Gene Dosage Analysis in Azotobacter vinelandii

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ABSTRACT

For more than a decade, Azotobacter vinelandii has been considered a polyploid bacterium on the basis of physical studies of chromosome size and DNA content per cell. However, as described in the present work, many genetic operations can be performed in A. vinelandii without the constraints expected in a polyploid bacterium: (i) reversion of transposon-induced mutations is usually associated with loss of the transposable element; (ii) revertants retaining the transposon always carry secondary transpositions; (iii) heterozygotic transconjugants and transformants are unstable and segregate homozygotic colonies even in the absence of selection. Physical monitoring of segregation, achieved by colony hybridization, indicates that phenotypic expression of an allele is always correlated with its physical presence, thus ruling out the existence of either threshold dosage requirements or transcriptionally inactive DNA. Chromosomal lac fusions constructed by double crossover with a linearized plasmid show a segregation pattern consistent with the inheritance of one or several chromosomes per daughter cell. Analysis of the delay required for the expression of recessive chromosomal mutations such as rif, nal and str provides further evidence that A. vinelandii is not a polyploid bacterium.

Azotobacter vinelandii is a soil-dwelling bacterium, widely known for its ability to fix nitrogen aerobically (Robson and Postgate 1980) and for the presence of several alternative nitrogenase systems (Bishop, Jarleneski and Hetherington 1980; Chisnell, Premakumar and Bishop 1988). Physical studies of genome organization have suggested that A. vinelandii is a polyploid bacterium containing 40–80 chromosomes per cell (Sadoff, Shimel and Ellis 1979; Nagpal et al. 1989), a genome redundancy which would make A. vinelandii unique in the eubacterial kingdom (Krawiec and Riley 1990). When the polyploidy of Azotobacter was first reported (Sadoff, Shimel and Ellis 1979), it seemed to explain a classical problem of Azotobacter genetics, namely the inability of investigators to isolate mutants exhibiting auxotrophic phenotypes commonly identified in other microorganisms. In a bacterium containing 40 or more chromosomes per cell, recessive mutants should certainly be elusive, because the long segregation required for their formation would cause loss of the mutation by dilution and recombinational repair.

However, many mutant strains of A. vinelandii have been isolated which do exhibit the phenotype of a recessive mutation. For example, strains deficient in nitrogen fixation (Nif⁻) were first described four decades ago (Wyss and Wyss 1950); since then, many Nif⁻ mutants have been isolated by chemical, UV or transposon mutagenesis, and their isolation is relatively easy (Kennedy and Toukdarian 1987). Other mutant types, including several classes of auxotrophs, are also available (Kennedy et al. 1986; Santero et al. 1986; Lúque et al. 1987; Phadnis and Das 1987; Kennedy and Toukdarian 1987; Contreras and Casadesus 1987; Toukdarian et al. 1990; Contreras, Maldonado and Casadesus 1991). The fact that certain types of auxotrophs have never been isolated, not even when directed selection and enrichment procedures are applied (Contreras and Casadesus 1987; Toukdarian et al. 1990), may have an explanation completely unrelated to genome structure: A. vinelandii may be unable to transport many amino acids into the cell, thereby turning the corresponding biosynthetic genes into essential genome components. This explanation, first suggested by Roberts and Brill (1981), is supported by two common observations: (i) “tryptone” auxotrophs that do not grow with any single component of an auxanography test are often isolated (Kennedy and Toukdarian 1987; Contreras and Casadesus 1987; Contreras, Maldonado and Casadesus 1991); (ii) auxotrophs which can be fed with short oligopeptides but not with their single components have been proven to exist (Kennedy et al. 1986; A. Contreras and J. Casadesus, unpublished data).

Besides the ease of obtaining Nif⁻, sugar non-users and other types of mutants, an independent observation which argues against extreme polyploidy was provided by UV sensitivity studies: A. vinelandii is extremely sensitive to UV light and its dose-response curve does not correspond to that of a polyploid bacterium (Terzaghi 1980). However, this kind of
evidence cannot be taken as conclusive, because single lethal events triggered by UV irradiation (e.g., prophage induction, bacteriocin release, endonuclease activation) might be involved in the process.

During the last decade, the introduction of transposon technology has provided further arguments against polyploidy. Mutants induced by transposons Tn5 and Tn10 are easily isolated and the selection of their dominant antibiotic-resistance markers facilitates many genetic operations (Kennedy et al. 1986; Phadnis and Das 1987; Kennedy and Toukarian 1987; Contreras and Casadesus 1987; Toukarian et al. 1990; Blanco et al. 1990; Contreras, Maldonado and Casadesus 1991). Of particular importance is the observation that transposon mutations are easily transferred from strain to strain and that the recombinants usually are homozygotes which only express the mutant allele (Kennedy et al. 1986; Contreras and Casadesus 1987; Toukarian et al. 1990; Contreras, Maldonado and Casadesus 1991). If heterozygotes are obtained, they are able to segregate homozygotic colonies of either parental type at extremely high frequencies (Contreras and Casadesus 1987; Toukarian et al. 1990). Another observation is that transposon mutagenesis has not increased the variety of mutant types found. Such results support the hypothesis that certain mutant types cannot be isolated because of the existence of essential metabolic requirements. In the last few years, more reports on gene transfer and strain construction have provided additional evidence that, in many aspects, the genetics of Azotobacter resembles that of haploid bacteria (Blanco et al. 1990; Toukarian et al. 1990).

The contradiction between physical studies which suggest the existence of a polyploid genome (Sadow, Shime1 and Ellis 1979) and the description of genetic procedures that do not exhibit the constraints expected in a polyploid bacterium (Contreras and Casadesus 1987) is further explored in this paper. We describe experiments specifically devised to measure gene dosage in A. vinelandii and the results strongly suggest that A. vinelandii is not a polyploid bacterium.

### MATERIALS AND METHODS

**Bacterial strains:** The A. vinelandii strains cited in this study are listed in Table 1. All derive from either the wild-type standard strain UW or its rifampicin-resistant derivative UW136, both obtained from W. J. Brill, University of Wisconsin, Madison. Escherichia coli HB101 is an E. coli K-12 × E. coli B hybrid (Boyer and Roulland-Dussoix 1969).

**Plasmids and transposons:** Plasmids were routinely maintained in E. coli HB101. pRZ102 (Km') is a ColE1 derivative carrying Tn5 (Rothstein et al. 1980a). pRZ131 (Tc') is a PRZ102 derivative carrying the tetracycline-resistant, non-transposing element Tn5-131 (Rothstein et al. 1980b). pMOB911-16 (Cm' Tc') is a PMOB45 derivative carrying the internal BglII fragment of transposon Tn10 (De la Torre et al. 1984). pCU101 is a Cm' Tra' plasmid that cannot replicate in A. vinelandii (Selvaraj and Iyer 1983). ColE1-derivated plasmids are efficiently mobilized into A. vinelandii by pCU101 (Contreras, Maldonado and Casadesus 1991). pB3J1 is a Km' derivative of the IncP plasmid R68.45 (Brewin, Benger and Johnston 1980). pLZ25 is a Tc' derivative of plasmid pB3J1, isolated after diethylsulfate mutagenesis (R. Maldonado and J. Casadesus, unpublished). pLZ25 is a pUC19 derivative carrying the internal HindIII fragment of Tn10; this fragment contains the tetracycline resistance gene. pLZ25 is a pUC19 derivative carrying the internal HindIII fragment of Tn5; this fragment contains the kanamycin resistance gene. Both pLZ52 and pLZ25 were constructed for this study; the prefix pLZ has been registered at the Plasmid Reference Center, Stanford University (Ledberg 1986). pUC19 (Ap', LacIz()) is a pBR322-M13mp19 hybrid (Yanisch-Perron, Vieira and Messing 1985). pDB154 is a pKT230 derivative containing a nif::lac fusion (Bennett, Jacobson and Dean 1988). Tn5-Mob is a transposition-proficient Tn5 derivative containing the mob region of plasmid RP4 (Simon 1984). Tn10HH104 is a "high-hopper" derivative of Tn10 (Foster et al. 1981).

#### Chemicals, culture media and growth conditions: Restriction enzymes were purchased from Boehringer Mannheim, Pharmacia and New England Biolabs. [32P]dATP was from Nuclear Ibérica. Chloramphenicol, kanamycin, tetracycline, rifampicin and nalidixic acid were all from Sigma Chemical Co. Growth conditions and antibiotic concentrations were as previously described (Contreras and Casadesus 1987). Minimal medium for A. vinelandii was Burk nitrogen-free medium, prepared according to Guerrero et al. (1973). Carbon sources other than sucrose were used at a final concentration of 5 g/liter. BSNB is Burk medium supplemented with 1 g/liter ammonium acetate, 5 g/liter tryptone and 2.5 g/liter yeast extract. Solid media (minimal and BSNA) were prepared with 15 g/liter of Difco agar. 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (henceforth, "Xgal") was purchased from Bethesda Research Laboratories and used as described by Bennett, Jacobson and Dean (1988).

**Matings:** Intergeneric matings E. coli x A. vinelandii were carried out by sucking the donor and the recipient strains onto the surface of a Millipore filter (0.2-μm pore size). The filters were placed on BSNB plates and the matings were allowed to proceed overnight. After conjugation, the mating mixtures were resuspended in BSNB containing at least 10^8 plaque-forming units of phage T4D (provided by E. P.

### Table 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS8</td>
<td>RifStr'</td>
<td>1</td>
</tr>
<tr>
<td>AS11</td>
<td>Nal'</td>
<td>This study</td>
</tr>
<tr>
<td>AS101</td>
<td>ade-1::Tn5 Rif'</td>
<td>2</td>
</tr>
<tr>
<td>AS115</td>
<td>met-1::Tn5 Rif'</td>
<td>2</td>
</tr>
<tr>
<td>AS116</td>
<td>mtl-1::Tn5 Rif'</td>
<td>2</td>
</tr>
<tr>
<td>AS145</td>
<td>ade-2::Tn10HH104 Rif'</td>
<td>3</td>
</tr>
<tr>
<td>AS151</td>
<td>gal-1::Tn10 (Rha- Gal-) Rif'</td>
<td>3</td>
</tr>
<tr>
<td>AS152</td>
<td>mtl-2::Tn10 Rif'</td>
<td>3</td>
</tr>
<tr>
<td>AS191</td>
<td>cyr-7::Tn5 Rif'</td>
<td>2</td>
</tr>
<tr>
<td>AS204</td>
<td>ura::Tn5-Mob Rif'</td>
<td>4</td>
</tr>
<tr>
<td>AS192</td>
<td>mtl-1::Tn5-131 Rif'</td>
<td>This study</td>
</tr>
</tbody>
</table>

GEIDUSCHEK, University of California, San Diego) and incubated 3–4 hr with shaking before plating on selective medium. A similar procedure was used for A. vinelandii interspecific mating, except that longer mating times were allowed (24 h) and T4D selection was omitted (for details, see CONTRERAS and CASADESUS (1987) and CONTRERAS, MALDONADO and CASADESUS (1991)).

Transposon replacements: Substitution of wild-type Tn5 by Tn5-Tet was achieved in triparental matings involving E. coli HB101/pCU101, E. coli HB101/pRZ151 and the corresponding A. vinelandii strain where the substitution was to be performed. Mating conditions and selection procedures were as described elsewhere (CONTRERAS, MALDONADO and CASADESUS 1991).

Transformation of A. vinelandii: Competent cells were prepared according to PAGE and VON TICHERSTROM (1979). Preparation of crude DNA extracts and transformation followed the procedures previously described (CONTRERAS and CASADESUS 1987).

Isolation, purification and digestion of A. vinelandii DNA: We followed the procedures described by CONTRERAS and CASADESUS (1987). Genomic DNA preparations for hybridization were digested with restriction endonucleases EcoRI, BamHI, BglII, HindIII or SalI before electrophoresis and Southern transfer. EcoRI has a unique restriction site in Tn10 and Tn10HH104 (FOSTER et al. 1981; CONTRERAS and CASADESUS 1987); BamHI has a unique restriction site in Tn5 (ROTSTEIN et al. 1980b). Thus Tn10 and Tn10HH104 insertions digested with EcoRI and Tn5 insertions digested with BamHI will appear as two hybridization bands in Southern hybridization experiments with either pMOBglI-16 or pRZ102 as probes. In BglII-digested genomic DNAs containing either Tn10 or Tn5-151, hybridization against the internal HindIII fragment of Tn10 generates two bands for Tn10 and one band for Tn5-131 (FOSTER et al. 1981; ROTSTEIN et al. 1980b; CONTRERAS and CASADESUS 1987). A single band is also observed in HindIII-digested genomic DNAs containing Tn5 or Tn5-Mob when hybridized against the internal HindIII fragment of Tn5 (ROTSTEIN et al. 1980b; SIMON 1984).

Plasmid DNA isolation: The alkaline lysis method was used both for minipreparations and large-scale isolation of plasmid (MANIATIS, FRITSCH and SAMBROOK 1982). Amplification was achieved as follows: a 50-ml culture, grown to station, was used to inoculate 1.5 liter of the same medium. When the new culture reached an OD690 of 2, 2 mg/liter was used to inoculate the appropriate plates. Hybridization followed the procedures of HANAHAN and MELSELON (1980).

Nitrosoguanidine mutagenesis: Exponential cultures in BSNB, containing 5 x 107 to 109 cells/ml were centrifuged, washed with cold Tris-maleate buffer, pH 6.8, and resuspended in a solution of N-methyl-N'-nitro-N-nitrosoguanidine (Aldrich), made in the same Tris-maleate buffer. The final concentration of nitrosoguanidine was 5 mg/liter. After a 30-min incubation at 37° without shaking, cells were centrifuged and washed twice with cold Tris-maleate buffer. Survivors were outgrown in BSNB.

RESULTS

Reversion analysis of transposon-induced mutations: Reversion analysis was based on the following rationale: in a polyploid bacterium, reversion must occur in the presence of the mutant allele, thus giving rise to heterozygotic isolates. A corollary is that such heterozygotes can be expected to be stable only if the number of chromosomal copies is high. On these grounds, we analyzed the ability of insertion mutants to revert and then scored the maintenance of the mutant allele in the revertant colonies. Whenever the mutant allele proved to be present, we determined its fate upon segregation by single-colony isolation. In a number of cases, physical analysis (Southern hybridization with a transposon probe) was required to distinguish putative heterozygotic strains from isolates carrying second-site insertions, as described by CONTRERAS and CASADESUS (1987).

Strains AS115, AS145, AS152 and AS191 were chosen for reversion studies because each bears only one insertion of either Tn10 or Tn5 (CONTRERAS and CASADESUS 1987; CONTRERAS, MALDONADO and CASADESUS 1991). Reversion data can be summarized as follows:

I. The four mutants were able to revert, as detected by colony formation on minimal plates. If the mutants were grown nonselectively (in BSNB) before plating, the frequencies of revertants varied within a range over 200-fold; these jackpots presumably reflect random accumulation of revertants in the liquid culture (i.e., Luria-Delbrück fluctuation). Replica-printing of the revertants to antibiotic plates revealed that some colonies were antibiotic-resistant while others were antibiotic-sensitive. The proportion of each type largely varied (25–95%) from one experiment to another and from strain to strain as well.

II. When reversion was forced to occur on the plate by plating cultures grown selectively in antibiotic BSNB, the frequencies of reversion (per cell plated) were estimated around 10−6 for strain AS145, 10−7 for AS115 and AS152 and 10−8 for AS191. When these revertants were directly replica-printed to antibiotic medium, >95% proved to be antibiotic-resistant, suggesting that they were heterozygotes or had formed mixed colonies on the reversion agar. Microscopic observation of cultures grown in BSNB indicated that cell duplexes occurred at a frequency of 2–3% and aggregates containing more than two cells at frequencies below 1%; thus the possibility that some revertant colonies might have originated from more than one cell cannot be ruled out. However antibiotic-
sensitive revertants (1–5%) were also found. This result is important because it suggests that the revertant colony originated from a single cell containing only one chromosome.

III: When antibiotic-resistant revertants were streaked on minimal plates, a high proportion (50–99%) of the single colonies obtained proved to be antibiotic-sensitive. In turn, upon colony isolation on antibiotic BSNA, 10–30% of the antibiotic-resistant colonies were auxotrophic. These results confirmed that the antibiotic-resistant revertants were either heterozygotic or had formed mixed colonies. More important, it showed that stable isolates of either parental type arise at extremely high frequencies after single-colony isolation. In other words, if heterozygotic revertants exist, they must be unstable.

IV: Antibiotic-resistant revertants that never segregate antibiotic-sensitive colonies were also isolated. This fraction was rather constant for every strain: 5–10% for AS115, 1–3% for AS145, 7–14% for AS152 and 8–28% for AS191. Revertants of this class always carried one or more secondary “hops” of the transposon (see examples for AS145 in Figure 1). Furthermore, isolates that never generate antibiotic-sensitive revertants were easily isolated upon repeated subculture on media exerting double selection (minimal medium supplemented with the appropriate antibiotic); such isolates always carried secondary transposon insertions (see examples for AS145 in Figure 1). These results indicate that, if heterozygotes exist, they are unstable even when both alleles are simultaneously selected.

The overall conclusion from these experiments is that, if A. vinelandii heterozygotic revertants exist, they must have a transient existence during the reversion process. Our experiments would not distinguish between the formation of an heterozygotic strain and that of a mixed colony. However, because the actual result is that every isolate expressing the two alleles is unstable, we are forced to conclude that A. vinelandii revertants do not behave as polyploid bacteria with 40–80 chromosomes capable of replication and independent partition to the daughter cells.

Segregation of heterozygotic transformants: Transformation with chromosomal DNA provides an independent approach for the study of heterozygotes. Because transformants are assumed to originate by recombination of one copy of the incoming allele, heterozygotes must necessarily form in a polyploid bacterium, at least in the initial stages of the process (Contreras and Casadesus 1987; Contreras, Maldonado and Casadesus 1991). Thus the fate of these heterozygotes can be analyzed by segregation analysis.

If transposon insertions are used, transfer can be scored in two directions: (I) transformation of transposon-induced mutants with wild-type DNA, selecting for prototrophic growth; (II) transformation of the wild-type with DNA from a transposon-induced mutant, selecting for the antibiotic resistance encoded by the transposon. Process II offers the additional advantage of preventing the formation of mixed colonies, since the antibiotic-resistance markers of Tn10 and Tn5 are dominant and do not code for extracellular enzymes.

Strains with low reversion frequencies (AS116, AS151 and AS192 and AS204, all reverting at frequencies below 10^-3) were chosen for transformation experiments; spontaneous 'Tc' or Km' mutants are not found in A. vinelandii. Transformants always appeared at frequencies above 10^-6 (per recipient bacterium). When selection was carried out only for the incoming marker, replica-printing showed that >90% of the transformants were homozygotes that did not express the resident allele. However, when selection for both the incoming and the resident allele was carried out, all the transformants were heterozygotic (or mixed colonies) as indicated by the following observations: (1) 100% of the prototrophic transformants generated by process I were able to grow when replica-printed to antibiotic medium; (2) 100% of the antibiotic-resistant transformants generated by process II were able to grow when replica-printed to plates selecting for wild-type growth. An additional observation was that the percentages of transformants which express both alleles were not higher in process I than in process II, thereby suggesting that the formation of mixed colonies occurred at low frequency. Thus, most transformants obtained by double selection must be true heterozygotes.

When these heterozygotic transformants were streaked on plates exerting single selection, fast segregation was observed. Streaking on plates selecting for wild-type growth allowed the recovery of antibiotic-sensitive prototrophs at high frequencies (80–100%); selection on antibiotic plates led to the isola-
Segregation of heterozygotic transconjugants: To rule out the possibility that transient heterozygosis might be a peculiar feature associated to transformation (i.e., dependent upon decrease of chromosomal copy number in competent cells), conjugal mobilization of chromosomal markers was carried out. Chromosomal transfer mediated by plasmids pJB3J1 and pIZ25 occurred at frequencies ranging from $10^{-9}$ to $10^{-5}$ (Table 2). Transconjugants obtained under double selection also proved to be heterozygotes capable of fast segregation, thus indicating that unstable heterozygotes appear irrespectively of the gene transfer method employed.

A similar situation was found in transposon substitutions, for instance when a chromosomal wild-type Tn5 (Km') was replaced by Tn5-131 (Tc') by homologous recombination at the flanking IS50 sequences. These experiments involved three-parental crosses E. coli × A. vinelandii, as described by CONTRERAS, MALDONADO and CASADESUS (1991). A plasmid-borne Tn5-131 is introduced into an A. vinelandii recipient containing a chromosomal Tn5 insertion; the vector containing Tn5-131 cannot replicate in A. vinelandii, but its transient presence allows recombination between the incoming and the resident Tn5 elements. Tc' transconjugants were usually Km'. Selection of transconjugants resistant to both kanamycin and tetracycline allowed the isolation of heterozygotes. However, these Tc' Km' transconjugants segregated into Tc' Km' or Tc' Km' at frequencies of 50–99% after streaking for single-colony isolation on antibiotic plates; non-segregating colonies usually carried secondary “hops” of one of the elements (data not shown; see also CONTRERAS, MALDONADO and CASADESUS 1991). In other words, heterozygotes made of transposon insertions showed a segregation behavior similar to that of heterozygotes carrying the wild-type allele and a transposon insertion. When segregation was carried out in the absence of selection, about 99% of the segregants were either Km' or Tc' and the minority Km' Tc' colonies carried, again, secondary transposition events (data not shown).

Physical monitoring of segregation: The simplest hypothesis to explain the difficulty in maintaining heterozygotes in the absence of double selection and the accumulation of secondary “hops” when double selection is exerted is that gene dosage is low. How can this idea be conciliated with the existence or 40 or 80 chromosomes per cell? One possible explanation would be that a critical gene dosage is needed for...
phenotypic expression. Namely, alleles would be silent until the threshold dosage is attained. An alternative possibility is that *A. vinelandii* contains a large number of chromosomes per cell but only a few were phenotypically expressed. Both hypotheses can be tested by DNA hybridization, to ascertain whether the homozygote-like segregants obtained by reversion or gene transfer still contain the two alleles albeit only homozygote-like segregants obtained by reversion or gene transfer still contain the two alleles albeit only one is expressed. Two types of hybridization experiments were carried out, as described below.

**Southern hybridization of fresh homozygotic segregants:** Antibiotic-sensitive segregants (derived from prototrophic, antibiotic-resistant transformants of strains AS151, AS192, AS204 and AS116) were isolated by streaking on minimal (or mannitol) plates. Genomic DNAs from these fresh (just segregated) homozygotes were hybridized against the transposon-resistance gene, hybridization was only found. This result is an important one because it indicates that whenever an allele was physically de-selected the colony showed the corresponding phenotype. Thus the existence of either threshold dosage requirements or phenotypically silent DNA, which might conciliate a low functional dosage with the existence of 40–80 chromosomes, seems unlikely.

**Segregation of nifF::lac fusions:** Plasmid pDB154 contains the *E. coli lacZ* gene flanked on both sides by portions of the *A. vinelandii nifF* gene (Bennett, Jacobson and Dean 1988). In a previous study, pDB154 was used in transformation experiments to incorporate the *lacZ* gene into the *A. vinelandii* chromosome such that its expression was placed under the control of the nifF regulatory elements. The nifF gene is expressed under both nitrogen-fixing and non-nitrogen fixing conditions and is not required for diazotrophic growth (Bennett, Jacobson and Dean 1988). Because *A. vinelandii* does not have any endogenous β-galactosidase activity, use of plasmid pDB154 provided a convenient way to visually monitor segregation of the nifF::lacZ fusion in *A. vinelandii* following transformation and homologous recombination. pDB154 was linearized by restriction enzyme digestion and used to transform competent cells of *A. vinelandii*. Linearization of plasmid DNA was done to ensure that homologous recombination occurred only through double crossover (and not via a single crossover which would result in chromosomal duplication of the cloned portions of the *A. vinelandii* chromo-

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**TABLE 2**

Segregation analysis in *A. vinelandii* transconjugants

<table>
<thead>
<tr>
<th>Donor</th>
<th>Recipient</th>
<th>Selected markers</th>
<th>Frequency of transconjugants</th>
<th>Percent of homozygotic colonies (for the incoming allele)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UW/pJB35</td>
<td>AS116</td>
<td>Mtl&lt;sup&gt;+&lt;/sup&gt; Km&lt;sup&gt;+&lt;/sup&gt;</td>
<td>3 × 10&lt;sup&gt;-6&lt;/sup&gt;</td>
<td>82</td>
</tr>
<tr>
<td>UW/pJZ25</td>
<td>AS101</td>
<td>Ade&lt;sup&gt;+&lt;/sup&gt; Km&lt;sup&gt;+&lt;/sup&gt;</td>
<td>10&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>74</td>
</tr>
<tr>
<td>UW/pJB35</td>
<td>AS191</td>
<td>Cys&lt;sup&gt;+&lt;/sup&gt; Km&lt;sup&gt;+&lt;/sup&gt;</td>
<td>7 × 10&lt;sup&gt;-8&lt;/sup&gt;</td>
<td>94</td>
</tr>
<tr>
<td>AS145/pJZ25</td>
<td>AS8</td>
<td>Ade&lt;sup&gt;+&lt;/sup&gt; Tc&lt;sup&gt;+&lt;/sup&gt;</td>
<td>3 × 10&lt;sup&gt;-6&lt;/sup&gt;</td>
<td>89</td>
</tr>
</tbody>
</table>

<sup>a</sup>In addition, the plates contained either rifampicin or streptomycin to counterselect the donor. The phenotype written in first place is that conferred by the incoming allele.

<sup>b</sup>Per recipient bacterium.

<sup>c</sup>Homozygotic colonies: those lacking the phenotype conferred by the unselected allele. The percentages given are averages of three to four independent conjugation experiments. Every segregation cycle involves colony isolation selecting only one allele (which, in the examples shown, was always the incoming allele) and replica-printing for the detection of the unselected allele.

<sup>d</sup>Not tested.

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some). Following transformation, cells were immediately plated on minimal medium containing X-gal.

Transformants which had incorporated the *E. coli lacZ* gene were easily recognized by their blue color. Close inspection of the blue colonies revealed that most of them were sectored, showing either a 50:50% blue:white sectoring pattern or a 25:75% blue:white sectoring pattern. All sectors showed the same intensity of blue color. Individual colonies were then picked and spread on fresh minimal plates containing X-gal. The individual colonies that arose at this stage of the experiment were either entirely blue or entirely white, with the relative abundance of the colony types reflecting the original sectoring of the parental colony. Moreover, all blue colonies exhibited the same intensity of blue color. Subculturing of individual blue colonies or individual white colonies identified at this stage showed that their respective phenotypes were completely stable. That is, blue colonies did not segregate white colonies nor did white colonies segregate blue colonies. The results of these simple experiments are what is expected from cells having one or several chromosomes.

**Expression of recessive mutations:** In *A. vinelandii*, spontaneous mutations causing resistance to rifampicin and nalidixic acid occur at very low frequencies. Streptomycin-resistant mutants of spontaneous origin are not found. All these mutations can be induced by nitrosoguanidine. As a test for functional gene dosage, we determined the number of generations required for the expression of these recessive mutations after nitrosoguanidine mutagenesis of strain UW. One typical experiment is depicted in Figure 3: Rif' mutants appeared after 2 generations of outgrowth, whereas appearance of Nal' mutants required 4 generations. In independent experiments, significant variation was found in the absolute numbers of mutants, but the expression of rif mutations never required more than 2–3 generations and that of nal mutations never more than 5–6 generations.

Streptomycin-resistant mutants were invariably rare and required >10 generations of outgrowth. Because
6–8 generations are required (after mutagenesis) to express \textit{str} mutations in enteric bacteria (data not shown), 10–12 generations are still a "short" time to express an extremely recessive mutation in a highly polyploid bacterium. Thus we suggest that \textit{A. vinelandii} may have a ploidy level slightly higher than \textit{E. coli} or \textit{S. typhimurium}, but the number of active chromosomes must be much lower than 40 or 80. The success in isolating \textit{Str}' at all is, in fact, good evidence against the existence of extreme polyplody in \textit{A. vinelandii}.

One might still argue that early expression of these mutations might be facilitated by massive destruction of chromosomal copies during mutagenesis. To rule out this possibility, we determined the number of generations required for the expression of \textit{str}, \textit{nal} and \textit{rif} mutations after transformation. Donors were strains AS8 and AS11; recipient was strain UW. The transforming mixtures (50–100 µl competent cells and 30–60 µg crude DNA extract) were mixed and spread over a small area of nonselective agar; 2–5 ml BSNB were also added. Aliquots were periodically extracted and plated on selective plates; the same aliquots were used for plate counts. The results were similar to those reported above: \textit{nal} and \textit{rif} mutations were expressed after 2–6 generations of outgrowth, while \textit{str} mutations required a longer expression period (around 10 generations). These data provide further support for the existence of low gene dosage.

**DISCUSSION**

The controversy raised by CONTRERAS and CASADESUS (1987) indicating that \textit{A. vinelandii} does not behave as a polyploid bacterium in many genetic operations is further explored in this paper. Gene dosage estimations are inferred from segregation analysis in several types of heterozygotic isolates. Heterozygotes are genetically identified as isolates harboring a pair of mutually exclusive alleles; they are easily obtained using transposon insertions, since these provide a dominant phenotype on antibiotic medium. An additional advantage of transposon-tagged alleles is that they can be physically detected by DNA hybridization.

The basic assumption is that, in a polyploid bacterium, heterozygosity is an obligate step in every process of reversion or recombination; thus heterozygotes can be expected to be common and relatively stable, at least under double selection. However, our observations for \textit{A. vinelandii} do not support this assumption: (i) when reversion of a transposon-induced mutation is forced to occur on the plate, many revertants are heterozygotic or had formed mixed colonies, but all are unstable and undergo fast segregation toward the parental type selected; (ii) segregation, mostly towards prototrophy, occurs even in the absence of selection; (iii) double selection transiently maintains the heterozygotic isolates, but strongly favors the detection of secondary "hops"; (iv) recombinants generated by genetic transfer are mostly homozygotic, unless double selection is applied; (v) under double selection, gene transfer yields heterozygotic recombinants but these are, again, unstable. Thus the overall conclusion is that \textit{A. vinelandii} behaves as an haploid or moderately polyploid bacterium and does not seem to contain >40 active chromosomes per cell.

Recessive mutations which confer a selectable phenotype provide an independent approach for the study of gene dosage. Expression of mutations such as \textit{str}, \textit{nal} and \textit{rif} require the turnover of proteins belonging to the transcription/translation apparatus before being expressed; thus the number of generations required for their expression must be always higher than the average number of chromosomes per cell. Our data indicate that expression of these muta-
tions in \textit{A. vinelandii} requires a rather short outgrowth; these results are thus consistent with the segregation data found for dominant mutations, since they indicate that heterozygotes \textit{Str'/Str^a} and \textit{Rif'/Rif^p} are unstable.

Segregation experiments carried out with \textit{nifF::lacZ} fusions provide a direct visualization of heterozygote instability. The segregation pattern of such fusions is that expected from cells containing a low number of chromosomes. Namely, following fusion formation by a double recombination event and subsequent cell division, a single chromosome containing the \textit{nifF::lacZ} fusion is partitioned into a daughter cell. Moreover, the pattern of segregation is entirely incompatible with the presence of a large number of active chromosomes, a situation which would result in a much more complex segregation pattern. Also, because there is no apparent selective advantage or disadvantage for the presence of the \textit{nifF::lacZ} fusion, its copy number in transformed cells should be variable if there are indeed 40–80 chromosomes per cell. However, there is no recognizable variability in the intensity of blue color in transformed cells.

How to reconcile the genetic evidence that \textit{A. vinelandii} has a low gene dosage with the proposed existence of 40 or more chromosomes? One can consider the possibility that the \textit{A. vinelandii} genome might indeed contain $>40$ chromosomal copies but only some of them were expressed. Lack of expression might be caused by threshold gene dosage requirements or by the existence of transcriptionally inactive DNA, perhaps in a similar fashion to the non-complementary diploids of \textit{Bacillus subtilis} (Hotchkiss and Gabor 1980). However, this possibility was ruled out by Southern hybridization, because a strict correlation between the presence of an allele and its phenotypic expression was found in all cases; in other words, all the homozgygote-like colonies derived from heterozygote transformants were true segregants which lacked the unexpressed allele. Even more compelling are the results provided by colony hybridization experiments, where physical and genetic segregation are simultaneously monitored. Thus, phenotypically silent DNA does not seem to exist in \textit{A. vinelandii}, unless it is maintained in a modified state that does not allow standard (NaCl + NaOH) DNA denaturation.

Other tentative explanations seem even more unlikely. One might conceive an odd, budding-like mechanism for chromosome segregation, generating haploid cells that would later increase their chromosome number up to $>40$ copies. However, the absence of mixed colonies among the segregants obtained without selection argues against this hypothesis. The possibility that extrachromosomal genetic information might account for the excess DNA seems also unlikely, because bacterial plasmids usually represent a minor fraction of the DNA content per cell (Grinsted and Bennett 1988). Actually, indigenous plasmids have been described in certain strains of \textit{A. vinelandii} (Maia, Sanchez and Vela 1988), but the standard strain UW used in our studies is plasmid-free (Kennedy and Toukhdarian 1987). Even more surprising would be the presence of introns or any other type of noncoding sequences. Actually, a number of chromosomal regions of \textit{A. vinelandii} have been sequenced (Raina et al. 1988; Bennett, Jacobson and Dean 1988; Morgan, Lundell and Burgess 1988; Toukhdarian et al. 1990); their gene arrays are typically prokaryotic and do not show any intervening sequences that might account for the excess DNA. On the other hand, the possibility that the \textit{A. vinelandii} genome might contain repetitive, satellite-like DNA is ruled out by the complexity studies of Sodoff, Shemel and Ellis (1979) which showed that the \textit{A. vinelandii} genome is only made of unique sequences. The gene dosage studies described in this paper strongly support the previous findings of Contreras and Casadesus (1987) that \textit{A. vinelandii} does not exhibit the segregation pattern expected of a polyploid bacterium. Thus, these results provide a direct challenge to the hypothesis that \textit{A. vinelandii} has a highly reiterated number of independently segregating chromosomes.

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LITERATURE CITED


LEDERBERG, E. M., 1986 Plasmid prefix designations registered

CONTRERAS, A., and J. CASADESUS, 1987 Tn10 mutagenesis in

CONTRERAS, A., R. MALDONADO and J. CASADESUS, 1991 Tn5
mutagenesis and insertion replacement in Azotobacter vinelandii.

DE LA TORRE, J. L., J. ORTIN, E. DOMINGO, J. DRLAMARTER, B.
ALLET, J. DAVIES, J. P. BERTRAND, L. W. WRAY and W. S.
REZNIKOFF, 1984 Plasmid vectors based on Tn10 DNA: gene

FOSTER, T. J., M. A. DAVIS, D. E. ROBERTS, K. TAKESHITA and N.
KLECKNER, 1981 Genetic organization of transposon Tn10.


GUERRERO, M. G., J. M. VEGA, E. LEADBETTER and M. LOSADA,


LUQUE, F., E. SANTERO, J. R. MEDINA and M. TORTOLERO,


MORGAN, T. V., D. J. LUNDELL and B. K. BURGESS,


