 8821 (7), was cloned with pCP13 as a vector. This DNA fragment was cloned on the rationale that the *Hin*dIII-*Hin*dIII insert from pAD1 complements *argH* mutation as shown previously (7). Plasmid pVD11 was isolated after triparental mating of a *P. aeruginosa* 8822 genomic library in *E. coli* AC80, made in the broad-host-range cosmid vector pCP13 (*mob*$^+$ *tra*) with *P. aeruginosa* PAO2003 as a recipient and pRK2013 (*tra*$^+$) in *E. coli* HB101 as a mobilizing helper plasmid. pVD11 was isolated from Tc^r

Arg^+ transconjugants. When pAD1 and pVD11 were compared, no discernible difference in restriction nuclease maps was observed. pVD11, although derived from the nonmucoid strain 8822 chromosome, also conferred *alg-22* complementing ability and caused slow induction of alginate production in *P. aeruginosa* PAO strains.

When pVD11 was used as a hybridization probe to detect potential differences in gene structure, we observed different hybridization patterns with restriction nuclease digests of total chromosomal DNA from mucoid variant VD2003M and nonmucoid strain PAO2003 (Fig. 1). DNA from mucoid strain VD2003M showed much stronger hybridization than DNA from PAO2003, though approximately equal amounts of DNA were probed. In addition, the intensity of hybridization shown in all lanes b of Fig. 1, which contain VD2003M DNA, varied from band to band in an uneven fashion. Those bands indicating stronger hybridization could also be distinguished on the gel stained with ethidium bromide (data not shown). This result suggested that gene amplification occurred in a chromosomal region involved in alginate biosynthesis (7).

The relative extent of amplification was determined by a dot blot analysis (Fig. 2A). DNA from VD2003M was amplified four- to sixfold compared with the corresponding region from the parental PAO2003 chromosome as determined by liquid scintillation counting of filtered areas (Fig. 2A). Approximately 0.8 to 1.2 μg of VD2003M and 5 μg of PAO2003 DNA were binding the same amount of ^{32}P-labeled pVD11 probe. Figure 2B shows hybridization of the same DNAs with a plasmid pVD8 containing a chromosomal Km^r determinant from the PAO2003 strain cloned into cosmid pHC79 (unpublished results). In this case, no significant difference was observed, suggesting that the amplification did not involve the Km^r gene known to map at 36 to 39 min on the PAO chromosome (32), i.e., 17 to 20 min distant from the *alg-22* mutation. Similar results were also obtained when plasmids complementing *cys-59* or *proB* mutations were used as probes (data not shown). Thus, amplification did not involve the Km^r gene or the chromosomal region previously demonstrated to be of importance for the instability of the mucoid phenotype (16).

Figure 3A shows all the *Bgl*II fragments present in the

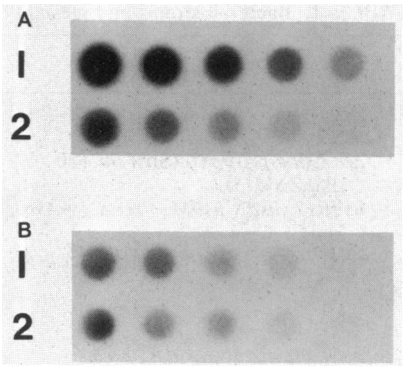

FIG. 2. Determination of the extent of DNA amplification. Dot blot analysis was done by immobilizing on nitrocellulose twofold serial dilutions of chromosomal DNA starting from 5 μg. Filters were probed with pVD11 (A) and pVD8 (B) DNA. Subsequently, filters were cut, and bound radioactivities were determined (see text). Rows 1, Total DNA from strain VD2003M; rows 2, total DNA from strain PAO2003.

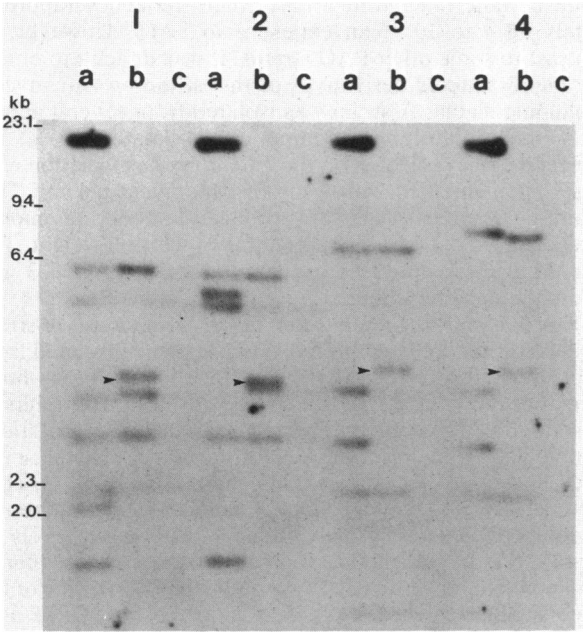

FIG. 1. Hybridization patterns of DNA from strains PAO2003 and VD2003M. Plasmid and chromosomal DNAs were digested, electrophoresed on 0.75% agarose gel, and after subsequent blotting, hybridized to nick-translated plasmid pVD11 DNA. Sets 1, 2, 3, and 4 correspond to *Bgl*II plus *Hin*dIII, *Bgl*II, *Eco*RI plus *Hin*dIII, and *Eco*RI digests, respectively, of 50 ng of plasmid pVD11, (a) and 0.5 μg of strain VD2003M (b) and strain PAO2003 (c) total DNA. Arrows indicate J fragments (see text for explanation).

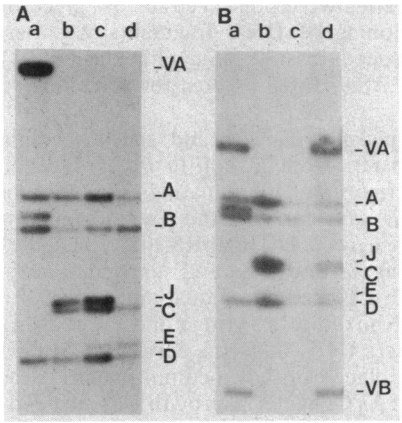

FIG. 3. Comparison of pVD11-specific *Bgl*II fragments present in the total DNA from strains PAO2003, VD2003M, and the nonmucoid revertant VD1/64. All DNAs were digested with *Bgl*II. Southern blot analysis was done as described in the legend to Fig. 1. Nick-translated pVD11 DNA was used as a probe. (A) Lanes a, b, c, and d contained 50 ng of plasmid pVD11, 0.5 μg of strain VD2003M, 2 μg of strain VD2003M, and 2 μg of strain PAO2003 DNA, respectively. (B) Lanes a, b, c, and d contained 50 ng of pVD11 DNA, 1 μg of VD2003M, 1 μg of PAO2003 DNA, and 1 μg of DNA from nonmucoid revertant VD1/64, respectively. VA, Vector (pLAFR1) A band; VB, vector B band (carrying λ *cos* site). The unlabeled band in plasmid lanes (lanes a) was shown to represent *Bgl*II fragment C linked to the portion of pCP13 or pLAFR1 vector carrying *oriV*. Other bands are described in the text.

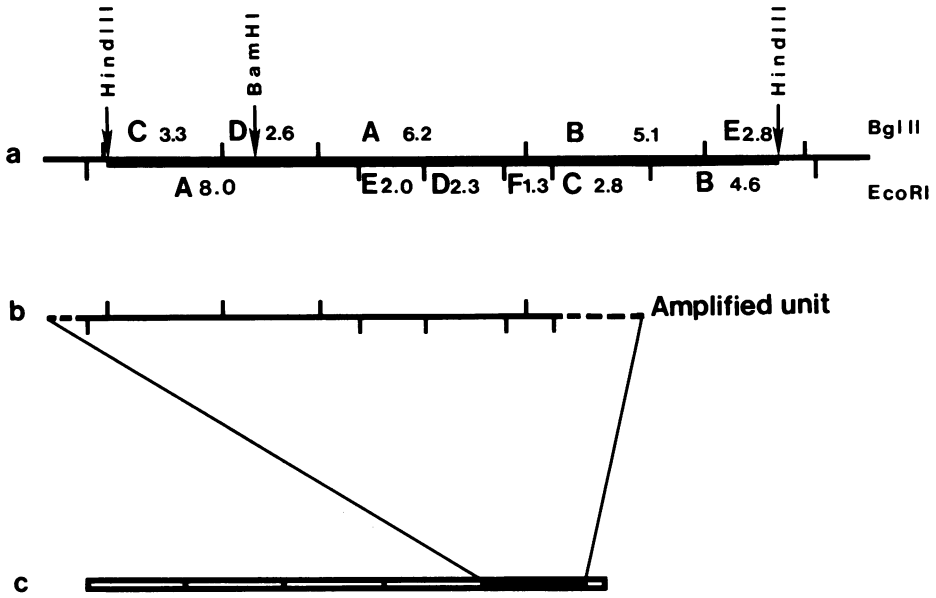

FIG. 4. Schematic representation of amplification of the *alg-22* region. (a) Restriction nuclease map of the PAO2003 chromosome hybridizing to sequences present in pVD11 (bold line). Numbers indicate lengths of fragments in kilobases. Capital letters are designations for DNA fragments. (b) The solid line represents the amplified region, and the dashed line indicates uncertainty in the left and right endpoints of the amplification unit. Vertical lines correspond to *Bgl*II sites (upward lines) and *Eco*RI sites (downward lines). The minimal unit length (16.8 kb) ends at the extreme right. (c) Chromosomal region with an array of five tandemly repeated units as estimated from results given in Fig. 2.

chromosome from VD2003M that hybridize to plasmid pVD11. As expected, there were at least two bands in the chromosomes different from the pattern obtained with the plasmid DNA, since pVD11 was generated by cloning *Hin*dIII fragments. In addition, the VD2003M chromosome had one band that was absent in the parental, PAO2003 chromosome. This band, J, appeared to be the expected novel fragment corresponding to the junction created in the case of the direct tandem repeat of the same unit (35). The band designated C was identified in the chromosomal lanes as the one hybridizing to the 4.6-kilobase (kb) *Hin*dIII-*Bam*HI portion (Fig. 4) from pVD11 (data not shown). Chromosomal band E was absent in the pVD11 lane due to its linkage to the pCP13 (7) vector moiety. The rest of the hybridizing fragments had counterparts in the plasmid lanes. From the intensity of bands it could be concluded that the only *Bgl*II fragments that had not undergone amplification were B and E. As can be seen from the restriction map (Fig. 4), the right endpoint of the amplification might be within *Bgl*II fragment B. *Eco*RI digestion confirmed the *Bgl*II results, since *Eco*RI fragments A, E, D, and F showed increased hybridization signals relative to *Eco*RI fragments C and B. *Eco*RI fragment F appeared amplified on the Southern blots, indicating that the rightmost point of the amplification unit must be beyond that fragment, i.e., within *Eco*RI fragment C. *Bgl*II and *Eco*RI junction fragments J, of 3.4 and 3.6 kb, respectively, were unaffected by cleavage with *Hin*dIII (Fig. 1, lanes 1b and 3b) and strongly hybridized with the pVD11 probe. Gels stained with ethidium bromide did not reveal any prominent bands in the lanes with VD2003M DNA except those hybridizing with the pVD11 probe. The expected size of *Eco*RI J fragment should be smaller than that of *Bgl*II J fragment if no other *Bgl*II sites are present between the leftmost point of amplification and the extreme left *Bgl*II site on the displayed map (Fig. 4a and b). In contrast, the *Eco*RI J fragment is larger than the *Bgl*II J fragment. The length of the amplified unit might not exceed a total of 16.8 kb, including 15.3 kb of the bands that hybridized to the pVD11 probe and 1.5 kb outside the cloned region to account for the apparent discrepancy in the lengths of the *Eco*RI and *Bgl*II J fragments (Fig. 4).

Reversion to Alg$^-$ phenotype as the consequence of reduction in the copy number of the amplified chromosomal region. We attempted to obtain a nonmucoid revertant from strain VD2003M. In contrast to some CF mucoid isolates that showed a great deal of instability of the mucoid character, it was difficult to obtain nonmucoid revertants from VD2003M strain under conditions described previously (18) as promoting reversion to Alg$^-$ phenotype. Because of observations that mucoid strains are more sensitive than nonmucoid variants to tetracycline (20), a Tc^r plasmid was introduced into strain VD2003M to allow its growth on tetracycline-containing media. The rationale was that among Tc^r survivors, some selection against mucoid strains would be exerted. This was achieved by mating *E. coli* AC80 carrying plasmid pLAFR1 with *P. aeruginosa* VD2003M in triparental mating with *E. coli* carrying pRK2013. Nonmucoid revertants were obtained at a frequency between 10^{-5} and 10^{-4} among Tc^r transconjugants. The Southern blots of the chromosome from nonmucoid revertants, one of which was designated VD1/64, revealed reduction in the copy number of the amplification unit with the J fragment still being present (Fig. 3B).

Cloning of the novel DNA junction. To prove that a new DNA junction was created in strain VD2003M, the cloning of the corresponding chromosomal region was undertaken. The *Bam*HI genomic library of the VD2003M chromosome was constructed by using cosmid vector pHC79 that allows cloning of DNA fragments over 40 kb in size. The clones containing sequences homologous to the pVD11 insert were

selected by colony hybridization. As a probe, the plasmid designated pVD12 was used. pVD12 was a subclone of pVD11 corresponding to the chromosomal *Eco*RI fragment A inserted into the *Eco*RI site of ps1EMBL plasmid. The ps1EMBL vector, a derivative of pUC9 and naturally occurring plasmid R6K (33), was shown to be useful for the colony hybridization screening technique due to the lack of homology to pHC79. A large number of positive colonies were observed (approximately 3%), as expected in the case of gene amplification. One of the colonies was shown to contain a plasmid with *Bgl*II and *Eco*RI fragments of the same size as corresponding J fragments from VD2003M chromosome. This plasmid designated pVD13 (43 kb) also contained chromosomal *Bgl*II fragments A, C, and D, as shown by restriction endonuclease mapping, Southern hybridization, and *alg-22* complementation ability.

*Eco*RI fragment J was subcloned from pVD13 into ps1EMBL vector (plasmid pVD14) and used to probe mucoid VD2003M, parental PAO2003, and revertant VD1/64 chromosomes. The results of this hybridization are displayed in Fig. 5. The hybridization with *Bgl*II fragment B of pVD11 and chromosomal fragments of the same mobility confirmed the proposed rightmost end of gene amplification (Fig. 4). A strong hybridization with another 2.1-kb *Bgl*II fragment (Fig. 5, band X) present in all chromosomes probed was also observed. This DNA fragment must be conferring the left endpoint of the amplification unit. Slight cross-hybridization of pVD14 and another DNA fragment was observed (Fig. 5, band X'). Vector ps1EMBL showed some homology to pRK290-derived plasmids like pLAFR1 and pCP13, which resulted in hybridization signals with plasmid-containing DNA fragments in the gel shown in Fig. 5. However, when *Eco*RI fragment J directly isolated from plasmid pVD13 served as a probe, essentially the same hybridization pattern was observed with DNA in lanes with chromosomal DNA. Therefore *Bgl*II fragment X' did not hybridize to ps1EMBL DNA but may have some sequences in common with *Bgl*II fragment X that are not present in chromosomal sequences cloned in pVD11 DNA. *Eco*RI fragment J also showed strong hybridization with *Eco*RI fragment C and another *Eco*RI fragment of 8 kb (data not shown). These results confirmed that the new DNA junction had been formed as a part of the amplification process.

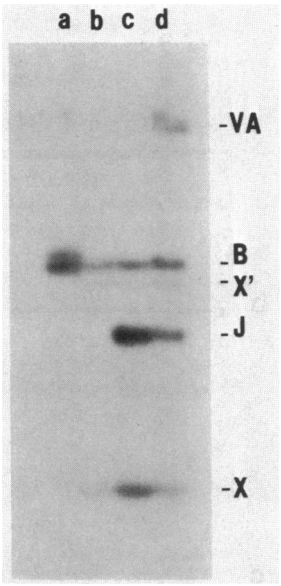

FIG. 5. Hybridization of cloned novel DNA junction with plasmid and chromosomal DNAs. Southern blot analysis was done with 50 ng of plasmid pVD11 (lane a), 1 μg of strain PAO2003 (lane b), 1 μg of strain VD2003M (lane c), and 1 μg of strain VD1/64 (lane d) total DNAs digested with *Bgl*II and probed with pVD14 nick-translated DNA. X and X' are bands described in the text. Other bands are described in the legend to Fig. 3.

DISCUSSION

The emergence of mucoid strains of *P. aeruginosa* is poorly understood. It is known that besides transition to the mucoid phenotype in CF lungs, *P. aeruginosa* can become mucoid under experimental conditions upon exposure to antibiotics (20), phages (29), or methylating agents such as EMS. These isolates phenotypically resemble naturally occurring strains. The difficulties observed in this study in obtaining mucoidy in *rec-2 P. aeruginosa* upon exposure to kanamycin in contrast to the ease in formation of alginate-producing derivatives of the same strain upon exposure to EMS may reflect the complexity of the processes leading to this genetic transition.

Recently, several genes complementing different mutations in the alginate biosynthetic pathway have been cloned (7, 8, 17). This procedure provided a powerful tool for screening potential DNA rearrangements causing alginate production. Gene amplification of the part of the *P. aeruginosa* chromosome around the *alg-22* marker might be either one mechanism by which *P. aeruginosa* becomes mucoid or a step in more complex processes involved in the full induction of alginate synthesis taking place in recombination-proficient strains. Other evidence suggests that the copy number of the DNA region complementing the *alg-22* mutation might play a role in the control of alginate production. It was observed that introduction of plasmid pAD1 into certain nonmucoid strains resulted in slow alginate production (7). This alginate induction occurs even when the chromosomal segment is derived from a nonmucoid strain such as 8822(pVD11). The increased gene dosage of chromosomal sequences involved in alginate biosynthesis, when placed on a plasmid with an estimated copy number of 5 to 6 such as pCP13 (7), might mimic antibiotic-promoted gene amplification. However, the relatively low level of alginate production and the stability of the mucoid phenotype peculiar to VD2003M are not characteristic of most CF isolates. Strain VD2003M produced a relatively small amount of alginate (7 to 15 μg/mg of wet cell weight). By contrast, some very mucoid laboratory strains and fresh CF isolates produced as much as 200 μg of alginate per mg of wet cell weight. In addition, strain VD2003M was stably mucoid in character, even under conditions promoting conversion to a nonmucoid phenotype in most of the natural mucoid isolates (18). It has been shown that, in contrast to their emergence and prevalence in CF lungs, mucoid *P. aeruginosa* strains phenotypically revert to the nonmucoid form in vitro at a high rate (18). Since the gene amplification was observed in the *rec-2* background of the parental PAO2003 strain, lack of homologous recombination at the repeated sites might be the basis of the apparent stability of the alginate production observed in the VD2003M strain. Gene amplification in the *lac* region of *E. coli* ranging from 7 to 37 kb was shown to be stabilized in the *recA* background (35). On the other hand, the *recA* function is not required for DNA amplification in *E. coli* and *Salmonella typhimurium*, when regions of 10 to 20 kb are involved (1, 12). A recently published study regarding a *recA* mutation in *P. aeruginosa* FRD (31) shows that this recombinational system has no effect on the instability of

mucoid FRD strains. At the same time, the authors of that study pointed out that other systems for homologous recombination are still operating in *recA* mutants (retaining 5% of the capacity of Rec^+ strains).

Gene amplification resulting in increased alginate production obtained as a result of growth in the presence of kanamycin also supports the idea that antibiotic therapy may provoke the mucoid phenotype in CF lungs (23, 28). At this point it might be too early for speculation on how antibiotics, EMS, or phages might increase the frequency of mucoid isolates. A 1.5-fold increase in the MIC of tobramycin, an aminoglycoside similar to kanamycin, has been reported for mucoid strains (20). The question remains whether such a slight difference merely selects for alginate-producing strains or whether antibiotic stress actually triggers in vivo a genetic response such as amplification which leads to alginate production.

The gene amplification described here remains to be studied further. Natural mucoid isolates maintained under laboratory conditions do not demonstrate amplification of the *alg-22* region (unpublished observations). More extensive studies will focus on the specific role of amplification in the induction of mucoidy as well as its relation to *rec-2* mutation. In addition, it will be interesting to determine what important function is coded by the *alg-22* region, since its amplification is enough to induce detectable alginate biosynthesis.

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