Mechanistic Basis of Branch-Site Selection in Filamentous Bacteria

David M. Richards¹*, Antje M. Hempel¹,²*, Klas Flär dh², Mark J. Buttner¹, Martin Howard¹

Introduction

The ability to break symmetry and establish an axis of polarity is crucial for the function and development of almost all cell types. In bacteria, such symmetry-breaking is often mediated by cytoskeletal elements inside the cell that direct new cell wall synthesis. Many rod-shaped bacteria (including Escherichia coli, Bacillus subtilis and Caulobacter crescentus) grow solely through the isotropic insertion of new cell wall material throughout the length of the lateral walls [1,2]. Here, cell wall growth is directed by MreB, the bacterial ortholog of eukaryotic actin [3–6], whereas cell division is mediated by the bacterial tubulin ortholog, FtsZ. In these rod-shaped bacteria, polarity systems are required to identify and differentiate cell poles that remain inert during cell elongation. However, many other organisms enlarge by hyphal growth, a strategy that has proved successful for the exploitation of soil and other environments. Hyphal growth has evolved independently in both eukaryotic and prokaryotic microbes, including fungi and Gram-positive bacteria of the genus Streptomyces [7,8]. A functional DivIVA-EGFP fusion localizes to tips and marks new branch points well before visible lateral outgrowth [9,10]. Deletion of divIVA is lethal, whereas overexpression leads to greatly increased numbers of DivIVA foci along the lateral wall and de novo cell wall outgrowth at these foci [8–10]. These data suggest that DivIVA can direct cell polarity and recruit the machinery for cell wall synthesis. Additionally, cytoskeletal components may also be involved (for example, Scy [11]), together forming a tip-organizing complex. However, regardless of whether there are additional components, we can use DivIVA-EGFP as a marker to monitor the dynamics of the tip-organizing complex as a whole.

The branch-site selection mechanism that localises DivIVA to new sites along the lateral wall, from which branches subsequently emerge, was previously unknown. We therefore used the DivIVA-EGFP fusion to monitor the dynamics of the tip-organizing complex in S. coelicolor by live cell time-lapse imaging. These experiments revealed that the new DivIVA foci that initiate lateral branches arise predominantly by a novel tip focus-splitting mechanism that bypasses the necessity for initial nucleation or de novo branch-site selection. We develop a mathematical model for DivIVA-dependent growth and branching, involving DivIVA focus-formation by tip-focus splitting, focus growth, and the initiation of new branches at a critical focus size. We quantitatively fit our model to the experimentally-measured tip-to-branch and branch-to-branch length distributions. The model predicts a particular bimodal tip-to-branch distribution results from tip-focus splitting, a prediction we confirm experimentally. Our work provides mechanistic understanding of a novel mode of hyphal growth regulation that may be widely employed.

Abstract

Many filamentous organisms, such as fungi, grow by tip-extension and by forming new branches behind the tips. A similar growth mode occurs in filamentous bacteria, including the genus Streptomyces, although here our mechanistic understanding has been very limited. The Streptomyces protein DivIVA is a critical determinant of hyphal growth and localizes in foci at hyphal tips and sites of future branch development. However, how such foci form was previously unknown. Here, we show experimentally that DivIVA focus-formation involves a novel mechanism in which new DivIVA foci break off from existing tip-foci, bypassing the need for initial nucleation or de novo branch-site selection. We develop a mathematical model for DivIVA-dependent growth and branching, involving DivIVA focus-formation by tip-focus splitting, focus growth, and the initiation of new branches at a critical focus size. We quantitatively fit our model to the experimentally-measured tip-to-branch and branch-to-branch length distributions. The model predicts a particular bimodal tip-to-branch distribution results from tip-focus splitting, a prediction we confirm experimentally. Our work provides mechanistic understanding of a novel mode of hyphal growth regulation that may be widely employed.

Citation: Richards DM, Hempel AM, Flär d h K, Buttner MJ, Howard M (2012) Mechanistic Basis of Branch-Site Selection in Filamentous Bacteria. PLoS Comput Biol 8(3): e1002423. doi:10.1371/journal.pcbi.1002423

Editor: Christopher V. Rao, University of Illinois at Urbana-Champaign, United States of America

Received November 28, 2011; Accepted January 26, 2012; Published March 8, 2012

Copyright: © 2012 Richards et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: We receive core support from the BBSRC. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: klas.flar dh@biol.lu.se (KF); martin.howard@jjc.ac.uk (MH)

These authors contributed equally to this work.
Author Summary

Amongst the great variety of shapes that organisms assume, many grow in a filamentous manner and develop at least partly into a network of branches. Examples include plant roots, fungi and some bacteria. Whereas the mechanisms of filamentous growth are partially understood in fungi, the same cannot be said in filamentous bacteria, where our knowledge of hyphal growth regulation is very limited. To rectify this we have studied the bacteria Streptomyces, which are an excellent model for all hyphal bacteria. The protein DivIVA is known to play a critical role in controlling filamentous growth in Streptomyces, forming large foci at branch tips and smaller foci that mark sites of future branch outgrowth. However, until now nothing was known about how these foci first appear.

We have shown experimentally that new foci appear via a novel mechanism, whereby existing tip-foci split into two clusters. The larger cluster remains at the growing tip, while the smaller cluster fixes onto the adjacent lateral membrane, where it grows in size, eventually initiating a new branch. By mathematically modelling how DivIVA foci grow, we show how this simple mechanism of focus formation can quantitatively capture the statistical properties of the entire hyphal branching network.

Results

Lateral DivIVA foci arise from splitting of apical foci

Our previous studies have shown that DivIVA foci are always present at new branch points before outgrowth occurs [9,10]. However, the origin of such DivIVA foci and the factors that determine their localisation have remained unclear [8]. To further understand the branching process, we have therefore studied more carefully how such foci are formed and traced their origin from time-lapse images. These experiments revealed that new small foci often arise from existing DivIVA foci at hyphal tips, by a process where a small cluster of DivIVA separates from the tip-focus and is left on the membrane just behind the tip. An example is shown in Figure 1 (see Video S1 for a movie of this figure). At around 12–18 minutes the focus of DivIVA at the tip splits and leaves behind a small focus on the adjacent membrane. As the tip continues to extend, the new focus remains fixed in place on the membrane and grows in size and intensity. In between 42 and 48 minutes a new branch is formed at the position of the new focus. Tip-focus splitting is only seen to occur from foci associated with extending tips; foci which have not yet initiated a branch, such as the smaller focus between 12 and 36 minutes in Figure 1, do not undergo splitting. We traced the origin of 52 nascent branches in time-lapse images and found that 42 of them (81%) were accounted for by tip-focus splitting events. Since only sufficiently large and intense DivIVA-EGFP foci are visible above the background fluorescence, some foci cannot be traced to their point of creation, and so this likely to be an underestimate of the real proportion of branching arising from tip-focus splitting [10]. Thus, tip-focus splitting, rather than other potential mechanisms, such as spontaneous nucleation, appears to be the predominant method for focus initiation in wild-type cells.

Measurements of hyphal growth and lateral branching

In order to quantitatively understand Streptomyces branch-site selection, we have measured two categories of distances from still images: the distance between the tip and the points where branches emerge, and the spacing between the branches themselves. Unlike the branch spacing, the tip-to-branch distance is not fixed: as the hyphae extend in length, the tip-to-branch distances increase. To avoid this difficulty we use our measurements to work out the tip-to-branch distance at the moment when the new branches appear, as discussed in Materials and Methods.

Unless care is taken when measuring the distributions from still images, it is easy to introduce biases that uncontrollably skew the data. For example, if only branching events relatively close to hyphal tips can be measured (as is inevitably the case for Streptomyces where individual hyphae cannot be traced into the dense mycelial clumps from which they emerge), then long branch-to-branch distances will never be recorded, even if they occur. As explained in Materials and Methods, we control for this effect by introducing a protocol so that all measured hyphae have effectively the same length, a distance we call the trim length. This is achieved by discarding hyphae which are shorter than the trim length and trimming those which are longer. This protocol does not eliminate measurement bias, but rather controls the bias so that our experimental measurements are unambiguous and can be precisely compared with data generated by our mathematical model (see below).

The measured tip-to-branch and branch-to-branch distributions with a 80 μm trim are shown in Figure 2. The tip-to-branch distribution has two distinct peaks, one between 0 – 5 μm and one at 40 – 45 μm (Figure 2A). This might suggest that two distinct mechanisms are involved in producing new branches. Surprisingly, however, our later analysis will show that a single mechanism can account for both peaks.

Minimal mathematical model of the growth of DivIVA foci

We assume that DivIVA foci, either on their own or as part of a tip-organizing complex, assemble the cell wall synthesis machinery to both extend hyphae and form new branches. Most new DivIVA foci do not immediately initiate a new branch (Figure 1). We assume this is a result of the small starting sizes of most foci. Foci must instead grow in size by accumulating DivIVA molecules from the cytoplasm until they contain enough molecules to initiate a new branch. To understand where new branches emerge we must therefore understand how the number of molecules, N, in a focus changes with time. We will refer to this number N as the tip-focus size. We consider simple cooperative binding where the rate of DivIVA molecules joining a focus is linearly dependent on both the cytoplasmic DivIVA density, ρ, and the focus size, N (alternative growth rules are considered in Supporting Text S1, but these alternatives give qualitatively similar results, with no better fit to the experimental data). Thus we have $N = \beta \rho N$, where $\beta$ is a parameter independent of N and ρ. Although, in the minimal model, we assume foci never lose DivIVA molecules, including this process again makes little or no difference (see Supporting Text S1). We also assume that the cytoplasmic DivIVA
density appearing in the above equation is the same for all foci (this assumption is justified by our full simulations, see Supporting Text S1). Thus we can replace \( \beta b \) by the single parameter \( b \), which we call the binding parameter, and consider 
\[
_{\text{NN}} N \sim b N_0.
\]
We assume that a focus starts with \( N_0 \) molecules and must reach \( N_{br} \) molecules before it can form a branch. We can easily solve the above equation for \( N \) to find the time taken, \( t \), for this growth from \( N_0 \) to \( N_{br} \). With an extension speed \( v \) for established tips, the distance \( L \sim v t \) behind the tip where a branch appears is
\[
L \sim \frac{v}{b} \ln \frac{N_{br}}{N_0}.
\]

By comparing images like Figure 1 at 12 and 42 minutes, we estimate a typical value for \( N_{br} \) as between 5 and 10, so that, to a rough approximation, \( L \approx \frac{2v}{b} \). The absolute value of \( N_{br} \) is difficult to determine, but since the fluorescence of a typical DivIVA focus is not dissimilar to that of an FtsZ ring, and since an FtsZ ring contains on the order of 10,000 molecules [13], we take \( N_{br} \) to be of a similar order of magnitude. The growth speed of an established tip, \( v \), is measured from time lapse images to be about 8 \( \mu \text{m} \text{/hr} \). Due to the trimming issues discussed above, measuring a typical value for \( L \) is not straightforward. In particular, using the average of a trimmed distribution, such as that in Figure 1A, will not give a good estimate. However, as explained in Materials and Methods, by studying the distributions over a range of trims, we estimate a value of about 65 \( \mu \text{m} \) under the growth conditions used, which implies that \( b \) should be about \( 7 \times 10^{-5} \text{s}^{-1} \). (See Figure S10 for a schematic of the colony morphology for different values of \( b \).)

*Streptomyces* produces branches at a range of distances behind tips, producing a distribution of tip-to-branch distances. In our model, this is due to fluctuations in the parameters in Eq. (1). Note that, although we vary these parameters, we do not model the growth of foci themselves stochastically (instead using a deterministic differential equation) due to the large number (thousands) of molecules involved. Each binding event will itself be stochastic but the overall process involving many thousands of such binding events will be well described deterministically.

The tip-focus splitting mechanism

So far we have been concerned with how the number of molecules in a pre-existing focus changes with time. We have not yet discussed the mechanism by which new foci are formed, the tip-focus splitting mechanism. Furthermore, after a tip-focus has undergone splitting, we are interested in the length of time before the focus can split again, which, after both foci have initiated new branches, will translate into the distance between branches. It is important to emphasise that, whereas the growth of foci controls the tip-to-branch distribution, it is the focus-splitting rules that control the branch-to-branch distribution.

---

**Figure 1. Evidence of tip-focus splitting, growth of foci and emergence of branches, in fluorescence-imaged *Streptomyces coelicolor* expressing divIVA-egfp.** The tip always contains a large DivIVA focus and established tips extend at an approximately constant speed. At about 12 minutes, the DivIVA tip-focus undergoes splitting, leaving behind a new focus (arrow). As the tip continues to extend, the new focus remains in place on the membrane and grows in intensity. After about 42 minutes a new branch is formed at the position of the new focus, with the new focus now sitting at the tip of the new branch. Both the new branch and the original branch now continue to extend in length. Time in hours:minutes. Scale bar: 3 \( \mu \text{m} \).

doi:10.1371/journal.pcbi.1002423.g001

---

**Figure 2. Comparison of histograms between minimal model and experimental data at 80 \( \mu \text{m} \) trim.** (A) Tip-to-branch distribution. Analytic prediction is also shown (curved line). 1097 experimental data points. (B) Branch-to-branch distribution. 858 experimental data points.

doi:10.1371/journal.pcbi.1002423.g002
The simplest assumption that could be made would be that the focus-splitting probability per unit time is constant, independent of when the tip-focus last split. This would describe a Poisson process and so imply an exponential distribution for the branch-to-branch distribution. However, as Figure 1B shows, for distances smaller than 10 μm the branch-to-branch histogram is not described by a decaying exponential; these shorter distances are measured much less frequently than implied by a Poisson distribution.

This suppression of short branch-to-branch distances shows that focus-splitting events are not independent of each other: a tip-focus that has just split is less likely to immediately split again. One potential explanation is that the probability of tip-focus splitting depends on the tip-focus size, such that smaller tip-foci are less likely to split. For this reason we implement a minimum tip-focus size (a critical mass), \( N_{\text{split}} \), below which the tip-focus cannot split, with some constant focus-splitting probability per unit time, characterised by the parameter \( \gamma \), for all tip-foci above \( N_{\text{split}} \). Splitting events cause the tip-focus to decrease in size and so, in some instances, such a splitting will cause the tip-focus size to drop below \( N_{\text{split}} \). In that case, only after the tip-focus has absorbed more DivIVA from the cytoplasm will it have sufficient size to split again. This time delay effectively reduces the number of short branch-to-branch distances.

Although it is difficult to analyse tip-focus splitting analytically, it is useful to note that, in the limit where \( \gamma \) is very large (compared to \( \beta \)), the branch-to-branch distance, \( d \), is given by

\[
d = \frac{v}{\beta} \ln \frac{N_{\text{split}}}{N_{\text{split}} - N_0}.
\]

a result which follows in a very similar way to Eq. (1).

Fitting the minimal model

In order to compare the minimal model with the experimental data, we developed a simulation which grows *Streptomyces* hyphae, implements tip-focus splitting and focus growth, performs the trim to the required length, and extracts the distributions (see Materials and Methods). We used the parameters listed in Table 1 with \( v, \beta, \) the mean initial focus size \( \langle N_0 \rangle \), and the mean focus size for branch initiation \( \langle N_{\text{br}} \rangle \) inferred from experiments (see above), and with the standard deviations in \( N_0 \) and \( N_{\text{br}} \), that is \( \delta N_0 \) and \( \delta N_{\text{br}} \), and \( \gamma \) fitted to the experimentally determined tip-to-branch and branch-to-branch distributions at 80 μm trim. We find that variations in just \( N_0 \) and \( N_{\text{br}} \) are sufficient to fit all the measured distributions. For simplicity we take \( N_0 \) and \( N_{\text{br}} \) to follow independent truncated Gaussian distributions, where the truncation ensures that \( N_0 \) and \( N_{\text{br}} \) are always positive. This is required since Gaussian distributions assign non-zero probabilities to all values, whereas biologically foci cannot contain fewer than zero molecules. The means \( \langle N_0 \rangle \) and \( \langle N_{\text{br}} \rangle \) and standard deviations \( \delta N_0 \) and \( \delta N_{\text{br}} \) are those for the truncated distributions, rather than the full Gaussians. However, as shown in Supporting Text S1, other distributions do not qualitatively change our results.

In our fitting, it was not immediately clear whether \( \langle N_{\text{br}} \rangle \) should be larger or smaller than \( N_{\text{split}} \). Note that although we allow the possibility that \( N_{\text{split}} \) is less than \( \langle N_{\text{br}} \rangle \) in the model, this does not mean that foci can split before they have initiated branches; DivIVA foci have only been observed to split when they are associated with a growing tip. However, \( \langle N_{\text{br}} \rangle \) smaller than \( N_{\text{split}} \) would imply that newly formed branches cannot normally produce their own branches until the tip-focus has grown further to size \( N_{\text{split}} \). This in turn results in a gap between where a branch emerges from its parent hypha and the position of its first offshoot.

We measured this distribution of distances and found no evidence for such a gap (see Supporting Text S1 and Figure S2), which implies that \( N_{\text{split}} \) is equal to (or smaller than) \( \langle N_{\text{br}} \rangle \). In our model we choose \( N_{\text{split}} = \langle N_{\text{br}} \rangle \), although smaller values of \( N_{\text{split}} \) make little qualitative difference.

As shown in Figure 2, there is excellent agreement between the minimal model fits and the experimental data. For the trimmed tip-to-branch distributions, our model is sufficiently simple that this distribution can be calculated analytically (see Supporting Text S1) without recourse to simulations. The analytic prediction is also shown in Figure 2A and agrees extremely well with the simulation data, as expected. Note that the reason the tip-to-branch distribution drops to zero at 80 μm is a consequence of the trimming protocol rather than any inherent property of *Streptomyces*. We chose a 80 μm trim as a trade-off between distribution width and amount of data, but it is also possible to compare the model and the experimental data at other trims. Figures S6 and S9 show that there is also good agreement at trims of 60 μm and 100 μm.

We have checked that the tip-to-branch and branch-to-branch distributions generated by the minimal model are robust to changes in all the parameters in Table 1. Further, we tested that adding fluctuations in the tip growth speed, \( v \), and the on-rate parameter, \( \beta \), also do not qualitatively change these distributions (see Supporting Text S1). There is little to be gained by also considering fluctuations in \( N_{\text{split}} \) since the stochastic nature of tip-focus splitting is already included via \( \gamma \), the tip-focus splitting parameter.

Verifying a model prediction in the tip-to-branch distribution

One of the most striking features of the experimentally measured tip-to-branch distribution, Figure 2A, is the peak at small distances. Naively it may be thought that a novel tip-focus splitting mechanism is required to account for this peak. However, our model predicts that this peak can be simply explained without additional assumptions. Since most new foci must attract more DivIVA molecules before they can initiate a new branch, the distributions of \( N_0 \) and \( N_{\text{br}} \) must be such that most new foci start with fewer than \( N_{\text{br}} \) molecules. However, there is a small tail to the distributions that causes a few foci to have \( N_0 \) above \( N_{\text{br}} \), i.e. when they are formed these foci already have enough DivIVA molecules to initiate branch outgrowth. These foci will cause branching

| Table 1. Main parameters and their values. |
|--------------------------------------------|
| **Parameter** | **Value** |
| Tip growth speed, \( v \) | 8 μm hr\(^{-1}\) |
| Binding parameter, \( \beta \) | 7 \times 10\(^{-5}\) s\(^{-1}\) |
| Mean initial focus size, \( \langle N_0 \rangle \) | 1,700 |
| Standard deviation in initial focus size, \( \delta N_0 \) | 1,000 |
| Mean focus size for branch initiation, \( \langle N_{\text{br}} \rangle \) | 10,000 |
| Standard deviation in focus size for branch initiation, \( \delta N_{\text{br}} \) | 2,600 |
| Minimum tip-focus size for tip-focus splitting, \( N_{\text{split}} \) | 10,000 |
| Tip-focus splitting probability per unit time, \( \gamma \) | 1 \times 10\(^{-3}\) s\(^{-1}\) |

We refer to an online version of this table for a detailed discussion of how the parameters were chosen and what they represent.
almost as soon as they are formed, very close to zero distance from the tip. We have directly observed such events and an example is shown in Figure 3 (see Video S2 for a movie of this figure). Furthermore, we also measured the total intensity of 25 newly-produced foci from time-lapse images: 12 from cases where the new branch appears next to the tip and 13 from normal tip-focus splitting events when the new branch appears much further back. In the first case the average intensity is almost three times greater than in the second case, supporting the hypothesis that events where the branch appears next to the tip correspond to the initial focus size, $N_0$, being much greater than average. The entire weight of the distribution with $N_0 \geq N_{br}$ will give effectively zero tip-to-branch distances, which then naturally explains the peak at the origin in Figure 2A. Consequently, our model predicts that if the distribution is analysed with bins of smaller width, then the peak at the origin will become even more dramatic. After reanalysing the measured data, this prediction is strikingly confirmed, as shown in Figure 4. Although the peak in the $0-1 \mu m$ bin matches well, the agreement is not perfect in the range $1-6 \mu m$. However, we believe this feature is an unavoidable artifact of how the data is analysed: the tip growth speed cannot be measured directly from still images, rather only the distribution of speeds is known, which necessarily slightly smears the data (see Materials and Methods and Supporting Text S1).

**Full model: curvature-dependent tip-focus splitting**

It has been shown that the DivIVA orthologue in *B. subtilis* preferentially assembles on negatively-curved membranes, and this appears to be an important factor in targeting of the *B. subtilis* protein to cell poles and septation sites [14,15]. Similarly, in *Streptomyces*, a preference for branches to emerge on the outer side of curved hyphae has been reported [10], which suggests, for example, that for tips that bend to the left, foci are more likely to form on the right inner membrane. Although the mechanism by which this occurs is not yet fully understood, it is possible to ask how such an effect impacts our model. To do so we developed and simulated a more detailed computational model (see Supporting Text S1), which implements hyphal growth in two-dimensional space. At each time step in the simulation, the direction of tip growth is randomly varied by a small amount, such that over sufficiently long distances (a few $\mu m$), memory of the previous growth direction is lost. We postulate that tip-foci with sizes above $N_{split}$ can split only when the local curvature near the tip is sufficiently high. Hence the earlier focus-splitting parameter, $\gamma$, is understood as an effective parameter that can be replaced by growth direction variation and a curvature threshold. However, it is worth noting that if curvature is the origin of $\gamma$, it must be quite a sensitive effect since during growth the mean curvature near the tip only changes by about 10%. The full model (see Supporting Text S1 for full details and parameters) produces colony dynamics that match well with the wild-type phenotype (for example, see Videos S3 and S4). In particular, the tip-to-branch and branch-to-branch distributions are practically identical to the minimal model, thereby justifying our earlier simplifying assumptions.

**Under- and overexpression of divIVA**

Since DivIVA is an essential protein, it cannot be completely removed. However, we can consider mild underexpression and various levels of overexpression. We first consider heavy overexpression. Previous work has examined hyphal morphology when *divIVA* was overexpressed in preformed hyphae to approximately twenty-five times its usual level [9,10]. Such overexpression resulted in increased levels of cytoplasmic DivIVA, swollen hyphal tips and lateral hyperbranching. Interestingly, after inducing increased DivIVA production, many of the new branches developed well behind the tip positions at the moment of induction. This observation is unexpected since, in the minimal model, foci can only be produced from the splitting of tip-foci. It is possible that these new branches are due to foci that were already present at the time of induction but were too small to be seen, and that overexpression subsequently caused them to develop into branches much more rapidly than normal. However, if this explanation were correct, wild-type *Streptomyces* would form many branches hundreds of microns behind the tips, a strategy which would be very inefficient in terms of nutrition acquisition. For this reason, we favour an alternative explanation, namely that these new branches arise from a separate mechanism of focus formation: spontaneous nucleation. In this process, due to the stochastic dynamics of molecules within the cytoplasm, occasionally a sufficient number of DivIVA molecules come together on the membrane and spontaneously form a cluster.

As is standard for nucleation dynamics [16], and as we confirmed by stochastic simulations, for cytoplasmic DivIVA densities below some threshold, the probability of spontaneous nucleation (involving the near simultaneous binding of multiple DivIVA molecules to overcome a nucleation barrier) is close to zero. Above this threshold, however, we find that the rate of nucleation rises approximately linearly with increasing cytoplasmic density. We assume that for the parameters chosen in Table 1, the DivIVA concentrations during wild-type growth fall well below this threshold and hence spontaneous nucleation does not occur. However, at 25-fold overexpression, this threshold is exceeded. In this latter case, we implemented spontaneous nucleation in our full model in the simplest possible way, by having a probability per unit length and time for spontaneously creating a new focus on the membrane, with a linear increase in nucleation probability with increasing cytoplasmic density above the threshold (see Supporting Text S1 for full details and parameters). We were then able to produce simulated colony dynamics which successfully matched the observed phenotype of 25-fold overexpression (for example, see Video S5).
In addition to heavy overexpression, we can also consider mild under- and overexpression. It was observed in [9] that underexpression seems to reduce the average tip-to-branch distance. It is important to realize that a change in DivIVA expression will probably not only affect the binding parameter $\beta$ (since $\beta \equiv p$, with $p$ the cytoplasmic DivIVA density and $\rho$ a constant), but also the tip growth speed $v$. This is because DivIVA is a critical component of the tip-organizing complex, which is present at all growing tips, and which is presumably important for tip extension. Since $N_0$ and $N_{br}$ are unlikely to depend strongly on DivIVA levels, Eq. (1) shows that it is the ratio $v/\beta$ which controls the average tip-to-branch distance. When DivIVA is underexpressed it is likely that both $v$ and $\beta$ decrease. Since in this case the average tip-to-branch distance decreases, this result suggests that $v$ proportionally decreases by more than $\beta$. In the case of overexpression $\beta$ will increase. However, it is less likely that $v$ will also increase. This is because the tip-organizing complex, which is responsible for tip extension, is likely to consist of many components, of which DivIVA is only one. Unless other components in addition to DivIVA are overexpressed, the effect on tip growth speed could be small, with $v$ remaining approximately constant. Thus we predict that mild overexpression of DivIVA will reduce $v/\beta$ and so decrease the average tip-to-branch distance. If this is the case, then both mild under- and overexpression of DivIVA will reduce the average tip-to-branch distance, with wild-type levels corresponding to the longest tip-to-branch distance.

**Discussion**

Streptomyces, like other bacteria, lack the motor proteins, vesicle transport systems, and polarisome components that are fundamental in eukaryotic cell biology. Thus, tip extension in *Streptomyces* is likely to be simpler than in, for example, filamentous fungi. Given that a complex of polarity proteins (including DivIVA) must presumably first gather at future branch sites, understanding branch-site selection in filamentous bacteria involves understanding where, when and how these proteins cluster together in sufficiently large groups. One surprising feature of wild-type *Streptomyces* is that this clustering of polarity proteins is not a random, spontaneous process. Rather, we have shown that new branch sites are predominantly created from the tips of previous branches, by a tip-focus splitting mechanism.

One important question concerns the benefit of producing foci, and hence branches, by tip-focus splitting rather than spontaneous nucleation. One possibility is that this provides a more efficient method of acquiring nutrients. Spontaneous nucleation will produce new branches at positions well behind the tips. This outcome would be suboptimal since regions far behind the tips are likely to have already been well-exploited, with few remaining nutrients. Tip-focus splitting, on the other hand, only generates new foci at tips and so biases branching towards the growing ends of hyphae, where nutrients are still more plentiful. Another potential advantage is that tip-focus splitting allows for a greater level of control over exactly where branching occurs. Unlike spontaneous nucleation where branches can appear anywhere, tip-focus splitting produces branches with an average tip-to-branch distance determined by parameters such as the initial tip-focus size and the binding parameter. By modifying these parameters, it is possible to respond to external stimuli. For example, under conditions when branching further from the tip would be favourable, we speculate that this could be achieved by modifying DivIVA (or other proteins that affect its assembly) so that the binding parameter is decreased (this would correspond to a shift from the morphology shown in Figure S10B to that in Figure S10A).

The morphology of branching organisms can be characterized by both the distance from the tip that new branches appear and the inter-branch distance. Counter-intuitively, our model shows that these distances are controlled by rather different processes. The tip-to-branch distance is governed by how long it takes new foci to gather enough molecules to initiate a new branch. This is related to the initial focus size, $N_0$, the size at which a new branch is initiated, $N_{br}$, the tip growth speed, $v$, and the binding parameter, $\beta$. In contrast, the branch-to-branch distance is governed by how often foci are formed (how long foci take to develop into branches is now irrelevant). This is dependent on a partly overlapping, but nevertheless distinct set of parameters: the minimum tip-focus size for splitting, $N_{split}$, the initial focus size, $N_0$, the tip growth speed, $v$, the binding parameter, $\beta$, and the tip-focus splitting parameter, $\gamma$.

We have focused on the control of branching during vegetative growth. However, there is a parallel question about how the first germ tube emerges from a spore. By imaging germinating spores expressing functional divIVA-EGFP, it has been shown that, exactly as in vegetative growth, a focus of DivIVA is first observed on the spore envelope, which then grows in size before initiating the first branch [9]. It is interesting to inquire how this first focus is formed. It is clear that the tip-focus splitting mechanism cannot be responsible since there are no previous DivIVA foci from which the first focus could arise. It is possible that other proteins, such as SsgA [17], aid DivIVA focus formation during spore germination. However, there is another possibility, that the spontaneous nucleation mechanism which plays a role when DivIVA is heavily overexpressed, is also responsible for the first DivIVA focus in a spore. If this is the case, then the DivIVA concentration within a spore would have to first rise high enough to overcome the nucleation barrier, an effect which may well be testable.

In fungi, branching also occurs at the cellular level and involves establishment of new cell poles at which apical growth will occur [18]. An apical cluster of vesicles and cytoskeletal elements named the Spitzenkörper has a prominent role in fungal tip extension. During branching, a new Spitzenkörper structure is established at the nascent branch tip, aided by proteins that direct cell polarity, cytoskeletal reorganisation, vesicle transport, and exo- and endocytosis (for reviews, see e.g. [18–21]). One of the components that appears to be involved in branch site selection prior to assembly of the Spitzenkörper structure is the protein complex termed the polarisome. Homologs of the budding yeast polarisome component Spa2p have been detected at hyphal tips in several fungi, and intriguingly, in *Neurospora crassa*, small foci of SPA-2-GFP were observed to detach from the major SPA-2 assemblies at elongating hyphal tips and subsequently give rise to new lateral.
branches [12]. This observation strongly suggests that, in addition to streptomycetes, tip-focus splitting mechanisms are also involved in the establishment of new hyphal branches in filamentous fungi. Streptomycetes appear to regulate hyphal growth and branching in a simple way. Indeed, we have found that a remarkably simple model can quantitatively explain the statistical properties of the entire hyphal network. Even the bimodal nature of the tip-to-branch distribution originates from a single mechanism of forming new foci, combined with variation in the parameter values. It is tempting to speculate that tip-focus splitting might be used by many filamentous organisms amongst fungi and Actinobacteria. In fact, focus splitting could turn out to be a general mechanism in situations where discrete foci must be generated in a growing organism.

Materials and Methods

Strains, general methods and microscopy

_Scoedoeletrum A3(2)_ strains M600 (SCP1− SCP2−), M145 (SCP1− SCP2−) and K112 [divIVA+ /Δ(divIVA− egfp)Hyb], which produces DivIVA-EGFP, were pregerninated and cultivated at 30°C in YEME medium [22]. Hyphae were prepared for microscopy as described previously [9]. Samples were observed through a DIC 63× objective of a Nikon Eclipse 800 microscope equipped with a Pixera ProES600 camera and still images were taken with Pixera software and processed with ImageJ (National Institute of Health USA).

Time-lapse imaging

Live cell time-lapse microscopy was performed essentially as described in [10]. In brief, hyphae of _Scoedoeletrum_ strains were grown on 1% agarose pads with Oxoid antibiotic medium no. 3. Pads were sealed to the bottom by an oxygen-permeable Lumox Biofoil 25 membrane (Greiner Bio-One) and to the top by a coverslip. Samples were incubated at 24°C to 27°C and observed using a Zeiss Axio Imager Z1 microscope, a 9100-02 EM-CCD camera (Hamamatsu Photonics), and Volocity 3DM software (Improvision). Images were captured every 6 minutes, processed by Velocitiy and analysed using ImageJ.

Measurement of tip-to-branch distances

Still images do not normally capture the exact instant at which a new branch emerges. To find the tip-to-branch distance at the moment the branch emerged, we measure the length of the new branch, calculate how long it has been growing for, and determine where the tip was when the new branch emerged. The calculation incorporates an initial speed for new branch growth of about half the base. (C) This focus develops into a branch after the tip has trimmed the base. (A) A growing branch which will be measured when it has emerged from the tip is included in the data set. The effect of trimming is to ensure that all measured hyphae are effectively of length _L_. As a consequence, both the tip-to-branch and branch-to-branch distributions explicitly depend on the trimming length _L_.

Estimation of average tip-to-branch distance

Estimating the average tip-to-branch distance from still images is complicated by the need to impose the trimming protocol on all measured data. The true average tip-to-branch distance is the average tip-to-branch distance at infinite trim. Distributions at progressively smaller trims have progressively smaller average tip-to-branch distances. The largest trim that we have a reasonable amount of data for is 120 μm, with an average tip-to-branch distance of 67 μm. It is not obvious that this trim is sufficiently high to give a good estimate of the true average tip-to-branch distance. However, by fitting the full distributions at 60 μm, 80 μm and 100 μm trims and extrapolating to infinite trim, this is seen to be a good approximation to the true average.

Simulation details

We give details of the minimal model simulation here; details of the full model simulation can be found in Supporting Text S1. We simulate the growth of a single hypha starting with a single DivIVA focus at the tip (initially of size _N_0) and keeping track of where branches appear. At each time step (Δt = 10−5 s), the hypha length is increased by _d_L_, the tip-focus is increased in size according to _ΔN_ = _β_Δt, and the tip-focus splitting rules are implemented (i.e. a tip-focus above _N_0 has a probability γΔt of splitting). If a new focus is created then its initial and final sizes, _N_0 and _N_0, are chosen at random from truncated normal distributions, after which Eq. (1) gives the tip-to-branch distance. After the hypha has grown to sufficient length (we grow the hypha to twice the trim length in order to effectively randomise the initial conditions), the tip-to-branch and branch-to-branch distances are measured if they satisfy the trimming protocol with trim _Λ_, i.e. tip-to-branch distances are recorded only if the branch appears within a distance _Λ_ of the tip, and branch-to-branch distances are recorded only if both branches are within a distance _Λ_ of the tip.

Supporting Information

Figure S1 Tip growth speed against time in Oxoid antibiotic medium for an established hypha and a newly formed branch. Error bars show the standard error of the mean.

Figure S2 Experimental distribution of distances from parent hypha to first offshoot at 35 μm trim. 44 data points.

Figure S3 Comparison of model histograms at 80 μm trim with _N_0 = 1,700 and _N_0 = 3,000. (A) Tip-to-branch distribution. (B) Branch-to-branch distribution.

Figure S4 Comparison of histograms at 80 μm trim for linear growth model (_N_ = _β_ _N_, parameters in Table 1) and constant growth model (_N_ = _β_0, _v_ = 8 μm hr−1, _β_0 = 0.29 s−1, _N_0 = 1,300, _δN_0 = 850, _⟨N_0⟩ = 10,000, _δN_0 = 3,000, _γ_ = 2.5 × 10−3 s−1, _N_0 = 10,000). (A) Tip-to-branch distribution. (B) Branch-to-branch distribution.

Figure S5 Analytic tip-to-branch distribution with infinite trim. This represents the “true” underlying distribution which can never be directly measured experimentally.

Figure S6 Requirement for a branch to be included in the data set. (A) A growing branch which will be measured when it has grown another _Δμm_. (B) A new focus is created at distance _x_ from the base. (C) This focus develops into a branch after the tip has
grown a further $L_\text{um}$, i.e. this branch has a tip-to-branch distance of $L_\text{um}$. (D) Only branches within A of the tip are used to collect data. So this branch will only be recorded if $x + L < \Lambda$.  

**Figure S7**  Behaviour of the mode of the tip-to-branch distance distribution as a function of various model parameters, for both an infinite trim (blue line) and an 80 $\mu_\text{m}$ trim (red line). The infinite trim line is always higher than the 80 $\mu_\text{m}$ trim line. The black dotted line shows the wild-type parameter value. (A) As a function of the binding parameter, $\beta$. (B) As a function of the mean initial focus size, $\langle N_0 \rangle$. (C) As a function of the mean focus size for branch initiation, $\langle N_b \rangle$.  

**Figure S8**  Comparison of distributions between the minimal model and experimental data at 60 $\mu_\text{m}$ trim. Analytic tip-to-branch distribution is also shown (curved line). (A) Tip-to-branch distribution. 1876 experimental data points. (B) Zoomed tip-to-branch distribution. 1215 experimental data points.  

**Figure S9**  Comparison of distributions between the minimal model and experimental data at 100 $\mu_\text{m}$ trim. Analytic tip-to-branch distribution is also shown (curved line). (A) Tip-to-branch distribution. 297 experimental data points. (B) Zoomed tip-to-branch distribution. 257 experimental data points.  

**Figure S10**  Schematic of colony morphology for various values of the binding parameter, $\beta$. Red dots represent DivIVA foci. (A) Small value of $\beta$. (B) Wild-type value of $\beta$. (C) Large value of $\beta$.  

**Text S1**  Supporting text.  

**References**

1. DePodo MA, Quintel JC, Holje JV, Schwarz H (1997) Murein segregation in Escherichia coli. J Bacteriol 179: 2823–2834.  
2. Daniel RA, Errington J (2003) Control of cell morphology in bacteria: two distinct ways to make a rod-shaped cell. Cell 113: 767–776.  
3. Margolin W (2009) Sculpting the Bacterial Cell. Curr Biol 19: R812–822.  
4. Garner EC, Bernard R, Wang W, Zhuang X, Rudner DZ, et al. (2011) Annu Rev Microbiol 65: 223–240.  
5. Dominguez-Escobar J, Chastanet A, Crevon AH, Fromion V, Wedlich-Soldner R, et al. (2011) Processive movement of MreB-associated cell wall biosynthetic complexes in bacteria. Science 333: 225–228.  
6. Garner EC, Bernard R, Wang W, Zhuang X, Rudner DZ, et al. (2011) Coupled, circumferential motions of the cell wall synthesis machinery and MreB filaments in B. subtilis. Science 333: 222–225.  
7. Flarkh K, Butter MJ (2009) Streptomyces morphogenesis: dissecting differentiation in a filamentous bacterium. Nat Rev Microbiol 7: 36–49.  
8. Flarkh K (2010) Cell polarity and the control of apical growth in Streptomyces. Curr Opin Microbiol 13: 756–765.  
9. Flarkh K (2003) Essential role of DivIVA in polar growth and morphogenesis in Streptomyces coelicolor A3(2). Mol Microbiol 49: 1523–1536.  
10. Hempel AM, Wang S, Letek M, Gil JA, Flarkh K (2008) Assemblies of DivIVA mark sites for hyphal branching and can establish new zones of cell wall growth in Streptomyces coelicolor. J Bacteriol 190: 7579–7583.  
11. Wadhwa J, Gillespie MD, Kekemen GH (2010) A novel coiled-coil repeat variant in a class of bacterial cytoskeletal proteins. J Struct Biol 170: 202–213.  
12. Arauzo-Palomares CL, Riquelme M, Castro-Longoria E (2009) The polarisome component SPA-2 localizes at the apex of Neurospora crassa and partially colocalizes with the Spitzenkörper. Fungal Genet Biol 46: 551–563.  
13. Lu C, Stricker J, Erickson HP (1998) FisZ from Escherichia coli, Azotobacter vinelandii, and Thermotoga maritima: quantitation, GTP hydrolysis, and assembly. Cell Motil Cytoskeleton 40: 71–86.  
14. Ramamurthi KS, Losick R (2009) Negative membrane curvature as a cue for subcellular localization of a bacterial protein. Proc Natl Acad Sci U S A 106: 13541–13545.  
15. Lenarcic R, Halbedel S, Visser L, Shaw M, Wu LJ, et al. (2009) Localisation of DivIVA by targeting to negatively curved membranes. EMBO J 28: 2272–2282.  
16. Howard J (2001) Mechanics of Motor Proteins and the Cytoskeleton. Massachusetts: Sinauer Associates. 384 p.  
17. Noens, et al. (2009) Loss of the controlled localization of growth stage-specific cell-wall synthesis pleiotropically affects developmental gene expression in an sgl mutant of Streptomyces coelicolor. Mol Microbiol 64: 1244–1259.  
18. Harris SD (2009) Branching of fungal hyphae: regulation, mechanisms and comparison with other branching systems. Mycologia 100: 823–832.  
19. Fischer R, Zekert N, Takeshita (2008) Polarized growth in fungi – interplay between the cytoskeleton, positional markers and membrane domains. Mol Microbiol 68: 813–826.  
20. Steinberg G (2007) Hyphal growth: a tale of motors, lipids, and the Spitzenkörper. Eukaryotic Cell 6: 351–360.  
21. Riquelme, et al. (2011) Architecture and development of the Neurospora crassa hypha – a model cell for polarized growth. Fungal Biol 115: 446–474.  
22. Kieser T, Bibb MJ, Butter MJ, Chater KF, Hopwood DA (2000). Practical Streptomyces Genetics. Norwich (UK): The John Innes Foundation. 613 p.