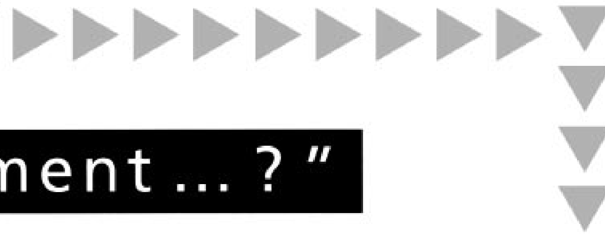

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The Editors hope that readers will take full advantage of this section and use it to raise matters that hitherto have been confined to a limited audience.

***Christopher M. Thomas*, Editor-in-chief**

Size, volume, length and the control of the bacterial division cycle

The statistical analysis of the relationships of length, diameter and volume of cells in an exponentially growing culture of *Escherichia coli* (5) raises an important question, specifically 'what, among these measurable elements of the cell, is most likely to be relevant to the control of events during the division cycle?' The conclusion reached by Grover & Woldringh (5) is that the cell cycle is more likely to be controlled by the length of the cell and that the volume of the cell is not, or is less likely to be, a controlling element. This is in opposition to the alternative *Volume* model, or the related constrained hoop model (2), that implicitly points to volume as the controlling element.

The key experimental results from their analysis of the dimensions of bacteria are (a) a failure to observe a negative correlation between length and diameter of cells in exponential culture, (b) finding a positive correlation between volume and diameter, and (c) observing that the coefficient of variation of the volumes of the cells is greater than the coefficient of variation of the diameters of the cells. Conclusions drawn from these observations are flawed, primarily due to the simple fact that the cell volume determination is dependent on diameter determination. The volume of a cell is proportional to the product of length, diameter and diameter. Diameter measurement affects the volume twice as often as it affects the diameter measurement itself. This simple geometric fact assures that observation (c) will be found, irrespective of any underlying biological controls. Similarly, the positive correlation between diameter and volume (point b) is foreordained by the fact that the volume of the cells is determined largely by the diameter value. If one measured the diameter and volume of absolutely identical spheres, any slight error in the measurements would lead to variation in diameter determination and an even larger variation in volume measurement. This would produce a corresponding positive correlation between diameter and volume, even without any correlation in reality.

The lack of an inverse correlation between the length and the diameter (point a) is to be expected if one does not have cells with a significant variation in diameter within the culture. The diameters of the cells used in this analysis are very likely constrained to lie within a very narrow range of diameters. The experimentally measured diameter variations would thus be due primarily to variations in experimental measurement. (This result is made more likely by the fact that the 50796 cells examined were from a culture growing slowly with a 113 min interdivision time in alanine minimal medium; these cells are small cells, and thus experimental error relative to actual biological sizes is increased; if larger, faster-growing cells had been used, the variation due to experimental measurement error would have been lessened.) If the experimental measurement error is large relative to the actual dimensions of the cell, the variation in diameter would be independent of cell length. Thus, not finding a negative correlation of length and diameter in a particular case does not eliminate explanations of results that do demonstrate a negative correlation of length and diameter.

Having noted their conclusion that there is no correlation between diameter and length, it is of interest to note that for one newborn set [1274 cells, Table 3 in (5)], and two total population sets [50796 cells and 1141 cells, Table 3 in (5)], there is a negative correlation of length and diameter. Thus, a deeper reading of the data shows that there are elements of the actual results that support the constrained hoop model and thus support volume as a mediator of the events of the cell cycle. As noted in Fig. 6.12 of (1), the *Volume* model predicts that the length–diameter correlation would be more negative in the newborn population than in the population as a whole. That the newborn cells have a general tendency toward a more negative correlation than the population as a whole supports the *Volume* model.

It should also be noted that the volume distributions for the newborn cells [Fig. 2b of (5)] indicate that the volume varies over a factor of 3–4. This spread in volumes is what one might expect for the total population of cells within a culture. In cells with no variation in sizes, the ideal variation would be over a factor of 2. With variation one might expect to see a variation over a factor of 3–4. But that newborn cells exhibit this variation suggests that there are large experimental errors in the measurements of the cell volume and thus large experimental errors in the

▶ *GUIDELINES*

Communications should be in the form of letters and should be brief and to the point. A single small Table or Figure may be included, as may a limited number of references (cited in the text by numbers, and listed in alphabetical order at the end of the letter). A short title (fewer than 50 characters) should be provided.

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Contributions should be addressed to the Editor-in-Chief via the Editorial Office.

measurements of lengths and widths. Furthermore, this volume distribution spread is not supported by electronic measurements of cell volume using a Coulter Counter. Specifically, the coefficient of variation of volumes of newborn cells (0·17, 0·18) are not very different from the coefficient of variation of volumes of the total population (0·27, 0·20). This suggests that there are internal problems with the experimental measurements themselves, thus weakening any critique of the *Volume* model using microscopic measurements of cell dimensions.

The constrained hoop model (2) – explaining published work on the negative correlation of length and width in exponentially growing cells (7) – was based on a culture that exhibited a negative correlation of length and width. If one does not find such a negative correlation it is likely to be due to the cells having a very narrow span of diameter variation. The absence of a negative correlation (although as noted above, there are some data showing such a negative correlation) is not a proof that the constrained hoop model is not correct; it is only a proof that one cannot see the correlation in a particular biological situation.

The analysis of Grover & Woldringh (5) would be improved if the cell volume were determined independently of the diameter measurement. If cells were somehow selected for volume by a method that did not involve diameter measurement, and then the diameters were measured on subgroups with different volumes, one could possibly conclude that there was a positive relationship between volume and diameter. This point is actually noted by Grover & Woldringh – 'In cells with the same length, the diameter volume correlations will always be large and positive…'. What is important to note here is that this fact, accepted by Grover & Woldringh, leads to a different interpretation of their data.

It should also be emphasized that the conclusion of Grover & Woldringh is that there is a systematic variation in diameter with cell age during the division cycle. As they put it, '…cell diameter is more likely to vary systematically with cell age than to conform the constraints of a rigid hoop', and one of the assumptions of their computer simulation is 'mean cell diameter decreases linearly with *L*[ength].' If the cellular diameter variation were the same as that of Trueba & Woldringh (7), one would expect a negative correlation between length and width. There is no indication (5) that such a negative correlation, or indeed any correlation, exists. According to the *Length* model, the diameter is proposed to decrease during the division cycle and then suddenly increase at division. The molecular biological problems with this analysis have been dealt with extensively (1) and those interested in this problem are referred to this reference for a complete and detailed analysis of shape at different growth rates as well as shape within the division cycle.

But trumping and overriding all of this discussion of length–width–volume in individual cells in a culture is the fundamental experimental basis of the volume control model, that the *Volume* model explains cell sizes over a wide range of growth rates (1, 6). This experimental result is completely ignored in the analysis of Grover & Woldringh (5). It is clear that the size of cells at initiation of DNA replication is a constant (3). Furthermore, there is evidence that the shape of the bacterial cell does not vary over a range of sizes (4). This means that over a range of cell sizes the length effect cannot be as good a measure of initiation as cell size. If cells vary over a fourfold range of volume, and this change correlates with the initiation size of a cell, the length will only range over the cube root of 4, or over a range of 1·6. Thus, length is not as good a measure of the triggering events as volume because it does not fit the experimental results of size variation over a range of growth rates. If a cell has a constant size at initiation, it cannot have a constant length at initiation. To put this idea more strongly, the size measurements of cells over a wide range of growth rates (based on actual dry weight or turbidity measurements) is stronger evidence for volume control than the subtle variations in cellular dimensions measured on individual cells.

Equally important is the overshoot in cell length following a shift-up, which is the basis for the constrained hoop model (2). The *Length* model would, in effect, counteract the overshoot phenomenon as the increased cell length would lead to increased cell division and thus lead to a cessation of the overshoot. As the overshoot is observed, this argues against the *Length* control model and for the constrained hoop model, and thus for the *Volume* control model.

In summary, the data supporting volume or cell mass as a controlling element is based on strong data and is far from being upset by the more problematic data on dimensions of cells in an exponential culture.

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Authors' reply

The order in which we address the items raised by Dr Cooper follows that of his letter above.

1. We do not calculate volume from length and diameter, we measure it. More specifically, a computer program determines the outline of the cell automatically using standard pattern recognition techniques, locates its major (usually curved) axis, takes the cell to be rotationally symmetrical about this axis, measures many transverse widths along its length and calculates cell volume from the resulting trapezoids. Such computations require no geometrical assumptions other than that a cell be locally radially symmetrical along its midline; thus, the volume of a cell as used here reflects its actual size and not the usual functional dependency on length and diameter on which Dr Cooper's objections are based.

2. Dr Cooper claims that the observed fluctuations in diameter are due primarily to variations in experimental measurements and that if faster-growing cells had been used, the variations would have been less. This is not borne out by actual data (Table 1). Clearly, faster-growing cells do not, in general, have less dispersion in their diameters.

If cv_D being due to experimental error is what makes the diameter–length correlation near zero then, in view of item 1 above, we would also expect the diameter–volume correlation to be near zero; in fact, it is very highly positive [Table 3 in (4)].

3. The diameter–length correlation in the total non-constricting population is predicted by both models to be negative and so cannot be invoked for or against either one.

The statement 'the newborn cells have a general tendency towards a more negative correlation than the [non-constricting] population as a whole' appears a little strange. Table 3 in (4) contains two coefficients of each kind: $-0{\cdot}117$ and $+0{\cdot}050$ for newborn cells and $-0{\cdot}120$ and $-0{\cdot}076$ for the corresponding non-constricting populations. The second newborn coefficient, $+0{\cdot}050$, is not only not more negative, it is actually positive. The first, $-0{\cdot}117$, is indeed more negative than $-0{\cdot}076$ but the difference is not statistically significant ($P > 0{\cdot}15$, one tail, as determined by using Fisher's *z*-

Table 1. Coefficient of variation of cell diameter (cv_D) at different culture doubling times ($\bar{\tau}$) [from Trueba & Woldringh (14)]

$\bar{\tau}$ (min)	CV_D (%)
22·5	7·2
60	9·0
109	8·3
125	7·8

transformation). Finally, −0·117 is less, not more, negative than the remaining coefficient, −0·120.

4. Cooper presents no experimental data and provides no references for these claims concerning the narrower volume distributions recorded by the Coulter Counter and so our response will have to be rather general. There are no quantitative studies in the literature comparing Coulter Counter and electron microscopic measurements on the same cells – none, not for newborn cells selected by membrane elution, not even for steady-state cultures. We are forced, therefore, to consider these techniques separately.

Electron microscopy has been investigated meticulously and extensively (15). A detailed comparison between the dimensions of *Escherichia coli* cells (of the strain used here) obtained from the electron microscope and those measured under the light microscope (13, 14) establishes the former methodology as accurate and reliable. The basis of Cooper's argument here is that 'the coefficient of variation of volumes of newborn cells (0·17, 0·18) are not very different from the coefficient of variation of volumes of the total [non-constricting] population (0·27, 0·20)'. But these are the very numbers that have been in the literature, unchallenged, for over 20 years (11).

On the other hand, volume determinations using the Coulter Counter are fraught with problems (5). Thus, size distribution plots appear in the literature comparing newborn cells with the steady-state cultures from which they were derived (8, 9), but no numbers are ever given. This may be due to the absence of a reliable method of calibration, a difficult procedure in general (6) and for non-spherical particles in particular (7).

5. There seems to be a basic misunderstanding here. In cells with the same length, the diameter–volume correlation will be large and positive; in cells with the same volume, the diameter–length correlation will be large and negative – this is a geometrical imperative, a mathematical necessity beyond all models and growth laws, from which no inferences can be drawn.

6. There is indeed an observed negative diameter–length correlation, not at specific cell-cycle events, but in the non-constricting population as a whole (−0·12), and it is very highly significant ($P < 0{\cdot}001$); in the phase-contrast data, it is also negative (−0·076) and highly significant ($P < 0{\cdot}01$).

'According to the *Length* model, the diameter is proposed to… suddenly increase at division.' Not at all. Not suddenly at division but gradually, over the entire T period: from onset of constriction to cell fission, at least 15 min in our culture (10) and probably closer to 20 (3).

7. The *Volume* model states that the diameter of an individual cell remains constant during its cell cycle in steady-state growth. Nonetheless, the strong negative correlation at any particular event in the cycle between diameter and length that is inherent in this model gives rise to an apparent decrease in diameter with cell age when expressed in terms of length. This effect of combining the contributions of separate subpopulations was first pointed out by Cooper himself (2) and termed 'correlated variables'. The same holds true in the *Length* model: the positive correlation at particular cell-cycle events between diameter and volume gives rise to an apparent increase in diameter with cell age when expressed in terms of volume.

8. In this article, we confined ourselves to steady state and to a single growth rate. And we do point out that the initiation of chromosome replication may indeed be regulated by cell volume, since we had no experimental marker for that event and so no way of testing either model. It should be noted, however, that initiation mass not only does not remain constant with growth rate, as Cooper claims, it actually doubles (1).

For a short while, cell shape – more properly (17) termed the 'aspect ratio', length/diameter; the 'shape' of a body refers to its geometrical form which, in the case of *Escherichia coli*, is roughly that of a right circular cylinder with hemispherical polar caps – cell shape was thought to be independent of growth rate (19), but that notion was soon discarded when a more rigorous study showed it to vary considerably in both B/r *A* and B/r *K* (16).

Actually, cell volume varies over an eight-fold not fourfold range (12), but there is no rational reason why a factor of 8 is required to regulate various features of cell growth and one of 1·6 will not do.

9. Again, we do not address effects of growth rate. Nonetheless, the *Length* model could serve equally well, perhaps even better, to explain the overshoot observed in shift-up experiments (17). Just as the *Volume* model could reflect a biochemical sensing for the concentration of an initiator protein, the *Length* model could reflect the physical or structural sensing of the presence of the nucleoid as inhibitor of the onset of constriction. During a nutritional shift-up, the increased amount of DNA per nucleoid due to re-initiation of DNA replication causes a lag in nucleoid segregation and a consequent postponement of cell constriction (18).

10. Length rather than volume is the controlling dimension at such cell-cycle events as onset of constriction and cell division. This is our thesis and we maintain that it has been established as far as steady-state growth at a single growth rate is concerned, which is what we set out to do. Furthermore, Dr Cooper has produced no evidence to suggest that it may be otherwise at other growth rates or during nutritional shift-up.

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Note

In the printed version of the article under discussion (4), the URL that appears in the legend to Fig. 1 is incorrect; it should read http://mic.sgmjournals.org/cgi/content/full/147/1/171/DC1

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Comment on reply of Grover & Woldringh

Reference (1) of Grover & Woldringh (their comment 8) actually concludes that within the period of constant C and D periods, initiation mass is constant. A similar critique of constant mass at initiation (Wold *et al*., 1994, *EMBO J* **13**, 2097–2102) has been answered (Cooper, 1997, *Mol Microbiol* **26**, 1138–1141, and references therein). Volume over the constant C and D period varies only over a factor of 4. Regarding the central point (their comment 1) of whether volume is 'measured' or 'calculated', taking the cell as 'rotationally symmetrical about its axis' means that the observed width of the cell is taken as the diameter of the cell; using the formula for the volume of a cylinder $[\pi(d/2)^2h]$ means that the diameter is entered into the calculation twice. Regarding their point 4, the data are not challenged, but the interpretation is challenged. Newborn cells should, in theory, have a much smaller CV for their volume than the total cell population because the total cell population contains cells that have grown, at a minimum, over a factor of 2. And lastly, regarding their point 6, the suddenness or gradual change in diameter during the division cycle is not the issue, but whether the biochemistry of the peptidoglycan can accommodate such a variation, whether sudden or gradual.

Stephen Cooper

Plasmids pEA29 and pEa34 in *Erwinia amylovora* are unrelated

As Vivian *et al*. (15) noted in a recent article in *Microbiology*, our knowledge of the role of plasmids in phytopathogenic bacteria is quite limited. The task of marshalling information on plasmids from five different genera was a daunting one. Unfortunately, the information concerning *Erwinia amylovora* indicated incorrectly that plasmid pEa34 is plasmid pEA29 carrying genes for streptomycin resistance on transposable element Tn*5393* (Table 2). In addition, strain CA11 was listed as the original source of pEA29, whereas in fact CA11 was the original source of pEa34. Since clearly the literature on this topic is complex to an outsider, we felt that it would be worthwhile to clarify our current knowledge of plasmids in *E. amylovora*.

Analysis of pEA29 was carried out initially to assess its role in pathogenicity. Two groups generated restriction maps for pEA29; one group worked with strain Ea7/74 and the other with strain CFBP1430 (4, 9). Working independently, each group reported slightly different size estimates and restriction maps, and as a result different names (pEA28 and pEA29) were assigned to the same plasmid. Both groups determined that the plasmid was non-transferable and exceptionally stable in naturally occurring strains of *E. amylovora*. Strains of *E. amylovora* with pEA29 or pEA28 evicted by incompatibility were still pathogenic but exhibited less virulence, suggesting that the plasmid may carry a virulence factor (5, 9). These strains also exhibited altered colony morphology on minimal medium without thiamin (5) or were auxotrophic for thiamin (9). Eviction of pEA29 from other strains confirmed the role of altered virulence and altered colony morphology on medium without thiamin (11). It remains to be established whether the change in morphology was due to the loss of the thiamin biosynthetic genes *thiO*, *thiG* and *thiF* found on pEA29. Also, it was found that pEA29 contains a putative iteron-containing theta-type origin of replication (11). The sequencing of pEA29 from *E. amylovora* strain Ea88 is providing a basis for studying the genetic organization of plasmids in other phytopathogenic *Erwinia* species. For example, a region of pEA29 from *E. amylovora* strain Ea88 and an 11 kb region of a large plasmid from *Erwinia pyrifoliae* strain Ep1/96, a novel pathogen of Asian pear that is similar but genetically distinct from *E. amylovora* (8), have been found to share some of the same genes (*aldD*, *ctpE*, *thiF*, *thiG*, *thiO*, *tnpR*, ORF15, and *betT*; G. C. McGhee & A. L. Jones, unpublished data). The genes and their orientation on the two plasmids are similar except that a 3·3 kb region of pEA29 containing *parA*, *parB* and ORF11 is absent in the corresponding region of the *E. pyrifoliae* plasmid. Further analysis is underway to determine if other regions of the *E. pyrifoliae* plasmid share extensive homology with pEA29.

Although pEA29 does not play a significant role in the induction of fireblight, it is ubiquitous in all strains of *E. amylovora*. This discovery led to the development of molecular assays to identify the species and diagnose fireblight (1, 4). PCR is the most widely used and sensitive method to identify colonies of *E. amylovora* and to detect *E. amylovora* in extracts from symptomatic and asymptomatic plants. The identification is based on the amplification of a *Pst*I fragment from pEA29 (4). As PCR assays came into wide use, size variation was observed in restriction digests of the pEA29 PCR product (10). Further analysis of this fragment by two groups revealed that a highly variable region consisting of short-sequence (8 bp) repeats was creating the polymorphism (7, 13). The repeats are located between genes *msrA* and *repA* and may be important in the replication and partitioning of pEA29 (11). Restriction patterns for pEA29 sometimes vary between strains, indicating that this plasmid is not homogeneous (11). The detection of insertion sequences, transposons and deletions in pEA29 provides insights into the molecular evolution of pEA29.

Analysis of pEa34 was carried out initially to assess its role in streptomycin (Sm) resistance. That pEa34 and pEA29 are unrelated was shown when pEa34 used as a probe did not hybridize with pEA29 from a large number of Sm^r field strains, including strain CA11 (2, 12). The identification of plasmid pEa34 in Sm^r strains of *E. amylovora* from Michigan apple orchards was of immediate interest because *E. amylovora* rarely carries plasmids other than pEA29, and because altered ribosomes were the sole known mechanism for Sm^r in *E. amylovora* in other regions of the world (6). A probe (pCPP505) initially cloned from Sm^r *Pseudomonas syringae* pv. *papulans* was used to establish that *E. amylovora* had acquired a determinant for Sm^r (2). Hybridization studies and sequence analysis of the Sm^r determinant in CA11 revealed that the tandem *strA–strB* genes were located within a newly discovered transposon Tn*5393* on pEa34 (2). The *strA–strB* genes on Tn*5393* and on the small broad-host-range plasmid RSF1010 are highly similar (2); thus, Sm^r genes found worldwide in Gram-negative animal and human pathogens had been detected in a phytopathogenic bacterium. Work from several groups indicated that the *strA–strB* genes found in *Pseudomonas*, *Xanthomonas* and *Erwinia* species, and in many Gram-negative Sm^r bacteria isolated from plant surfaces are commonly associated with Tn*5393* inserted into various large plasmids (2, 14). Some variants of Tn*5393* contain IS elements with promoters that increase the expression of the *strA–strB* genes (3, 14). By studying the plasmids found in a large pool of Sm^r Gram-negative bacteria from Michigan apple orchards, molecular evidence was obtained for natural horizontal transfer of pEa34 from *Pantoea agglomerans* (formerly *Erwinia herbicola*) into *E. amylovora* (2, 6).

This species is often ecologically associated with *E. amylovora* and is the only species in the pool of Sm^r bacteria found to harbour pEa34 with the same genetic arrangements of Tn*5393* commonly found in *E. amylovora*. Transfer of pEa34 from Sm^r *Pantoea agglomerans* donor strains to Sm^s *E. amylovora* and *Escherichia coli* in mating experiments is further evidence that this is the likely natural route by which *E. amylovora* acquired Sm^r genes (2, 6). Recently, Tn*5393* has been detected on the ubiquitous plasmid pEA29 and on the chromosome in a number of Sm^r field strains of *E. amylovora*, indicating that natural mobilization of the transposon results in further spread of Sm^r and increases plasmid diversity (6, 11, 12). The recognition of Tn*5393* has been invaluable in answering the basic question of how a common determinant for streptomycin resistance has developed on different plasmids and in different bacteria.

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