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Changes of Ploidy during the *Azotobacter vinelandii* Growth Cycle

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The size of the *Azotobacter vinelandii* chromosome is ~4,700 kb, as calculated by pulsed-field electrophoretic separation of fragments digested with the rarely cutting endonucleases *Spe*I and *Swa*I. Surveys of DNA content per cell by flow cytometry indicated the existence of ploidy changes during the *A. vinelandii* growth cycle in rich medium. Early-exponential-phase cells have a ploidy level similar to that of *Escherichia coli* or *Salmonella typhimurium* (probably ca. four chromosomes per cell), but a continuous increase of DNA content per cell is observed during growth. Late-exponential-phase cells may contain >40 chromosomes per cell, while cells in the early stationary stage may contain >80 chromosomes per cell. In late-stationary-phase cultures, the DNA content per cell is even higher, probably over 100 chromosome equivalents per cell. A dramatic change is observed in old stationary-phase cultures, when the population of highly polyploid bacteria segregates cells with low ploidy. The DNA content of the latter cells resembles that of cysts, suggesting that the process may reflect the onset of cyst differentiation. Cells with low ploidy are also formed when old stationary-phase cultures are diluted into fresh medium. Addition of rifampin to exponential-phase cultures causes a rapid increase in DNA content, indicating that *A. vinelandii* initiates multiple rounds of chromosome replication per cell division. Growth in minimal medium does not result in the spectacular changes of ploidy observed during rapid growth; this observation suggests that the polyploidy of *A. vinelandii* may not exist outside the laboratory.

The organization of the *Azotobacter vinelandii* genome was first investigated in H. L. Sadoff's laboratory (51). These classical studies can be summarized as follows. (i) Combined data from thermal denaturation and DNA renaturation kinetics experiments indicated that the *A. vinelandii* genome was made of unique sequences. (ii) Both the sedimentation rates and the $c_0t_{1/2}$ values were similar for *A. vinelandii* and *Escherichia coli* chromosomal DNAs, indicating that these chromosomes have similar sizes. (iii) *A. vinelandii* cells harvested during mid-exponential growth contained at least 40 times more DNA than *E. coli* cells. The overall conclusion from these data was that *A. vinelandii* must contain at least 40 chromosomes per cell (51). A decade later, the idea that *A. vinelandii* is a highly polyploid bacterium received further support from the studies of Nagpal et al. (39). Using a quantitative hybridization procedure, those authors measured the copy number of *leu* and *nif* genes in stationary-phase cultures of *A. vinelandii* and found an approximate copy number of 80 copies per cell. Similar copy numbers were obtained when a plasmid β-lactamase gene was integrated into the chromosome (39).

The polyploidy of *A. vinelandii* seemed to explain a classical problem of *Azotobacter* genetics, namely, the difficulty in isolating certain types of auxotrophs (reviewed in reference 22). However, the development of *A. vinelandii* genetics and the introduction of transposon technology provided several lines of evidence against polyploidy (discussed in references 6, 8, and 32). This controversy prompted the design of experiments specifically devised to measure gene dosage, which seemed to indicate that *A. vinelandii* is not a polyploid bacterium (32). The main arguments against polyploidy were as follows. (i) Heterozygotic transformants and transconjugants of *A. vinelandii* were unstable even in the absence of selection. (ii) Reversion of transposon-induced mutations was usually associated with loss of the transposable element. (iii) Chromosomal *lac* fusions constructed by double crossover with a linearized plasmid showed a segregation pattern consistent with the inheritance of one or several chromosomes per daughter cell. (iv) Recessive mutations induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine or introduced by genetic transfer were expressed after a small number of generations of outgrowth. Altogether, these experiments indicated that the behavior of *A. vinelandii* in many genetic experiments was not significantly different from that of haploid bacteria like *E. coli* or *Salmonella typhimurium* (32).

This paper may bring the controversy to a reasonable end, since it reconciles the biochemical data that support the polyploidy of *A. vinelandii* with the genetic studies that argue against the existence of a highly polyploid genome. We show that the DNA content of *A. vinelandii* cells changes during growth in rich medium: early-exponential-phase cells have low ploidy, but a continuous increase in DNA content is observed during exponential growth. Late-exponential- and stationary-phase cells are highly polyploid. However, the process is later reversed, since a dramatic reduction in DNA content occurs in old stationary-phase cultures, perhaps reflecting the onset of cyst differentiation. Cells with low DNA content are also formed when old cultures are diluted into fresh medium. The absence of ploidy changes in minimal medium casts doubts on the biological relevance of the phenomenon, suggesting that polyploid cells may be formed only when *A. vinelandii* is grown under certain laboratory conditions.

MATERIALS AND METHODS

Bacterial strains. The standard wild-type strain *A. vinelandii* UW and its rifampin-resistant derivative UW136 were obtained from W. J. Brill, University of Wisconsin, Madison. Mutant derivatives of strain UW carrying transposon inser-

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TABLE 1. Mutants of *A. vinelandii*[a]

Strain	Description	Reference or source
UW136	Rif^r	W. J. Brill
AS115	*met-1*::Tn*5* Rif^r	8
AS116	*mtl-1*::Tn*5* Rif^r	8
AS151	*rha-1*::Tn*10* Rif^r	6
AS154	*gal-4*::Tn*10* Rif^r	6
AS202	*mtl-3*::Tn*5*-Mob Rif^r	4
AS203	*srb-1*::Tn*5*-Mob Rif^r	4
AS204	*ura-1*::Tn*5*-Mob	4
AS206	*ogl-1*::Tn*5*-Mob	4
AS207	*ogl-1*::Tn*5*-Mob Rif^r	4
AS226	*ace-1*::Tn*5*-Mob *mtl-3*::Tn*5*-Gen^r Rif^r	4
MV556	*ntrC16*::Tn*5* Rif^r	C. Kennedy

[a] All are derived from the standard wild-type strain UW.

tions are listed in Table 1; all are derived from either UW or UW136 and were used only for pulsed-field characterization of the *A. vinelandii* chromosome (see below). Strain MV724 was provided by C. Kennedy, AFRC Institute of Plant Science Research, University of Sussex, Brighton, England. *E. coli* K-12 and *S. typhimurium* LT2 were from the strain collection of our laboratory.

Media and culture conditions. Minimal medium for *A. vinelandii* was Burk nitrogen-free medium, prepared as described by Guerrero et al. (11). BSNB is Burk medium supplemented with 1 g of ammonium acetate, 2 g of tryptone, and 1 g of yeast extract per liter. Minimal medium for *E. coli* and *S. typhimurium* was E medium containing 0.2% glucose (60). Rich medium for *E. coli* and *S. typhimurium* was Luria-Bertani broth (33). *A. vinelandii* was grown at 30°C; *E. coli* and *S. typhimurium* were grown at 37°C. Encystment of *A. vinelandii* was induced in the presence of either β-hydroxybutyrate (15, 27) or 2-butanol (31) as the sole carbon source. Although the terms cysts and encystment are routinely used throughout this paper, it should be noted that strain UW and its derivatives do not form wild-type cysts but rather form cystlike structures (43).

Extraction and purification of chromosomal DNA from *A. vinelandii*. Preparations of intact chromosomes were obtained by the method described by Itaya and Tanaka for *Bacillus subtilis* (16), with slight modifications. A saturated culture of *A. vinelandii* made in BSNB was diluted to obtain an optical density at 560 nm of 1.0. An aliquot of 1 ml was centrifuged in an Eppendorf tube. The pellet was resuspended in 10 ml of RNase A (1 mg ml^{-1}) and 250 ml of sucrose-EDTA solution (16); 1 ml of 1.5% low-melting-point agarose (type VIII; Sigma) prepared in Tris-EDTA (TE) was then added. After homogenization, the melted mixture was transferred to a mold (Bio-Rad) and incubated at 4°C for 15 min to allow solidification of the agarose plugs. After 2 h of incubation at 37°C, the plugs were transferred to a 10-ml plastic tube; 5 ml of proteinase K solution (16) was added per every six plugs. The tube containing the plugs treated with proteinase K was incubated for 17 h, and then the plugs were washed four times (10 ml of TE per every six inserts) and incubated for 1 h at room temperature in the presence of 0.1 mM phenylmethylsulfonyl fluoride (prepared in TE). Finally, the plugs were washed three times with TE and kept at 4°C.

Digestion of *A. vinelandii* chromosomal DNA with rarely cutting endonucleases. Plug slices, each 2 mm thick, were prepared and incubated on ice for 30 min in 200 μl of digestion buffer. Complete digestion was achieved by using 10 to 20 enzyme units for 16 h. The enzymes used were from Boehringer Mannheim (*Swa*I, *Spe*I, *Ssp*I, and *Xba*I) and New England Biolabs (*Ase*I, *Not*I, and *Pac*I); the buffers were those supplied by the manufacturers.

Separation of fragments by pulsed-field electrophoresis. The agarose slices were inserted into the gel wells; these wells were then filled with low-melting-point agarose at 0.75% (prepared in TE). The gel was made with 1% agarose (type II-A; Sigma) in 0.5× Tris-borate-EDTA buffer. A CHEF-DR II system (Bio-Rad) was used for electrophoresis, as described by Chu et al. (5). The conditions used varied depending on the size ranges of the fragments to be separated (2); typical running conditions were 200 V and 0.08 A (constant voltage). Pulse times were ramped between 80 and 110 s during 24 h for DNA preparations digested with *Swa*I and between 10 and 40 s during 21 h for preparations digested with *Spe*I.

After electrophoresis, gels were stained with ethidium bromide and photographed on a UV transilluminator. Band sizes were determined by interpolation, using DNA size standards (phage λ concatemers [*cI857 Sam7*; Bio-Rad no. 170-3635] and *Saccharomyces cerevisiae* chromosomes [Bio-Rad no. 175-3605]) in the same gel.

Preparation of cell samples for flow cytometry and estimation of the relative DNA content per cell. Liquid cultures of *A. vinelandii* UW, *E. coli* K-12, and *S. typhimurium* LT2 were prepared; growth was monitored by optical density at 560 nm. Aliquots of 1 ml were harvested by centrifugation and resuspended in 1 ml of sodium phosphate buffer (pH 7.4) (52). To harvest cells from low-density (e.g., early-exponential-phase) cultures, larger aliquots (e.g., 10 ml) were used; the final number of cells harvested ranged from 10^5 to 10^7. *A. vinelandii* cysts were harvested from 1-week-old cultures grown with either β-hydroxybutyrate (50) or 2-butanol (31). The cell (or cyst) suspensions were then centrifuged, resuspended in 1 ml of 70% ethanol, and kept at −20°C for 30 min. These suspensions were centrifuged again, resuspended in 0.5 ml of saline phosphate buffer, and kept at 4°C.

For staining, the cells (and the *A. vinelandii* cysts) were centrifuged and resuspended in 0.5 ml of filter-sterilized sodium citrate (0.5 M). The cell and cyst preparations were diluted 5 to 20 times, if necessary. The suspensions were then treated with RNase A (final concentration, 100 μg ml^{-1}) for 2 h at 37°C. Propidium iodide was then added to a final concentration of 8 μg ml^{-1} (41). The samples were incubated in the dark for 30 min at room temperature before being examined with the flow cytometer.

Estimation of the relative DNA content per cell by flow cytometry. The samples were analyzed with a FACScan flow cytometer (Becton Dickinson). The excitation laser wavelength used was 488 nm. Fluorescence was measured at 639 nm. Data were processed in a Hewlett-Packard computer, using the Lysis II program (version 1.0.2) from Becton Dickinson.

Rifampin treatments. For rifampin treatments we used the method of Skarstad et al. (53), with slight modifications. A 5-ml aliquot was extracted from a bacterial culture, and rifampin was added at a final concentration of 150 μg ml^{-1}. The culture was incubated at the usual growth temperature (30°C for *A. vinelandii* UW and 37°C for *E. coli* K-12 and *S. typhimurium* LT2). Incubation in the presence of rifampin was carried out for 2 h. The cells were then harvested, washed, and stained as described above.

RESULTS

Computer analysis of DNA sequences from *A. vinelandii* and choice of rarely cutting endonucleases. Nucleotide sequences

TABLE 2. *A. vinelandii* DNA sequences collected from gene banks

Gene(s)	Size (bp)	Reference
anfHDGK	6,108	20
cydA, *cydB*	3,387	38
Dihydrolipoyl-transacetylase	2,142	12
glnA	1,950	59
hoxK, *hoxG*	3,800	36
Lipoamide dehydrogenase, succinyl transferase	1,860	62
mutS, *fdxA*	3,465	26
nfrX	2,790	7
nifA	2,026	1
nifB, *nifQ*	3,787	18
nifH, *nifD*, *nifK*, *nifT*, *nifY*, *nifE*, *nifN*, *nifX*, *nifU*, *nifS*, *nifV*, *nifW*, *nifZ*, *nifM*, *nifF*	28,793	17
nifL	1,870	3
ntrA (*rpoN*)	1,994	37
vnfA	2,957	19
vnfHDGK	6,557	21

representing a total of 73,486 bp of *A. vinelandii* DNA were collected from gene banks (Table 2). These DNA sequences were treated as a single unit and subjected to Markov chain analysis to determine the target frequencies for known, commercially available restriction enzymes (35, 45, 46). The enzymes *Pac*I, *Spe*I, *Ssp*I, *Ase*I, *Xba*I, *Swa*I, and *Not*I were chosen as potential rarely cutting endonucleases because their targets were found at low frequencies in the *A. vinelandii* DNA sequences analyzed (31).

Digestion of *A. vinelandii* chromosomal DNA with rarely cutting endonucleases and calculation of chromosome size. Preliminary pulsed-field fragment separation experiments indicated that *Pac*I digestion caused massive DNA degradation of the *A. vinelandii* UW chromosome, while digestions with *Ase*I, *Ssp*I, *Not*I, and *Xba*I yielded an undesirably large number of fragments (data not shown). Only digestions with *Spe*I and *Swa*I yielded an appropriate number of fragments. Gel photographs are presented in Fig. 1 and 2, respectively; diagrams summarizing the wild-type band patterns obtained are shown in Fig. 3. The *Swa*I restriction pattern found was identical to that described by Manna and Das (34), except that our digestions lacked an ~15 kb fragment (Fig. 2 and 3). The molecular size of the *A. vinelandii* chromosome was determined by adding the sizes of all fragments. Addition of the sizes of the *Spe*I bands suggests a size of 4,795 kb, while the size determined from *Swa*I digestions is 4,580 kb. The variation found is intrinsic to the method employed; for instance, the size of the *E. coli* chromosome has been estimated to be around 4,700 kb by *Sfi*I digestion (56) and 4,595 kb by *Not*I digestion (13). For the purpose of this paper, the relevant conclusion was that the size of the *A. vinelandii* chromosome is similar to that of the *E. coli* chromosome, as first reported by Sadoff et al. (51) and recently confirmed by Manna and Das (34).

A side observation in these experiments was that a number of transposon-induced mutants showed altered restriction patterns. The occurrence of genomic rearrangements is known to be common in *A. vinelandii* strains carrying wild-type copies of transposons Tn*5* and Tn*10* (6, 8).

Evolution of DNA content per cell in rich medium. Given the similar sizes of the *A. vinelandii* and *E. coli* chromosomes, the DNA contents of individual cells of *A. vinelandii* UW, *E. coli* K-12, and *S. typhimurium* LT2 could be compared by flow cytometry. Although the flow cytometer does not measure the absolute DNA content, relative comparisons between independent cell preparations are easily obtained (30, 41, 53, 55, 57, 58). The use of two enterobacterial species was intended as an internal control: since *E. coli* and *S. typhimurium* have similar chromosome sizes (13, 23, 28, 29, 56) and similar growth cycles (9, 25), they must have similar DNA contents per cell in all stages of growth examined. This prediction was confirmed in all experiments (data not shown); henceforth, only data corresponding to one enterobacterial species (*S. typhimurium*) will be presented.

Since the staining dye used (propidium iodide) is known to have residual affinity for RNA (42, 57), cell suspensions were treated with RNase A prior to being stained. Actually, the FACscan histograms obtained were blurred and flat whenever the RNase treatment was omitted, indicating that RNA was a

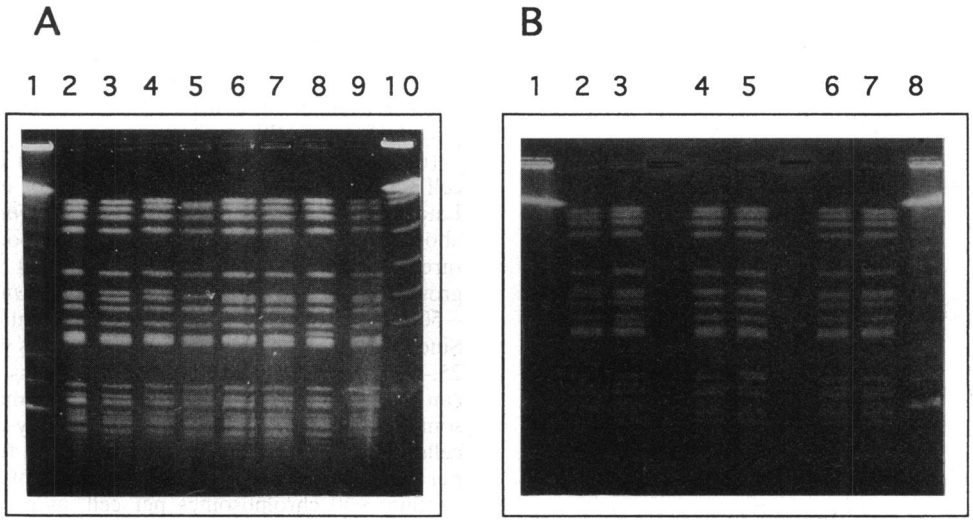

FIG. 1. Pulsed-field electrophoretic separation of fragments generated by *Spe*I digestion of the *A. vinelandii* chromosome. (A) Lanes: 1, phage λ concatemers; 2, AS115; 3, AS116; 4, AS202; 5, AS206; 6, AS207; 7, AS226; 8, MV556; 9, UW; 10, *S. cerevisiae* chromosomes. (B) Lanes: 1, phage λ concatemers; 2, AS154; 3, AS151; 4, AS203; 5, AS204; 6, UW136; 7, UW; 8, phage λ concatemers. Note that 5 of 11 insertion mutants (AS151, AS154, AS204, AS206, and MV556) show banding patterns different from that of the wild type.

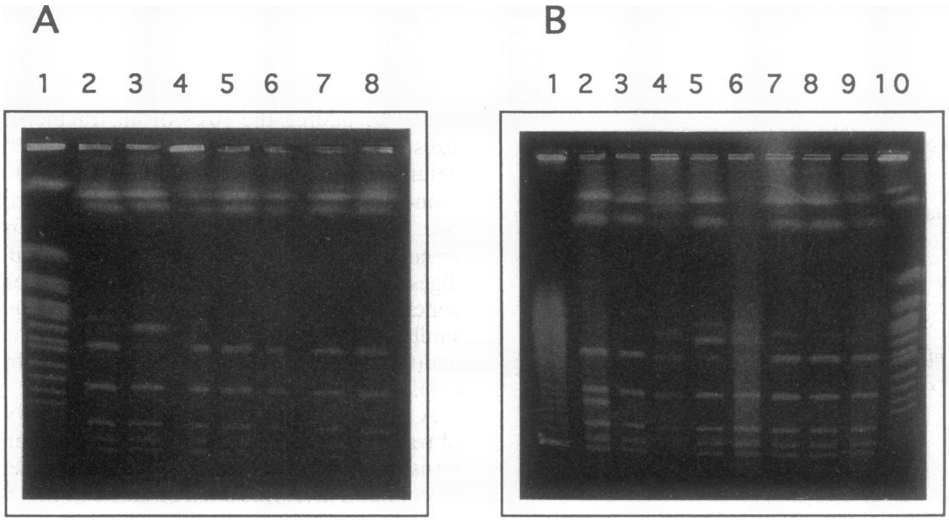

FIG. 2. Pulsed-field electrophoretic separation of fragments generated by *Swa*I digestion of the *A. vinelandii* chromosome. (A) Lanes: 1, *S. cerevisiae* chromosomes; 2, AS203; 3, AS204; 4, AS205; 5, MV556; 6, AS154; 7, UW136; 8, UW. (B) Lanes: 1, phage λ concatemers; 2, AS115; 3, AS202; 4, AS203; 5, AS204; 6, AS154; 7, AS207; 8, MV556; 9, UW; 10, *S. cerevisiae* chromosomes. Note that 4 of 11 insertion mutants (AS115, AS154, AS203, and AS204) show banding patterns different from that of the wild type.

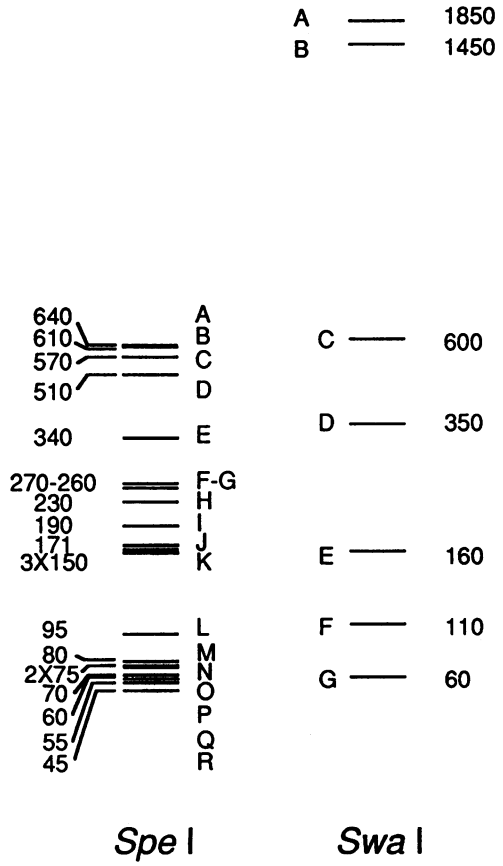

FIG. 3. Diagrams of *Spe*I and *Swa*I digestions of the *A. vinelandii* chromosome. Bands are designed with capital letters; the size (in kilobases) of each band is also indicated. Data were inferred from strain UW and its mutant derivatives lacking visible chromosomal rearrangements.

substantial component of the propidium iodide-stained material under these conditions (data not shown). After RNase A treatment, samples of 10^4 cells were examined with the flow cytometer. Growth of *A. vinelandii* in BSNB resulted in low frequencies of two-cell aggregates (2 to 3%) and larger aggregates (<1%); thus, the vast majority of the *A. vinelandii* cells examined with the flow cytometer can be expected to be single cells. Because of their large size (2 μm or more in diameter), *A. vinelandii* cells are especially suitable to be examined with the flow cytometer; for this reason, their FACscan histograms are sharper than those of enteric bacteria (see Fig. 4 and 5). The histograms obtained for cultures at different stages of growth in rich medium (LB for *S. typhimurium* and BSNB for *A. vinelandii*) are presented in Fig. 4; growth was monitored by both optical density and viable counts. The results can be summarized as follows.

(i) At the beginning of the exponential stage, *A. vinelandii* and *S. typhimurium* contained similar amounts of propidium iodide-stained material (DNA) per cell. Since the size of the *A. vinelandii* chromosome is similar to that of enteric bacteria, it seems reasonable to conclude that *A. vinelandii* cells were not polyploid during this period.

(ii) As the cultures grew, an increase in DNA content per cell was observed in *A. vinelandii* but not in *S. typhimurium*. Late-exponential-phase cultures of *A. vinelandii* contained about 10 times more DNA than early-exponential-phase cultures (and than *S. typhimurium* cultures at the same stage of growth). Stationary-phase cultures of *A. vinelandii* contained ~50 times more DNA than early-exponential-phase cultures. Since the ploidy level of enteric bacteria is well known (9, 14, 25, 63), direct extrapolation from the peaks of the histograms can tentatively translate DNA content to number of chromosomal equivalents: (i) *A. vinelandii* early-exponential-phase cells, like *S. typhimurium*, may contain ca. four chromosomes per cell; (ii) late-exponential-phase cells of *A. vinelandii* may contain ~40 chromosomes per cell, as previously suggested (39, 51); and (iii) stationary-phase cells of *A. vinelandii* may contain >100 chromosome equivalents per cell.

(iii) The observation that the ploidy level of *A. vinelandii* continued to increase at the beginning of the stationary stage

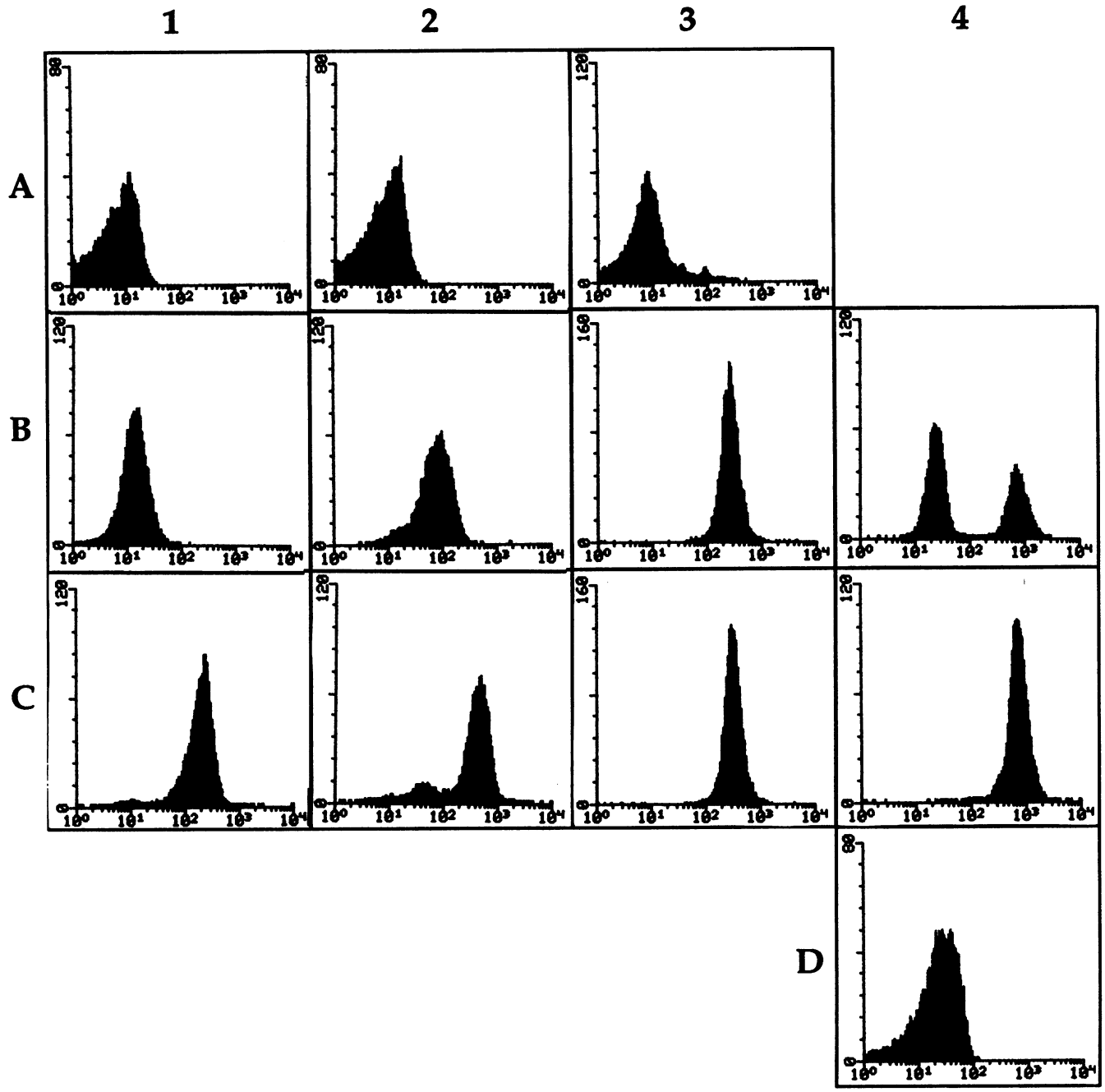

FIG. 4. FACscan histograms of cultures of *A. vinelandii* and *S. typhimurium* grown in rich medium. These histograms represent the frequency distributions of DNA concentrations; each histogram corresponds to the examination of 10^4 individual cells. The horizontal axis indicates DNA content; the vertical axis indicates number of cells. (A) *S. typhimurium* LT2; (B) *A. vinelandii* UW; (C) rifampin-treated cultures of *A. vinelandii* UW; (D) *A. vinelandii* cysts; (1) early-exponential-phase culture; (2) late-exponential-phase culture; (3) stationary-phase culture (>2 days); (4) old stationary-phase culture (>7 days).

suggests that exponentially growing *A. vinelandii* cells initiate multiple rounds of chromosome replication per cell division; these rounds can be completed when the cells cease to divide. This hypothesis is supported by the results obtained with rifampin treatment (see below).

In old stationary-phase cultures, a dramatic change was observed: the highly polyploid population of *A. vinelandii* cells segregated cells with a low DNA content, probably reflecting the onset of cyst differentiation (the DNA content of this fraction was similar to that of a cyst preparation [Fig. 4B4 and D]). The observation that *A. vinelandii* cysts contain less DNA than vegetative cells was first reported by Sadoff et al. in 1971 (50).

(v) If polyploid *A. vinelandii* cells are transferred to fresh medium, cells with low DNA contents are formed (Fig. 4B1; data not shown). This result was obtained whenever polyploid

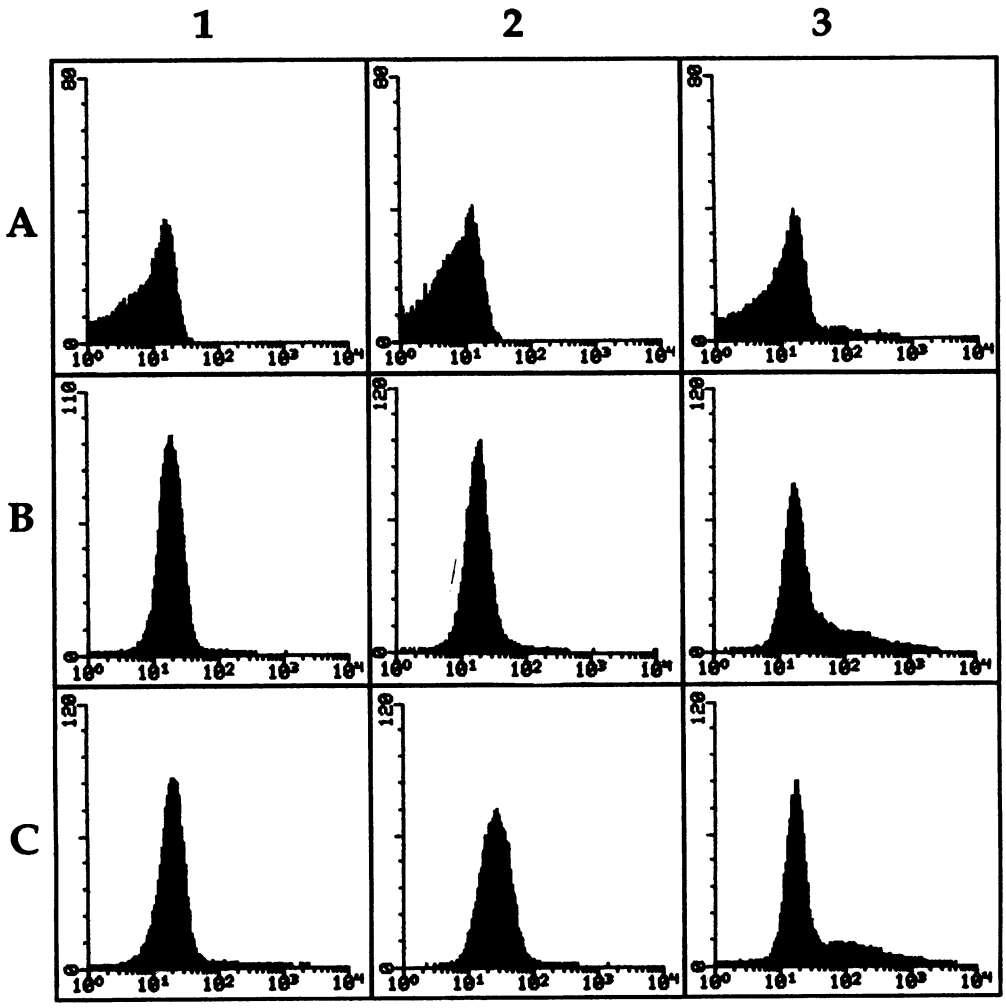

FIG. 5. FACscan histograms of cultures of *A. vinelandii* and *S. typhimurium* grown in minimal medium. These histograms represent the frequency distributions of DNA concentrations; each histogram corresponds to the examination of 10^4 individual cells. The horizontal axis indicates DNA content; the vertical axis indicates number of cells. (A) *S. typhimurium* LT2; (B) *A. vinelandii* UW; (C) rifampin-treated cultures of *A. vinelandii* UW; (1) early-exponential-phase culture; (2) late-exponential-phase culture; (3) stationary-phase culture (>3 days).

cells (in either late logarithmic or stationary phase) were diluted into fresh medium. Thus, at the beginning of every growth cycle, *A. vinelandii* polyploid cells seem to reverse the asynchrony between DNA replication and cell division (or may even divide without initiating new rounds of DNA replication).

Effect of rifampin on the DNA content per cell. Addition of rifampin to *A. vinelandii* cultures caused a sudden cessation of growth, as observed by both spectrophotometric monitoring of optical density and plate counts of CFU (data not shown). These observations are consistent with the well-known inhibition of bacterial transcription by binding of rifamycins to RNA polymerase (61). The effect of rifampin on the replication of the *E. coli* chromosome is also well known: addition of rifampin inhibits the initiation of chromosome replication but permits the completion of ongoing replication rounds (24, 57). Our rationale for investigating the effects of rifampin on the ploidy level of *A. vinelandii* was that if ploidy increase is caused by the initiation of more than one replication round per cell cycle, rifampin should increase the ploidy level of *A. vinelandii* when added to growing cultures. This observation was fully confirmed (Fig. 4C2).

As expected, rifampin did not increase the ploidy of nondividing cultures (late-stationary-phase cells) (Fig. 4C3). A side observation was that if added before a critical time, rifampin could prevent the formation of low-ploidy cells (putative cyst precursors) in old stationary-phase cultures (Fig. 4C4). Although this phenomenon was not studied in depth and is cited only as a preliminary observation, the inhibition of cyst formation by rifampin seems logical because cyst differentiation can be expected to require transcription.

Evolution of DNA content per cell in minimal medium. Estimations of DNA content per cell, analogous to those described above, were carried out with minimal media (nitrogen-free Burk medium for *A. vinelandii* and E medium for *E. coli* and *S. typhimurium*). The doubling time of *A. vinelandii* UW in BSNB is about half of that seen in nitrogen-free Burk medium (data not shown). The main conclusion from these experiments, summarized in Fig. 5, was that *E. coli*, *S. typhimurium*, and *A. vinelandii* contained similar amounts of propidium iodide-stained material throughout the growth cycle. Slight differences (like those shown in Fig. 5) might indicate, at most, a two- to fourfold difference in DNA content (for

instance, compare Fig. 5A2 and B2). Rifampin caused only a slight ploidy increase when added to growing cultures of *A. vinelandii*; thus, the asynchrony between initiation and cell division must be small during slow growth. Two main conclusions can be drawn from these data: (i) *A. vinelandii* does not become highly polyploid in minimal medium, and (ii) during slow growth, *A. vinelandii* initiates a smaller number of rounds of chromosome replication per cell cycle and may contain, at most, ca. eight chromosomes per cell. As an extrapolation of these conclusions, the possibility that *A. vinelandii* is highly polyploid outside the laboratory seems unlikely.

DISCUSSION

Physical analysis of the *A. vinelandii* chromosome by digestion with rarely cutting endonucleases and separation of fragments by pulsed-field gel electrophoresis allowed us to estimate the size of the *A. vinelandii* chromosome to be around 4,700 kb. This chromosome size is similar to that of *E. coli* and *S. typhimurium*, as reported by Sadoff et al. (51) more than a decade ago and recently confirmed by Manna and Das (34). Given their similar chromosome sizes, *A. vinelandii* and enteric bacteria can be expected to have similar amounts of DNA per cell unless they differ in chromosome number.

The technique used to compare the DNA contents of individual cells of *A. vinelandii*, *E. coli*, and *S. typhimurium* was flow cytometry. In the flow cytometer, individual cells pass one by one through the focus of a fluorescence microscope in which excitation light of a suitable wavelength is transmitted (57, 58). Thus, the DNA contents of individual cells can be measured; the resulting histograms represent frequency distributions for the cells with respect to their DNA content (53, 54, 55, 57). Since the strain used, *A. vinelandii* UW, is plasmid free (22), the occurrence of artifacts caused by massive accumulation of plasmid DNA can be ruled out. The possibility that changes in the amount of propidium-iodide stained material might correspond to substances other than DNA seems unlikely, although it is certainly possible that the preparations still contained a certain amount of RNA (57). However, the possibility of a major artifact from RNA contamination was ruled out by rifampin treatments, which caused an increase in the amount of propidium-iodide stained material in spite of inhibiting RNA polymerase (and thus RNA production [see below]).

The amount of propidium iodide-stained material (DNA) found in individual cells of *A. vinelandii* underwent major changes during growth in rich medium. Early-exponential-phase cells had low ploidy, similar to that of enteric bacteria. However, unlike those of *E. coli* and *S. typhimurium*, the DNA contents of individual *A. vinelandii* cells showed a swift increase during growth in rich medium. It should be emphasized that the conditions employed do not permit the discrimination of slight changes in DNA content per cell (30, 55); for instance, the expected reduction of from ca. four to one or two chromosomes per cell is not clearly observed in *S. typhimurium* stationary-phase cultures (Fig. 4). Thus, the chromosome numbers suggested for *A. vinelandii* (~4 for early-exponential-phase cells, ~40 for late-exponential-phase cells, >100 for stationary-phase cells, and ~4 for cysts) should be regarded as approximate. Given the large space needed to accommodate >100 chromosomes, it is not surprising that large pleomorphic cells (up to 8 μm in diameter) are formed when *A. vinelandii* is grown in rich media (44).

The rapid formation of two discrete, nonoverlapping cell populations in old stationary-phase cultures suggests that the rise of low-ploidy cells results from a "decision" taken by single cells, rather from a gradual evolution of polyploid cells towards low ploidy. Whether the low-ploidy cells formed might correspond to the "germinal" filterable cells described by González López and Vela (10) can be a matter of speculation. Other possibilities (e.g., biased segregation or massive DNA turnover) can be also considered.

Although the spectacular changes of ploidy observed during the *A. vinelandii* growth cycle have no precedents in the bacterial world (9, 23), the accumulation of chromosomes in *A. vinelandii* can be tentatively explained by analogies with the enterobacterial cell cycle. *E. coli* and *S. typhimurium* growing at rapid rates contain several (ca. four) chromosomes per cell (9, 14, 25, 55); however, when the culture enters the stationary stage and the growth rate declines, the ploidy decreases to one or two chromosomes per cell (14, 40, 55). The higher ploidy of enterobacterial cultures growing at rapid rates is caused by the initiation of more than one round of chromosome replication per cell cycle (9, 14). Initiation of chromosome replication is known to be inhibited by rifampin (24); this antibiotic inhibits RNA polymerase and thus also blocks cell division (57, 61). When rifampin was added to exponential-phase cultures of *A. vinelandii*, a rapid increase in DNA content was observed; actually, the DNA content of exponential-phase cells treated with rifampin resembled that of late-exponential- or stationary-phase cells (Fig. 4). By analogy with the enterobacterial cell cycle, this increase can be attributed to the completion of ongoing replication rounds, thereby indicating that *A. vinelandii* cells initiate multiple rounds of chromosome replication per cell cycle. The existence of a more severe asynchrony between replication and cell division provides a simple model to explain why *A. vinelandii* becomes more polyploid than enteric bacteria during rapid growth.

The biological significance of these ploidy changes is unknown; they may merely reflect an irrelevant phenomenon that occurs only in laboratory conditions. This view is supported by the finding that growth in minimal medium does not result in the appearance of highly polyploid cells. It may be interesting to recall that the general design of *A. vinelandii* metabolism is different from that of enteric bacteria. For instance, *A. vinelandii* does not transport many nutrients (e.g., certain amino acids) into the cell; others may be imported but later degraded (22, 48). Moreover, *A. vinelandii* cells grown in soil dialysates show a morphology different from that seen in standard laboratory media (64). Growth in rich medium can thus be viewed as an unnatural situation for *A. vinelandii*; under these conditions, it is not surprising that the mechanisms that regulate the initiation of chromosome replication can be seriously perturbed. However, the existence of other bacterial species harboring polyploid genomes (47, 49) leaves open the possibility that bacterial polyploidy plays a physiological role in nature.

For practical purposes, the data presented in this paper throw light on the controversy about the ploidy degree of *A. vinelandii*, since they reconcile the biochemical quantitations of DNA content per cell (39, 51) with the genetic data which argue against polyploidy (6, 8, 32). *A. vinelandii* is polyploid in full-grown batch cultures but can behave as a haploid bacterium in genetic experiments because the start of every growth cycle involves a drastic reduction of chromosome number. Such a reduction permits fast segregation of heterozygotes and expression of recessive alleles, two phenomena which had been presented as strong arguments against polyploidy (32).

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