

How *Listeria* Exploits Host Cell Actin to Form Its Own Cytoskeleton. II. Nucleation, Actin Filament Polarity, Filament Assembly, and Evidence for a Pointed End Capper

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Abstract. After *Listeria*, a bacterium, is phagocytosed by a macrophage, it dissolves the phagosomal membrane and enters the cytoplasm. The *Listeria* then nucleates actin filaments from its surface. These newly assembled actin filaments show unidirectional polarity with their barbed ends associated with the surface of the *Listeria*. Using actin concentrations below the pointed end critical concentration we find that filament elongation must be occurring by monomers adding to the barbed ends, the ends associated with the *Listerial* surface. If *Listeria* with tails are incubated in G actin under polymerizing conditions, the *Listeria* is translocated away from its preformed tail by the elongation of filaments attached to the *Listeria*. This experiment and others tell us that in vivo filament assembly must be tightly coupled to filament capping and cross-bridging

so that if one process outrips another, chaos ensues. We also show that the actin filaments in the tail are capped on their pointed ends which inhibits further elongation and/or disassembly in vitro. From these results we suggest a simple picture of how *Listeria* competes effectively for host cell actin. When *Listeria* secretes a nucleator, the host's actin subunits polymerize into a filament. Host cell machinery terminate the assembly leaving a short filament. *Listeria* overcomes the host control by nucleating new filaments and thus many short filaments assemble. The newest filaments push existing ones into a growing tail. Thus the competition is between nucleation of filaments caused by *Listeria* and the filament terminators produced by the host.

ALTHOUGH actin is a major constituent of most cells, how the length, polarity, and distribution of individual filaments that comprise the cytoskeleton are regulated remains poorly understood. Obviously actin-associated proteins must be involved, but to decide what role each of the 40 or so actin binding proteins thought to be present in most cells plays is a difficult task, particularly as several may work in concert. The problem becomes even more bewildering because something must orchestrate these proteins so that they in turn can control the position, length, and polarity of the filaments. Even worse the cell is not static, but changes its form depending on the cell cycle, external stimuli, etc.

To try to determine how the cytoskeleton forms and is regulated, we have turned to a simplified system. Our choice is the bacterial pathogen, *Listeria monocytogenes*. This pathogen invades macrophages and other mammalian cells and nucleates actin filament assembly from its surface (Tilney et al., 1990). Being a definable unit, one can concentrate on what it does and how it does it, ignoring the greater complexity of the host cell.

In this paper we define the polarity of the filaments nucleated from the surface of *Listeria* and show that actin

monomers add to the end of the filament (barbed end) associated with the surface of *Listeria*. We then show that actin assembly is coupled to actin filament capping and cross-bridging resulting in the construction of a cross-linked actin-based tail.

Materials and Methods

Bacterial Strains and Growth Conditions

Listeria monocytogenes strain 10403S (Bishop and Hinrichs, 1987) and mutant DP-L1054 (Sun et al., 1990) were used in this study. Mutant DP-L1054 is a transposon mutant containing a Tn917 insertion within a gene encoding phosphatidylinositol specific gene for phospholipase C (Camilli et al., 1991).

Tissue Culture and Growth Medium

These methods are presented in the accompanying paper in this issue (Tilney et al., 1992).

Cytochalasin and Chloramphenicol

Cytochalasin D (Sigma Chemical Co., St. Louis, MO) was dissolved in DMSO and applied to infected macrophages at a final concentration of 0.5

$\mu\text{g/ml}$. Chloramphenicol (Sigma Chemical Co.) was used at a concentration of 20 $\mu\text{g/ml}$ (Tilney et al., 1990).

Detergent Extraction, Addition of Actin, and Decoration with Subfragment 1 of Myosin

To examine the polarity and/or elongation of actin filaments polymerized on the surface of *Listeria* we infected macrophages with either wild-type *Listeria* or the mutant for 30 min, then washed 3 times, and 1 h after the beginning of infection added cytochalasin D for an additional 3 h. The macrophages were then detergent extracted for 20 min at 4°C in 1% Triton X-100 containing 3 mM MgCl_2 and 50 mM phosphate buffer at pH 6.8. The Triton solution was decanted and a solution was added containing recently centrifuged G actin in 50 mM phosphate buffer at pH 6.8, 1 mM MgCl_2 , and 60 mM KCl. In later experiments, to determine to which end monomers were added, we added to detergent extracted, infected cells G actin (at a concentration below the pointed end critical) in a solution consisting of 20 mM Tris, 60 mM KCl, 1 mM MgCl_2 at pH 7.5, rather than phosphate because phosphate lowers the pointed end critical concentration. The plate was placed on a rocker table at room temperature for 20 min. The solution was then decanted and the plate washed with buffer containing MgCl_2 and KCl and then incubated in 5 mg/ml subfragment 1 of myosin (S1) in 0.1 M phosphate buffer at pH 6.8 for 10 min at room temperature and then 20 min at 4°C. After decoration with S1 the petri plates were washed and fixative was added.

We also examined the polarity of the actin filaments from the mutant DP-L1054 that had been grown in macrophages for 4 h. The infected macrophages 4 h after the beginning of infection were extracted in a solution containing 1% Triton X-100 in 3 mM MgCl_2 , 50 mM phosphate buffer at pH 6.8 for 20 min at 4°C. The plate was then incubated in S1 as outlined above.

G actin was obtained by extracting acetone powders of rabbit skeletal muscle by the method of Spudich and Watt (1971) and gel filtering it (Weber et al., 1987). The actin was stored in aliquots in liquid nitrogen until needed at a concentration of 7 mg/ml. The morning before experimentation the actin solution was thawed and centrifuged at 120,000 g for 2 h to remove any small aggregates of F-actin oligomers that had spontaneously formed during freezing that would act as nuclei and induce polymerization. This centrifugation technique gives the same results as gel filtering the actin solution just before use and is much more rapid. Only the top half of the centrifuged actin solution was used and it was pipetted out carefully.

To determine if the filaments comprising the tail of *Listeria* were capped, infected macrophages were washed with Triton, incubated in G actin under polymerizing conditions, decorated with S1, and then fixed, all as outlined above.

To test whether *Listeria* grown in liquid media nucleate actin assembly, we grew wild-type *Listeria* in suspension at 37°C in 10 ml LB broth in a 50-ml conical tube. The *Listeria* were then carefully pipetted on grids coated with formvar with a light carbon coat for 2 min. The excess fluid was removed with a small piece of filter paper and a drop of fluid added which contained 5 μM G-actin, 1 mM MgCl_2 , 60 mM KCl, and 50 mM phosphate buffer at pH 6.8. After 15 min incubation in this medium, the grids were negatively stained with 1% uranyl acetate. To be sure that the monomeric actin was polymerizable and displayed the pointed and barbed end critical concentrations expected based on their protein concentrations, we added to the monomeric actin *Limulus* acrosomes which act as nuclei to induce polymerization of actin (Coluccio and Tilney, 1984). These acrosomes polymerized actin filaments off their ends as would be expected.

Electron Microscopy

Methods are as described in the accompanying paper (Tilney et al., 1992).

Results

Actin Filament Assembly Takes Place from the Surface of *Listeria* Growing In Vitro

When, by mistake, *Listeria* were grown in broth which was not shaken and not aerated and then incubated in gel filtered G-actin under polymerizing conditions, some actin filaments

were now found extending from the *Listeria* (Fig. 1). The number of actin filaments extending from *Listeria* was small, never exceeding a dozen.

The Polarity of the Actin Filaments that are Nucleated from the Surface of *Listeria*

Shortly after the *Listeria* have entered the cytoplasm of the macrophage by dissolving the vacuolar membrane of the phagosome, the *Listeria* coats itself with numerous short actin filaments (Tilney and Portnoy, 1989). In fact, there are so many filaments in this coat that the polarity of individual filaments cannot be determined. Furthermore, if infected macrophages are treated with cytochalasin and then after 2 1/2 h detergent extracted and incubated in 3 μM G actin in vitro under polymerizing conditions, so many filaments assemble from the surface of *Listeria* that again we cannot determine the filament polarity (Tilney et al., 1990).

There are two ways to reduce the number of filaments assembled from *Listeria*. One is to induce polymerization in vitro using very low concentrations of actin, concentrations of 1 μM or less (see Fig. 3), the other is to use a mutant in which the number of filaments assembled from *Listeria* is drastically reduced (Fig. 2).

Mutant DP-L1054 is a mutant in which a gene coding for phosphatidylinositol-specific phospholipase C (Camilli et al., 1991) is interrupted. This ultimately results in the assembly of only a few actin filaments on the surface. The reason behind this will be the subject of a subsequent publication (Camilli, A., L. G. Tilney, and D. A. Portnoy, unpublished observations). Macrophages were infected with this mutant and after 3 h were detergent extracted and the actin filaments decorated with S1. In all cases the filaments attached to *Listeria* display unidirectional polarity with the "arrowheads" pointing away from the *Listeria* surface (data not shown). The barbed ends are always associated with the *Listeria* surface.

We also determined the polarity of the actin filaments assembled from the *Listeria* in vitro. Macrophages were infected with wild type and/or the mutant, then after 1 h cytochalasin D was added to prevent further assembly. After 3 h had elapsed in cytochalasin D, the cells were detergent extracted to remove endogenous actin and exogenous G actin was added under polymerizing conditions. The newly assembled actin filaments were subsequently decorated with S1 before fixation (Figs. 2 and 3). Again the filaments assembled from *Listeria* have unidirectional polarity with the "arrowheads" pointing away from the *Listeria*. We examined many hundreds of micrographs and scored only those that are physically connected to the *Listeria*. Over 95% show an identical polarity.

To Which End of the Filament Do Monomers Add?

Although filaments assemble from the surface of the bacterium with their barbed ends associated with the bacterium, elongation of the filament could occur by the addition of subunits to either the barbed or pointed end or both. Two different experiments were carried out; both of these are consistent with addition of monomers exclusively to the barbed ends of the filaments, the ends associated with the surface of *Listeria*. The first experiment is included in this subhead-

1. Abbreviation used in this paper: S1, subfragment 1 of myosin.

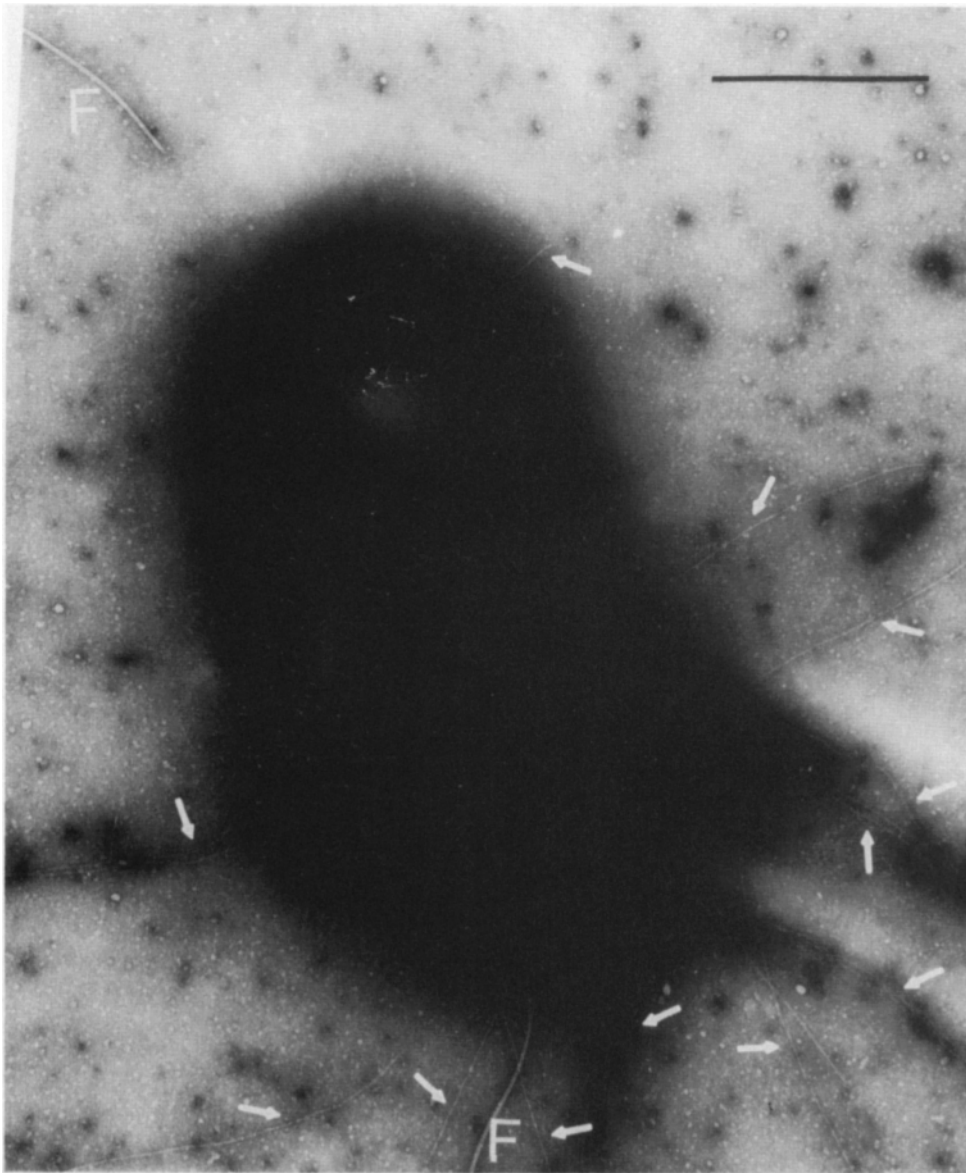


Figure 1. Listeria which had been incubated in monomeric actin under polymerizing conditions, then negative stained. Extending perpendicularly from the surface of this bacterium are a few actin filaments (*arrows*) and a flagellum (*F*). Bar, 1 μ m.

ing. The second is presented in the last section of Results. The critical concentration for assembly of actin in vitro is different on each end, 0.1 μ M for the barbed end, 0.7–0.9 μ M for the pointed end (Bonder and Mooseker, 1983; Coluccio and Tilney, 1984; Pollard, 1986). What was done was to treat infected macrophages with cytochalasin for 3 h, then extract them with detergent and add 0.5 μ M actin, a concentration below the pointed end critical but above the barbed end critical, under polymerizing conditions in Tris buffer containing $MgCl_2$ and KCl. What we observed was that assembly of filaments occurred off the *Listeria* surface. Thus the monomers must be adding to the barbed or high affinity end, the end in contact with the surface of the *Listeria*.

Listerial Protein Synthesis is Required for the Elongation of a Tail

We infected macrophages with *Listeria* and then incubated the macrophages for 3 h in cytochalasin. Under these cir-

cumstances nucleating material accumulates on the surface of *Listeria*, but assembly cannot occur (Tilney et al., 1990). We then washed the cells and returned them to culture medium containing chloramphenicol, a potent inhibitor of bacterial, but not host, protein synthesis, for 3 h before fixation. In this way we could study the assembly of actin on *Listeria* during the cytochalasin treatment. When we examined our infected macrophages we found some *Listeria* with actin filaments coating their entire surfaces and some with very short tails resulting from filaments polymerized from the finely fibrillar material secreted during cytochalasin treatment, but no *Listeria* with long tails. The fact that long tails were not found indicates that even though the amorphous material nucleates actin filament assembly, it is not incorporated to form into a normal appearing tail. As a control, infected cells were incubated in cytochalasin D, then washed to remove the cytochalasin and allowed to recover in media without chloramphenicol for 3 h and then fixed.

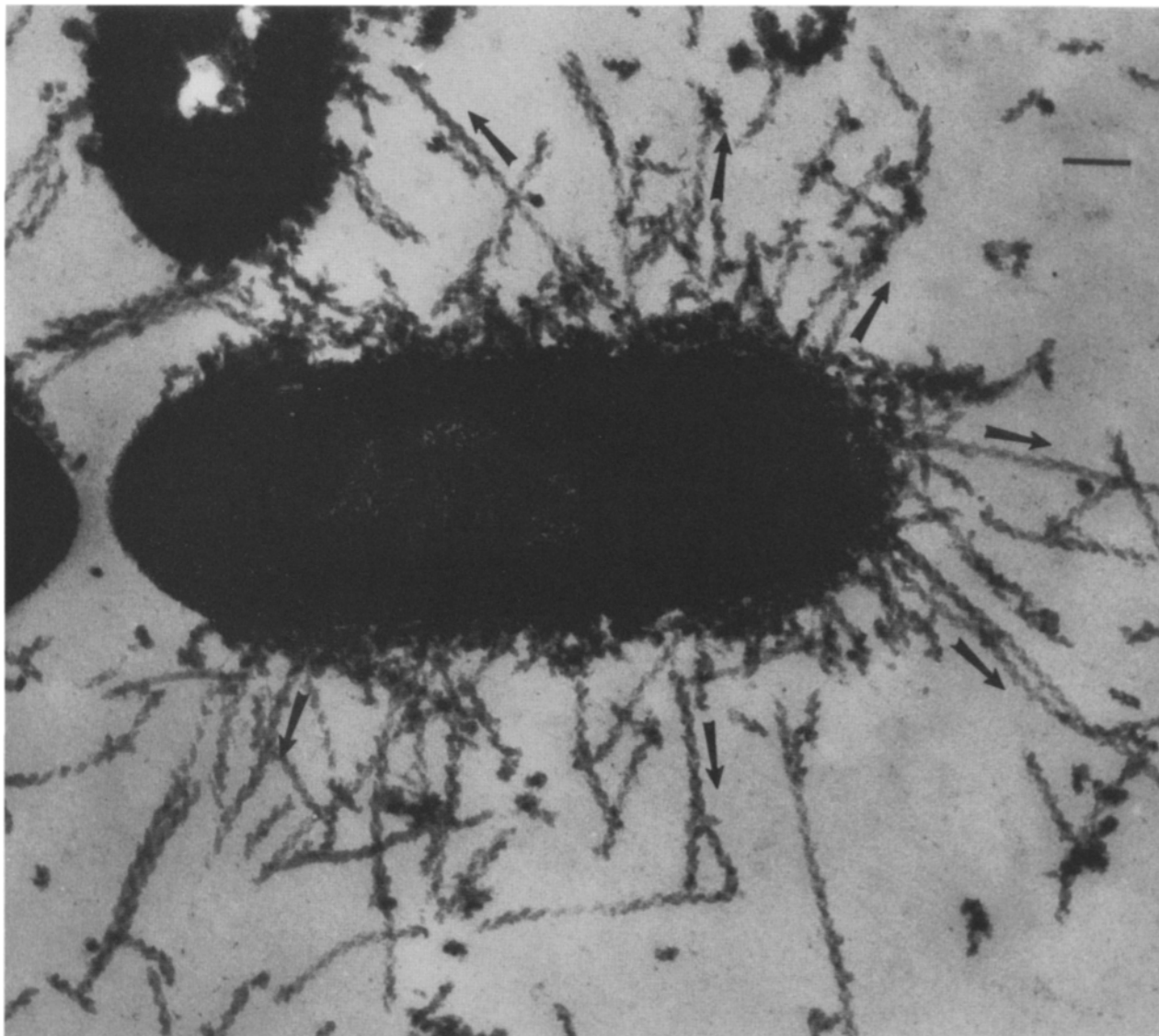


Figure 2. Mutant *Listeria* DP-L1054 was phagocytosed by a macrophage and 1 h later cytochalasin D was added. 3 h later the macrophage was detergent extracted and then incubated in 3 μ M G actin under polymerizing conditions for 20 min, then washed and the newly polymerized filaments decorated with S1. The arrows indicate the polarity of the filaments that polymerized from the surface of the *Listeria*. Note that their barbed ends are nearest the *Listeria*. Bar, 0.1 μ m

Fig. 4 documents the length of the tails seen in infected macrophages fixed after 3 h in cytochalasin (Fig. 4 *a*), after cytochalasin and then chloramphenicol (Fig. 4 *b*), and after cytochalasin and recovery in media (Fig. 4 *c*). The "tails" present on *Listeria* fixed after 3 h in cytochalasin (Fig. 4 *a*) represent only an aggregation of the "finely fibrillar material." No actin filaments are present (Tilney et al., 1990). The tails in Fig. 4 *b* represent both the finely fibrillar material and actin filaments that polymerize from it. The tails are all very short. In contrast, if *Listerial* protein synthesis is allowed, the tails become longer (Fig. 4 *c*), some up to 3 μ m in length. The mean length of tail (compare the dashed lines in Fig. 4, *b* and *c*) is clearly longer.

In another group of experiments, an extension of the findings reported earlier (Tilney et al., 1990), we treated *Listeria* with chloramphenicol at varying times after infec-

tion. What we find is that chloramphenicol seems to "freeze" the *Listeria* at the stage that it is in, it cannot progress to the next stage. Thus, if added at 2 1/4 h and examined later, no *Listeria* are seen in pseudopods even though the controls at 2 1/2 h begin to form pseudopods and by 3 1/2 h pseudopods with *Listeria* extend from most cells.

Addition of Actin Monomers to *Listeria* with Tails

Infected macrophages were cultured for 3 h, a time when many would have long tails. They were then detergent extracted and incubated in 2 μ M G actin under polymerizing conditions, washed, decorated with S1, and fixed. This concentration of actin (2 μ M) is 20 times higher than the barbed end critical concentration and three times higher than the pointed end critical concentration. This experiment should

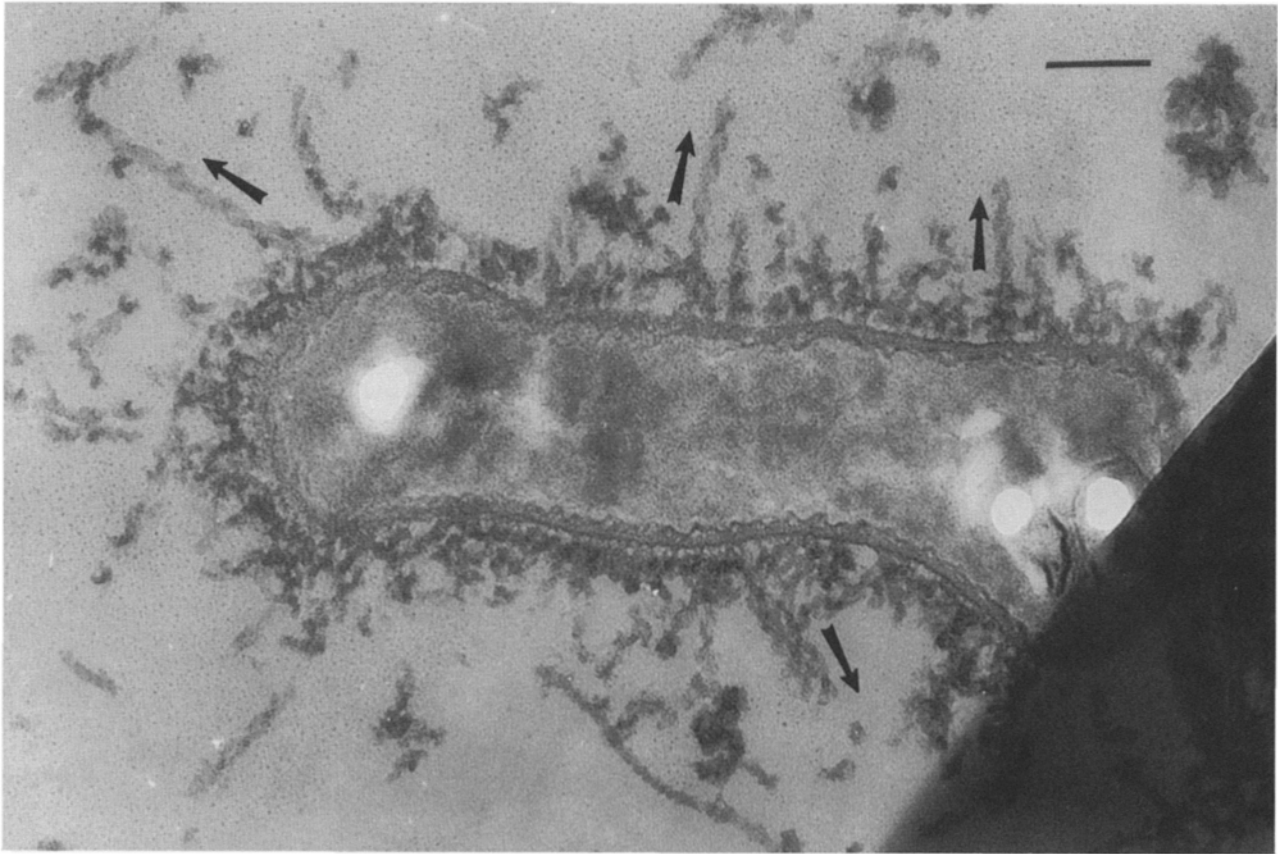


Figure 3. Infected macrophages with wild-type *Listeria* were treated with cytochalasin D for 3 h, then detergent extracted and incubated with 0.5 μM G actin under polymerizing conditions. The preparation was then washed and newly assembled actin filaments decorated with S1. Short filaments extend from the surface of *Listeria*. All have their barbed ends nearest the surface of *Listeria* (see arrows). Bar, 0.1 μm .

give us information as to which filaments are capped and which end of the filament is capped. The results of this experiment were unexpected. The *Listeria*, which is normally situated at one pole of the tail, is translocated forward away from the tail proper by the assembly of a number of long, parallel actin filaments (Figs. 5 and 6).

As demonstrated in the accompanying paper, short actin filaments extend from the lateral and distal surfaces of *Listeria*, but not from the anterior end, the end formed during the preceding division. It is as if the *Listeria* sits in a goblet composed of numerous cross-bridged actin filaments. Thus, when macrophages containing *Listeria* with long tails are detergent extracted and G actin added under polymerizing conditions, assembly of monomers occurs on the end of the filaments that were in the process of elongation before detergent extraction. Thus, actin filaments explosively assemble on the barbed ends which are associated with the surface of the *Listeria*. The net result is that the *Listeria* are propelled forward out of the goblet. In this case one would predict that the long filaments extending from the translocated *Listeria* down towards the goblet would have unidirectional polarity with their barbed ends nearest the bacterium. This is what is observed in every case (Fig. 6).

In essence what this experiment has shown us is that under these conditions where we add to a *Listeria* that is in the pro-

cess of elongating its tail, a large excess of monomeric actin, actin assembly outstrips controls of actin filament length and cross-bridging leading to the rapid translocation of the *Listeria* away from the tail.

Additional Evidence That the Actin Filaments Making up the Tail Are All Short. In an earlier publication (Tilney and Portnoy, 1989) and in the accompanying paper (Tilney et al., 1992) we concluded that the filaments in the tail are all short, less than 0.3 μm . The possibility remains, however, that the filaments may spiral around the tail and thus in any thin section, no matter what the orientation is, only short filaments would be seen. This seems unlikely because if the filaments in the tail are decorated with S1, one sees in longitudinal sections what looks like filament ends at the margins of the tail (see Fig. 8 in the accompanying paper). If they were to spiral around the tail, these filaments would have to bend sharply. We can eliminate such an unlikely possibility with the following observation. When macrophages containing *Listeria* with long tails are detergent extracted and then incubated in G actin under polymerizing conditions, filaments elongate as mentioned in the preceding section, but also filaments in the tail elongate. Overall, the tail no longer presents a smooth outline, but instead filaments project out from the tail (Figs. 5 and 7). This could not occur if the filaments in the tail were spirally arranged as the only ends of

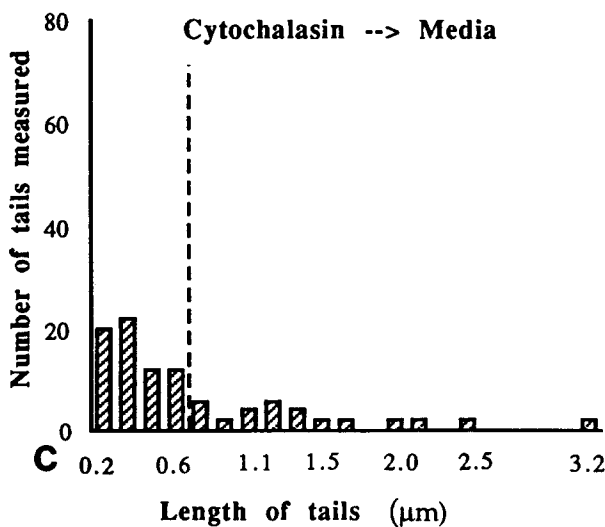
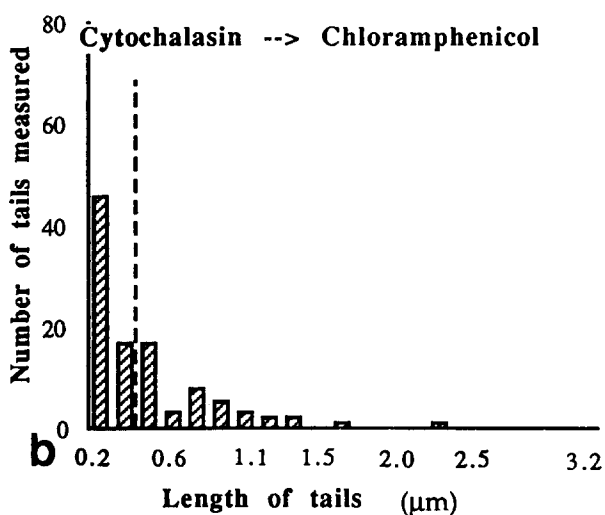
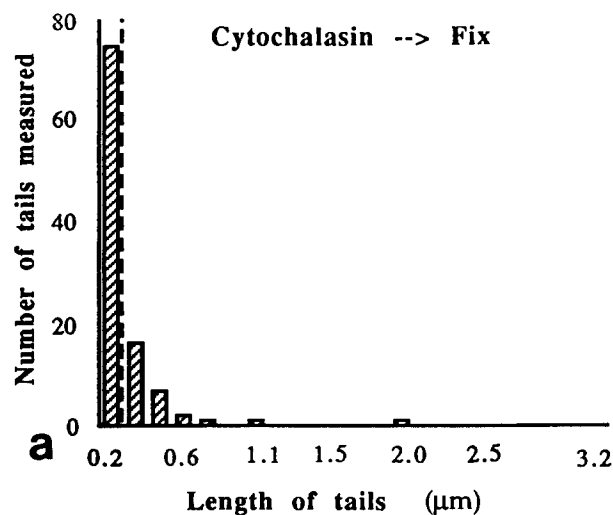


Figure 4. Bar graphs depicting the tail length of *Listeria* in the macrophage cytoplasm. (a) 3 h in cytochalasin D before fixation. The dotted line indicates the mean tail length. (b) After 3 h in cytochalasin D, the infected macrophages were incubated in chloramphenicol,

the filaments would be at the tip of the tail. These observations confirm our earlier speculations that the filaments making up the tail are all short, seldom exceeding $0.2 \mu\text{m}$ in length.

The Actin Filaments in the Tail Are Capped on Their Pointed Ends. Careful examination of the polarity of those filaments that project out from the tail reveals an additional fact, namely, that the portion of the filament that extends from the tail proper into the medium after being incubated in monomeric actin under polymerizing conditions has the barbed end located out in the medium. We almost never find filaments connected to the tail that have opposite polarity. To be more quantitative, we examined thin sections of 14 different tails and in those we were able to determine the polarity of 178 of the filaments that extend out perpendicular to the long axis of the tail. 176 of these had the barbed end out in the medium with the pointed end inserted into the tail. Only two had a different polarity. This result is surprising because in the accompanying paper we demonstrated that the filaments in the tail have their pointed ends located at the margins of the tail so one would have predicted pointed end growth with high concentrations of exogenous actin. What this observation means is that there must be a pointed end capper.

Additional Evidence That Monomers Add to the Barbed Ends of the Filaments. Since there is a pointed end capper, yet the filaments associated with the surface of *Listeria* elongate when incubated in $2 \mu\text{M}$ actin under polymerizing conditions, addition of monomers must be occurring on the barbed end of the filament or that end associated with the surface of *Listeria*. A similar conclusion was obtained by incubating *Listeria* that had been in the cytoplasm of a macrophage with a concentration of actin below the pointed end critical concentration (see above).

Discussion

Nucleation of Actin Filaments from the Surface of *Listeria*

We demonstrated that the actin filaments that extend from the surface of a wild-type or mutant *Listeria* after decoration with S1 of myosin display unidirectional polarity with their barbed ends invariably associated with the bacterium. We also showed in two ways that the actin monomers add to the barbed ends of these filaments or the end nearest the bacterium. The first was by using concentrations of monomer below the pointed end critical concentration. Since filaments form, they must do so by adding to the barbed end. The second way was by adding monomers to *Listeria* with tails. In this case the filaments associated with the surface of *Listeria* elongated even though all the filaments are capped on their pointed ends. Thus both nucleation of actin assembly and ad-

col, an inhibitor of bacterial protein synthesis. The mean length increase is due to the assembly of actin filaments from nucleator that was secreted before chloramphenicol was added. There are now a number of *Listeria* with tails of $1 \mu\text{m}$ or more. (c) After 3 h in cytochalasin D the infected macrophages were placed in media for 3 h before fixation. The mean length has increased and a number of *Listeria* have long tails.

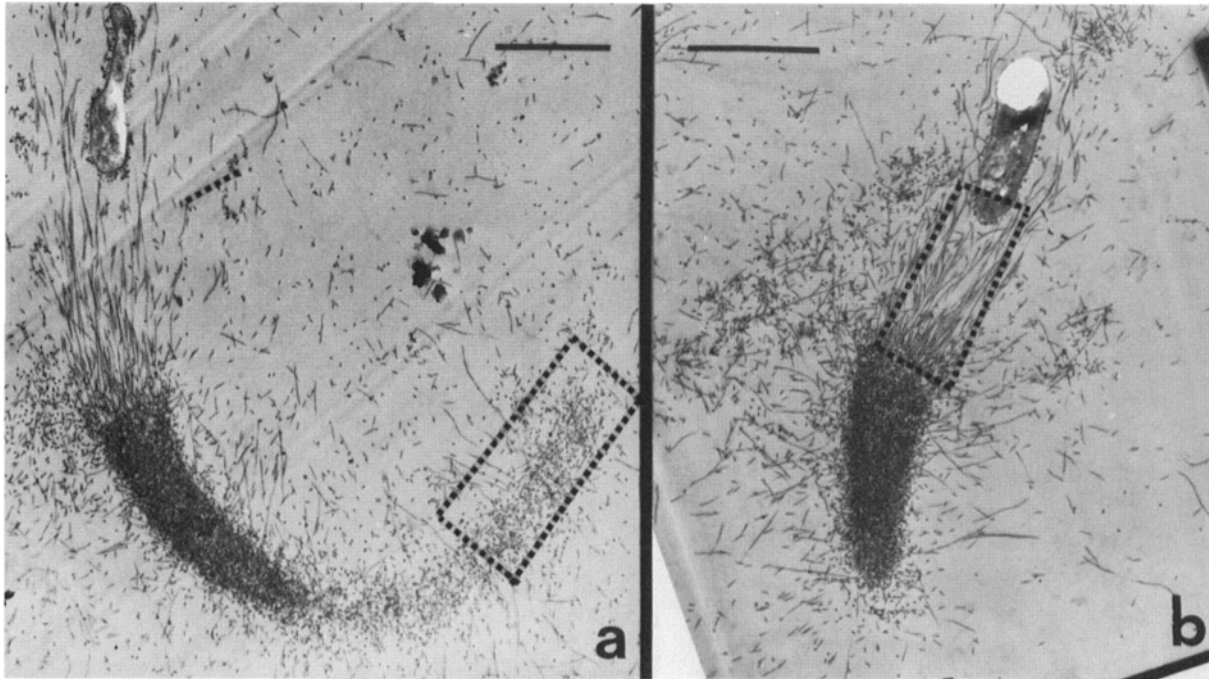


Figure 5. Macrophages were infected with *Listeria*. After 3 h they were detergent extracted and incubated in 2 μ M monomeric actin under polymerizing conditions. Subsequent to washing, the actin filaments were decorated with S1. In both of these sections the *Listeria* are translocated forward away from their tails by the assembly of actin filaments that polymerize from the surface of the bacteria. The boxed region on *a* is shown at higher magnification in Fig. 7; that on *b*, in Fig. 6. Bars, 1 μ m.

dition of monomers must be occurring at the surface of *Listeria*.

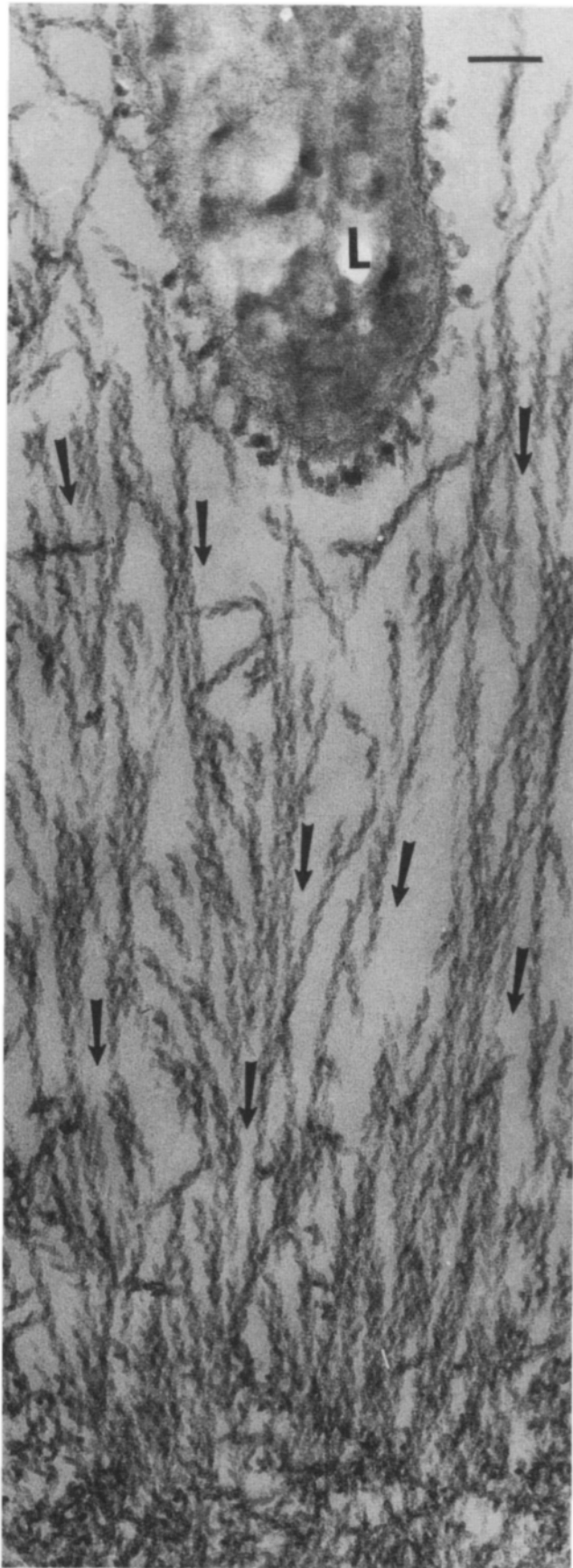
Since the filaments that are nucleated from *Listeria* often extend perpendicularly from the surface (see data here, data in the accompanying paper, and in Tilney et al., 1990), it seems reasonable to conclude that the nucleator is probably not like ponticulin, a nucleator isolated from *Dictyostelium* by Shariff and Luna (1990), because ponticulin binds to the lateral surfaces of the actin subunits allowing monomer addition to either end. Thus if the *Listeria* nucleator is like ponticulin the actin filaments should connect to the *Listeria* at a grazing angle, not at 90°. What remains unsubstantiated is how firmly attached the filaments are to *Listeria* and what molecule or molecules are nucleating assembly.

In an earlier publication (Tilney et al., 1990) we presented evidence that the *Listeria* synthesizes and secretes on its surface an actin filament nucleator. At that time we could not prove this point as one might argue that what the *Listeria* is actually doing is synthesizing a "glue" that attracts a nucleator from the host cytoplasm. In this publication we present more evidence that the *Listeria* is secreting a nucleator of actin filament assembly. When *Listeria* that are not aerated and shaken are incubated in monomeric actin under polymerizing conditions, a few filaments assemble from its surface, e.g., about a dozen (Fig. 1). Since these *Listeria* were never in the cytoplasm of a host cell, the assembled filaments must have been nucleated from the surface of the bacterium. Both reviewers of this manuscript were concerned that only a few filaments are nucleated from *Listeria* in vitro. It is possible that there might be another explanation for these few filaments, e.g., some junk that stuck to the bacterium that nucleates actin assembly. Although this explanation remains a

possibility, aerated bacteria never nucleate actin assembly (see Tilney et al., 1990), so there is something different about unshaken and unaerated bacteria. We have not yet isolated a nucleator.

Evidence for a Pointed Capper

We reported an experiment in which *Listeria* with a tail was incubated with monomeric actin under polymerizing conditions at a concentration three times above the pointed end critical concentration. The pointed ends of the filaments did not elongate, but the barbed ends did. This was a surprising result because at the periphery of the tail, the pointed ends are more common than barbed ends (data is presented in the accompanying paper). It is true that the barbed end of an actin filament, by being the preferred end for addition of monomers, will have longer filaments extending from them at any concentration of monomeric actin (Coluccio and Tilney, 1984), but in this case we see no pointed end elongation (2 cases out of 178), even though this is the most available end. This has to mean that the pointed end is capped. The fact that there must be a pointed end capper in the tail of *Listeria* is interesting as there are no identified pointed end cappers in nonmuscle cells although one suspects that there must be one associated with thin filaments of skeletal muscle. At one time acumentin (Southwick and Hartwig, 1982) appeared to be an interesting candidate for a pointed end capper, but it now must be classified not as a pointed end capper, but as a barbed end capper (Southwick, F. S. and A. Weber, unpublished observations). For completeness we should mention (Tilney and DeRosier, unpublished data) that both a pointed and barbed end capper are present and at-



tached to the actin filaments in mature hair cells of the cochlea of birds so that, in fact, we know that such a capper exists, but what it is remains to be determined.

Tropomyosin cannot be the pointed end capper. While muscle tropomyosin reduces the rate of depolymerization from the pointed ends of purified actin filaments that are capped on the barbed ends by villin (Broschat et al., 1989), it does not prevent addition of monomers to the pointed end. We have shown that the pointed ends of the actin filaments in the tail are not available for subunit addition by exogenous actin. This was the same experiment that was carried out on the chick cochlea.

Actin Filament Nucleation, Cross-bridging, and Control of Filament Length Are Tightly Coupled

We presented two sets of experiments from which we conclude that actin filament nucleation and elongation must be tightly coupled. The first was to treat infected macrophages with cytochalasin for 3 h, then wash out the cytochalasin with chloramphenicol present to inhibit bacterial protein synthesis. Polymerization occurred only from nucleating material secreted during the treatment with cytochalasin, but true tails did not form. Thus a true tail will not just spring out from a pile of components, but must form in sequential stages. The second experiment was more direct. In that experiment detergent extracted macrophages containing *Listeria* with tails were incubated in exogenous G actin under polymerizing conditions. What happens is that the *Listeria* is translocated forward away from its tail by the assembly of long filaments. The newly assembled filaments are many times longer than the filaments making up the tail, parallel in orientation, unlike those in the tail, and unidirectionally polarized with their barbed ends near the *Listeria*. These observations are consistent with the interpretation that actin assembly is occurring on those filaments in the *Listeria* tail that were in the process of elongating before the detergent extraction and incubation in a large excess of G actin. They continue to elongate but their growth is uncontrolled, e.g., cappers are not present and further cross-bridging is not occurring. If this were to occur in vivo so that one process outstrips another, chaos would ensue. From these experiments we conclude that nucleation, capping, and cross-bridging must be coupled to achieve controlled growth such as occurs when *Listeria* is growing its tail in vivo.

How Does Listeria Circumvent the Host Cell Controls to Build Its Own Cytoskeleton?

The host cell usually has a substantial store of unpolymerized actin; for example, in a fibroblast the concentration of actin is $\sim 100 \mu\text{M}$ (Bray and Thomas, 1975). What percentage of this is monomeric and what percentage of that is polymerizable is not known, but it cannot be high or the actin would spontaneously polymerize if not tightly regulated by the host. Thus the host must be suppressing extraneous actin

Figure 6. Higher magnification of the boxed region of the tail in Fig. 5 b. Of interest is that the actin filaments responsible for translocating the *Listeria* (L) anteriorly are unidirectionally polarized with their barbed ends nearest the *Listeria*. The polarity of some of the filaments is indicated by the arrows. Bar, 0.1 μm .

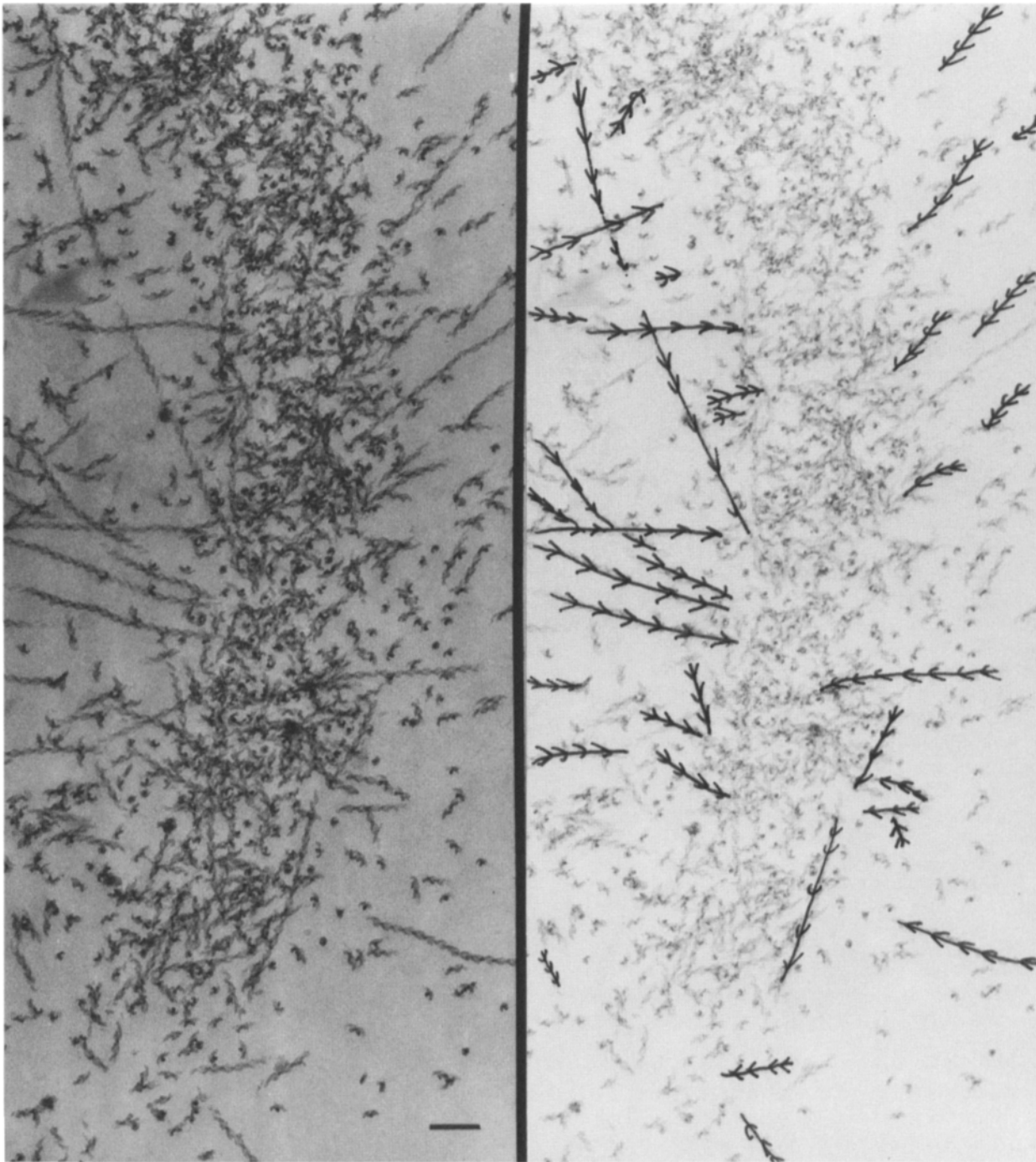


Figure 7. Higher magnification of the boxed region of the tail in Fig. 5 a. Newly assembled filaments extend from the tail proper. Their polarity is indicated on the light print on the right. Bar, 0.1 μm .

filament nucleation and growth. The *Listeria*, on the other hand, are trying to produce a cytoskeleton and are therefore competing with the host for the monomeric actin. We propose the following: the bacterium generates sites of nucleation which initiate actin filament polymerization. The host cell stops the elongation of the filaments, e.g., by using barbed end cappers. At this point the bacterium would be “out of business,” but it avoids this problem by simply nucleating new filaments that are in turn terminated by the host. The result is a tail made of many short filaments; a tail that gradually increases in length by the nucleation of more filaments (Fig. 8).

This conclusion is similar to that proposed recently by Theriot and Mitchison (1991) for fibroblasts.

Listeria Is an Excellent Experimental System to Isolate and Characterize an Actin Filament Nucleator and a Pointed End Capper

As mentioned in the introduction, investigations on the cytoskeleton of *Listeria* seem worthwhile as it is a simplified system in which a particle enters the cytoplasm, builds around it a simple, yet predictable cytoskeleton that is used in a

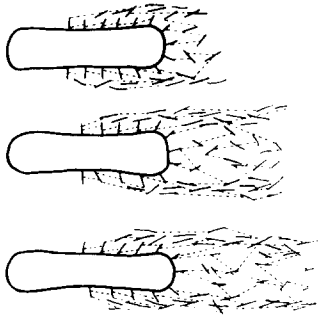


Figure 8. Drawing illustrating stages in the growth of the tail. Actin filaments are nucleated from the basal and lateral surfaces of a *Listeria*. They become cross-bridged and fall off the surface. New filaments are then nucleated and the process continues. Thus a tail composed of short filaments is generated.

prescribed way. It is not like the cytoskeleton of a eucaryotic cell which is simultaneously involved in many actin-based motile functions such as streaming, amoeboid movements, phagocytosis, cytokinesis, shape changes, etc. Furthermore, we know that at least one of the essential components of the *Listeria* cytoskeleton seems to be produced and secreted by the *Listeria*, a bacterium which has a simplified genome relative to the host, a genome that can be explored by mutants, many of which already exist.

Because of the relatively small genome size and the fact that *Listeria* makes a cytoskeleton which potentially can be isolated (the tail), we should be able to either biochemically or by molecular biological approaches isolate and characterize the nucleator and the pointed end capper. Both of these would be important to isolate, particularly as barbed end cappers such as villin and gelsolin will nucleate actin assembly in vitro, although they have not been shown to be involved in actin filament nucleation in vivo. Likewise no information is available on pointed end cappers (acumentin is no longer considered a pointed end capper) and, although one must exist in skeletal muscle, little is known about it. Thus *Listeria* seems to be a beautiful system to exploit to get chemical and structural information on these two elusive cytoskeletal components, nucleators, and pointed end cappers.

Unsolved Mysteries

We have included this chapter here because to ultimately understand what actin filaments are doing in the cytoplasm of a cell, it is necessary to relate the static images that we see in the electron microscope to the behavior of living cells, and this behavior to the action of purified proteins in vitro. At this moment there are a number of observations that "don't jibe." This does not mean that the observations are faulty, only that our understanding is flawed by seeing only a part of the picture. We are hoping that by pointing out these discrepancies investigators in and outside of the field will be spurred on to resolve these issues.

First, we presented data here and in earlier publications (Tilney et al., 1990; Tilney et al., 1992) that chloramphenicol, an inhibitor of bacterial, but not host cell protein synthesis, blocks the elongation of a tail or formation of a pseudopod with *Listeria* in it. If chloramphenicol is applied 2 h after infection when a cloud of actin filaments and short tails have formed and the infected cells fixed 3 h later, it is as if the cytoskeleton is "frozen" in place. The cloud and short tails remain the same length. If the actin filaments in the tail are turning over every 33 s as demonstrated by Theriot et al. (1992) and if bacterial protein synthesis is inhibited by chloramphenicol, continual bacterial protein synthesis is not

necessary for filament nucleation. Therefore, the rate-limiting step is controlled by the bacterium, not the host.

Second, we demonstrated that the pointed ends of the actin filaments in the tail are capped. If these filaments turn over every 33 s, what is the purpose of a pointed end capper? To state this another way, the presence of a pointed end capper is exactly contrary to what the cell needs if the actin filaments turn over so rapidly.

Third, if the *Listeria* is moving through the cytoplasm at up to 1 $\mu\text{m/s}$ by actin assembly, then there must exist at the bacterial surface a concentration of polymerizable actin that is extraordinary, e.g., 50 μM . It is almost as if there must be enzymes present to facilitate the process.

Fourth, if the turnover of actin is every 33 s and the movement of the bacterium through the cytoplasm is caused by actin assembly, one would expect the filaments at the tip of the tail to be shorter than those at the base. This is not the case as shown in the accompanying paper (Tilney et al., 1992), so one wonders exactly where turnover is occurring and how it occurs.

Fifth, because we can isolate *Listeria* with tails that are stable in vitro, there must exist in the cytoplasm local mechanisms to depolymerize filaments.

Sixth, examination of the tails of *Listeria* in living host cell cytoplasm with fluorescent actin (Dabiri et al., 1990) or by phase contrast (Theriot et al., 1992) or fixed cells examined with rhodamine phalloidin (Sanger, J. M., F. S. Southwick, and J. W. Sanger. 1990. *J. Cell Biol.* 111:390 *Abstr.*) reveal tails that can be up to 40 μm in length. In thin sections examined by EM tails longer than 3.1 μm were very rare and none longer than 3.5 μm were found.

Finally, in the accompanying manuscript we concluded that the age of the surface of *Listeria* is related to the rate of tail formation. Although we have not quantitated this, it appears that the density of filaments emanating from the surface of *Listeria* is the same if the *Listeria* is in the cloud state or if it has a long tail except, of course the new surface formed during septation. How are these two observations related? Are there, for example, a limited number of "pores" in the cell surface for secretion of presumed nucleators or does the number of pores increase and thus the rate of assembly increases. Since nucleators, cappers, and cross-bridges are coupled, how is this related?

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