Cell shape and chromosome partition in prokaryotes or, Why *E. coli* is rod-shaped and haploid

William D. Donachie, Stephen Addinall and Ken Begg

Summary

In the rod-shaped cells of *E. coli,* chromosome segregation takes place immediately after replication has been completed. A septum then forms between the two sister chromosomes. In the absence of certain membrane proteins, cells grow instead as large, multichromosomal spheres that divide successively in planes that are at right angles to one another. Although multichromosomal, the spherical cells cannot be maintained as heterozygotes. These observations imply that, in these mutants, each individual chromosome gives rise to a separate clone of descendant cells. This suggests a model in which sites for cell division form between pairs of sister chromosomes at the time of segregation, but are not used in spherical cells until further rounds of replication have taken place, thus ensuring clonal ('hierarchical') segregation of chromosomes into progeny cells. The role of the morphogenetic membrane proteins is to convert the basically spherical cell into a cylinder that is able to divide as soon as replication and segregation have been completed, and thus to maximise the number of viable cells per genome.

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Introduction

An unsolved biological mystery is how prokaryotic cells ensure the equipartition of their genomes at division. Intensive study of the 'model' prokaryote, *Escherichia coli*, has shown that the process is very efficiently carried out, but has not yet revealed how it is done. The precision of division is reflected in the narrow range of cell sizes in growing populations (showing that cells always divide at approximately the same size) and the efficiency of chromosome partition can be inferred from the fact that cells lacking chromosomes are very rarely produced. In addition, each cell starts to divide almost immediately after it has replicated its single chromosome, so that each newborn sister cell receives only one copy of the chromosome[1]. Chromosome partition therefore differs from that of those high copy number plasmids which are simply distributed at random between sister cells at division[2]. Moreover, we can show that the prokaryotic method of chromosome partition differs fundamentally from that of eukaryotes in that it prevents the maintenance of genetically heterozygous lines, even in polyploid cells. To explain this last observation, we suggest that sister chromosomes are automatically assigned to different cell lines by the formation, at the time of their separation, of a potential division site[3] between them. As every potential division site will eventually be used, each descendant cell will contain only identical copies of a single ancestral chromosome.

What follows is a review of the evidence for this hypothesis.

Separation of sister chromosome-centres is rapid

In actively growing cells, chromosomal DNA is largely dispersed throughout the cytoplasm[4] but this DNA rapidly condenses into tightly packed 'nucleoids' if RNA or protein synthesis is inhibited. The condensed nucleoids can then be counted and their positions measured within each cell. When this is done, it is found that most cells have either a single, centrally located nucleoid, or two, well-separated nucleoids[1,5]. Very few cells in an asynchronous, log-phase

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population have two nucleoids that are separated by less than half a cell-length. It is clear therefore that the organisational centres (i.e. the points around which the dispersed DNA condenses when protein synthesis is inhibited) of sister chromosomes must move apart, by about half a cell-length, during an interval which is very short relative to the generation time of the cells themselves.

'G_2' is very short

Completion of chromosome replication has been shown to take place about 20 minutes before the completion of cell division, independent of the growth rate (for strain B/r growing at rates between about 1 and 3 generations/hour at 37°; see ref. 6). It is therefore possible to calculate the proportions of cells having either 1 or 2 chromosomes, for populations growing at different rates. These calculated proportions are identical to the observed proportions of cells having 1 or 2 nucleoids. From this it can be inferred that sister chromosomes separate from one another within two or three minutes after replication is complete[1]. Each nucleoid in a normal cell therefore consists of a single chromosome. (This chromosome will usually be in the process of replication in rapidly growing cells: see Fig. 1.)

Nucleoids separate over a fixed distance

In normal cells, which are short rods, pairs of sister nucleoids are found to be separated, on average, by about half the length of the cell[1,7]. The *average* length of cells in asynchronous populations increases with increasing growth rate[8,9] and cell length at the time of partition has been estimated for cells with different growth rates. The length of a cell at partition (although *not* its volume), and therefore the distance by which sister chromosomes are moved apart during partition, was found to be constant at all growth rates[1] (Fig. 1). Is this separation distance determined by the size of the cell, or does it reflect an innate property of the partition apparatus itself?

To answer this question, we[10] inhibited DNA synthesis in cells but allowed them to grow in length until they were 2-4 times longer than normal. We then allowed chromosome replication to resume. Under such conditions, multiple rounds of chromosome replication are initiated in quick succession until the normal DNA/cell mass ratio is restored[10-13]. During this period of rapid DNA synthesis, the nucleoid increases in size but remains in the cell centre. When the normal DNA complement has been restored, individual nucleoids can be seen to move away from the central mass of DNA, but their separation is limited to a distance similar to that by which sister nucleoids move apart in normal, short cells. Consequently, the nucleoids remain clustered together in the central region of the long cells (Fig. 2A). (As

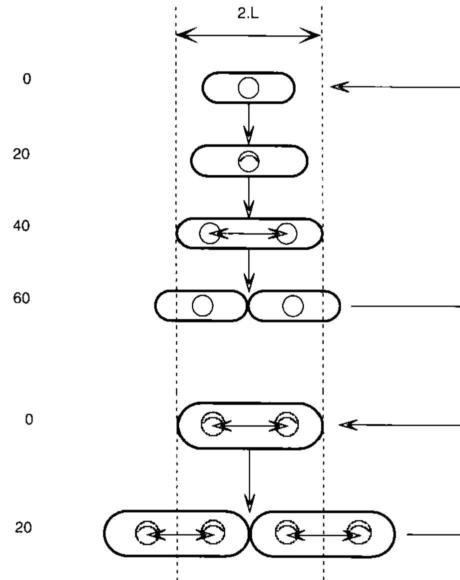

Fig. 1. Cell size and shape, chromosome replication and position during the normal cell cycle of *E. coli*. The top section of the figure shows the cycle for cells growing with a doubling time of 60 minutes, and the bottom shows the cycle for a doubling time of 20 minutes. Cells are drawn to scale (where 2.L, or two unit lengths, is about 2.8 μm). At any constant growth rate, a cell grows only by elongation, without change in width: *however, average cell* volume increases with growth rate (although the relative proportions of length to width remain constant). The chromosome is drawn as a small circle (the actual circumference of the single DNA molecule, when spread out, is about one thousand times the length of the cell itself) that replicates bidirectionally from a site at the top of the circle. The time taken for replication forks to travel from origin to terminus is about 40 minutes at both growth rates. At all growth rates completion of replication of a pair of sister chromosomes takes place (40 minutes after initiation) when a cell reaches a fixed length (2.L), and sister chromosome-centres then separate by a fixed distance (1.L). At this time septum formation begins at the cell centre and division is complete about 20 minutes later (at all growth rates).

the cells continue to grow and make more DNA, they also divide, producing mixtures of cells of various lengths and DNA content, including, initially, a majority of cells without any DNA at all; see ref. 10). Our conclusion from such experiments is that sister nucleoids are able to separate by only a limited distance during partition, and that this distance is approximately equal to half the length of a normal cell at the time of partition. Our estimate of the length of cells at partition is about 2.8 μm[8] and therefore our estimate of the separation distance of sister chromosomes during partition is about 1.4 μm[1].

Nucleoid partition *requires* a minimum cell length

In normal cells, nucleoid partition takes place when the cells are about 2.8 μm long (Figs 1, 2). Can partition take place in cells that are shorter than this?

To answer this question, we took a culture of cells and inhibited protein synthesis (by amino acid starvation). Under such conditions, cells that have already started to replicate their chromosome are able to complete this repli-

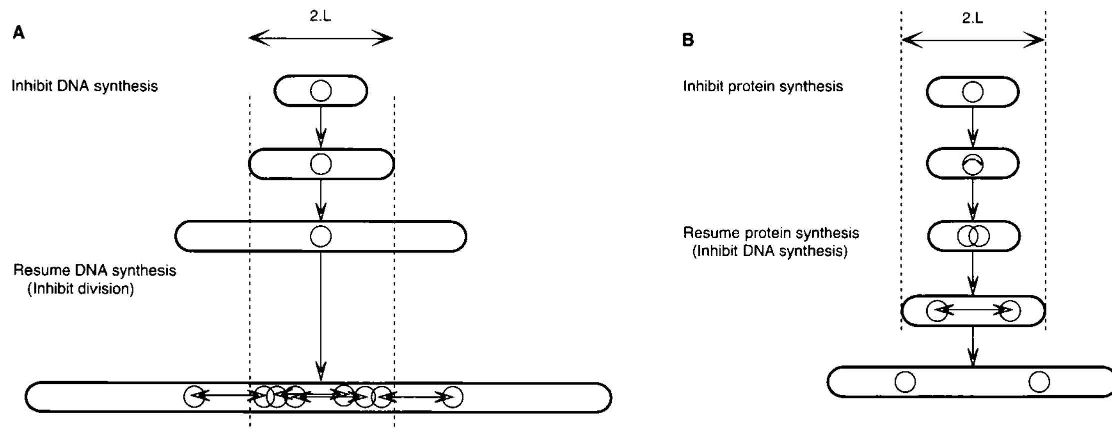

Fig. 2. (A) Sister chromosomes can move apart by only a fixed distance, and (B) only in cells that have reached a minimum length (2.L). (A) If DNA replication is inhibited, cells continue to grow and the unreplicated chromosome remains in the cell centre. If DNA synthesis is then allowed to resume, all the 'missed' replication cycles are initiated in rapid succession, so that the normal DNA/mass ratio of the cell is restored: however, the chromosomes remain crowded together near the cell centre, with single 'outliers' at a distance of about 1.L from the central mass of DNA. (B) If protein synthesis is inhibited, chromosome replication can proceed to completion, but separation can take place only when the cell has resumed growth and reached the required minimum length (2.L).

cation[14,15] but are unable to divide without further protein synthesis. After a period of amino acid starvation, therefore, almost every cell will contain two complete sister chromosomes, but all cells will be shorter than the length at which partition normally occurs. (Every cell that already had two chromosomes before the start of amino acid starvation would have divided during the period of amino acid starvation and therefore no cell in the population would be as long as 2.8 μm; see refs 8 and 15). When this experiment was carried out, it was found that the number of nucleoids per cell did not increase, showing that partition could not take place in these non-growing cells even when two sister chromosomes were present. The cells were then given back the amino acids that they required for growth, but further DNA synthesis and cell division were inhibited at the same time. The cells resumed growth and the average number of nucleoids began to increase immediately, showing that post-replication protein synthesis is necessary for partition[1,7]. However, the number of nucleoids did not increase simultaneously in every cell; instead, the total number of nucleoids increased by a factor of 2 (as expected), but this increase took place over a period during which the total cell mass (and length) of the population of cells also increased by a factor of 2[1]. These are the kinetics expected if partition of replicated sister chromosomes can take place *only* in cells that have reached a required minimum length (2.8 μm). (During subsequent cell growth, nucleoid number did not increase further, again as expected, because further chromosome replication had been blocked.) Fig. 2B illustrates this experiment.

Spherical cells are delayed in division

Mutation of either the *rodA* or *pbpA* gene causes *E. coli* cells to become spherical. The RodA and PbpA(PBP2) proteins are transmembrane proteins that together modify the peptidoglycan sacculus of the growing cell so that it has a cylindrical shape[16]. The spherical mutant cells grow and divide regularly but division is delayed, so that fast-growing mutant cells can be as much as sixfold larger in volume than normal rods[17]. Division in spherical cells always starts at a single point on the surface, from which an ever-deepening furrow spreads around the circumference of the cell[17]. Measurement of the mutant cells showed that their average diameter was almost the same as the average length of rod-shaped cells growing at the same rate[17]. The DNA content of the mutant cells was compared with that of normal rods. It was found that the ratio of DNA/mass (or DNA/volume) is identical in wild type and mutant (showing that the control of initiation of DNA replication is linked to cell mass in the same way as in normal cells, see ref. 13). However, since the mutant cells are much larger, this means that they contain more chromosomes per cell than normal rod-shaped bacteria. In normal cells, we saw that cell division begins almost immediately after each chromosome has completed replication and the sister chromosomes have been separated by partition. In the spherical mutants, however, cell division is delayed until the cell contains many chromosomes. This originally led us to propose that a minimum distance between cell poles is required before division can be initiated[17]. This could explain the actual sizes and chromosome complements of both rod-shaped (Rod$^+$) and spherical (Rod$^-$) cells in exponential growth. However, we have now found (S. Addinall, unpublished observations) that Rod$^+$ and Rod$^-$ cells have similar volumes in stationary phase, showing that the spheres can indeed divide when smaller, at least under conditions of

slow growth. Thus it seems that division is delayed, rather than prevented altogether, in spherical cells that have diameters less than the minimum length that is required for division in rods. Eventually, a septum is still formed between every pair of sister chromosomes.

It remains to be explained why division is delayed in spherical cells and indeed, why division starts at a point on the cell surface, rather than as a ring around the whole circumference as in rods. One possible reason could be that one or more of the cell division proteins are limiting in cells of greatly increased diameter[18]. FtsZ would of course be a prime candidate because it is known to form a ring around the cell at division in normal cells[19]. Increasing the amount of FtsZ protein has however been found not to correct asymmetric division in Rod⁻ cells, and has even proved to be detrimental to the division of large spherical cells, so that this may not be the correct explanation for the division delay (K. Begg, unpublished observations). If the hypothesis that one or more division proteins are limiting for division in spherical cells were correct, however, then the function of the RodA and PBP2 proteins would be to reduce the cell diameter so that division can take place immediately after chromosome partition. According to this hypothesis, the cell shape proteins are true cell cycle proteins required to maximise the number of viable cells per genome.

It should be noted that sister chromosomes could still separate by the same fixed distance in spheres as in rods, if this distance were measured along the surface of the cells (i.e. as arcs rather than chords: see Fig. 5). Observed cell dimensions and DNA contents are consistent with this suggestion for both rods and spheres, and this idea has been incorporated into our model for chromosome partition and division (see below).

Cell division in spherical cells is therefore delayed after the completion of each round of chromosome replication, for reasons that are not yet clear. The result is that Rod⁻ cells in log phase are large and multichromosomal.

Prokaryotic chromosome partition is not like mitosis, because chromosomal heterozygosity cannot be maintained

In normal rod-shaped cells of *E. coli*, sister chromosomes can move apart only along the long axis of the cell and, as soon as they do, a septum begins to form between them. Consequently, a mutation arising in one sister chromosome will immediately segregate from its wild-type allele, and it is clear why no heterozygous cells can arise. (In very rapidly growing cells, each chromosome will have multiple replication forks and mutations arising in replicated segments may take up to two generations to be segregated. Even under such conditions, however, a heterozygous cell could exist for no more than two cell cycles.) In contrast to rod-shaped cells, rapidly growing Rod⁻ mutant cells each contain multiple chromosome copies (above) and we were therefore able to ask whether heterozygous cell lines can be maintained in such strains.

In a spherical cell, sister chromosomes might, (A) separate in random directions (Random Segregation), or (B) always separate in a direction perpendicular to the plane of cell division (Mitotic Segregation), or (C) be partitioned in such a way that a mutation arising in any one copy would come to be segregated into a pure mutant line (Hierarchical Segregation).

Segregation of sister chromosomes in a random direction (mode A) would give efficient segregation in rod-shaped cells, because the narrowness of the cell lumen would force nucleoids to separate along the long axis, but would lead to random partitioning of different chromosome 'lines' in large spherical cells. Mode B is formally equivalent to mitotic segregation, and would give rise to stable heterozygous lines of spherical mutant cells, although rod-shaped cells could give rise only to homozygous lines. Mode C would give rise only to homozygous lines in cells of any size or shape. The three modes of segregation are illustrated in Fig. 3.

These three fundamentally different modes of chromosome segregation cannot be distinguished by studying narrow, rod-shaped cells, for the reasons mentioned above. However, they should be distinguishable in spherical mutants, because these cells are multichromosomal and have no obvious geometric constraint on the direction of separation of sister chromosomes within them. To do this, we made spherical cells heterozygous for the *leu* locus and then followed the appearance of segregating homozygous lines[20].

Reciprocal transductions were made between *leu*⁺ and *leu::Tn10*, *rodA*⁻ strains, using phage P1 (Fig. 4). (Large, exponentially growing recipient cells were used, in order to ensure that all cells were multichromosomal.) Selection for transduced cells (either Leu⁺ or tetracycline-resistant phenotypes) gave numerous transductant clones. When these were analysed, however, it was found that all transductant clones were homozygous for the *leu* allele that had been selected initially. (A strain harbouring an intrachromosomal duplication of the *leu* locus gave true heterozygous clones after such transduction.) This result effectively eliminates mode B, the equivalent of a mitotic mode of chromosome segregation.

Random partition of sister chromosomes (mode A) would also be expected to give rise to segregating homozygous lines, but unstable heterozygous lines should be maintainable by constant selection for the presence of both *leu* alleles. Reciprocal transductions were therefore carried out as before, except that selection was applied for the abil-

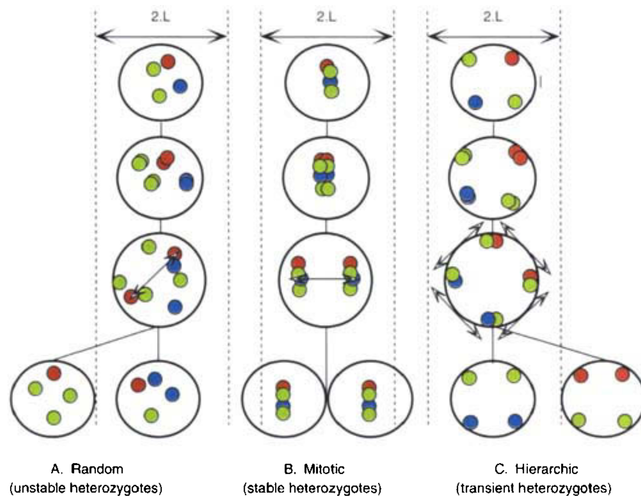

Fig. 3. Segregation of a mutation in Rod⁻ cells. Rod⁻ cells are large, spherical and carry multiple chromosome copies. Because the direction of separation of sister chromosomes is not restricted by the narrowness of the cell (as in Rod⁺ cells), sister chromosomes could, in principle, segregate in any one of three different ways. (A) If the direction of separation is not fixed, division of the cell after replication would result in random assortment of chromosomes. (B) If sister chromosomes always separated at right angles to the plane of cell division, each sister cell would receive one copy of each chromosome 'line', as in mitosis in diploid and polyploid eukaryotes. (C) If sister chromosomes separated and reattached along the cell surface, different chromosome lines could rapidly segregate into different cell lines.

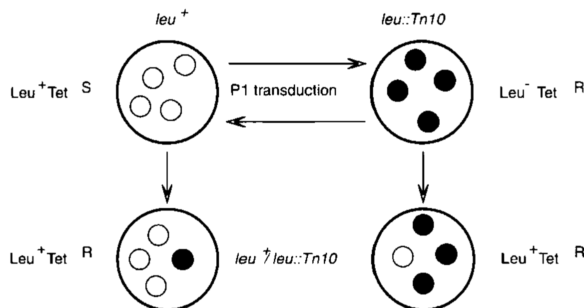

Fig. 4. Test to determine the mode of chromosome segregation. Two Rod⁻ strains (*rodA*^{am} *sup*^{o}) were constructed, one *leu*⁺, and one with a transposon (*Tn10*, carrying a gene for tetracycline-resistance) inserted into the *leu* gene. P1 phage grown on each strain was used to transduce the other strain. In both cases heterozygous transductants (*leu*⁺/*leu::Tn10*) will be produced that should be able to grow without leucine in the presence of tetracycline (Leu⁺ Tet^{R}). The stability of these heterozygotes provides a means of discriminating between the three modes of chromosome segregation shown in Fig. 3.

ity to grow in the presence of tetracycline without added leucine (i.e. direct selection for *leu*⁺/*leu::Tn10* heterozygotes). Again, selection for transductants that had received either *leu* allele alone gave numerous colonies, but no transductant colonies at all were obtained under double selection. (The strain with the internally duplicated *leu* locus gave the same number of transductant clones whether single or double selection was applied, thus demonstrating that the selection method is valid.) The fact that not even microcolonies could be obtained under double selection seems to us to eliminate mode A (random segregation) as a mechanism of partition.

A criticism of this line of reasoning is the possibility that, after plating on selective medium, newly transduced cells might first divide several times without growing, so as to become monochromosomal before resuming growth and forming a colony. We are unable to completely eliminate this theoretical possibility for our strains of *E. coli*, although we consider it unlikely; however, excellent support for a non-mitotic and non-random chromosome-segregation mechanism comes from extensive work with two other bacterial species. Thus, mutations segregate very rapidly to give homozygous clones of cells of *Azotobacter vinelandii* and *Micrococcus radiodurans*[23-25], despite the fact that these two species have been shown to have multiple chromosomes, both in log-phase (about 10/cell in fast-growing *M. radiodurans*[23] and up to 80/cell in *A. vinelandii*[26]) and in stationary-phase (4/cell in *Micrococcus radiodurans*[23] and 10/cell in *A. vinelandii*[27]). Indeed, the rapid segregation of mutations has been used as an argument against the reality of the measured chromosome contents of *A. vinelandii* cells[28]. Our model of hierarchical chromosome segregation (below), however, predicts homozygotisation of mutations in cells with 40-80 chromosomes in 5-6 generations, as is actually observed[28].

Thus, surprisingly, even large cells with multiple chromosomes, and without any apparent geometric limitation to the direction of chromosome segregation relative to the division plane (*M. radiodurans*, like our Rod⁻ *E. coli*, is a coccus with alternating division planes, and *A. vinelandii* grows either as spheres or large ovoid cells) could not be forced to maintain two different chromosome lines. (Only the internal duplication of a segment of the chromosome allows two alleles to be maintained stably in the same cell.) How can we explain the obdurate homozygosity of multichromosomal bacterial cells?

In rod-shaped cells of *E. coli*, cell division begins at a point midway between sister chromosomes, soon after they have moved apart. Even if cell division is prevented at this time (e.g. in a cell division mutant) a site of potential cell division remains at this location, and this site can be used at a later time, when the inhibition of division is relieved[3]. If cell division is blocked for a period, without preventing cell growth or DNA replication, long cells with evenly spaced pairs of sister chromosomes will form[13]. If division is then allowed to restart, septa will form, compartmentalising each chromosome, at the same locations as they would have occupied had division not been blocked. Division and chromosome segregation in Rod⁻ cells can be understood in the same way (Fig. 5). What we suggest is that a potential division site (PDS: see ref. 3) is laid down midway between each pair of sister chromosomes at the

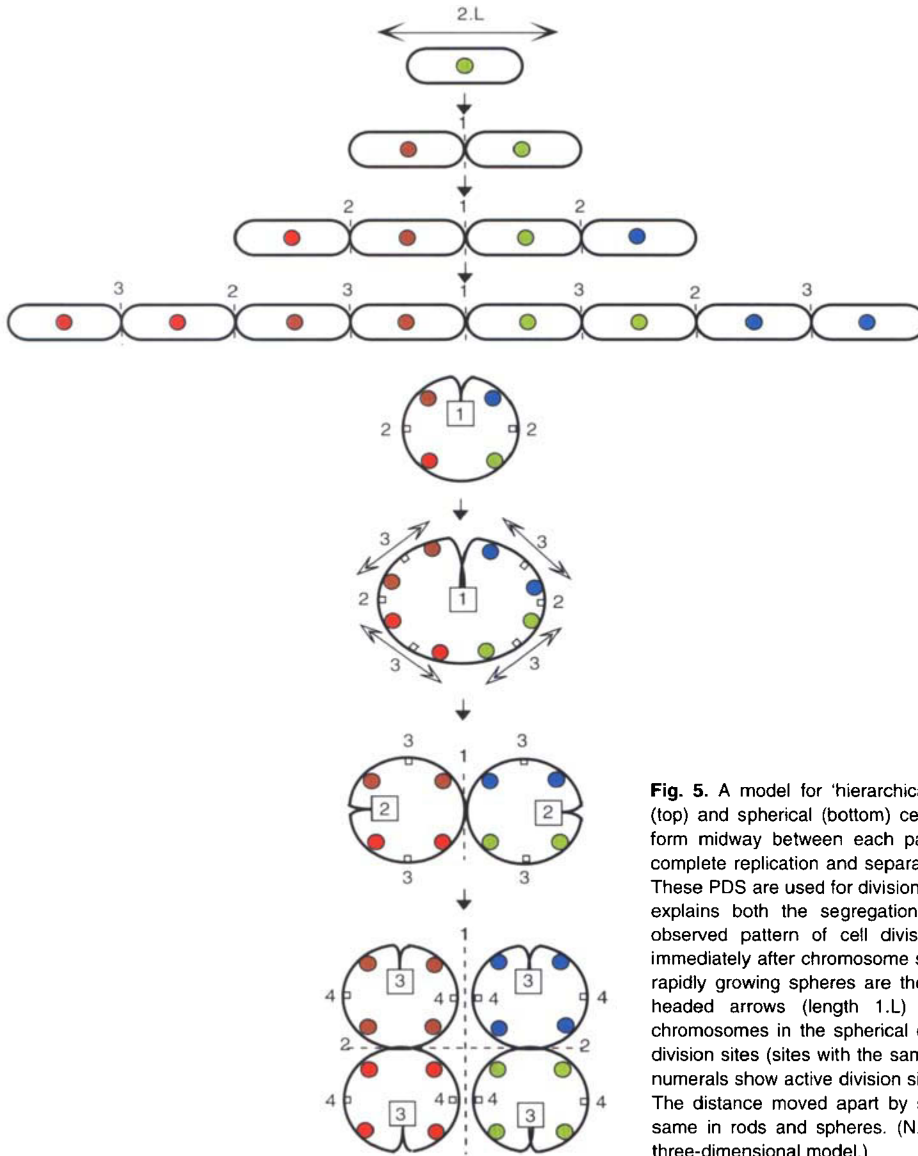

Fig. 5. A model for 'hierarchical' chromosome segregation. In both rod-shaped (top) and spherical (bottom) cells, a potential division site (PDS) is assumed to form midway between each pair of sister chromosomes at the time that they complete replication and separate (by a fixed distance, L, along the cell surface). These PDS are used for division in the same order as they have been formed. This explains both the segregation of mutations into homozygous lines, and the observed pattern of cell division in rods and spheres. Division takes place immediately after chromosome separation in rods but is delayed in spherical cells: rapidly growing spheres are therefore multichromosomal. The lines with double-headed arrows (length 1.L) show the directions of segregation of sister chromosomes in the spherical cell line. The ordinal numerals show the potential division sites (sites with the same numeral were formed at the same time). Boxed numerals show active division sites. Dashed lines show completed division planes. The distance moved apart by sister chromosomes along the cell surface is the same in rods and spheres. (N.B. This is a two-dimensional representation of a three-dimensional model.)

time that they separate. The spherical Rod$^-$ cells therefore each have, not only multiple chromosome copies, but also multiple PDS. In order to explain the rapid segregation of transduced alleles, there must be a regular spatial relationship between chromosome copies and PDS, just as there is in long cylindrical cells. A possible spatial arrangement is shown in Fig. 5. In this model the chromosomes are arranged evenly around the inside of the cell membrane and move apart along the curved surface. The actual arrangement of chromosomal DNA within spherical cells is unknown at present, although EM-sections of cells of spherical mutants show the DNA arranged as a shell around the cell surface[21,22]. Our proposal is that a PDS forms midway between each pair of sister chromosomes at the time that they separate from one another, just as in rod-shaped cells. The spherical cell, like a long rod-shaped cell with the same volume and DNA content, would have a hierarchy of PDS of different ages that would be utilised in turn. Immediately after division at the oldest PDS, each newborn spherical cell (assuming that it had four chromosomes) would have three PDS, two formed in the previous cell cycle, and one that had been formed in the cycle before that. After chromosome replication in the next cycle, partition would take place and four new PDS be produced. According to this model, the locations of successive divisions would reflect the previous positions of chromosomes. The same formal model would apply to cells with larger numbers of chromosomes and larger numbers of PDS.

Although Fig. 5 is two-dimensional, we suppose that multiple chromosomes would be arranged in three dimensions around the spherical cell. As first reported by Iwaya *et al.*[22], we have found that divisions of spherical *E. coli* cells immobilised on agar do indeed take place in successive planes that are perpendicular to one another (Fig. 6).

We have devised a model of division that is consistent with the observed division pattern of spherical (and rod-shaped) cells, and which will result in hierarchical chromosome segregation (Fig. 5). This is a modification of the model of Iwaya *et al.*[22], with incorporation of our later knowledge that spherical cells are multichromosomal.

This model for chromosome segregation and PDS formation applies equally to rod-shaped and spherical cells, and would explain why multichromosomal bacteria are always genetically haploid. We presume that our model will also apply to bacteria of other sorts, including natural coccal species. Many coccal species show alternating planes of cell division (e.g. *Micrococcus radiodurans*[32], *Neisseria sp., Lampropedia sp., Sarcina sp.*), while others are known to be multichromosomal (e.g. *Azotobacter vinelandii*[27,29], *Micrococcus radiodurans*[23,24]) but show rapid homozygotisation of mutations[24-26,28]. Our most surprising finding is that *E. coli*, a natural rod-shaped species, appears to have the same innate pattern of chromosome segregation and division as do natural coccal species. This pattern is concealed, and *E. coli* cells made monochromosomal, by the action of the cell-shape proteins, RodA and PBP2. The mode of chromosome segregation that we propose for *E. coli* may therefore be a general one for prokaryotes. This mode, 'hierarchical chromosome segregation', is totally different from the mitotic system of eukaryotes, and is therefore yet another fundamental point of distinction between the two Kingdoms.

Predictions of the model

Our model specifies a particular mode of chromosomal DNA localisation and movement within cells and also describes the time of formation, position and order of utilisation of potential division sites. Although we believe that this, or a similar model, is required to explain the facts of bacterial genome segregation as already known, further tests of the hierarchical model for chromosome segregation are difficult because of the limitations of present tech-

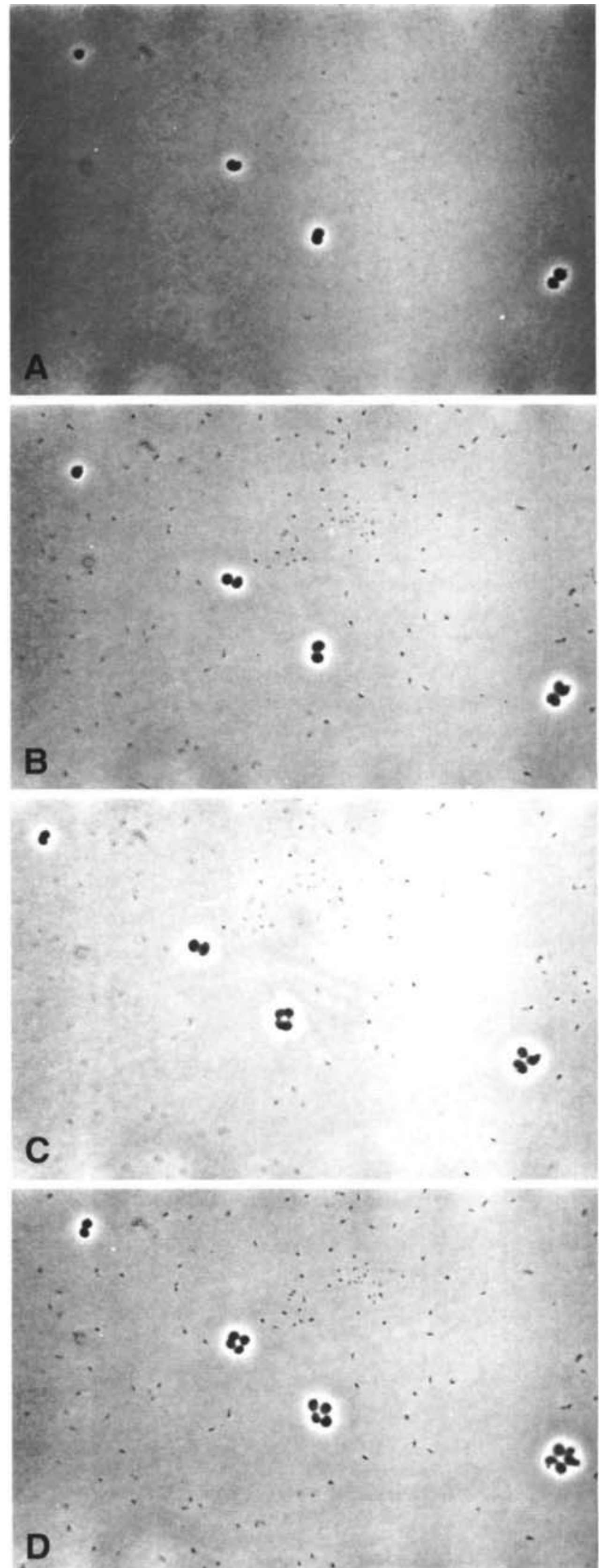

Fig. 6. Alternating planes of division in spherical *E. coli* cells. Spherical *rodA*^{am} *sup*^{o} cells were placed on nutrient agar and observed during subsequent growth and division. Four cells and their progeny are shown at progressively later stages (from left to right) in the growth and division cycle. (A), (B), (C) and (D) were photographed 0, 50, 70 and 90 minutes, respectively, after placing the cells on the agar surface. As can be seen, the second division plane is always perpendicular to the first. (The plane of the third division, seen beginning in line D, is difficult to determine, probably because growth of individual cells in the microcolony pushes cells into new alignments.)

nology. Thus, we do not have a way, as yet, to observe chromosome localisation and movements in living cells directly (partly because the DNA in growing cells is spread out throughout the cell and because we do not have a way to locate separately the supposed 'organisational centres'). It is possible to predict the proportions of cells homozygous for each of the two alleles of a gene in a colony arising from a newly transduced multichromosomal cell under non-selective conditions, but the low frequency of gene transfer by P1 transduction (about $1/10^5$ phages will transduce a given gene) would require that 10^7 unselected colonies be individually screened to find about a hundred such clones! A much more efficient means of gene transfer is therefore required for this experiment.

It is possible to make testable predictions about the pattern of cell division itself. The hierarchical model states that all division planes must be medial, to give two equal sisters. This predicts that if division is inhibited during a period of growth of Rod^- cells, then the resulting large cells should recommence division medially and continue in successive alternating planes, just as in smaller spherical cells. This behaviour is in contrast to that of similarly treated Rod^+ cells which, at the end of a period of growth without division, recommence at any of a number of linearly arranged potential division sites and therefore often first divide unequally[3,11-13]. Similarly, inactivation of the *minB* locus allows Rod^+ cells to use cell poles as additional division sites[30,31], but our model predicts that *minB rodA* double mutants will divide exactly like *minB*$^+$ *rodA* cells, because these cells have no poles (or alternatively consist entirely of large poles; possibly a purely semantic distinction). Tests of these predictions are underway.

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William D. Donachie, Stephen Addinall and **Ken Begg** are at the Institute of Cell and Molecular Biology, University of Edinburgh, Edinburgh EH9 3JR, Scotland.