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Segregation characteristics of multiple chromosomes of *Azotobacter vinelandii*

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Abstract. The genome of *Azotobacter vinelandii* has been tagged *in vivo* with transposons. The cells have then been allowed to divide and the pattern of segregation of the genomes has been studied. The results suggest the presence of multiple (possibly identical) copies of the genome in *A. vinelandii*. Only a fraction of the total number of genomes seem to have been tagged with transposon and an equilibrium between alleles of the same gene with and without the transposon was evident during random segregation.

Keywords *Azotobacter vinelandii*; multiple genomes; segregation of genomes.

1. Introduction

Single cells of *Azotobacter vinelandii* from a mid-exponential culture, contain $1{\cdot}5 \times 10^{-13}$ gm DNA, a value approximately 40 times that of *Escherichia coli* (Sadoff *et al* 1971; also our unpublished observation). Nevertheless, the sedimentation coefficient of gently prepared folded chromosomes of both bacteria are about the same, 1700 S for *A. vinelandii* and 1600 S for *E. coli*. In addition, a comparison of *cot* values of DNA from the two microorganisms, has revealed the presence of about the same unique sequence lengths of DNA in them (Sadoff *et al* 1979). It has therefore been thought that each *A. vinelandii* cell contains at least about 40 copies of its genome.

These observations lead us to two related questions. Are all the genome copies identical and functional? Do these copies segregate randomly during cell division?

We reported earlier the selection of clones from a gene library of *Azotobacter vinelandii* that rendered *E. coli* HB 101, a leucine auxotroph with lesion in the *leu B* gene, independent of leucine (Medhora *et al* 1983). We computed, from the total size of the genome of *A. vinelandii* and the average size of inserts in the library, that the probability (at 98·8%) of encountering, in the library, a sequence which may be present in 40 copies in a cell would be 1 in 400.

When 400 such clones in *E. coli* HB 101 were checked, we detected 3 cases where *E. coli* did not require leucine for their growth, and this was because of the presence of *Azotobacter* genes. This result suggested that the '*leu B*' gene is present in *A. vinelandii* in at least as many copies as the number of genomes, but does not necessarily let us conclude that each copy of the genome has a *leu B* gene. All these

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copies function in *E. coli*, but again, this is not enough evidence to suggest that this is so in *A. vinelandii*. All of them surely have the potential to express in *A. vinelandii*, but most of them may still be repressed by some negative control element.

We have sought to obtain information about the questions raised, by tagging the genomes with transposons and studying their segregation during cell division.

2. Materials and methods

Bacteria and plasmids: The bacterial strains and plasmids used in this study are described in table 1.

Manipulations of bacteria: Culture of bacteria, their mating and transformation with plasmids were done as described earlier (Phadnis and Das 1987).

Isolation of plasmid: Plasmid DNA was isolated according to Kahn *et al* (1979).

Nick translation: ^{32}P-labelled pUC9 :: Tn10 DNA was prepared using (α-^{32}P) deoxycytidine 5′-triphosphate (New England Nuclear, USA) as described by Rigby *et al* (1977).

Dot blot: DNA samples (2 μg) were applied to nitrocellulose membranes (Schleicher and Schuell, type BA 85) according to Thomas (1980). Hybridization of the membrane-bound DNA with ^{32}P-labelled pUC9 :: Tn10 probe and subsequent washing were done as described by Southern (1975) and Denhardt (1976).

Table 1. Bacterial strains and plasmids

	Description	Reference
Bacteria		
Escherichia coli HB 101	*pro*, *leu*, *thi*, *lac Y*, str^r, *end A*, *hsd R* (r_k m_k) *hsdM*	Boyer and Roullard-Dussiox (1969)
Azotobacter vinelandii UW	Non-gummy derivative of wild type	Shah *et al* (1973)
Plasmids		
pRK 2013 :: Tn3	Col E1 replicon (Kan^r) :: Tn3 (Amp^r)	Phadnis and Das (1987)
pRK 2013 :: Tn10	Col E1 replicon (Kan^r) :: Tn10 (Tet^r)	Phadnis and Das (1987)
pUC 9 – Cm^r	Col E1 replicon (Cm^r)	G Ditta, San Diego, Cal., USA (personal communication)
pUC 9 :: Tn10	Col E1 replicon (Cm^r) :: Tn10 (Tet^r)	G P Dimri (unpublished work)

Denatured linearized unlabelled pUC9 DNA (200 μg/ml) was also included in the hybridization mixture.

The washed and dried membrane was exposed to Indu X-ray film (Hindustan Photo Film Company Ltd., India) with Kiran Intensifying Screen (Kiran X-ray Screens Ltd., India) and the film was developed according to manufacturer's specifications.

Isolation of chromosomal DNA: DNA from *A. vinelandii* was isolated by the method of Sadoff *et al* (1979).

Results and discussion

Segregation behaviour of tagged genomes

Mutant phenotypes have been rare to come by in *Azotobacter vinelandii* (Sadoff *et al* 1979; Ramos and Robson 1985). No amino acid auxotroph, for example, is known so far. We decided to look into the segregation behaviour of genes involved in amino acid biosynthesis in general. The following three assumptions have been made:

(i) The genes for the amino acid biosynthetic pathways are present in each chromosome of *Azotobacter vinelandii*, which is in at least 40 copies. A direct corollary of this would be that all the 40 copies of the genome are identical, at least with respect to these genes.
(ii) All these copies are expressed in *A. vinelandii*.
(iii) The segregation of the genomes on cell division is random.

The rationale behind the experiment is as follows: tagging with a transposon, to start with, would inactivate only one allele of any gene. During subsequent cell division and random segregation of the genomes, some progeny cells are likely to have a larger number of some alleles (but not all) of the same gene with a transposon in them. These cells would grow poorly on media lacking amino acids. On the other hand, in the absence of amino acids, a reverse selection pressure would come into play and segregation of alleles without the transposon in them would be favoured.

We have earlier (Phadnis and Das 1987) demonstrated the suitability of using the plasmid pRK 2013 as a vehicle for transposition in *A. vinelandii*. Cells of *A. vinelandii* were conjugated with *E. coli* HB101 containing pRK 2013::Tn3 and plated on a medium containing ampicillin and BNF (Page and Sadoff 1976) supplemented with a mixture of all amino acids except leucine and proline. Since *E. coli* HB101 is auxotrophic for leucine and proline, this would be selected against. About a thousand of the *Azotobacter* exconjugant colonies were screened for failure to grow on BNF-ampicillin plates, but none were found. Several colonies, however, did grow poorly on BNF-ampicillin, but grew quite well on BNF-ampicillin-amino acids. Seven such colonies were purified by isolating single colonies on amino acid supplemented BNF-ampicillin media and tested again on media lacking amino acids. Once again they showed poor growth on BNF-ampicillin media, but on further successive transfers on BNF medium, they exhibited near-independence towards amino acids. These colonies could nevertheless be shown to be resistant to

ampicillin. The nature of the results remained unaltered if the amount of ampicillin was raised to 400 μg/ml, or if the cells were allowed many more divisions in amino acid supplemented BNF-ampicillin media.

There is thus an equilibrium between the alleles of the same gene with and without the transposon in it. This equilibrium can be shifted considerably either way, depending on the presence or absence of amino acids in the medium. The position of the equilibrium would determine whether in the absence of the amino acids a cell would grow well or poorly.

The predicted pattern of segregation of genes containing transposon has been further tested. *Azotobacter* ex-conjugants, after mating with *E. coli* containing pRK 2013 :: Tn3, were selected on plates containing ampicillin in BNF medium. The *Azotobacter* colonies appeared at the expected frequency (*cf* Phadnis and Das 1987). These colonies were patched again on two different plates, BNF only and BNF-ampicillin. Almost 90% of the colonies failed to grow this time on BNF-ampicillin but nevertheless all grew on BNF alone. The colonies that had grown on BNF alone were now tried again on BNF-ampicillin. Again, they all grew. The results are presented in figure 1. Of the 26 colonies obtained on first transfer to BNF-ampicillin plates, only 12 grew in BNF-ampicillin after the second transfer.

These results thus suggest that the assumptions made on the identity and functionality of the genomes and their random segregation are valid. When a transposon gets inserted in one copy of the *Azotobacter* chromosome, it might inactivate the allele of an essential gene in that chromosome. The cell, however, would continue to grow and divide, since the essential gene product would be formed as a result of expression of the alleles of the same gene located in other identical copies of the chromosome. Due to segregation in presence of the

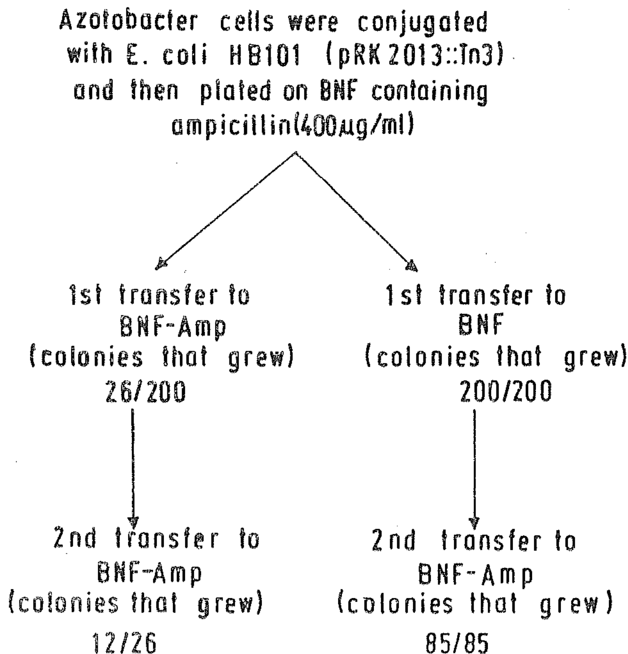

Figure 1. Behaviour of *A. vinelandii* containing Tn3.

antibiotic, cells with more and more copies of the chromosome containing the alleles of the gene that have the transposon in them, would accumulate. Since the segregation is random, it would take very long to have the transposon in the alleles of the gene present in all the copies of the chromosome. The cell would be able to divide continuously and form a colony during this period. Most of the cells of this colony would, however, contain multiple copies of the allele with the transposon. If expression of all the alleles of a particular gene, or of a good majority of them are needed for cell growth and division, cells having a few copies of transposon would also fail to grow, if patched again on a BNF-ampicillin plate. When the selection pressure is taken off (ampicillin withdrawn) the selection in reverse direction would set in. Thus, all the cells which failed to grow after the first transfer to ampicillin-BNF plates, would grow and divide in plates containing BNF only.

If the concept of random segregation is correct, one should never lose the transposon from a colony of cells. The recovery of ampicillin-resistant cells from colonies grown without ampicillin in both the experiments, supports this view.

Molecular analysis

The concept has also been tested by molecular analyses using Tn10. Tn10 was transposed on to the chromosome of *A. vinelandii* from pRK2013 :: Tn10. Colonies were selected for growth in amino-acid-supplemented BNF containing tetracycline (1 μg/ml) and for failure to grow in the same medium where tetracycline has been replaced with kanamycin (25 μg/ml). These cells were successively subcultured 12 times in the medium containing tetracycline, 1 μg/ml, and then twice more in the presence of tetracycline, 5 μg/ml (an increase of antibiotic concentration to 10 μg/ml results in the partial lysis of the cells). DNA was isolated from a part of these cells. Some of these cells were inoculated in BNF medium containing no tetracycline or amino acid. These cells were subcultured twice more after full growth in the same medium with no tetracycline or amino acids (all these cells could still be shown to be resistant to tetracycline). DNA was isolated from these

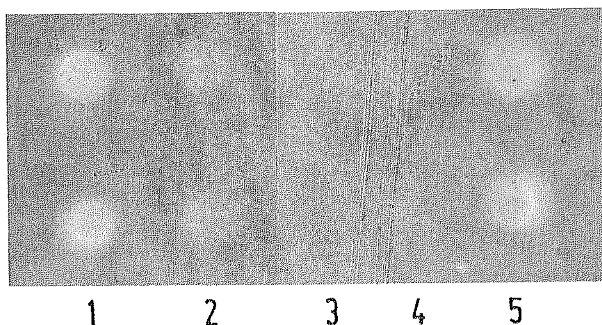

Figure 2. 'Dot blot' analysis of DNA from *A. vinelandii* for number of copies of Tn10 in the chromosome. All dots are in duplicate. Dot 1: DNA (2 μg) from cells cultured in presence of tetracycline (5 μg/ml). Dot 2: DNA from cells from which antibiotic pressure has been withdrawn. Dot 3: DNA from *A. vinelandii* UW which do not have any Tn10. Dot 4: 100 pg pUC9 :: Tn10 plus 2 μg *A. vinelandii* UW DNA. Represents 2 μg DNA from cells containing 1 copy of Tn10 per cell. Dot 5: 500 pg pUC9 :: Tn10 plus 2 μg *A. vinelandii* UW DNA. Represents 2 μg DNA from cells containing 5 copies of Tn10 per cell.

cells too. Both the DNA samples (2 μg each) were applied on to cellulose nitrate membrane. Also applied was DNA (2 μg) from *A. vinelandii* UW cells, which have no Tn10 in them. Apart from these, mixtures at specific proportions of pUC9 :: Tn10 and DNA from *A. vinelandii* were also applied. The membrane was then subjected to hybridization with ^{32}P-labelled pUC9 :: Tn10 containing large excess of unlabelled pUC9. The result of this 'dot blot' assay has been presented in figure 2. It is obvious that the number of copies of Tn10 in cells under pressure of tetracycline are more than that in cells wherefrom the pressure of the antibiotic has been withdrawn. Tn10 is not known to undergo tandem amplification like Tn9. Hence, the results obtained are in conformity with the concept of random segregation and equilibrium between alleles of a gene with and without a transposon in it.

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