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*Caenorhabditis elegans*

## Plague bacteria biofilm blocks food intake

Bubonic plague is transmitted to mammals, including humans, by the bites of fleas whose digestive tracts are blocked by a mass of the bacterium *Yersinia pestis*<sup>1</sup>. In these fleas, the plague-causing bacteria are surrounded by an extracellular matrix of unknown composition<sup>2</sup>, and the blockage depends on a group of bacterial genes known as the *hmsHFRS* operon<sup>3</sup>. Here we show that *Y. pestis* creates an *hmsHFRS*-dependent extracellular biofilm to inhibit feeding by the nematode *Caenorhabditis elegans*. Our results suggest that feeding obstruction in fleas is a biofilm-mediated

process and that biofilms may be a bacterial defence against predation by invertebrates.

A biofilm is a population of adherent bacteria enclosed by a matrix<sup>4</sup>. When *C. elegans* is exposed to *Y. pestis* or to the closely related bacterium *Yersinia pseudotuberculosis*, biofilms become visible on the worms' heads within 1 h. The biofilms increase in size with continuing exposure, eventually covering the mouth completely (Fig. 1a). Bacterial cells rarely come into direct contact with the worms — they are not found at all in interior tissues (Fig. 1a, b) and there is no other evidence of infection.

*C. elegans* and many other invertebrates eat bacteria, and microbial synthesis of a biofilm that inhibits predatory feeding therefore has obvious survival value. To demonstrate that the biofilm produced by *Yersinia* prevents feeding by *C. elegans*, we allowed biofilms to accumulate on *phm-2* mutant nematodes<sup>5</sup>, which pass many unlysed bacteria through the pharynx when feeding. We placed the worms on lawns of *Escherichia coli* expressing green fluorescent protein (GFP) and monitored the presence of GFP in the gut, which indicated an ability to feed.

The intestines of 99.0% ( $n=238$ ) of unexposed controls (Fig. 1c) were positive for GFP, whereas only 2.9% ( $n=171$ ) of worms exposed to *Y. pseudotuberculosis* (Fig. 1d) and 46.1% ( $n=107$ ) of those exposed to *Y. pestis* were positive. The lesser

inhibition by *Y. pestis* was due to the organism's nutritional defects<sup>6</sup> — when grown on a richer medium, it allowed only 7.8% ( $n=166$ ) of worms to feed.

*C. elegans* grows through four larval stages to adulthood, but animals that are deprived of food fail to develop normally. Starting with eggs of uniform age, 99.4% of animals grown on an *E. coli* control strain developed to the fourth larval (L4) stage within 2 days. For worms exposed to *Y. pestis* and *Y. pseudotuberculosis*, the proportions were 6.9% and 43.9%, respectively. With *Y. pestis* grown on richer medium, only 2.4% of nematodes reached the L4 stage.

Random screening produced a transposon-insertion mutant of *Y. pseudotuberculosis* that allowed normal nematode growth and failed to produce a biofilm. The mutation was in *hmsT*, which was previously identified as a positive regulator of the *hmsHFRS* operon in *Y. pestis*<sup>7</sup>. We created *Y. pestis* and *Y. pseudotuberculosis* strains with mutations in *hmsHFRS* and found that these bacteria failed both to produce biofilms and to inhibit nematode growth.

Homologues of HmsF and HmsR are required for the synthesis of polysaccharide components of biofilms by *Staphylococcus epidermidis*<sup>8,9</sup>. HmsR is 39% identical to staphylococcal IcaA, a glycosyltransferase that catalyses the assembly of large polymers of *N*-acetyl glucosamine residues<sup>8</sup>. HmsF is 23% identical to IcaB, a predicted staphylococcal polysaccharide deacetylase.

In fleas, plague bacteria blocking the digestive tract are surrounded by an electron-dense matrix, the composition of which has not been described<sup>2</sup>. Because flea blockage and biofilm formation on nematodes both require HmsHFRS proteins, our results suggest that the matrix in fleas also includes a bacterially synthesized biofilm. Because biofilms are highly resistant to flushing with fluids, the presence of a biofilm may help to explain why *Y. pestis* is not dislodged from the flea gut by the pumping and threshing action of the insect's digestive system<sup>10</sup>.

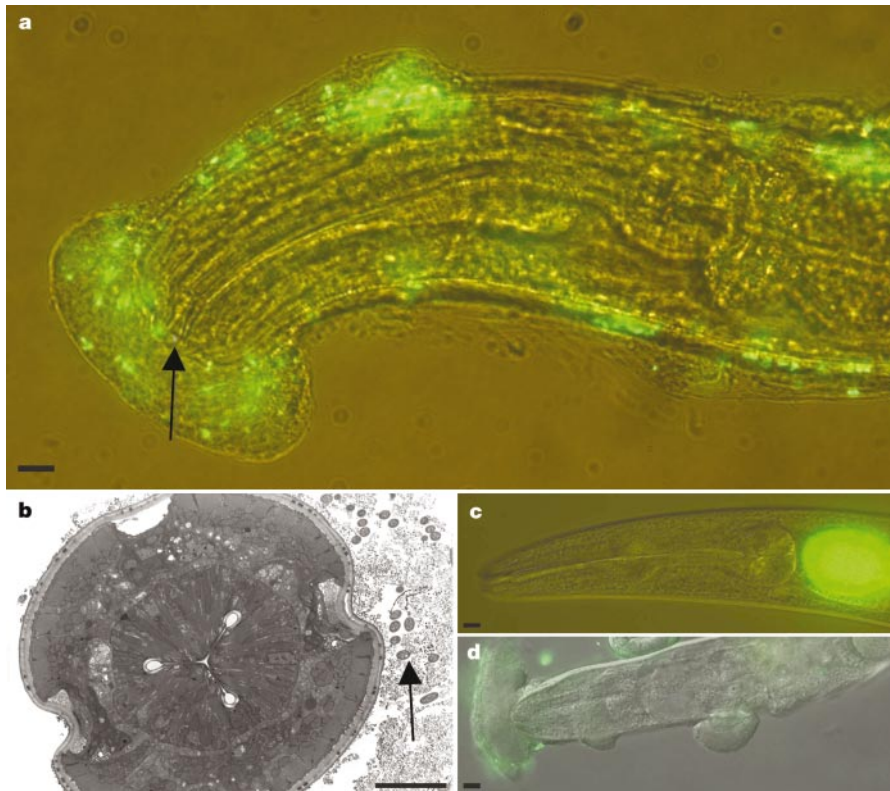
Genetic analysis of both sides of the bacteria–nematode interaction is feasible, as *C. elegans* mutants that are resistant to biofilm formation can readily be obtained by chemical mutagenesis (C.D., unpublished results). The *Yersinia*–*C. elegans* model therefore offers a potentially useful experimental system in which to investigate biofilm-mediated interactions between bacteria and invertebrates.

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**Figure 1** *Yersinia pseudotuberculosis* biofilms on *Caenorhabditis elegans*. **a**, *Y. pseudotuberculosis* expressing green fluorescent protein (GFP) embedded in a biofilm on the surface of *C. elegans*. Arrow shows the position of the worm's mouth. **b**, Transverse section through the *C. elegans* head. The biofilm has a granular appearance; arrow denotes a cluster of bacteria within the film. **c**, Control *C. elegans* without a biofilm after feeding on *Escherichia coli* expressing GFP; bright signal is in the anterior of the intestine. **d**, *C. elegans* after attempting to feed on *E. coli* expressing GFP in the presence of a biofilm produced by *Y. pseudotuberculosis*. Scale bars, 5  $\mu$ m.

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Phylogeny

## A non-hyperthermophilic ancestor for Bacteria

The first phyla that emerge in the tree of life based on ribosomal RNA (rRNA) sequences are hyperthermophilic, which led to the hypothesis that the universal ancestor, and possibly the original living organism, was hyperthermophilic<sup>1</sup>. Here we reanalyse the bacterial phylogeny based on rRNA using a more reliable approach, and find that hyperthermophilic bacteria (such as Aquificales and Thermotogales) do not emerge first, suggesting that the Bacteria had a non-hyperthermophilic ancestor. It seems that Planctomycetales, a phylum with numerous peculiarities, could be the first emerging bacterial group.

rRNA is a useful tool for investigating the universal phylogeny of life, particularly for uncultured organisms<sup>2</sup>. The phylogeny of Bacteria based on rRNA is congruent

with genomic data, such as phylogenetic trees based on multiple genes<sup>3,4</sup>. This suggests that rRNA is rarely transferred and indicates that species phylogeny can be deduced by using this marker.

However, rRNA phylogenies can be seriously affected by artefacts of tree construction<sup>5</sup>. As the most slowly evolving positions are less prone to confounding factors such as multiple substitutions, they probably retain an ancient phylogenetic signal<sup>6</sup>. Phylogenies constructed by examining these positions are less affected by artefacts. We used the ‘slow–fast’ method<sup>7</sup> to identify these positions, which has proved efficient in analysing eukaryotic phylogeny based on rRNA<sup>5</sup>.

There are several discrepancies between the standard phylogeny<sup>2</sup> and the one we have deduced using the most conserved positions (Fig. 1). Our version shows hyperthermophilic Bacteria (Aquificales and Thermotogales) to be monophyletic, which is consistent with large-scale studies<sup>3,4</sup>, instead of paraphyletic as in classical versions. More important, our phylogeny shows the late emergence of hyperthermophiles, as they are clustered with Fusobacteria within a vast multifurcation containing almost all the phyla.

Surprisingly, Planctomycetales emerge at the base of the Bacteria. Whereas the support for the early branching of hyperthermophilic Bacteria is low (bootstrap value about 20%), slowly evolving positions (Fig. 1) provide reasonable, albeit inconclusive, support for the early emergence of Planctomycetales (bootstrap value about 70%). The basal position of hyperthermophilic phyla is found only when examining noisy (that is,

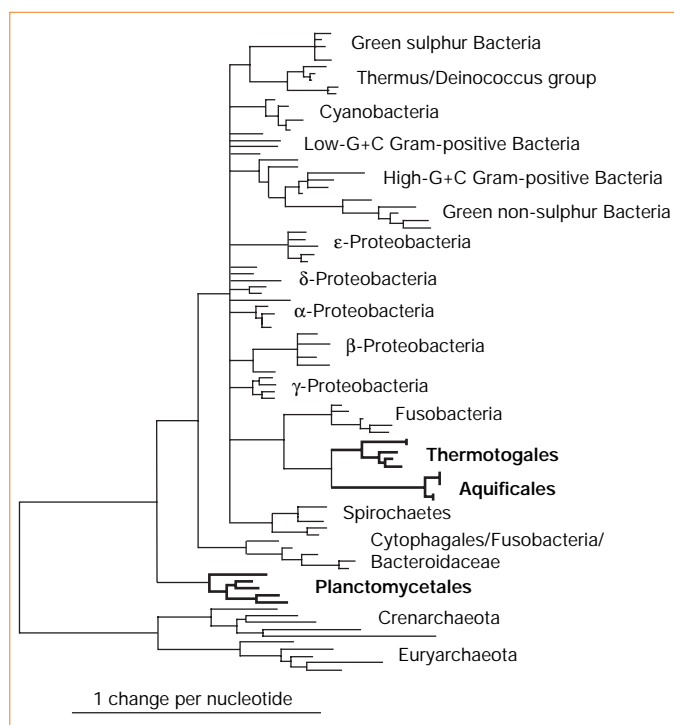
fast-evolving) positions, and is thus probably artefactual. The artefactual attraction of the long branches<sup>8</sup> of Archaea and hyperthermophilic Bacteria, and the high guanine and cytosine content of rRNA in most Archaea, Aquificales and Thermotogales<sup>9</sup>, could explain why hyperthermophiles are often found to branch early in Bacteria.

The emergence of hyperthermophilic Bacteria among mesophiles suggests a secondary adaptation to life at very high temperature for these organisms, which may have been facilitated by massive gene transfer from the Archaea<sup>10</sup>. The most convincing support for this idea is provided by the enzyme reverse gyrase, which is specific to hyperthermophiles<sup>11</sup>. This enzyme, which introduces positive supercoils in DNA and is thought to stabilize it at high temperatures, has been independently acquired by *Aquifex* and *Thermotoga* from Archaea<sup>11</sup>. Consistent with the ancestral guanine and cytosine content of rRNA<sup>9</sup>, our results indicate that the most recent common ancestor of Bacteria was not hyperthermophilic.

We find that Planctomycetales are the first branching bacterial group (Fig. 1). This is a significant, although understudied, division of Bacteria, whose members share several peculiar traits, such as a budding mode of reproduction and the lack of peptidoglycan in their cell walls<sup>12</sup>. The most intriguing feature of this group is the existence of a single or double membrane around the chromosome in *Gemmata* sp. and *Pirellula* sp., respectively, which has been compared to the eukaryotic nucleus<sup>12</sup>. However, evolutionary homology of these structures with the eukaryotic nucleus has not been proved. An early emergence of Planctomycetales, as inferred from our work, must be confirmed by a careful phylogenetic analysis of genome sequences from several apparently early-branching Bacteria. If our finding is verified, the origin of Bacteria should be seriously reconsidered.

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**Figure 1** Prokaryotic phylogenetic tree based on conserved positions in ribosomal RNA. The ‘slow–fast’ method<sup>7</sup> was used to estimate the rate of evolution at each position as the sum of the number of substitutions within 19 predefined phyla. Alignments were constructed for various thresholds (0–15) and the most parsimonious was inferred using PAUP 4b8 (alignments and trees are available from the authors). The relative positions of Planctomycetales, Aquificales and Thermotogales remain very similar for all thresholds from 1 to 10. The phylogeny shown here is based on the 751 positions with a threshold of fewer than 5 substitutions, which represents 45% of informative positions and is thus a valid compromise between the quality and the quantity of phylogenetic information.

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