



Expression of bovine annexin A4 in *E. coli* rescues cytokinesis blocked by beta-lactam antibiotics

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ARTICLE INFO

Keywords:

Annexin
Cytokinesis
Bacteria
Beta-lactam antibiotics

ABSTRACT

Treatment of bacteria with beta-lactam antibiotics can impair the process of cytokinesis, the final step in cell division, leading to the formation of a filamentous form of the bacteria. The expression of a mammalian calcium-dependent, membrane-binding protein, bovine annexin A4, in *E. coli* was found to reverse the inhibitory effects on cytokinesis of the beta-lactam antibiotics ampicillin, piperacillin, and cephalexin. This novel activity of the annexin was blocked by mutation of calcium binding sites in the annexin, indicating roles for calcium binding to the annexin and the binding of the annexin to membranes in restoring cytokinesis. The filamentous form of the bacteria has been reported to be more resistant to phagocytosis by cells of the immune system in eukaryotic hosts. Therefore, expression of annexins in pathogenic bacteria, by promoting the breakdown of the bacterial filaments, might serve as an adjuvant to enhance the efficacy of beta-lactam antibiotics.

1. Introduction

E. coli and many other bacteria under conditions of stress reduce the frequency of undergoing cytokinesis while continuing to grow and replicate their genome. The result is transformation of the bacteria into a filamentous form [1]. For infectious bacteria this may have survival value by preventing consumption of the bacteria by phagocytic cells of the immune system of the host organism, and the formation of biofilms that protect bacterial colonies. This report describes the unexpected discovery that the expression of mammalian annexin A4, a calcium-dependent, phospholipid-binding protein, in *E. coli* tends to restore the ability for bacteria undergoing antibiotic stress to undergo cytokinesis and leads to a return to a normal morphology.

The annexins constitute a highly conserved family of calcium-binding proteins in plants, animals, and protists [2,3]. However, they are not expressed in *E. coli*. Motifs similar to the canonical fourfold calcium- and membrane-binding motifs in eukaryotic annexins [4] have been discovered in proteins in some strains of bacteria [5,6]. Their occurrence in bacteria is rare, however. The bioinformatic analysis of Kodavali et al. [6] detected annexin motifs in only 17 bacterial species among the thousands of genomes analyzed. It is unclear if the bacterial annexin-like proteins evolved independently from eukaryotic annexins or if genetic material encoding annexin motifs was acquired by non-genetic transfer from the eukaryotic relatives. The bacterial annexin-like proteins are unusual in that they do not generally follow

the pattern of the fourfold domain repeat found in the eukaryotic examples as they have as few as a single annexin motif and up to 6 copies of the repeats. None of the bacterial proteins containing annexin motifs have yet been isolated or characterized biochemically so it is not known if they bind calcium and membranes, although the sequence similarities to the eukaryotic proteins suggest this may be the case. Nor is there evidence for any particular function of the bacterial annexins obtained, for example, by gene deletion or modification experiments. Therefore, the ability of the eukaryotic annexin to modulate a specific function in bacteria, cytokinesis, was not anticipated.

The affinity of eukaryotic annexins for calcium is modulated by the coincident binding of phospholipids [7,8] so the annexins are implicated in many activities regulated by calcium that occur with or on membranes. It has been reported that inhibition of the expression of a particular eukaryotic annexin, annexin A11, in human A431 and HeLa cells leads to the failure of cytokinesis in its final stage during which the neck of cytoplasm between daughter cells undergoing mitosis collapses and the membranes fuse, separating the cells [9]. It is not known what the precise role for the annexin may be in this process, but regulation by calcium, and the ability to pull two membranes together to promote membrane fusion could provide an explanation consistent with the known *in vitro* properties of the annexins [10].

In 1940 Gardner reported that, while working with Chain, Florey, and others at Oxford, he observed that many types of bacteria exposed to penicillin at low concentrations formed long filaments [11,12].

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<https://doi.org/10.1016/j.bbrep.2023.101553>

Received 26 March 2023; Received in revised form 1 October 2023; Accepted 3 October 2023

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Subsequently it was determined that this morphological change was caused by the binding of penicillin or other beta-lactam antibiotics to proteins that play essential roles in bacterial cell division [13–15]. For example, in *E. coli* penicillin binds to penicillin binding protein 3 (PB3) which functions in peptidoglycan synthesis in the bacterial cell wall, including in the septa between dividing cells. This process is inhibited by the binding of penicillin resulting in continued growth of the bacteria without cell division, producing filaments containing multiple copies of the bacterial genome.

It has been suggested that the formation of these filaments may provide a selective advantage to bacteria under attack by phagocytic cells of the immune system of eukaryotic organisms which are unable to encapsulate the extended bacterial filaments [1,16]. To this extent the formation of bacterial filaments may be of medical importance in that they may impair treatment of bacterial infections with beta-lactam antibiotics. The result is that bacteria gain resistance to the host defenses precisely when the antibiotic is administered with the intention to inhibit bacterial growth. It is possible that small molecule drugs that could inhibit the filamentation process might provide an effective adjuvant treatment to enhance antibiotic efficacy, but no such compounds have been identified. The results reported here suggest that expression of annexins in pathogenic bacteria might serve such a prophylactic benefit.

2. Materials and methods

2.1. Materials

Components of the bacterial growth medium, Luria broth, (10 gm/liter tryptone, 5 gm/liter NaCl, yeast extract 10 gm/liter) were obtained from Fisher (Fair Lawn, New Jersey). Bacterial agar for petri plates was from Acros Organics (The Hague, Netherlands), ampicillin from Fisher (BP1760), piperacillin from Sigma-Aldrich (P8396) (St. Louis, Missouri), cephalexin from Alpha Aesor (J63172) (Haverhill, Massachusetts), IPTG from Fisher, and glutaraldehyde from Fisher. Stock concentrations of the antibiotics were prepared as follows and stored at minus 20° C: ampicillin 100 mg/ml in 1:1 ethanol:H₂O; piperacillin 2 mg/ml in ethanol; and cephalexin 25 mg/ml in 1:1 ethanol:H₂O. Other standard chemicals were of reagent grade obtained from Fisher or Sigma.

E. coli strain BL21(DE3) was obtained from Thermofisher Scientific (Waltham, Massachusetts) and the expression plasmid pET11d from Millipore Sigma (Burlington, Massachusetts).

Annexin expression plasmids were constructed as described [17,18]. Since the plasmids were originally sequenced manually in the early 1990's the inserts in all plasmids used in this study were resequenced with high fidelity automated Sanger sequencing by Azenta Life Sciences, (Burlington, Massachusetts). All designed mutations were confirmed and no spurious mutations were found.

Unique reagents will be provided by the author upon reasonable request.

2.2. Methods

Use of dark field light microscopy to monitor antibiotic-driven bacterial filament formation. In bright field light microscopy the filaments formed by bacteria in the presence of beta-lactam antibiotics are difficult to distinguish from “floaters” in the eyes of the microscopist. This problem is eliminated by using dark field microscopy because the visual floaters do not scatter light from the microscope condenser into the objective lens and are therefore invisible. The visual floaters also do not appear in micrographs taken by photography or videography in either brightfield or darkfield. The light micrographs in this report were all obtained by darkfield light microscopy. This technique also enhanced the contrast of the bacteria against a dark background and led to easy visualization of areas of difference of refractive index such as the difference between the cell wall and cytoplasm. This was particularly

useful for monitoring overall shape and real-time growth of bacterial cells, however, intracellular structures were generally not resolved. Therefore, transmission electron microscopy was also used in some cases for visualization of cell ultrastructure.

Darkfield light microscopy of bacterial cultures was performed with a Nikon Labophot microscope (Nikon Instruments, Melville, New York) with a dark field condenser (dry) and an e plan40X/0.65 Nikon objective. Micrographs were recorded with an Amscope Microscope digital camera MU 1803-HS 18mp Aptina color CMOS (Irvine, California) using software supplied with the camera.

Cell culture and microscopy. Protein expression from the plasmid pET11d can be regulated by the addition of the inducer IPTG (Isopropyl β-D-1-thiogalactopyranoside) [19]. However, protein expression from this plasmid can have significant baseline expression without the inducer. It was seen in SDS gels of extracts of bacteria expressing annexin A4 that basal expression of the annexin appeared to be 5–10 % of expression seen with the inducer [20]. In preliminary tests for the present study it was found that including the inducer (concentration 0.4 mM, previously used for maximum protein production) led to formation of inclusion bodies of insoluble protein, distortion of bacterial morphology and partial lysis and aggregation of the bacteria. Accordingly, all of the imaging and experiments described in this report were performed without adding the inducer, which avoided these problems. In the future it may be possible to determine a more detailed dose response relationship for the annexin effect by titrating the levels of the inducer.

Five ml precultures in LB medium with 200 µg/ml ampicillin were seeded with colonies from LB-ampicillin agar plates and incubated on a rotator overnight at 34° C. Fifty µl samples of the saturated cultures were diluted into 5 ml of LB medium with the specified antibiotics in 17 × 100 mm clear polystyrene culture tubes with loose fitting caps that permitted gas exchange (Fisher Healthcare 14-956-6B) and the growth of the cultures at 34° C was monitored by dark field microscopy and measurement of absorbance at a wavelength of 600 nm. For the microscopy, 20 µl samples of the culture were placed on a glass slide (Fisher, 12-550123 1 × 3 inches, 1 mm thickness, precleaned by the manufacturer) and the drop of culture fluid covered with a cover slip (Fisher 12540B 22 × 22 mm, 0.17–0.25 mm thickness). Slides were rested for 2–5 min to allow the cells to settle before observation. Nonetheless, the cells were not tightly adherent to the slides and occasionally some cells moved slightly during imaging resulting in a double image. Cultures were checked and micrographs taken at 5–20 min intervals for up to 4 h. Occasionally during this period the culture tubes were briefly inserted into a Genesys 20 spectrophotometer (Thermospectronic, Rochester, NY) and the absorbance at 600 nm was recorded to measure culture density. The culture tubes have a circular cross section with an inside diameter of 1.3 cm, so the absorbances recorded were approximately 30 % higher than would have been measured in a square cuvette with a one cm pathlength.

For preparation of the micrographs in Fig. 1 through 3 comparing the morphology of the different transformed strains, each test culture (5 ml) was inoculated at the same time with 50 µl of the saturated overnight preculture. For display in the Figures, photographs taken after approximately the same period of growth of each of the parallel cultures were obtained when 20 µl samples provided a microscope image containing a broad sampling of the cell morphologies seen without overcrowding. Each experiment illustrated was repeated at least three times with comparable results.

Measurement and statistical analysis of filament lengths. The lengths of the bacterial filaments were measured using the Image J program (NIH) with the dark field light micrographs. Particles that did not have any linear dimension greater than 2 µm were not measured because of uncertainty as to whether they represented bacterial cells, broken cells, or other debris in the light micrographs. The longest dimension of each cell or filament was taken to represent the “length” for the analysis. Forty to 90 filaments were measured from each of the

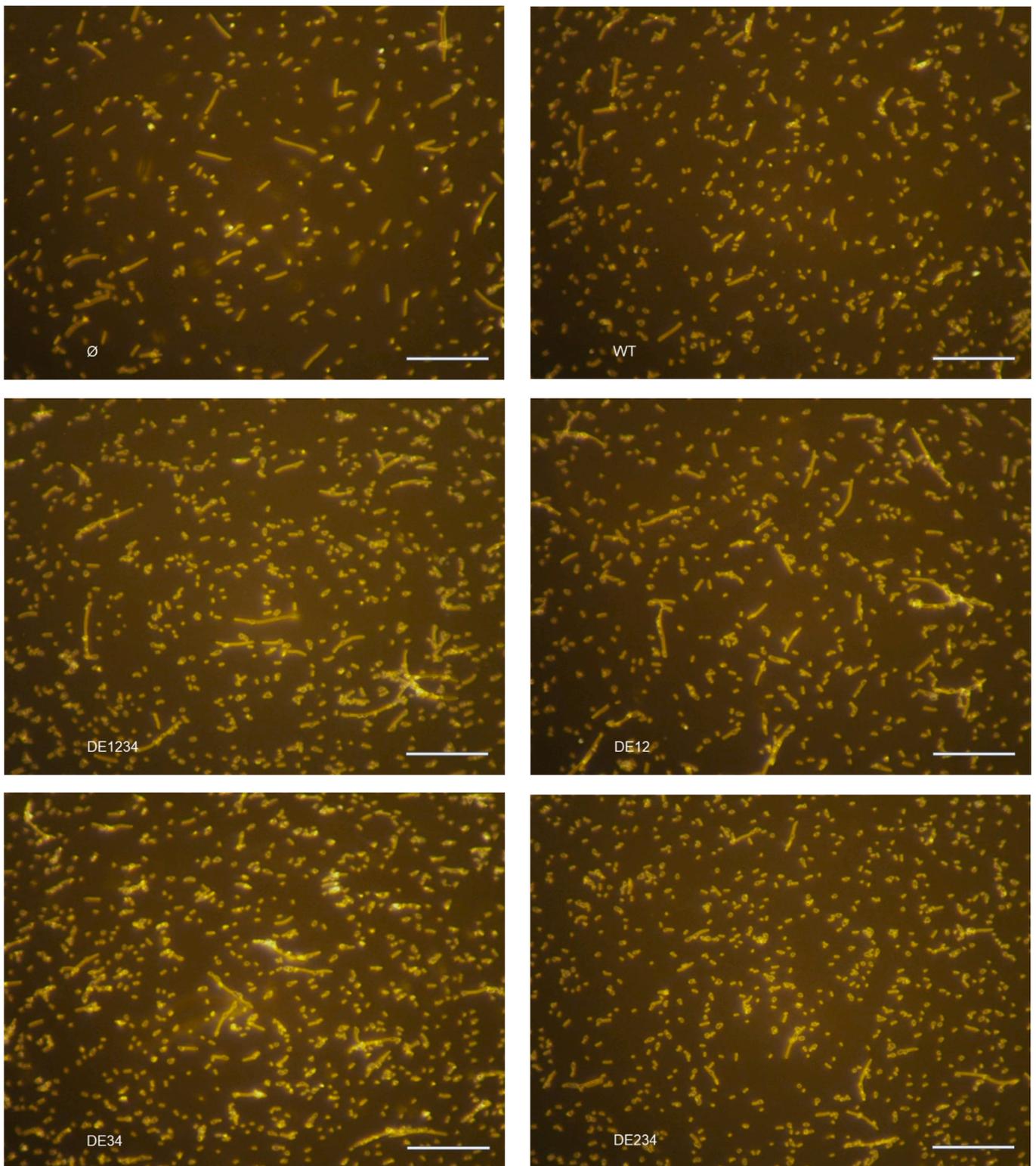


Fig. 1. Dark field light micrographs of *E. coli* grown in AMPICILLIN (200 $\mu\text{g}/\text{ml}$) and expressing wild type or mutant annexin A4

1A: Ø, control plasmid with no insert

1B: WT wild type annexin A4

1C: DE1234 mutant annexin A4

1D: DE12 mutant annexin A4

1E: DE34 mutant annexin A4

1F: DE234 mutant annexin A4

Calibration bar on the bottom right of each panel = 50 μm .

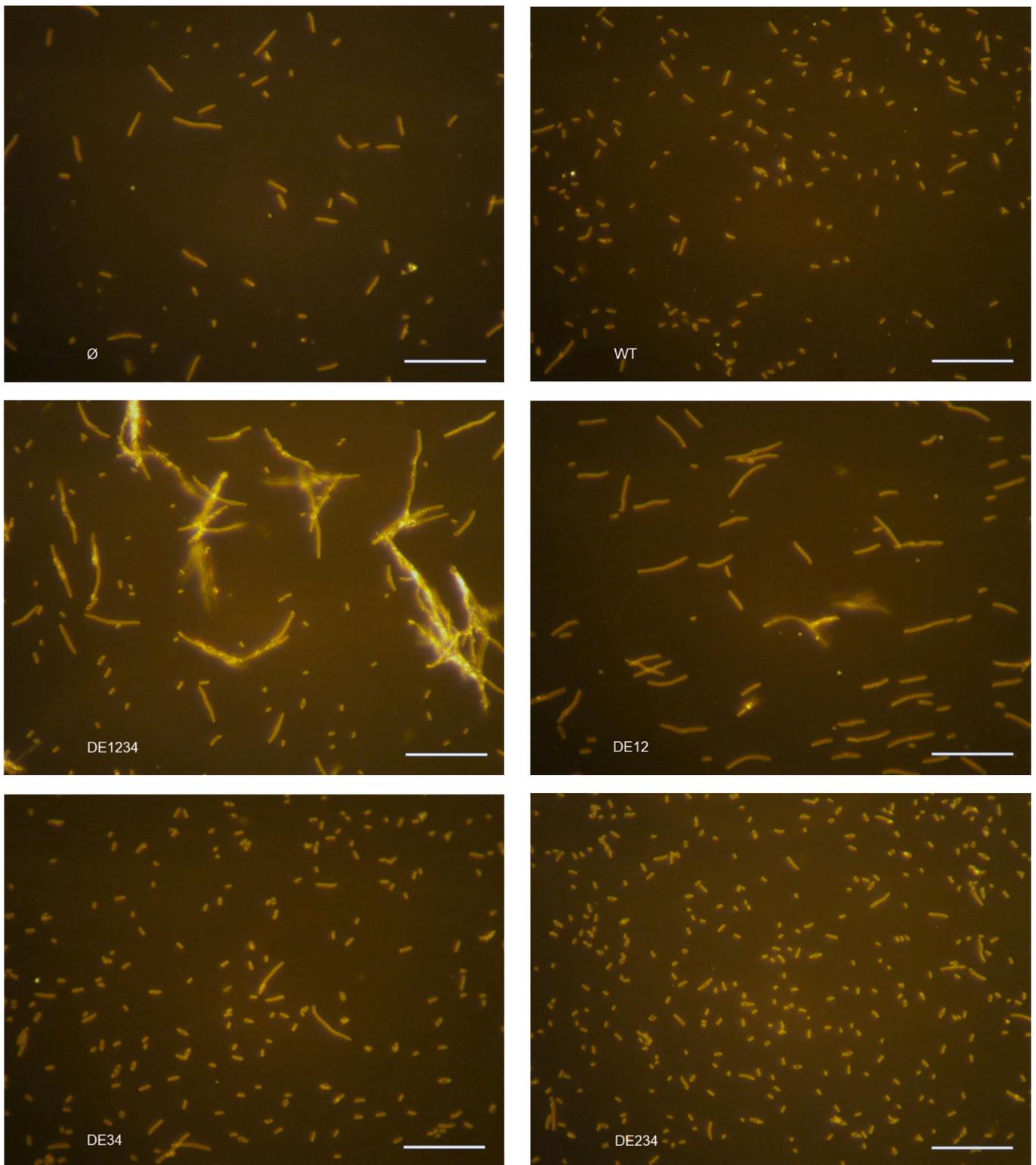


Fig. 2. Dark field light micrographs of *E. coli* grown in AMPICILLIN (200 $\mu\text{g}/\text{ml}$) PLUS PIPERACILLIN (4 $\mu\text{g}/\text{ml}$) and expressing wild type or mutant annexin A4

2A: \emptyset , control plasmid with no insert

2B: WT wild type annexin A4

2C: DE1234 mutant annexin A4

2D: DE12 mutant annexin A4

2E: DE34 mutant annexin A4

2F: DE234 mutant annexin A4

Calibration bar on the bottom right of each panel = 50 μm .

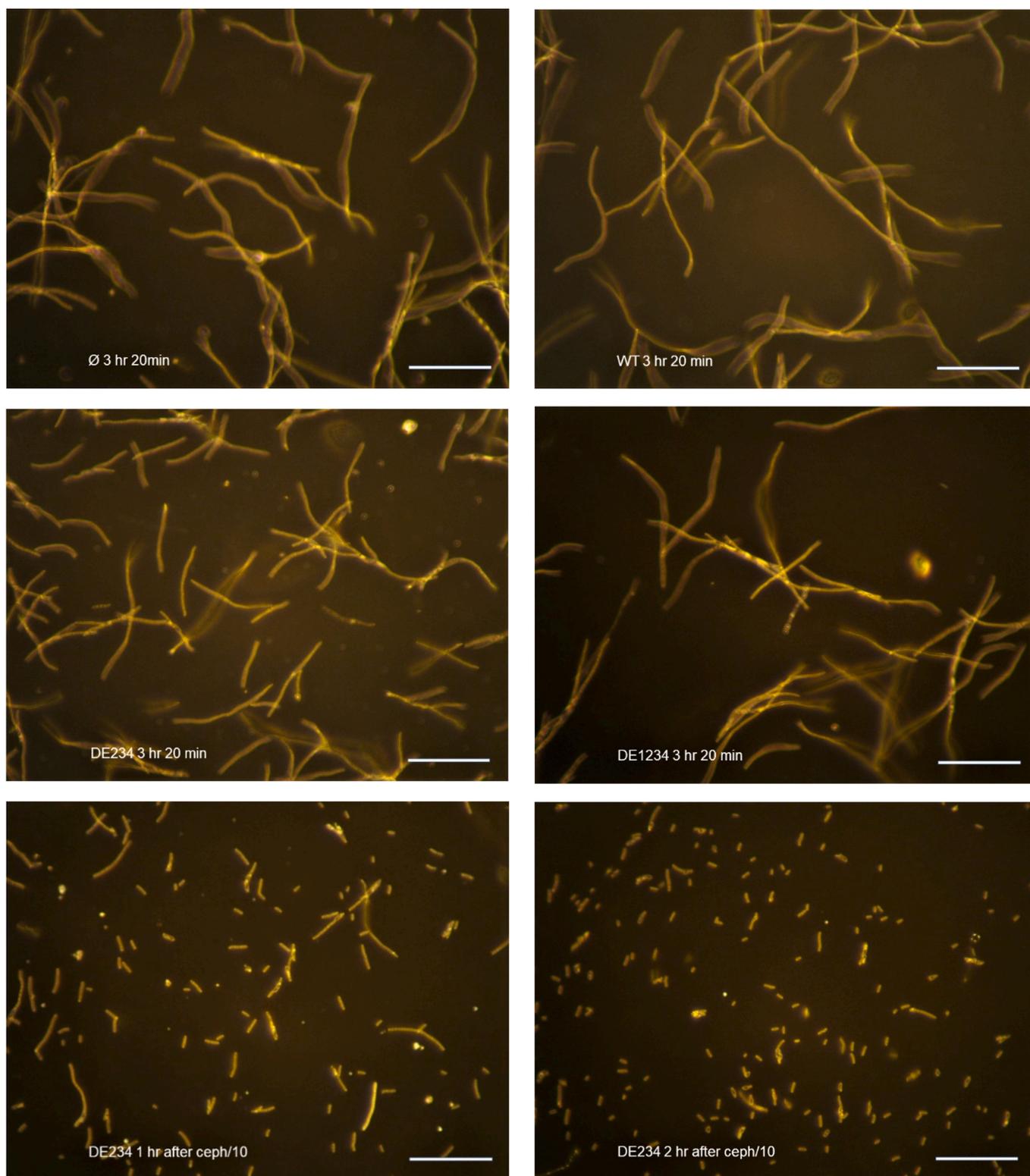


Fig. 3. Dark field light micrographs of *E. coli* grown in AMPICILLIN (200 $\mu\text{g}/\text{ml}$) PLUS CEPHALEXIN (25 $\mu\text{g}/\text{ml}$) and expressing wild type or mutant annexin A4
 3A: \emptyset control plasmid with no insert; 3 h 20 min, time after initial inoculation of the culture
 3B: WT wild type annexin A4; 3 h 20 min, time after initial inoculation of the culture
 3C: DE234 mutant annexin A4; 3 h 20 min, time after initial inoculation of the culture
 3D: DE1234 mutant annexin A4; 3 h 20 min, time after initial inoculation of the culture
 3E: DE234 mutant annexin A4; 1 h after ceph/10 (this is the culture from Fig. 3C after diluting the cephalosporin 10-fold and continuing the incubation for 1 h)
 3F: DE234 mutant annexin A4; 2 h after ceph/10 (this is the diluted culture from Fig. 3E after waiting another hour during which cytokinesis continues – see text for additional details).

Calibration bar on the bottom right of each panel = 50 μm .

sub-images in Figs. 1 and 2. In Fig. 3 the lengths were not determined because most filaments extended beyond the dimensions of the images and were greater than 200 μm . It was instead recorded if these filaments were stable, or broke up into shorter filaments over time, as seen in some of the panels in Fig. 3.

The average lengths of the filaments and the sample standard deviations were calculated in Excel. When comparing the average lengths of the filaments in different samples, P values for statistical significance were calculated in the GraphPad program using a *t*-test with a two-tailed distribution.

Electron microscopy. *E. coli* samples selected for ultrastructural analysis were processed for electron microscopy using technical support and instrumentation in the Advanced Microscopy Facility at the University of Virginia School of Medicine. The samples were fixed in glutaraldehyde and embedded in epon. Ultrathin sections were stained with uranyl acetate and lead citrate and visualized with a JEOL JEM1230 transmission electron microscope.

3. Results

Morphology of *E. coli* expressing wild type and mutant annexins A4 (the “DE” mutants). In 1993 Michael Nelson described the construction of a combinatorial library of mutant annexin A4 cDNAs that was designed to facilitate study of the roles of each of the four homologous calcium and membrane binding sites in bovine annexin A4 in biochemical studies [17,18]. The annexin cDNAs in the library were expressed in *E. coli* using the prokaryotic expression vector pET11d. Each calcium binding site contains an important acidic amino acid residue, aspartate or glutamate, that contributes to the coordination of calcium in the cation binding site. Replacement of these residues with alanine by site directed mutation was used to create a library of mutant proteins with reduced calcium and membrane binding properties, which correlated with the specific combinations of mutations present. These mutants were called the “DE” mutants, for replacement of aspartate (D) or glutamate (E). The sites of the mutations are denoted by the domains harboring the mutations. For example, the DE1 mutant has the calcium binding site in the first domain mutated. The DE12 mutant has the calcium binding sites in the first and second domains mutated, and so forth up to the DE1234 mutant which has all four sites mutated. The specific residues mutated in the constructs used in the present study are listed in the paper by Nelson and Creutz [17]. These mutations reduced the calcium sensitivity of the annexin by up to an order of magnitude in two types of assays: binding of the annexin to a biological membrane (chromaffin granule membrane) and aggregation of chromaffin granule membranes. The calcium sensitivities were altered depending upon which mutations, or combination of mutations were introduced. The measured calcium sensitivities of the mutants used here are given in Nelson and Creutz [17], Table 4. The concentration of calcium leading to half-maximal binding to a biological membrane (bovine chromaffin granule (secretory vesicle) membrane) were as follows: wild type, 5.0×10^{-6} M; DE12, 4.0×10^{-5} M; DE34, 4.9×10^{-5} M; DE234, 1.1×10^{-4} M; DE1234 – no binding. However, it should be noted that the calcium binding affinities of annexins depend on a lipid headgroup participating as a ligand for calcium in the calcium binding pocket. Therefore, the exact affinity for calcium depends on the specific lipid headgroups participating in the binding. Although the annexins generally bind lipids with negatively charged headgroups, the specific lipids in *E. coli* that are bound by the annexin have not been determined. Therefore, the binding affinities in *E. coli* are not known precisely, but are likely to be similar. SDS gel analysis of bacterial extracts indicated that all of the mutants in the library were expressed in the bacteria to comparable levels (Nelson and Creutz (17)).

As demonstrated in the sections below, the use of selected mutants from this library revealed that the ability of the annexin to promote cytokinesis in bacteria was dependent on the competence of some of these sites.

Control cultures grown in ampicillin (200 $\mu\text{g}/\text{ml}$) without expression of the annexin (Fig. 1A). Dark field light microscopy of cultures of *E. coli* strain BL21DE3 harboring the expression plasmid pET11d without an annexin cDNA insert grown in LB-ampicillin media reveals a broad distribution of bacterial cell lengths from 2 to 25 μm (average 9.645 μm , SD (sample standard deviation) 0 5.449 μm , $n = 81$).

Bacterial cultures grown in ampicillin expressing wild type annexin A4 (Fig. 1B). When the bacteria were transformed with the expression plasmid containing the wild type annexin A4 cDNA, the number of longer, filamentous forms of the bacteria were reduced relative to the shorter forms (average 5.279 μm , SD 2.535 μm , $n = 62$) although the optical density (A600) of the cultures with or without the annexin were similar, suggesting net growth of bacterial mass was not greatly altered.

Bacterial cultures grown in ampicillin expressing the DE1234 mutant annexin A4 (Fig. 1C). When bacteria were transformed with the expression plasmid containing the DE1234 mutant annexin, with all four calcium binding sites mutated, a number of longer filaments were formed that were up to 37 μm long (average 8.486 μm , SD 6.580 μm , $n = 61$) – similar to the ones that formed when the bacteria were transformed with the empty expression vector (Fig. 1A). It therefore appeared that the annexin in which all the calcium binding sites had been mutated had a similar effect to removing the entire annexin cDNA from the vector.

Bacterial cultures grown in ampicillin expressing the DE12 mutant annexin A4 (Fig. 1D). When bacteria were transformed with a plasmid expressing the DE12 mutant, which is defective in the calcium binding sites in domains 1 and 2, but has normal calcium binding sites in domains 3 and 4, the cultures had a distribution of sizes (average 9.139 μm , SD 4.104 μm , $n = 65$) similar to control cells transformed with the empty plasmid (Fig. 1A). This suggests that domains 1 and 2 play a dominant role in mediating the cytokinesis promoting effect, while domains 3 and 4 play a minor role in promoting this effect.

Bacterial cultures grown in ampicillin expressing the DE34 mutant annexin A4 (Fig. 1E). When the bacteria were transformed with the plasmid expressing the DE34 mutant, which has the calcium binding sites in domains 3 and 4 mutated but retains wild type calcium binding sites in domains 1 and 2, there were fewer long filaments and short 1–5 μm bacteria were more prominent (average 5.038 μm , SD 2.938 μm , $n = 61$). The culture therefore resembled cultures transformed with the plasmid expressing the annexin with wild type calcium binding sites in all four domains (Fig. 1B). The calcium binding sites in the first two domains of the annexins are the most highly conserved in sequence and the results suggest these two sites provide the major contribution to the cytokinesis promoting activities of the unmutated protein.

Bacterial cultures grown in ampicillin expressing the DE234 mutant annexin A4 (Fig. 1F). When bacteria were transformed with the construct expressing the DE234 mutant the culture was similar to cultures expressing the wild type annexin (average 4.643 μm , SD 2.577 μm , $n = 60$). This result is notable in that this mutant only has one functional calcium-binding domain – domain 1 – so this single domain appears to be sufficient to support the cytokinesis promoting activity of the intact annexin.

Statistical summary of filament lengths of cells grown in ampicillin (200 $\mu\text{g}/\text{ml}$). Summary statistics for filaments grown in ampicillin are given in Table 1. P values for the differences between filament lengths of cells expressing different annexin constructs are given in Table 2. The behavior of the constructs fell into two distinct groups: Constructs that promoted cytokinesis (wild type annexin, DE34, and DE234) and constructs that did not promote cytokinesis (\emptyset (the null vector), DE12, and DE1234). The first group all expressed an annexin with a native (unmutated) first domain calcium binding site. The second group all had a mutated or absent first domain calcium binding site. The P values for comparisons of members of the first group with members of the second group were all highly significant: P values of 0.0005 or less.

Studies with ampicillin (200 $\mu\text{g}/\text{ml}$) plus piperacillin (4 $\mu\text{g}/\text{ml}$)

Table 1

Filament lengths in Fig. 1 (ampicillin). Micrograph: Individual images in Fig. 1. Construct: Annexin expression construct: \emptyset , null vector with no annexin insert; wt, wildtype annexin A4; DE1234, etc -specific DE mutants with the mutated domains (1 through 4) indicated. n: Number of filaments measured in the designated micrograph. AVG μm : average length of the measured filaments; SD μm : sample standard deviation of the lengths of filaments; MAX μm : length of the longest filament in the micrograph.

Micrograph	Construct	n	AVG μm	SD μm	MAX μm
Fig. 1A	\emptyset	81	9.645	5.449	24.88
Fig. 1B	wt	62	5.279	2.535	15.03
Fig. 1C	DE1234	61	8.486	6.580	37.41
Fig. 1D	DE12	65	9.139	4.104	23.49
Fig. 1E	DE34	61	5.038	2.938	19.32
Fig. 1F	DE234	60	4.643	2.577	15.26

Table 2

Statistical analysis of the differences in filament lengths in Table 1 (ampicillin). Each entry in the table is organized as is the data in the top left entry: \emptyset -wt compares the lengths of filaments harboring the null vector (\emptyset , no annexin) and filaments expressing the wild-type annexin (wt); 9.645, 5.279 compares the lengths of the two types of filaments; $P < 0.0001$, P value for the significance of the difference in lengths. Calculations made from data in Table 1.

\emptyset -wt					
9.645, 5.279					
$P < 0.0001$					
\emptyset -DE1234	wt-DE1234				
9.645, 8.486	5.279, 8.486				
$P = 0.2536$	$P = 0.0005$				
\emptyset -DE12	wt-DE12	DE1234-DE12			
9.645, 9.139	5.279, 9.139	8.486, 9.139			
$P = 0.5359$	$P < 0.0001$	$P = 0.5023$			
\emptyset -DE34	wt-DE34	DE1234-DE34	DE12-DE34		
9.645, 5.038	5.279, 5.038	8.486, 5.038	9.139, 5.038		
$P < 0.0001$	$P = 0.6269$	$P = 0.0003$	$P < 0.0001$		
\emptyset -DE234	wt-DE234	DE1234-DE234	DE12-DE234	DE34-DE234	
9.645, 4.643	5.279, 4.643	8.486, 4.643	9.139, 4.643	5.038, 4.643	
$P < 0.0001$	$P = 0.1720$	$P < 0.0001$	$P < 0.0001$	$P = 0.4336$	

(Fig. 2). Ampicillin binds penicillin binding protein 3 (PB3) [15]. A related beta-lactam antibiotic, piperacillin, binds PB3 with higher specificity for PB3 than other beta-lactam antibiotics and at low concentrations affects the formation of septa between dividing bacterial cells to a greater extent than elongation of cells [21]. Piperacillin was tested in this study as it was thought it might be more effective at promoting filament formation by the bacteria and therefore provide a better model for detecting the effects of the annexin on cytokinesis. As described in the sections below, differences between the DE mutants in affecting cytokinesis were also apparent in cultures treated with ampicillin plus piperacillin.

Control bacterial cultures in ampicillin plus piperacillin without expression of annexin A4 (Fig. 2A). As shown in Fig. 2A, bacteria harboring the empty expression plasmid and grown in 200 $\mu\text{g}/\text{ml}$ ampicillin supplemented with 4 $\mu\text{g}/\text{ml}$ piperacillin predominantly formed intermediate length filaments 10–40 μm long, with very few bacteria of near normal 2–4 μm length (average 10.71 μm , SD 5.520 μm , $n = 43$).

Bacterial cultures in ampicillin and piperacillin expressing wild type annexin A4 (Fig. 2B). When the bacteria were transformed with the plasmid expressing the wild-type annexin A4 (Fig. 2B) the filament lengths were predominantly reduced to a length of 2–10 μm (average 5.052 μm , SD 1.594 μm , $n = 81$).

Bacterial cultures in ampicillin and piperacillin expressing the DE1234 mutant annexin A4 (Fig. 2C). When the bacteria were transformed with the plasmid expressing the DE1234 mutant (Fig. 2C), the cells formed longer filaments than were seen with the plasmid without an insert, suggesting the DE1234 quadruple mutant had a slight dominant-negative effect on cytokinesis. However, these longer

filaments tended to aggregate, which may have been due to partial lysis of the cells, and this prevented a precise statistical analysis of the filament lengths (Fig. 2C).

Bacterial cultures in ampicillin and piperacillin expressing the DE12 mutant annexin A4 (Fig. 2D). When the bacteria were transformed with the plasmid expressing the DE12 mutant in medium containing ampicillin plus piperacillin, the filaments were significantly longer than normal, typically 10–36 μm (average 18.17 μm , SD 8.633 μm , $n = 60$) (Fig. 2D). Therefore, the loss of the first two calcium binding sites apparently significantly impaired the ability of the annexin to promote cytokinesis in the presence of ampicillin and piperacillin.

Bacterial cultures in ampicillin and piperacillin expressing the DE34 mutant annexin A4 (Fig. 2E). When the bacteria were transformed with the expression vector for the DE34 mutant the predominant length of the filaments was reduced to 5–10 μm (average 5.941 μm , SD 2.241 μm , $n = 72$) in piperacillin plus ampicillin, indicating, as before with ampicillin alone, the mutants with intact first and second calcium binding domains alone were more effective at promoting cytokinesis than mutants that had only the intact third and fourth calcium binding domains.

Bacterial cultures in ampicillin and piperacillin expressing the DE234 mutant annexin A4 (Fig. 2F). When the bacteria were transformed with the plasmid expressing the DE234 mutant, the cells formed predominantly short filaments of 2–8 μm length (average 4.601 μm , SD 1.723 μm , $n = 64$) in ampicillin plus piperacillin, indicating once again, that the presence of only a single functional calcium binding site in domain 1 was sufficient to promote cytokinesis comparable to, or slightly greater than the extent that was promoted by the wild type annexin.

Statistical summary of filament lengths of cells grown in ampicillin (200 $\mu\text{g}/\text{ml}$) plus piperacillin (4 $\mu\text{g}/\text{ml}$). Summary statistics for filaments grown in ampicillin and piperacillin are given in Table 3. P values for the differences between filament lengths of cells expressing different annexin constructs are given in Table 4. The behavior of the constructs fell into two distinct groups: Constructs that promoted cytokinesis (wild type annexin, DE34, and DE234) and constructs that did not promote cytokinesis (\emptyset (the null vector), DE12, and DE1234). The first group all expressed an annexin with a native (unmutated) first domain calcium binding site. The second group all had a mutated or absent first domain calcium binding site. The P values for comparisons of members of the first group with members of the second group were all highly significant: P values of 0.005 or less.

Studies with ampicillin (200 $\mu\text{g}/\text{ml}$) plus cephalixin (25 $\mu\text{g}/\text{ml}$) (Fig. 3). The antibiotic cephalixin has one of the highest affinities among the beta-lactam antibiotics for binding to penicillin binding protein 3 [15] and was found in this study to have the greatest propensity to promote the formation of long, stable filaments, compared with ampicillin and piperacillin. When bacteria transformed with the empty expression plasmid (Fig. 3A), or with the wild type annexin

Table 3

Filament lengths in Fig. 2 (ampicillin plus piperacillin). Micrograph: Individual images in Fig. 2. Construct: Annexin expression construct: \emptyset , null vector with no annexin insert; wt, wildtype annexin A4; DE1234, etc -specific DE mutants with the mutated domains (1 through 4) indicated. n: Number of filaments measured in the designated micrograph. AVG μm : average length of the measured filaments; SD μm : sample standard deviation of the lengths of filaments; MAX μm : length of the longest filament in the micrograph. Note: Measurements were not made of filaments in Fig. 2C because of aggregation of the filaments (see text).

Micrograph	Construct	n	AVG μm	SD μm	MAX μm
Fig. 2A	\emptyset	43	10.71	5.520	29.79
Fig. 2B	wt	81	5.052	1.594	9.472
Fig. 2C	DE1234	–	–	–	–
Fig. 2D	DE12	60	18.17	8.633	36.28
Fig. 2E	DE34	72	5.941	2.241	13.25
Fig. 2F	DE234	64	4.601	1.723	8.718

Table 4

Statistical analysis of the differences in filament lengths in Table 3 (ampicillin plus piperacillin). Each entry in the table is organized as is the data in the top left entry: \emptyset -wt compares the lengths of filaments harboring the null vector (\emptyset , no annexin) and filaments expressing the wild-type annexin (wt); 10.71, 5.052 compares the lengths of the two types of filaments; $P < 0.0001$, P value for the significance of the difference in lengths. Calculations made from data in Table 3.

\emptyset -wt			
10.71, 5.052			
$P < 0.0001$			
\emptyset -DE12	wt-DE12		
10.71, 18.17	5.052, 18.17		
$P < 0.0001$	$P < 0.0001$		
\emptyset -DE34	wt-DE34	DE12-DE34	
10.71, 5.941	5.052, 5.941	18.17, 5.941	
$P < 0.0001$	$P = 0.0050$	$P < 0.0001$	
\emptyset -DE234	wt-DE234	DE12-DE234	DE34-DE234
10.71, 4.601	5.052, 4.601	18.17, 4.601	5.941, 4.601
$P < 0.0001$	$P = 0.1048$	$P < 0.0001$	$P = 0.0002$

(Fig. 3B), or the DE1234 mutant (Fig. 3D) were grown in ampicillin supplemented with cephalixin, long filamentous cells were formed over time, suggesting cytokinesis was blocked nearly completely. Many of the filaments extended beyond the field of view in the micrographs and the lengths were not measured. However, the filaments formed with the DE234 mutant (Fig. 3C) were of limited length at the same time point (3 h and 20 min after seeding the cultures) and were estimated to be 15 to at least 100 μm in length. These filaments were observed over time to be growing more slowly than the other constructs, apparently because both growth and cytokinesis were occurring simultaneously.

Although the length of the filaments of the DE234 mutant cells grew more slowly than the lengths of the other constructs, in all cases the optical densities of the cultures increased at approximately the same rate, plus or minus approximately 20 %. Apparently the mass of total bacterial cells in the culture was increasing at a similar rate independent of the presence of the mutations, but the mass was differentially distributed between cells in the extended, filamentous form and the more normal shape as shorter rods, without having a large effect on the relationship between optical density and mass of cell material present.

Tenfold dilution of the cephalixin concentration results in an increase in cytokinesis in the filaments formed with the DE234 mutant, but not in the filaments formed with other mutants (Fig. 3E and F). After 3 h and 20 min of growth in the presence of ampicillin and cephalixin the cultures were diluted tenfold in fresh medium containing ampicillin but no cephalixin, resulting in a cephalixin concentration reduced from 25 to 2.5 $\mu\text{g}/\text{ml}$ to test the stability of the filaments. The filaments formed by bacterial cultures harboring the empty plasmid, wild type annexin, or the DE1234 quadruple mutant were completely stable for the next 2 h, unaffected by the reduction in cephalixin concentration (not shown). However, the cells expressing the DE234 mutant, containing only one functional calcium binding site, began to undergo cytokinesis to form shorter filaments after 1 h, and further divided by 2 h after the dilution. (Compare Fig. 3C with 3E and 3F). This activity of the DE234 mutant parallels the superior ability of the same mutant to promote cytokinesis in ampicillin alone or in ampicillin plus piperacillin seen in Figs. 1 and 2 above.

Electron microscopy of cells expressing wild type or mutant annexins A4. Cultures of the BL21(DE3) cells harboring the empty expression plasmid or plasmids expressing wild-type or selected mutant annexins A4 were grown in LB medium plus 200 $\mu\text{g}/\text{ml}$ ampicillin and 4 $\mu\text{g}/\text{ml}$ piperacillin and processed for electron microscopy of ultrathin sections of pelleted cells to determine if the expression of the annexin altered the ultrastructure of the cells, particularly at sites of cell division. No unique morphology was apparent at sites of cell division with or without annexin expression. There was no apparent increase of protein density at division sites as might be expected if the annexin were preferentially accumulated at sites of cell division and membrane fusion.

However, in all of these experiments the expression level of the annexin was necessarily kept low since high levels of annexin expression resulted in disruption of membrane structure and aggregation of the bacteria, as described in the Methods section.

Although no unique ultrastructure was seen in cells expressing the annexin, the changes in filament length due to the annexin, as seen by light microscopy, were confirmed in the electron micrographs. Fig. 4A shows the morphology of cells harboring the empty expression plasmid and therefore without any annexin. This representative figure shows that there are both short bacterial cells present and filamentous forms, as was seen in the light micrographs of these cultures. Fig. 4B shows cells expressing the wild type annexin A4. In this case the number of short cells exceeds the number of extended filamentous forms due to the cytokinesis enhancing activity of the annexin. Similarly, in Fig. 4C the number of short cells exceeds the filamentous forms due to the cytokinesis promoting activity of the DE234 mutant, while in Fig. 4D the filamentous forms exceed the short forms due to the enhancement of filamentous forms due to the action of the DE1234 mutant. In these electron micrographs of ultrathin sections it is not possible to determine if what appear to be short cells in the electron micrographs are not actually cross sections of the filamentous forms. However, light micrographs of similar cultures (e.g. in Fig. 2) show the complete cells and support the relative abundance of short and long forms in the cultures as interpreted in the electron micrographs.

4. Discussion

This study reports the finding that a eukaryotic calcium- and membrane-binding protein, annexin A4, can enhance cytokinesis in bacteria in which cytokinesis is partially inhibited by beta-lactam antibiotics. The processes of cytokinesis in eukaryotes and in prokaryotes have in common characteristic morphological steps. The dividing cells pinch down into a neck between the forming daughter cells, the membranes in the neck fuse together and the cells separate. The molecular events underlying these morphological changes are under intense study in both cell types [9,22–24]. Different, unrelated sets of proteins undergo complex interactions in each of these types of organisms to mediate cytokinesis, but there is little similarity or known homology between the sets of proteins. In prokaryotes like *E. coli* a master protein appears to be FtsZ which has some features in common with the eukaryotic protein tubulin in that it has a GTPase activity and undergoes polymerization [25]. The FtsZ polymer forms a ring around the middle of the bacterium that contracts to form the neck between the dividing cells leading to membrane fusion and separation of the cells. However, to accomplish this the protein interacts with numerous other proteins, some of which, like penicillin binding protein 3 (PB3), bind beta-lactam antibiotics. The set of proteins responsible for cytokinesis in eukaryotes is also complex and the complete sequence of protein interactions is not fully worked out. Beta-lactam antibiotics do not interact with the proteins participating in cytokinesis in eukaryotic cells, indicating a significant contrast between the mechanisms in the two types of cells. These differences raise the question of how a single protein, annexin A4, can influence cytokinesis processes involving such distinct protein machineries.

The answer may be that the annexin interacts with two critical elements common to both types of cells: intracellular calcium and phospholipids [6]. The importance of the interaction with calcium in bacteria is demonstrated in this report since disabling the calcium binding sites in the annexin reduces its ability to promote cytokinesis. The interaction of the annexin with calcium is intimately linked to its interactions with phospholipids since both of these ligands participate in forming the high affinity calcium and phospholipid binding site in the annexin [8]. Since the binding of calcium and phospholipid has been demonstrated in the simplest model systems, involving only an annexin, calcium, and lipid bilayers, to lead to bilayer fusion [26], no other proteins may be necessary to support membrane fusion in cells. However, evidently other

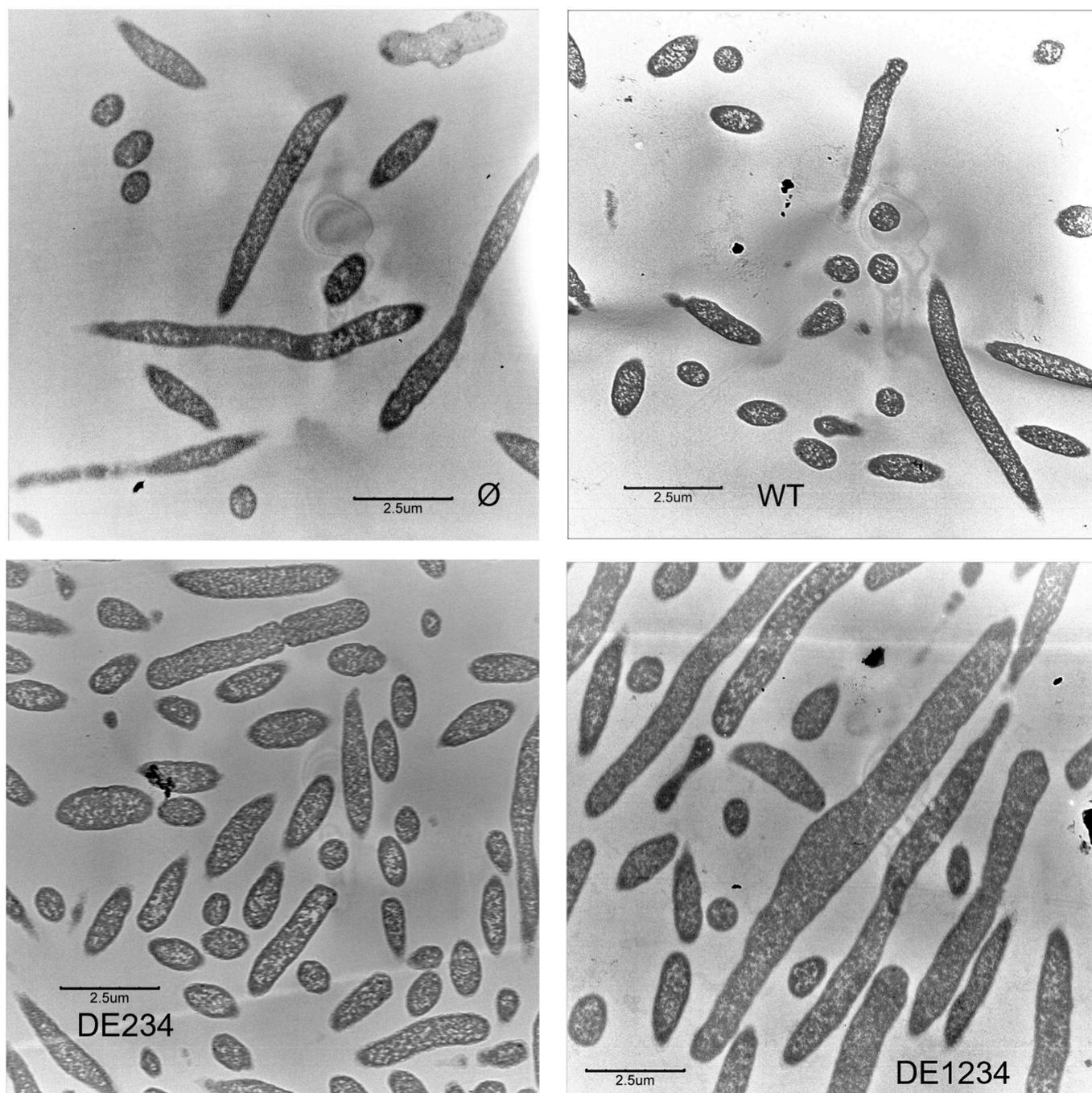


Fig. 4. Electron micrographs of *E. coli* grown in AMPICILLIN (200 $\mu\text{g}/\text{ml}$) PLUS PIPERACILLIN (4 $\mu\text{g}/\text{ml}$) and expressing wild type or mutant annexin A4
 4A: \emptyset control plasmid with no insert
 4B: WT wild type annexin A4
 3C: DE234 mutant annexin A4
 3D: DE1234 mutant annexin A4
 Calibration bar in each panel = 2.5 μ

proteins are important in both eukaryotes and prokaryotes for membrane fusion to be utilized in the biological context of cytokinesis.

On the other hand, annexins are not absolutely required for cytokinesis in prokaryotes as demonstrated by the absence of annexins in the majority of bacteria [6]. A few eukaryotes, such as the yeast *S. cerevisiae*, can also carry out cytokinesis without depending on annexin homologues since they are not expressed in this yeast. Other mechanisms must be able to substitute for the role of the annexins in these counterexamples.

Although the exact mechanism by which the annexin promotes cytokinesis in bacteria is not clear, this should not prevent consideration of possible biotechnology applications of bacterial expression of annexins. As discussed in the INTRODUCTION, disassembly of bacterial filaments formed in the presence of antibiotics may promote the effectiveness of antibiotic chemotherapy by enabling cells of the immune system to destroy the non-filamentous forms of the bacteria [1,16]. Among the proteins studied here, the DE234 mutant annexin A4 might be the most effective potential therapeutic because of its efficacy in

promoting cytokinesis in the bacterial filaments.

The possibility of using annexins to suppress the deleterious effects of pathogenic bacteria will be dependent on the ability to express the eukaryotic annexins in the bacteria while they are invading a host. Multiple approaches may be available, including transfection by expression plasmids as used in this study, or introduction through phage-based transfer of genetic material [27].

5. Conclusions

A eukaryotic calcium- and membrane-binding protein, bovine annexin A4, is shown here to promote cytokinesis in bacterial cells in which this process has been partially inhibited by beta-lactam antibiotics. The expression of annexins in bacteria may therefore be a useful adjuvant in bacterial chemotherapy as it might make the shortened bacterial cells more susceptible to attack by cells of the immune system. This should be tested in animal models of bacterial infection. The similar calcium- and membrane-binding properties of other annexins suggest that promotion of cytokinesis might be a common property of all annexins. It will be important to determine by gene deletion or mutagenesis whether the known bacterial proteins that have sequence similarities to the annexin family of proteins function in bacterial cytokinesis.

Funding

Financial support was provided by the Office of the Vice President for Research, University of Virginia, and the Department of Pharmacology, University of Virginia.

Declaration of competing interest

The University of Virginia has filed a preliminary patent application for the use of annexins as an adjuvant in antimicrobial chemotherapy.

Data availability

Data will be made available on request.

Acknowledgements

Hitoshi Sohma (Sapporo Medical University) kindly provided replacement samples of the DE234 and DE1234 annexin A4 mutants after these samples in the University of Virginia collection were lost.

Thurl Harris and Michael Nelson (University of Virginia) provided helpful advice and discussions throughout this study, and Daniel Stornetta (University of Virginia) provided consultation on the Image J program for image analysis and on statistical methods.

Stacey Criswell, Natalia Dworak, and Helen Park of the Advanced Microscopy Facility, University of Virginia School of Medicine, provided technical assistance for the electron microscopy.

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