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Multiple Chromosomes of *Azotobacter vinelandii*

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The number of copies of the genes *leuB*, *nifH*, *nifD*, and *nifK* per cell of *Azotobacter vinelandii* has been determined to be about 80. A β-lactamase gene was integrated into the *A. vinelandii* chromosome by single-point crossover. Subsequently, we have been able to detect nearly 80 copies of this β-lactamase gene per cell of *A. vinelandii* when cultured for a large number of generations in the presence of ampicillin. The multiple copies of the β-lactamase gene do not seem to be present on a single chromosome, as evident from the fragment obtained by digestion of cellular DNA with the appropriate restriction endonuclease. The kinetics of renaturation of DNA of *A. vinelandii* is suggestive of complexity similar to that of *Escherichia coli*. The DNA content of *A. vinelandii*, however, is 40 times that of *E. coli*. All these indicate the presence of multiple chromosomes, possibly as many as 80, in *A. vinelandii*.

Azotobacter vinelandii, a gram-negative bacterium that reduces dinitrogen aerobically (18), has the remarkable feature that it contains a large amount of DNA, as much as 40 times that in *Escherichia coli* (22; also, our unpublished observation with the strain we have been using). How is this huge amount of DNA organized? Are the genes in *A. vinelandii* reiterated? If so, are they present in a long single chromosome or are there multiple copies of the chromosome? We have directly titrated, with cloned probes, the number of copies of several genes and also determined the distribution of an external gene after its integration into the *A. vinelandii* chromosome. Besides, we have assessed the complexity of DNA of the strain we have been using by determination of its renaturation kinetics. Our inference is that a cell of *A. vinelandii* has nearly 80 chromosomes.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains and plasmids used in this study have been listed in Table 1.

Media and culture conditions. *A. vinelandii* was grown at 30°C on a rotary shaker in modified Burk nitrogen-free medium (25). Ammonium acetate (0.11%) was included in the medium when faster growth was desired. *E. coli* was grown similarly at 37°C in Luria-Bertani medium (15).

Isolation of DNA. *A. vinelandii* chromosomal DNA was isolated from log-phase cultures by the method of Sadoff et al. (23). *E. coli* chromosomal DNA was also isolated from log-phase cultures by a method described by Ditta (10) for the isolation of *Rhizobium* DNA. Plasmid DNA was isolated by the alkaline lysis method (1).

Restriction analysis of DNA. DNA digested with restriction endonuclease in appropriate buffer (15) was electrophoresed in a 0.8% agarose gel in 88 mM Tris–88 mM boric acid–25 mM EDTA (pH 8.3). Staining was done with ethidium bromide (1 μg/ml). To determine the amount of DNA solution to be electrophoresed, the A_{260} was measured and 1 absorbance unit was assumed to represent 50 μg of DNA per ml.

Isolation of DNA fragment, labeling by nick translation, and use as a hybridization probe. The DNA was cleaved with the relevant restriction endonuclease and electrophoresed on an agarose gel, and the desired fragment was eluted into a strip of Whatman DE-81 paper. The fragment was recovered from the paper by treatment with 10 mM Tris (pH 8.0)–2 mM EDTA–1 M NaCl. Nick translation was done by the method of Rigby et al. (19). DNA was blotted by the method of Southern (24), and DNA was hybridized on the membrane with nick-translated probe as described by Botchan et al. (5) and Denhardt (9).

Determination of DNA content per cell. DNA was estimated by the method of Burton (6). Cells were counted in a hemacytometer counting chamber. *A. vinelandii* contained 1.35×10^{-13} g of DNA per cell, and *E. coli* contained 3.4×10^{-15} g. Assuming the *E. coli* DNA to be equivalent to 4×10^3 kilobase pairs (kb), we have computed *A. vinelandii* DNA to be 1.6×10^5 kb.

Determination of renaturation kinetics of DNA. DNA solution (50 μg/ml) was subjected to ultrasonic treatment in the MSE Soniprep 150 for 30 s for *E. coli* and for 2 min for *A. vinelandii*. The time of treatment was arrived at to obtain fragments of similar lengths from the DNAs of both the microorganisms. The average size for both DNAs, as seen by agarose gel electrophoresis, was 700 base pairs with a spread from 300 to 1,000 base pairs. The DNA was precipitated with ethanol and dissolved in 0.12 M sodium phosphate buffer (pH 7.0) to a final concentration of about 20 μg/ml. The DNA solution was degassed under vacuum. Its A_{260} was noted in a Shimadzu UV-190 double-beam spectrophotometer at room temperature. The temperature was raised to 99.9°C with the help of the temperature controller, and absorbance of the fully denatured DNA was noted. After 5 min at 99.9°C, the temperature was brought down immediately to $T_m - 25$°C (i.e., 66°C for *E. coli* and 72°C for *A. vinelandii*) and held there. The A_{260} was noted every 5 min for 1 h. Renaturation time was plotted against the fraction of residual denatured DNA, $(A_d - A_n)/(A_i - A_n)$, where A_d is A_{260} of fully denatured DNA, A_n is A_{260} of native DNA, and A_i is A_{260} of DNA at intermediate stages of renaturation.

Transformation of *A. vinelandii*. *A. vinelandii* cells were transformed with plasmid DNA by the method of Glick et al. (11).

RESULTS

Determination of the number of copies of the *leuB* gene per cell of *A. vinelandii*. The plasmid pMM113 contains a 1-kb fragment from the *A. vinelandii* genome in the *Bam*HI site of pHC79 (13). pMM113 is a deletion derivative of pMP113 (16)

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TABLE 1. Strains and plasmids used

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
E. coli K-12 W3110	F^- prototrophic and lambda lysogenic	ATCC 14948
A. vinelandii UW	Nongummy derivative of wild type	2
UW(pMP2)	Amp^r; plasmid pMP2 integrated into the chromosome	This study
UW(pMM113)	Amp^r; the plasmid pMM113 integrated into the chromosome	This study
Plasmids		
pHC79	Amp^r Tet^r; pBR322 with the lambda cohesive end (cos) fragment	12
pMP2	Amp^r; pHC79 carrying a fragment (~35 kb) from *A. vinelandii* containing the conventional *nif HDK* genes	15
pMM113	Amp^r; pHC79 carrying a fragment (~1 kb) from *A. vinelandii* containing the *leuB* gene	15

and complements the *leuB* mutation in *E. coli*. The insert in pMM113 has restriction sites for *Hin*dIII, *Pst*I, *Sal*I, and a second *Sal*I, in that order. If the *leuB* gene is present in one copy in an *A. vinelandii* cell, a synthetic mixture of the same composition could be made by mixing 7.4 μg of pMM113 (size, 7.4 kb) DNA with 1.6×10^5 μg of calf thymus DNA. The total DNA content of *A. vinelandii* has been assumed to be 1.6×10^5 kb per cell (see Materials and Methods). Similarly, synthetic mixtures were made equivalent to 6-copy and 40-copy situations. DNAs from *A. vinelandii* UW and each of the synthetic DNA mixtures were digested with *Eco*RI, electrophoresed in agarose, transferred to a nitrocellulose membrane (24), and hybridized with the ^{32}P-labeled *A. vinelandii leuB* probe isolated from pMM113. The intensity of the signal obtained with *A. vinelandii* chromosomal DNA was almost double that with the synthetic mixture equivalent to the 40-copy situation (Fig. 1). We have found later that the intensity of the signals of the standard samples remains unchanged, even if calf thymus DNA is omitted.

Determination of the number of copies of the *nif* genes. We have, in a similar manner, determined the number of copies of the three nitrogenase structural genes, *H*, *D*, and *K*. The Southern blot of *A. vinelandii* chromosomal DNA probed with a *nifD* fragment isolated from pMP2 (16) is shown in Fig. 2. The additional faint high-molecular-weight signal in lane 4 is presumably the result of incomplete digestion with *Eco*RI. The intensity of the signal obtained with *A. vinelandii* chromosomal DNA was nearly the same as that with the relevant standard plasmid DNA equivalent to the 80-copy situation (Fig. 2). The result obtained with the *nifK* probe was very similar (data not shown). With the *nifH* probe, two additional *Bgl*II bands (30 and 7 kb) could also be seen, which presumably represented genes of the alternative pathways (3, 4, 8, 14). The main band (4.5 kb) however, was almost as intense as the band representing the standard plasmid DNA equivalent to the 80-copy situation (data not shown).

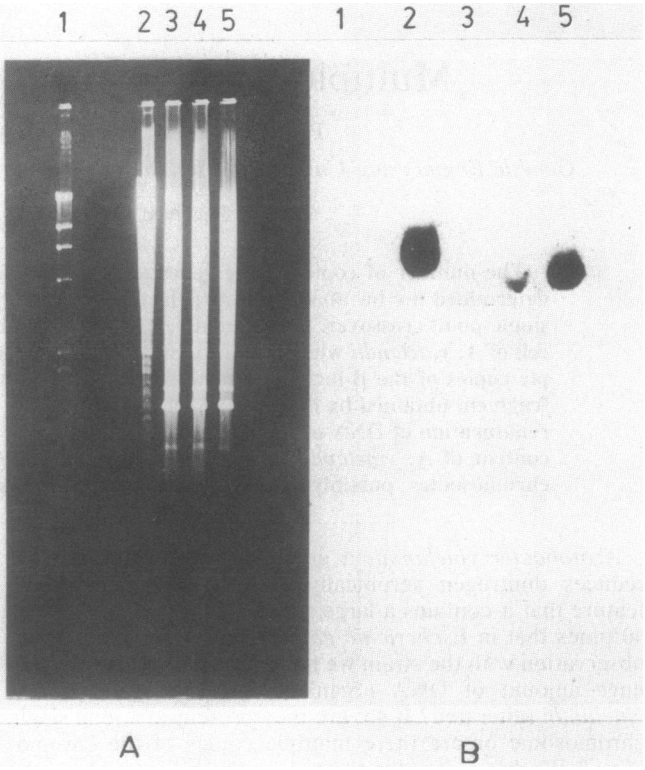

FIG. 1. (A) *Eco*RI-digested *A. vinelandii* chromosomal DNA and synthetic DNA mixtures after agarose gel electrophoresis. Lane 1, Lambda DNA cleaved with *Hin*dIII; lane 2, *A. vinelandii* chromosomal DNA (5 μg) digested with *Eco*RI; lane 3, synthetic mixture (5 μg) of pMM113 and calf thymus DNA (cleaved with *Eco*RI) equivalent to 1 copy of the *leuB* gene per cell of *A. vinelandii*; lane 4, 6-copy situation; lane 5, 40-copy situation. (B) Autoradiogram of the Southern blot of the gel after hybridization with a nick-translated 370-bp *Hin*dIII-*Sal*I fragment of *A. vinelandii leuB* gene.

We conclude that the *leuB* and the *nifHDK* genes are present in the *A. vinelandii* cell in nearly 80 copies. Are all the copies of these genes present on a single huge chromosome, or are they present as single copies on 80 different chromosomes contained in each cell? An external drug resistance gene like that of β-lactamase, if integrated into the chromosome of *A. vinelandii* by single-point crossover after homologous pairing, would be in only one copy if there was a large single chromosome. On the other hand, if there are 80 identical chromosomes of proportionately smaller size and if a very large number of cell divisions are allowed in the presence of ampicillin (50 μg/ml), segregation of the chromosomes would be expected to lead to a situation in which many of the surviving cells would have the β-lactamase gene in nearly all the 80 copies of the chromosome.

Integration of pMM113 and pMP2 into the *A. vinelandii* chromosome. Both pMM113 and pMP2 have the ColE1 replication origin, which has been found to be unstable in *A. vinelandii* (17). Both the plasmids have a β-lactamase gene that is not a part of any transposon. This gene is situated almost adjacent to, but not inside, the cloned fragments of the *A. vinelandii* chromosome. We transformed *A. vinelandii* cells with both these plasmids separately and selected for resistance to ampicillin (50 μg/ml). Since these plasmids are incapable of free existence in *A. vinelandii*, ampicillin-resistant cells can originate only if homologous recombination involving single-point crossover takes place, resulting in

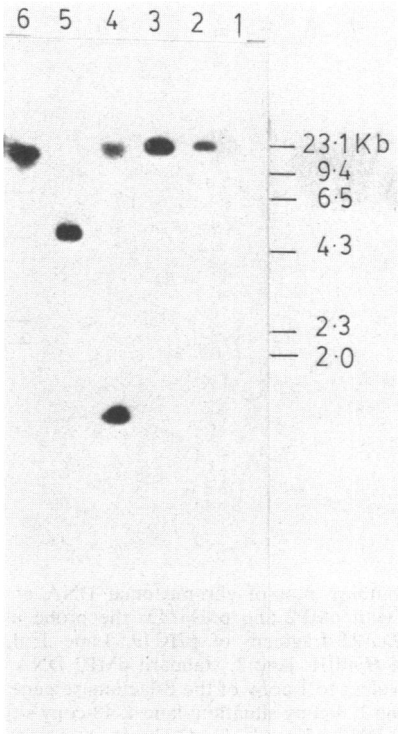

FIG. 2. Southern blot of chromosomal DNA of *A. vinelandii* probed with the ^{32}P-labeled 0.8-kb *Kpn*I fragment (*nifD*) from pMP2. Lane 1, Standard pMP2 DNA digested with *Hin*dIII equivalent to 8 copies of the *nifD* gene per cell of *A. vinelandii*; lane 2, 40-copy situation; lane 3, 80-copy situation; lane 4, chromosomal DNA (3 μg) of *A. vinelandii* digested with *Eco*RI; lane 5, chromosomal DNA digested with *Bgl*II; lane 6, chromosomal DNA digested with *Hin*dIII.

integration of the entire plasmid into one of the copies of the bacterial chromosome. This integration would take place at a position adjacent to the bacterial genes that have been cloned in the plasmid. This possibly happens in a manner analogous to that conceived in the Campbell model (7) for integration of the lambda genome into the *E. coli* chromosome (Fig. 3). This is different from a two-point crossover, in which only the cloned fragment in the plasmid is exchanged

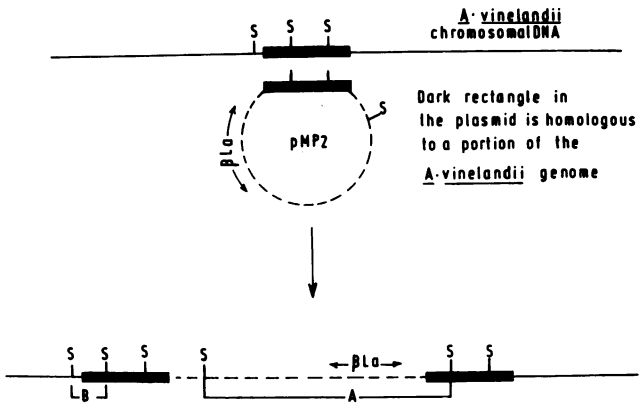

FIG. 3. Postulated mode of integration of recombinant plasmid into the *A. vinelandii* chromosome by single-point crossover. βLa, β-Lactamase; S, *SalI*; B and A, fragments that would hybridize with the probe whose construction is shown in Fig. 4.

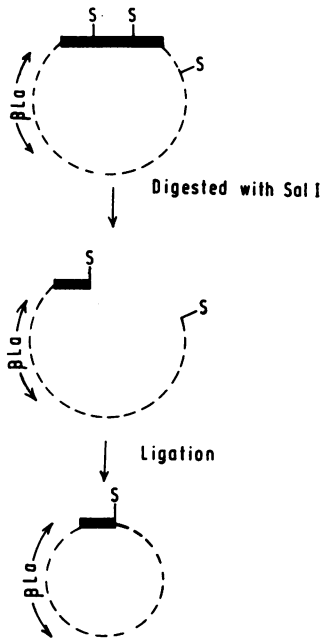

FIG. 4. Principle of construction of truncated derivatives from pMP2 and pMM113 for use as probes in the experiment represented by Fig. 5.

with the homologous segment in the bacterial chromosome (cf. reference 21). The ampicillin-resistant phenotype was stable in spite of five consecutive subculturings in medium devoid of the antibiotic. We also failed to detect any plasmid (1) in the transformed cells cultured in the presence of ampicillin (50 μg/ml).

Verification of single-point crossover. A single-point crossover, as envisaged here, would result in the appearance in the chromosome of a second copy of the DNA segment that was present as in insert in the transforming plasmid along with the plasmid vehicle. This has been verified experimentally. DNA isolated from stationary-phase cultures of *A. vinelandii* transformed with pMM113 and pMP2 (ampicillin resistant) and also from untransformed cells (ampicillin sensitive) were digested with *SalI*, electrophoresed in agarose, and Southern blotted onto cellulose-nitrate membranes. *SalI* was chosen because it was found to cleave the *A. vinelandii* DNA very frequently and it had only one site in the vehicle pHC79. The probes to be used were constructed by digesting pMM113 and pMP2 with *SalI*, purifying the main fragment by agarose gel electrophoresis, and religating (Fig. 4). The presence of the vehicle and the β-lactamase gene in these truncated constructs was verified by transforming *E. coli* HB101 and selecting for ampicillin (50 μg/ml) resistance. These constructs were nick translated (19) and used as probes. The probes were designed this way to ensure the presence of a much larger proportion of the cloning vehicle in them and thus make the detection of the vehicle in the chromosome absolutely sure. While only one band hybridized with DNA from the untransformed cells, an additional band hybridized and exhibited a very strong signal with DNA from the ampicillin-resistant transformed cells (Fig. 5).

Determination of copy number of the integrated β-lactamase gene. The copy number of the β-lactamase gene was next determined in stationary-phase *A. vinelandii* cells transformed with pMM113 and pMP2. These cells had undergone many subculturings in medium containing ampicillin over a

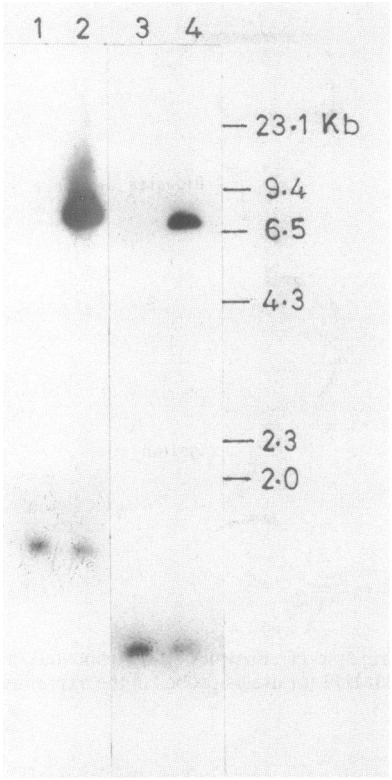

FIG. 5. Southern blots of chromosomal DNAs of untransformed cells and cells transformed with pMP2 and pMM113 cleaved with *Sal*I and probed with the truncated derivatives described in the legend to Fig. 4. Lane 1, DNA from untransformed cells probed with the pMP2 derivative; lane 2, DNA from cells transformed with pMP2 and probed with the pMP2 derivative; lane 3, DNA from untransformed cells probed with the pMM113 derivative; lane 4, DNA from cells transformed with pMM113 and probed with the pMM113 derivative.

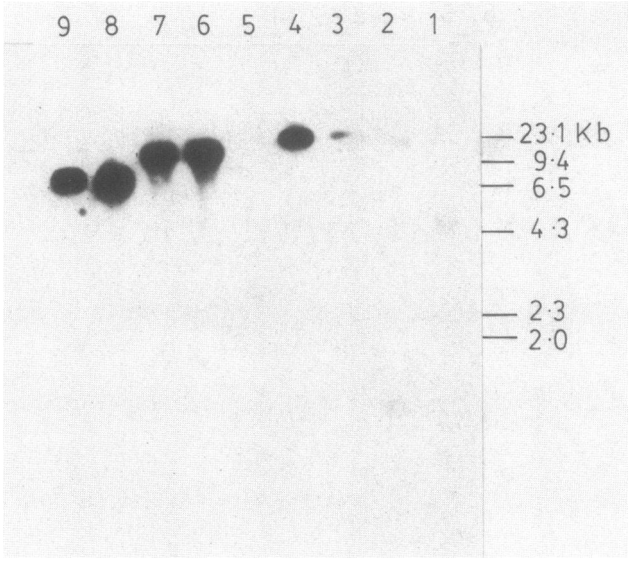

FIG. 6. Southern blot of chromosomal DNA of *A. vinelandii* transformed with pMP2 and pMM113, the probe used being the 0.7-kb *Pst*I-*Eco*RI fragment of pHC79. Lane 1, Lambda DNA digested with *Hin*dIII; lane 2, standard pMP2 DNA digested with *Hin*dIII equivalent to 1 copy of the β-lactamase gene per cell of *A. vinelandii*; lane 3, 8-copy situation; lane 4, 40-copy situation; lane 5, chromosomal DNA of *A. vinelandii* (3 μg) untransformed with any plasmid, digested with *Hin*dIII; lane 6, chromosomal DNA from *A. vinelandii* (3 μg) transformed with pMP2 (isolate 1) digested with *Hin*dIII; lane 7, same as lane 6 but with isolate 2; lane 8, chromosomal DNA from *A. vinelandii* (3 μg) transformed with pMM113 (isolate 3) digested with *Hin*dIII; lane 9, same as lane 8 but with isolate 4.

long period of time after transformation. The probe used in this case was the *Eco*RI-*Pst*I fragment containing only the β-lactamase gene isolated from the vehicle pHC79, which was free from any other DNA. The chromosomal DNA was digested with *Hin*dIII. In at least one isolate out of two of each kind, the copy number has been found to be nearly 80 (Fig. 6). Only one band hybridized in both the cases. The sizes of the hybridizing bands from both isolates of the same transformant were identical. This confirmed that the integration of a particular plasmid was at the same site of the chromosome in both the isolates.

When the same experiment was performed with DNA from *A. vinelandii* soon after transformation, fewer copies of the β-lactamase gene were found per cell. Figure 7 represents the results of such an experiment in which the transformation has been done with pMM113 and the chromosomal DNA has been digested with *Pst*I. Similar results were obtained when *Hin*dIII was used (data not shown).

One large chromosome or multiple chromosomes? We would like to presume that the 80 copies of the β-lactamase gene are present in as many copies of the *A. vinelandii* chromosome, 1 copy in each chromosome. It is, however, possible that by some hitherto unknown mechanism, the β-lactamase gene gets reiterated in tandem on a single chromosome. The plasmid pMM113 does not have any site for the restriction endonucleases *Kpn*I and *Bam*HI. DNA isolated from *A. vinelandii* transformed with pMM113 (the same as that used for the experiment represented by Fig. 6) was digested separately with *Kpn*I and *Bam*HI, electrophoresed, Southern blotted, and probed with nick-translated

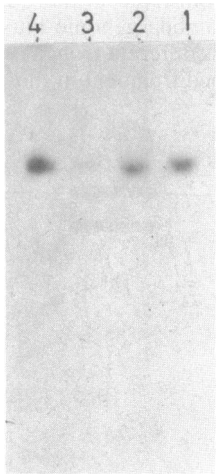

FIG. 7. Southern blot of chromosomal DNA (3 μg) isolated soon after transformation of *A. vinelandii* with pMM113, cleaved with *Pst*I, and probed with the 0.7-kb *Pst*I-*Eco*RI β-lactamase gene fragment from pHC79. Lanes 1 and 2, DNA from transformants; lane 3, plasmid pMM113 (standard) cleaved with *Pst*I, in an amount equivalent to 10 copies of β-lactamase per *A. vinelandii* cell; lane 4, 40-copy situation.

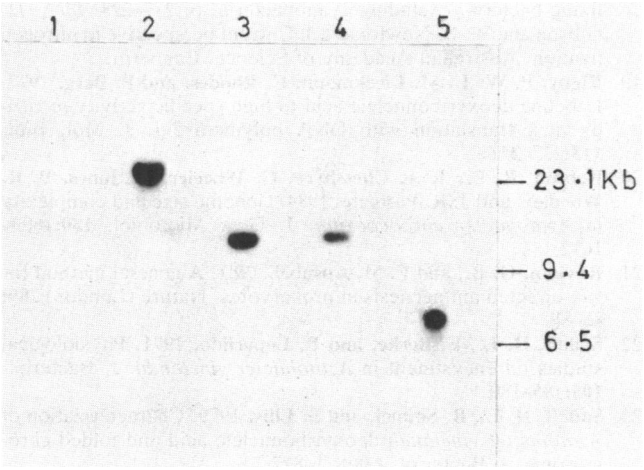

FIG. 8. Southern blot of chromosomal DNA from *A. vinelandii* cells transformed with pMM113 (same as used for the experiment represented by Fig. 6), cleaved separately with *Kpn*I and *Bam*HI, and probed with the 0.7-kb *Pst*I-*Eco*RI β-lactamase gene fragment from pHC79. Lane 1, Lambda DNA cleaved with *Hin*dIII; lane 2, DNA from transformed cells cleaved with *Bam*HI; lanes 3 and 4, different amounts of same DNA cleaved with *Kpn*I; lane 5, plasmid (standard) pMM113 cleaved with *Eco*RI.

pMM113. The sole signal appeared at around 12 kb for *Kpn*I and around 24 kb for *Bam*HI (Fig. 8). In a case of tandem reiteration, we would have expected the size of the signal-giving band to be 80 times the size of pMM113, i.e., about 600 kb. On the other hand, if the β-lactamase gene had been integrated at 80 different locations in a large chromosome, nearly as many bands would have been visible.

Renaturation kinetics. We have looked at the kinetics of renaturation of denatured DNA from the strain of *A. vinelandii* being used by us and compared it with that from *E. coli* K-12 W3110. The slopes of the plot of the fraction of residual denatured DNA ($A_d - A_n/A_i - A_n$) against time are measures of the complexity of the genomes, and these appear very similar (Fig. 9). A more or less identical observation has been made (23) with another strain.

DISCUSSION

A 40-fold-higher DNA content in *A. vinelandii* than in *E. coli* and similar complexities suggest that there might be 40 chromosomes per cell of *A. vinelandii*, the assumption being that the sizes of the chromosomes in *E. coli* and *A. vinelandii* are the same. Results of direct titration of several chromosomal genes presented here, however, show that an *A. vinelandii* cell contains nearly 80 chromosomes. One way to reconcile these results would be to postulate that the *A. vinelandii* chromosome is in reality half as big as that of *E. coli*, a situation analogous to that in *A. chroococcum* (20). On the other hand, it is quite possible that under the conditions of culture (rich medium), each *E. coli* cell, in fact, had two chromosomes, each similar in size to each of the multiple chromosomes of *A. vinelandii*. The data on renaturation kinetics would favor this alternative.

To conclude, an *A. vinelandii* cell in a stationary-phase culture contains nearly 80 chromosomes, possibly all of them identical. Our results do not tell us anything about whether all these copies of chromosomes are functional simultaneously. It is, however, of interest that *A. vinelandii* has one of the highest respiratory quotients known (12). This might suggest the presence of an unusually large amount of respiratory enzymes that can be produced only from multiple copies of genes.

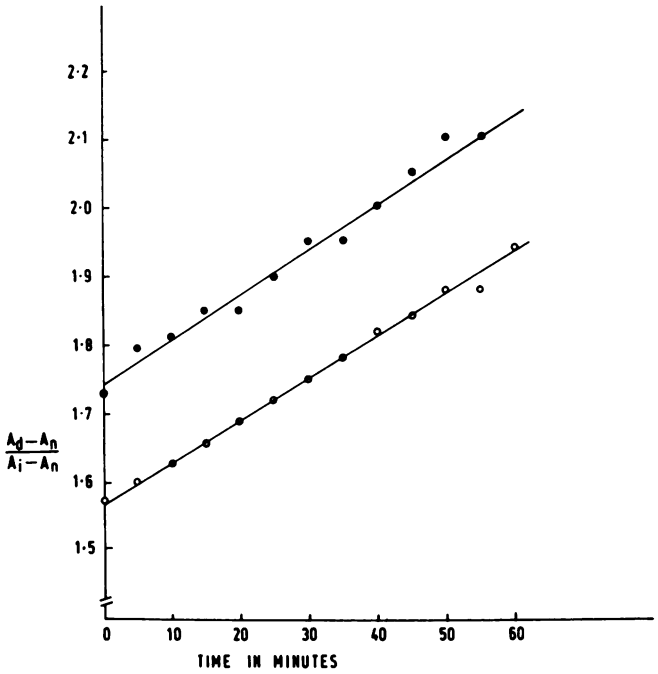

FIG. 9. Renaturation kinetics of DNA from *A. vinelandii* (●) and *E. coli* (○). A_n represents the A_{260} of native DNA, while A_d is that of fully denatured DNA. A_i denotes absorbance at intermediate stages, as renaturation takes place. Renaturation temperature (T_m − 25°C) for *A. vinelandii* was 72°C and for *E. coli* was 66°C.

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