Statistical analysis of the growth and morphology of the filamentous microbe *Streptomyces coelicolor*

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1 Introduction

Streptomycetes are commonly occuring soil bacteria that grow through extension and branching of hyphal filaments (Figure 1). This morphological growth pattern, which is rare for bacteria and interesting in its own right, is thought to be connected to the onset of secondary metabolite production. Streptomycetes are a rich source of biologically active secondary metabolites, some of which are potent antibiotics, growth promotors or anti-cancer agents (see Flardh and Buttner 2009 for a full description of the organism's life cycle). *Streptomyces* is therefore not only a fascinating model system for morphological studies but also of high pharmaceutical importance for its natural products.
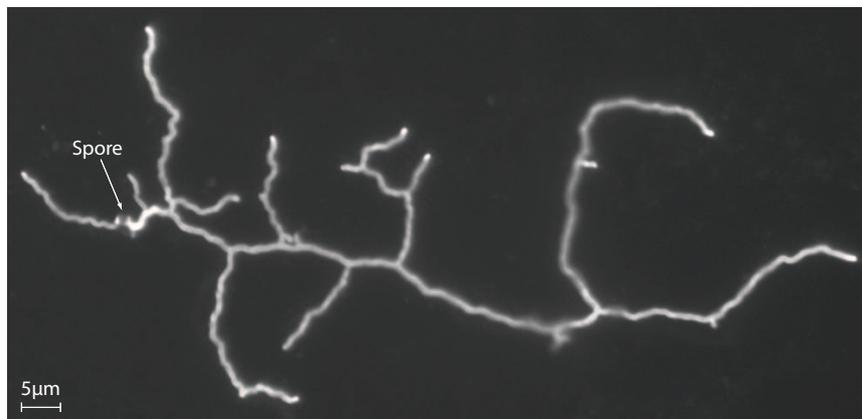

Figure 1: Example image of *S. coelicolor* (wild-type strain) grown on solid media for 16 hours.

Our research has focussed on understanding and modelling the early-stage growth and morphology of the model organism *Streptomyces coelicolor*. Part of this work has involved collecting thousands of microscope images and developing techniques to gather and analyse statistics from them. The data produced by this work is being used to study the differences between wild-type and mutant strains, as well as informing and validating spatial growth models.

2 Data gathering

Samples of *S. coelicolor* were grown on cellophane discs laid over solid media, an approach which encourages 2-dimensional growth. Preparation for microscopy involved methanol fixing and staining with a fluorescent wheat germ agglutinin (WGA) conjugate and propidium iodide

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(PI). The former binds to bacterial cell wall components and provides good contrast profiles of the organism's shape; the latter binds to nucleic acids, allowing easy identification of spores. Fluorescence microscopy was carried out using a Zeiss AxioImager upright microscope with 63x and 100x oil-based lenses. Channel images were saved as PNG files.

Spores were innoculated and incubated for 6 to 18 hours and samples were taken at 2 hour intervals. Imaging this process thus provides a series of snapshots of spatial patterns from which we aim to recover the morphological development of the growing bacteria.

3 Image analysis

We have developed software to provide semi-automatic analysis of images of filamentous microbes. This software has been used successfully for processing *Streptomyces* growth pattern images but it is of sufficient generality to be applicable for a wide range of similar image analysis problems, for instance fungal growth, moss development, root hairs, veins, and nerves.
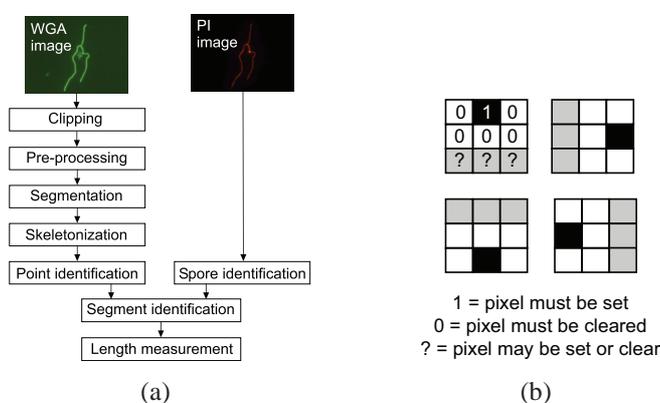

Figure 2: Image processing: (a) The steps involved in processing images and extracting morphological data. (b) Masks used for locating tip points.

Each microscope image was loaded into the software and underwent an 8-stage process, as depicted in Figure 2a. In the pre-processing stage, the image is cropped and a Gaussian filter applied to smooth the image and remove noise (see, for example, Sonka *et al.* 1999). Threshold segmentation (see also Sonka *et al.* 1999) is used to find the organism's shape and to remove image background. This results in a black and white image with white pixels for bacteria and black pixels for background. The image is then thinned to single pixel width by applying two common skeletonization algorithms in sequence - firstly that of Zhou *et al.* 1995, then that of Zhang and Suen 1984.

Once this process has produced a skeleton image, algorithms are applied to identify tip points and branch points. For branch points, we perform a raster scan of the skeleton image, looking for any white pixel which has three or more neighbours in the 8-neighbourhood. Tip points are identified by running a set of 3-by-3 pixel templates over the image which seek out characteristic patterns (Figure 2b). The spore point has to be manually selected by the user, but this is easily recognised from the propidium iodide channel image.

The skeleton is then divided up into segments - that is, a piece of connected filament bounded by any two key points (branch, spore or tip). A path following algorithm (Figure 3a) is applied to build up segment information by tracing the skeleton path between points. Using this information, the software calculates segment lengths with a standard arclength calculation and scaling factors provided with the microscope.

Path following algorithm

1 Mark all skeleton pixels as *unvisited*.
2 Remove the next tip point from the list of tip points and set *currentPosition = tipPoistion*.
3 Look in the 8-neighbourhood around *currentPosition* for an unvisited pixel. If the pixel is a tip point or branch point, proceed to step 6.
4 Mark pixel as visited and set *currentPosition = pixelPosition*.
5 Repeat steps 3-5.
6 If last pixel is a branch point, check if it needs to be re-classified by counting the number of *unvisited* pixels in the 8-neighbourhood.
7 Repeat steps 2-7 until no more tip points.

(a)

Filament classification algorithm

1 Mark all segments as *unvisited*.
2 Set *startPoint = sporePoint* and *d* = 0.
3 Increment *d*.
4 Build a list of all paths from *startPoint* to all tip points, travelling along only unvisited segments.
5 Find longest path in list and mark each segment of it as *visited* and with depth of *d*
6 For each branch point in the longest path, set *startPoint = branchPoint* and repeat steps 3-6.

(b)

Figure 3: Two algorithms implemented in the software: (a) Path following algorithm used for segment identification. (b) Filament classification algorithm used to determine the filament depth.

4   Calculation of biometrics and statistics

With all key points in an image identified (Figure 4a) and with lengths measured, the software calculates a range of biometrics and statistics. Basic biometrics include the total hyphal length, the number of tips, the area covered by the bacteria and the number of spore germ tubes. Histograms are generated of internal segment lengths (the lengths between branch points), distances from tip to nearest branch point and a range of other metrics. Line graphs are plotted of mean values of metrics against time, while scatter plots are used to plot individual bacteria metrics against their total hyphal length (a property which is proportional to growth time).

Spore germ tubes are classified as primary filaments, their children as secondary filaments, their grandchildren as tertiary filaments. We use the term *filament depth* to describe this - a depth of 1 relates to a primary filament, 2 a secondary filament and so on. The relative amounts of filament at each depth is important, as it can be an indicator of the degree to which a bacterium has colonised its enviornment, as well as an indication of various morphological features.

Because our images don't show the development of the same bacterium over time, we cannot classify filaments as they emerge; instead we assume (as is commonly understood) that all tips extend at a constant, rate and apply the algorithm in Figure 3b to classify segmemts according to depth. We can also use the depth data to calculate a measure of hyphal balance. In *Streptomyces*, there are typically two germ tubes, the second emerging later than the first and extending in the opposite direction. Adding up the hyphal length on each side of the spore enables insights into the symmetry of the organism.

The positioning of branches within filaments is considered of biological significance. Our software looks through the list of tip points, identifying any where the distance to the nearest branch point is less than a threshold value (typically 1.5μm). This tip is classed as an emerging tip and the nearest branch point provides the point of branching on the parent filament. This parent branch point will be connected to a previous branch point which represents either its own birth from another filament, or the site of its previous child branch. New branches tend to form between the tip and the previous branch, so the parent branch point is usually connected to a tip point which represents the parent filament's active tip. The distances from the parent's active tip to the child branch site and from the child branch site to the previous branch point are useful indicators of branching behaviour. We can use this information to plot a histogram showing the distribution of new branch locations on the parent branch (Figure 4b).

The software also seeks to identify apical branching. In wild-type *S. coelicolor*, new branches predominantly form behind a tip, but in some fungi and mutants, the growing tip can split into two. Such cases are identified by looking for two emerging tips which share the same parent branch point.
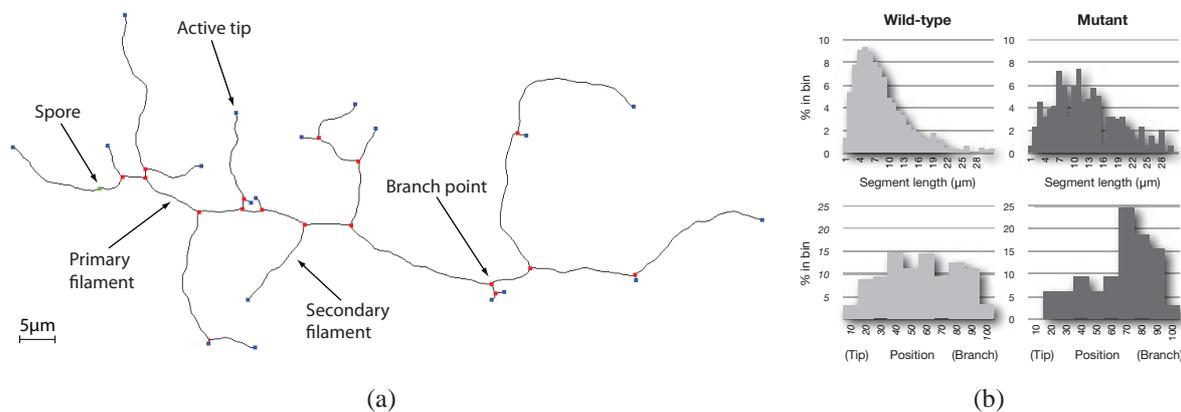

(a)　　　　　　　　　　　　　　　(b)

Figure 4: Example results: (a) The bacterium from Figure 1 after image processing and analysis. (b) Histograms for segment length and position of child branch for the wild-type and a mutant.

5 Discussion

We have presented the methodology underlying our automated filamentous growth image analysis software tool. The main developments include algorithms for noise reduction, skeletonization, tip and branch point identification, as well as routines to perform statistical analyses on the derived data. Using the tool, we have begun to analyse a large collection of data from wild-type *S. coelicolor* and from a number of mutants which display markedly different branching (Figure 4). The data generated so far with our software is enabling us to highlight a number of morphological differences which had not previously been obvious and to provide quantitative analysis of anecdotal observations, with the goal of producing spatial models of growth. We are in the process of developing ideas about the biological mechanisms involved and, in so doing, hope to shed light on the function of the genes which are affected in the mutants.

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