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## ***Rickettsia peacockii*, an endosymbiont of *Dermacentor andersoni*, does not elicit or inhibit humoral immune responses from immunocompetent *D. andersoni* or *Ixodes scapularis* cell lines**

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### Abstract

*Ixodes scapularis* and *Dermacentor andersoni* cell lines were stimulated with heat killed *Escherichia coli* and *Micrococcus luteus* to investigate whether infection by *Rickettsia peacockii*, an endosymbiont of *D. andersoni*, modifies humoral immune responses. Radial diffusion assays, western blotting, flow cytometry, and quantitative reverse-transcription PCR were used to determine if expression of bacteriolytic peptides, including lysozyme and defensin, was upregulated by bacterial stimulation or infection with *R. peacockii*. The *I. scapularis* line IDE12 upregulated expression of lysozyme and defensin following stimulation. The *D. andersoni* cell line DAE15 also expressed defensin and lysozyme, but only lysozyme was upregulated by bacterial stimulation. *R. peacockii* infection alone, or in cells stimulated with bacteria, did not modify defensin or lysozyme expression in either cell line. These results suggest tick endosymbionts may avoid recognition by the tick immune system, and infection may not affect humoral immune responses to bacteria not normally associated with ticks.

### Keywords

endosymbiont; immune response; defensin; lysozyme; tick cell culture; rickettsia; real-time PCR; immune evasion

### Introduction

Ticks are important vectors of bacteria that cause disease in humans and animals. Factors underlying tick success as vectors are poorly understood, but the tick immune system may play a role in determining vector capacity (1). Bacterial challenge causes immunocompetent tissues to produce several soluble antimicrobial factors, including defensin and lysozyme. Defensins are small cationic antimicrobial peptides with activity against gram positive bacteria (2,3) that have been identified in several hard ticks including *Dermacentor variabilis* (3) and *Ixodes scapularis* (4,5). Lysozymes hydrolyze the 1-4  $\beta$  linkage between the N-acetyl-muramic acid and N-acetyl-D-glucosamine residues that make up peptidoglycan in bacterial cell walls (6). Immunoresponsive c-type lysozymes have been identified in *Ornithodoros moubata* (7,8), *D.*

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*variabilis* and a *D. andersoni* cell line (9). Lysozyme-like immunoreactivity has been reported in *I. ricinus* hemocytes (10) and protein extracts from several soft and hard ticks (11). Lysozymes may synergize antimicrobial peptides, including defensins, by degrading the peptidoglycan cell wall and permitting better access to the bacterial cell membrane (3,12,13).

Ticks are persistently infected with nonpathogenic rickettsia-like bacteria (14,15). Immune interactions between ticks and endosymbionts are difficult to assess *in vivo*, and it is not known whether endosymbionts elicit immune responses similar to those induced by *Escherichia coli* or *Micrococcus luteus*. *Rickettsia peacockii* is a rickettsial endosymbiont of *D. andersoni*, and is found within the interstitial cells of the ovaries, and developing oocytes (16). It is not known why *R. peacockii* appears to be restricted to the female reproductive tissues, but host immune responses may limit rickettsial distribution. *R. peacockii* can infect and grow in cell lines from a variety of arthropods (17) and tick cell culture may help determine whether the tick immune system recognizes and responds to endosymbionts in the same way it responds to bacteria that are not normally present within the tick hemocoel.

Previous research demonstrated the cell lines IDE12 and DAE15 from *Ixodes scapularis* and *D. andersoni*, respectively, phagocytosed and killed *Borrelia burgdorferi*, and that prior infection by *R. peacockii* slightly diminished phagocytic activity (18). In the studies reported here, we investigated whether these tick cell lines produced soluble antimicrobial factors, and examined expression of these factors following bacterial stimulation. Additionally, we examined whether *R. peacockii* alone induces a humoral response or modifies the humoral response to heat inactivated *E. coli* and *M. luteus*. Our results demonstrate *E. coli* and *M. luteus* stimulated IDE12 and DAE15 cells upregulated expression of a lysozyme-like protein that was not upregulated by *R. peacockii* infection. IDE12 cells upregulated defensin expression following stimulation with heat inactivated *E. coli* and *M. luteus*, but *R. peacockii* infection alone did not induce upregulation or modify expression following stimulation. DAE15 cells expressed defensin, but expression was not upregulated by stimulation or infection. These results indicate cultured tick cell lines respond to stimulation by *E. coli* and *M. luteus*, but not *R. peacockii*, with upregulated humoral immune responses. Thus, our results suggest rickettsial endosymbionts avoid activating the humoral immune responses of their tick host.

## Materials and Methods

### Cell lines and culture conditions

The *I. scapularis* lines IDE12 (19) and ISE6 (20), and the *D. andersoni* line DAE15 (17) were grown in 25 cm<sup>2</sup> CellStar tissue culture flasks (Greiner Bio-One Inc, Longwood, FL) containing 5 mL L15B (21) supplemented with 5% fetal bovine serum, 5% tryptose phosphate broth (Becton Dickinson, Franklin Lakes, NJ), and 0.1% cholesterol concentrate (MP Biomedicals, Irvine, CA). New cultures were established by 1:5 dilutions (1 part cell-containing medium to 4 parts fresh medium) of confluent cultures into fresh flasks. Cultures were maintained at 34°C and fed fresh medium weekly. IDE cell lines have been shown to be chronically infected with the St. Croix River virus (Reoviridae: Orbivirus) (22). However, this does not appear to impair their phagocytic ability, since the ISE6 line from the same tick species, but which does not carry that virus (H. Attoui, pers. comm.), shows much less phagocytic activity than IDE lines (18).

Cultures were infected with host-cell free preparations of *R. peacockii* (*Rp*) made by filtering *Rp*-containing medium from heavily infected ISE6 cultures through a 5.0 µm syringe filter (Millex-SV) (Millipore, Bedford, MA). Isolated rickettsiae were added to uninfected cultures one week after subculture with an estimated multiplicity of infection of 100 rickettsiae per cell.

The infection status of cultures was monitored by comparing the proportion of infected cells to uninfected cells in Giemsa-stained Cytospin preparations (Shandon, Pittsburg, PA).

*E. coli* (D21) and *M. luteus* were grown in LB medium (MP Biomedicals, Irvine, CA) overnight in a shaking water bath at 37°C. Stock cultures of *E. coli* and *M. luteus* were collected by centrifugation, the pellets were washed with Hanks' balanced saline solution (HBBS) (Invitrogen, Carlsbad, CA) and resuspended in 10 mL of PBS. Bacteria were inactivated by autoclaving cultures for 20 minutes at 121°C. Autoclaved bacteria were washed once more as described above and resuspended in HBSS (pH 6.8), counted using a Petroff-Hauser counting chamber, and the density adjusted to  $1 \times 10^9$ /mL. Bacterial stocks were stored at 4°C.

### Bacterial challenge and harvest of cells

Cultures were seeded into Falcon® 12.5 cm<sup>2</sup> flasks (Becton-Dickinson) at a density of approximately  $5 \times 10^5$  cells per mL and maintained at 32°C in 2.5 mL of medium. One week later, half of the cultures were infected with *R. peacockii* as described above. Uninfected and infected cultures were grown for one week and the infection status assessed. A  $2 \times 2$  factorial design with three replicates per treatment was used to determine the effects of bacterial stimulation and *R. peacockii* on expression of antimicrobial peptides. Cultures were divided into four groups: unstimulated and uninfected cultures (control), unstimulated and infected cultures (*Rp* infected), uninfected and bacteria stimulated cultures (stimulated), and *R. peacockii* infected and bacteria stimulated cultures (*Rp* infected and stimulated). Cultures were fed with 2.0 mL of fresh medium the day prior to stimulation. Cells were counted by comparing the number of cells per five randomly chosen microscopic fields per flask to ensure each flask contained similar numbers of cells. Cultures were inoculated with 10 *E. coli* and 10 *M. luteus* per cell for a total of 20 bacteria per cell and incubated 48 hours at 32°C.

Antimicrobial factors present in spent culture medium and cell lysate were measured by radial diffusion assays, flow cytometry, and quantitative real-time PCR (qRT-PCR). Spent medium was removed 48 hours post stimulation and transferred to microfuge tubes. Medium samples were centrifuged at  $21,000 \times g$  for 20 minutes at 4°C, and filtered through 0.22 µm syringe filter prior to concentration using YM-3 Centricon centrifugal filters (Millipore, Billerica, MA). Samples were centrifuged at 4°C for 110 minutes at  $4890 \times g$  in a refrigerated centrifuge (Hettich, Tuttlingen, Germany), concentrating them from 1.2 mL to approximately 200 µL. Concentrated medium samples were stored at -20°C. Remaining cell layers were washed with PBS and resuspended in 3 mL of PBS with a blunt-tip, 14 g cannula attached to a 5 mL syringe. Aliquots containing 0.25 mL of cells, equivalent to  $3.5 \times 10^5$  cells, were removed from each culture. The 0.25 mL aliquots were pooled within replicates for a total volume of 0.75 mL per treatment, centrifuged at  $300 \times g$  for 8 minutes and the supernatant was removed. Cells were lysed by vortexing in 1 mL of sterile water, and centrifuged at  $21,000 \times g$  for 20 minutes at 4°C to pellet insoluble cell fragments. Clarified lysate was stored at -20°C until concentrated as above, and the concentrated lysate was stored at -20°C. A 1 mL aliquot of cells in PBS was removed from each flask, centrifuged at  $300 \times g$  for 8 minutes, and the PBS was removed. The cells were resuspended in 50 µL of RNAlater (Ambion, Austin, TX) and frozen at -70°C. The remaining volume of cells in PBS (approximately 1.75 mL) was kept on ice until fixed, permeabilized and stained for assay by flow cytometry.

### Assays for antimicrobial activity in cells and culture medium

**Radial diffusion assays**—An overnight culture of *M. luteus* was measured at 600 nm and diluted to an optical density of 0.01 in 0.8% LB-agarose equilibrated at 45°C. Plates were prepared by pouring 15 mL of bacteria-containing LB-agarose into 150 mm Petri dishes. Wells were punched in the solidified agarose with the sterilized tip of a 14 g cannula attached to a 5 mL syringe, and 4 µL of concentrated 48 hr medium or cell lysate was added to each well.

Plates were incubated overnight at 37°C and examined the following day. Wells surrounded by circular zones free of bacterial colonies were considered to be positive for antimicrobial factors. Images of the plates were taken using a Cohu CCD camera (Cohu Inc., San Diego, CA) and the diameters of the bacteria-free zones measured with Photoshop (Adobe Systems Inc., San Jose, CA). The diameter of bacteria-free zones was determined by comparing the diameter of the *M. luteus*-free zone to the diameter of the cannula-punched well (2 mm). Data from media samples are expressed as the mean diameter of zones from three replicate cultures  $\pm$  standard deviation.

### Nonreducing gel electrophoresis with *M. luteus* overlay

Nonreducing gel electrophoresis using a *M. luteus* overlay was adapted from protocols described by Cytrynska et al (23). Briefly, lysate from  $1 \times 10^5$  uninfected cells stimulated with bacteria, was prepared as described above and concentrated in a Savant Speed Vac (Thermo Electron Corporation, Waltham, MA) to 10  $\mu$ L volume. Samples were mixed with 3  $\mu$ L of 5X nonreducing loading buffer (50% glycerol, 31% 1M Tris-HCL [pH 6.8], 14% deionized water, and 5% of 1% bromophenol blue) and run on a 4-20% tris-tricine precast polyacrylamide gel (ISC BioExpress, Kayesville, UT) that had been run for 15 minutes at 150 V prior to addition of samples. Prestained molecular weight markers were omitted because they had deleterious effects on *M. luteus* growth. Electrophoresis running buffer included (w/v) 0.61% tris, 0.9% tricine, and 0.05% SDS. Lysates were loaded onto the gel and electrophoresed until the dye front had reached the bottom of the gel. The gel was washed for 30 minutes in 150 mL of 0.1% Triton-X-100 to remove the sodium dodecyl sulphate from the gel, proteins were refolded with one 30 minute incubation in 50 mM Tris-HCl (pH 7.0) at room temperature, and incubated in LB medium for 30 minutes. Gels were placed in a 150 mm Petri dish and overlaid with a thin layer of *M. luteus*-containing LB agarose (previously described) and incubated overnight at 37°C. Areas containing antimicrobial activity in the gel were indicated by zones without *M. luteus* growth.

### Western blotting

Aliquots containing  $1 \times 10^6$  cells were resuspended in 15  $\mu$ L of 2X protein extraction buffer (50% deionized water, 20% of 10% SDS solution, 12% of 0.5M Tris HCl [pH 6.8], 10% glycerol, 4% B-mercaptoethanol, and 4% of a 1% bromophenol solution), lysed by vortexing, and solubilized by heating in boiling water for 5 minutes. Insoluble material was removed by centrifugation at  $21,000 \times g$  for 15 minutes at 4°C. Sample volumes equivalent to  $1 \times 10^5$  cells were loaded into 4-20% tris-tricine precast gels (ISC BioExpress) and run at room temperature until the dye front reached the bottom of the gel. Proteins were transferred to Immobilon-P membranes (Millipore) and blocked with 3% powdered milk-PBS overnight at 4°C. A rabbit anti-lysozyme antibody (Lab Vision, Fremont, CA) was chosen for western blotting because it cross reacts with lysozyme from a broad range of vertebrate taxa, as well as chicken egg lysozyme (Fisher Scientific, Waltham, MA), and the lysozyme standard in the SeeBlue Plus2 prestained molecular weight marker (Invitrogen, Carlsbad, CA) (not shown). Membranes were reacted with antibodies diluted 1:1000 in 3% bovine serum albumin PBS (BSA-PBS) followed by several washes with PBS. Membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD) diluted 1:1000 in BSA-PBS and developed using the 3C Peroxidase Detection Kit (Kirkegaard & Perry Laboratories). Images of membranes were acquired on a flat bed scanner (UMax, Dallas, TX) at 300 dpi.

### Flow cytometric detection of intracellular lysozyme

Cells were harvested as described above, resuspended in PBS, and transferred to microfuge tubes for fixation using the Fix and Perm Kit (Invitrogen) with methanol modification. Briefly,

50  $\mu$ L of fixative was added to each tube, followed by 300  $\mu$ L of ice cold absolute methanol, and the cells were incubated on ice for 15 minutes. The fixed cells were pelleted, the fixative was removed, and the cells were washed once with 1.5 mL of PBS. Cells were treated with 50  $\mu$ L of permeabilization reagent containing primary antibody against lysozyme (diluted 1:50 in permeabilization buffer). Cells were incubated with antibody for 30 minutes at 37°C, washed once with 1.5 mL PBS, and stained for 30 minutes at 37°C with a 1:50 dilution of goat anti-rabbit FITC-conjugated secondary antibody (Pierce, Rockford, IL) in permeabilization buffer. The cells were washed once with 1.5 mL of PBS and the pellets were resuspended in 1 mL of PBS for analysis. Samples were run on a FACSCalibur flow cytometer (Becton Dickinson). Forward scatter and side scatter were used to gate on the main cell cloud and exclude debris and aggregated cells. The fluorescence of 10,000 events in the F11 channel was recorded and data were analyzed in CellQuest Pro (Becton Dickinson). Cytospin preparations were made of the remaining cells. Cell spots were sealed under glass cover slips using VectaShield Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector Labs, Burlingame, CA) to limit photobleaching and to counterstain the cell nuclei. Cells were examined on an Eclipse 400 fluorescence microscope (Nikon, Melville, NY) to visualize staining patterns. Images were taken using a DXM-1200 digital camera (Nikon) at 100 $\times$  using separate filters for FITC and DAPI.

### RNA isolation and DNase treatment

The cells stored at -70°C in RNAlater were diluted 1:5 with PBS, centrifuged for 10 min at 400  $\times$  g, and the supernatant was removed. Total RNA was isolated using the RNeasy RNA Isolation Kit (Qiagen, Valencia, CA) following kit protocols. RNA was eluted from the column with 35  $\mu$ L of RNase-free water and quantified by UV spectrophotometry with a BioPhotometer (Eppendorf AG, Hamburg, Germany). DNase treatment was done using the DNasefree DNase treatment kit (Ambion) following kit protocols. DNase-treated RNA was diluted to 15 ng/ $\mu$ L in DNase and RNase-free water and stored at -70°C.

### Sequencing of the defensin from the DAE15 cell line

Primers for reverse-transcription PCR (RT-PCR) were designed with the PrimerQuest application on the Integrated DNA Technology website (<http://scitools.idtdna.com/Primerquest/>) (Table 2). RT-PCR was done with DvDef fw and DvDef Rev primers developed from the sequence of varisin (Genebank accession AY181027), a defensin from *D. variabilis* to amplify a 543 bp sequence of the *D. andersoni* defensin. Amplification was done using DNase-treated RNA as template according to Access RT-PCR kit protocol (Promega, Madison, WI). Reactions were made to 25  $\mu$ L using 2.5 U AMV reverse transcriptase, 2.5 U *Tfl* polymerase, 5X buffer, 0.2 mM dNTP mix, 1.5 mM MgSO<sub>4</sub>, 0.25  $\mu$ M each primer and 30 ng total RNA template. Reactions were run on a Robocycler PCR Cyclyer (Stratagene, La Jolla, CA) with a heated top using the following program: 45 min at 48°C (reverse transcription), 5 min at 94°C (denaturation), 35 cycles of 94°C (30 s), 50°C (45 s), 72°C (1 min) and a final extension at 72°C for 5 minutes. Products were run on a 1.5% agarose gel stained with ethidium bromide, and a band of the predicted size excised and purified using a MinElute Gel Extraction Kit (Qiagen). Eluted products were cloned using the TOPO-TA Cloning Kit for Sequencing PCR Products (Invitrogen) and sequenced in the forward and reverse directions on an ABI 3700 automated sequencer (BioMedical Genomics Center, University of Minnesota). The 5' and 3' ends of the defensin cDNA were determined using the GeneRacer Kit (Invitrogen) according to kit protocols. The sequences of GeneRacer kit supplied primers are available at [www.invitrogen.com/content/sfs/manuals/3prime\\_race\\_man.pdf](http://www.invitrogen.com/content/sfs/manuals/3prime_race_man.pdf). The 5' end was determined by nested PCR reactions on GeneRacer (GR) ligated cDNA using the GR forward (fw) primer and var157 reverse (rev) primer followed by a second reaction using the nested GR fw primer and DaDefInt Rev primer to amplify a 190 bp fragment. The 3' sequence was determined by performing nested PCR on GR 3' oligonucleotide-ligated cDNA using the DaDefInt fw primer



in conjunction with the GR 3' rev primer followed by a nested reaction using var157 fw and nested GR rev primers. The 5' and 3' ends were cloned using the supplied Zero Blunt TOPO Cloning kit and sequenced at least six times. The 543 bp product produced by the DvDef fw and DvDef rev primer set was cloned and sequenced three times in the forward and reverse directions. Sequences were aligned and the consensus sequence, and deduced amino acid sequences generated using the BioEdit software package (24). Protein identity and similarity to other tick defensins was determined by BLAST (25), and aligned with BioEdit.

### Quantitative reverse transcription PCR

LUX primers (Invitrogen) for quantitative RT-PCR (qRT-PCR) were generated using the D-LUX designer software (<https://orf.invitrogen.com/lux/>) from the cDNA sequences of the *D. andersoni* defensin (this report), *I. scapularis* defensin (AY660970) and *I. scapularis*  $\beta$ -actin (AF426178) as template sequences. Primers were tested using standard RT-PCR to find primer sets that worked in multiplexed applications to simultaneously detect defensin and  $\beta$ -actin cDNA (internal control). Products were resolved on 3% NuSieve GTG agarose gels (BMA, Rockland, MD) stained with SYBR green (Cambrex Bioscience, Rockland, ME). Primer sets producing extra amplicons, or that yielded an amplicon intensity in multiplexed reactions which was different from individual primer-pair reactions, were not used. The primers selected for the *I. scapularis* defensin and  $\beta$ -actin were located within the protein-encoding open reading frame (ORF) of the corresponding gene. The fluorophore-labeled primer for the *D. andersoni* defensin was located within the 5' end of the protein-encoding ORF and the unlabeled primer was located in the non-coding cDNA sequence preceding the protein-coding ORF.

A master mix was prepared using the One-Step Supermix kit with ROX (Invitrogen) as described by the kit protocol and included 5  $\mu$ M each primer (unlabeled and fluorophore-labeled defensin and  $\beta$ -actin primers), 2 $\times$  buffer, polymerase cocktail and DNase-treated RNA template for a final reaction volume of 20  $\mu$ L. Reactions for DAE15 defensin used 30 ng of total RNA template whereas IDE12 reactions used 60 ng of total RNA template. Duplicate reactions were prepared using *Taq* polymerase (Promega) alone as a no reverse-transcriptase control to ensure template RNA was not contaminated with DNA and only the target was being measured. Plates were run on an ABI 9700HT real time PCR cycler (Applied Biosystems, Inc., Foster City, CA) using the following program: a reverse transcription step of 15 min at 50°C, 2 min at 94°C, and 50 cycles of 94°C (30s denaturation), 60°C (30s hybridization and extension). Melting curve analysis followed amplification cycles to ensure a single product was amplified for each primer set and to confirm the absence of primer dimers. Data were analyzed using the Sequence Detection System 2.1 software package (Applied Biosystems) and expressed as the fold change in relative transcript abundance from the untreated control using the method described by Livak and Schmittgen (26).

### Statistical analysis

Statistical analysis was performed with SigmaStat (SysStat Software, Inc., Point Richmond, CA). Comparisons of two means were done using the Student t-test. Comparisons of multiple treatments against uninfected, unstimulated controls were done using one way analysis of variance (one way ANOVA). Data with  $p < 0.05$  were considered statistically significant.

## Results

### Infection of IDE12 and DAE15 cells with *R. peacockii*

Preliminary experiments were performed to determine culture parameters suitable for detecting and measuring humoral immune responses due to *R. peacockii* infection or bacterial stimulation. Expression of lysozyme and defensin by confluent or overgrown DAE15 cell

layers was less uniform than that observed for less dense cultures (not shown). In addition, IDE12 cells were found to lift off the culture flask when grown past confluency. Consequently, we challenged cultures that contained approximately  $1 \times 10^6$  cells per mL and were 80-90% confluent.

One week post *R. peacockii* inoculation, at least 60% of IDE12 cells and 80% of DAE15 cells were infected when stimulated with the *E. coli*-*M. luteus* bacterial suspension. IDE12 cells contained fewer rickettsiae per cell than DAE15 cells. Cells infected with *R. peacockii* did not show of loss of adhesion or visible cytopathic effects. Cell-free *R. peacockii* were present in the medium of both lines at the time of inoculation with the *E. coli*-*M. luteus* bacterial suspension.

### Partial characterization of intracellular and secreted antibacterial proteins

We established four treatments to examine expression of bacteriolytic factors: control cultures of uninfected, unstimulated cells, *R. peacockii* infected cultures to test for the rickettsial effect on constitutive expression, *R. peacockii* infected cultures stimulated with heat killed *E. coli* and *M. luteus* to determine whether intracellular bacteria modulated the response to extracellular bacteria, and uninfected, bacteria stimulated cultures to examine the response of the cell lines to bacteria in the absence of rickettsiae. Nonreducing gel electrophoresis and gel overlays containing live *M. luteus* enabled us to detect intracellular antimicrobial peptides expressed by IDE12 and DAE15 cells. Lysates from IDE12 and DAE15 cells produced large bacteria-free zones (Fig. 1A). The large bacteria-free area was taken as evidence for the presence of lysozyme-like peptides (see below). The DAE15 lysate contained an additional faintly visible band with higher electrophoretic mobility than the main zone of inhibition (Fig. 1A, arrow). A 4.2 kDa defensin has been identified in *D. variabilis* (3,27) and this second zone may be caused by a defensin-like protein in DAE15 cells. No similar zone was detected in the IDE12 lysate.

A 14-16 kDa factor with immunologic identity to lysozyme was identified in both cell lines by Western blotting (Fig. 1B). This factor comigrated with the lysozyme molecular weight marker (not shown) and with recombinant hen egg lysozyme (Fig. 1B). The electrophoretic mobility of the lysozyme in Western blots was similar to the patterns seen in nonreducing gel electrophoresis, suggesting the large zones may have been caused by a bacteriolytic lysozyme-like protein.

### Antimicrobial activity in uninfected and *R. peacockii* infected tick cells

Radial diffusion assays were used to examine expression of soluble antimicrobial factors following bacterial stimulation or *R. peacockii* infection and whether these factors were secreted or retained. No zones of inhibition were produced by concentrated culture medium controls or deionized water (not shown). Zones of inhibition were produced by concentrated 48 hour medium and lysate from both IDE12 and DAE15 cells (Table 1). Differences were observed, however, in the diameters of bacteria-free zones and whether the majority of antimicrobial factors were present in the culture medium or cell lysate. Small zones of equivalent diameters were produced by uninfected and *R. peacockii* infected IDE12 cultures, and significantly larger zones were produced by uninfected and *R. peacockii* infected cultures stimulated with heat-killed bacteria (one-way ANOVA,  $F=17.04$ ,  $p < 0.001$ ) (Fig. 2). A detectable bacteria-free zone was produced only by lysate from stimulated IDE12 cells (Table 1). Concentrated culture medium and cell lysate from DAE15 cells also produced bacteria-free zones. Culture medium produced small zones with diameters that showed significant decreases in diameter when uninfected and *R. peacockii* infected cells were stimulated with bacteria (t-test,  $t = 4.480$ ,  $dof=4$ ,  $P = 0.011$  and  $t = 4.832$ ,  $dof = 4$ ,  $P = 0.008$ , respectively). Statistically significant differences in zone diameter were not observed between uninfected and *R.*

*peacockii* infected cells in the absence of heat killed *E. coli* and *M. luteus* (Fig. 2). Lysate from DAE15 cells produced zones of equivalent diameters that did not vary significantly by treatment.

Lysate:medium ratios were calculated by comparing the diameters of the zone produced by lysate (including the well diameter) with the diameters of wells produced by medium to determine whether cells secreted or retained the majority of their antimicrobial factors. We found IDE12 cells secreted most of their antimicrobial factors while most of the antimicrobial factors produced by DAE15 cells were retained intracellularly (Table 1). *R. peacockii* infection did not change where the majority of the antibacterial activity was located.

### Relative measurement of intracellular lysozyme by flow cytometry

Changes in intracellular lysozyme content in cells stained with an anti-lysozyme antibody, and then labeled with a FITC-conjugated secondary antibody, were measured by flow cytometry. Increase in cellular fluorescence relative to uninfected and unstimulated control cultures were taken an indicator of upregulated lysozyme expression. The fluorescence profiles showed uninfected and *R. peacockii*-infected IDE12 cells stimulated with bacteria contained significantly more lysozyme than unstimulated controls ( $F = 18.220$ ,  $p < 0.001$ ), but there was no significant difference between uninfected or *R. peacockii* infected cells in either line (Fig. 3). There was significantly more lysozyme in uninfected and *R. peacockii* infected DAE15 cells after bacterial stimulation than was present in unstimulated *R. peacockii* infected cells ( $F = 4.789$ ,  $p = 0.034$ ). Cells stained for flow cytometry and viewed by microscope had small cytoplasmic granules that stained with anti-lysozyme antibodies, and these granules increased in size and number when the cells were stimulated with heat-killed bacteria (not shown).

### Sequencing of the *D. andersoni* defensin and qRT-PCR measurement of expression

A 629 bp defensin-like transcript with similarity to varisin, a defensin from *D. variabilis* (3), was identified in the DAE15 line. A cDNA with 96% identity at the nucleotide level, containing an open reading frame encoding a 74 amino acid preprodefensin with 100% identity to the *D. variabilis* defensin, was sequenced (Fig. 4).

We used qRT-PCR to examine expression of defensins in IDE12 and DAE15 cells using fluorophore-labeled primers based on the defensin sequence from the DAE15 line, and an *I. scapularis* defensin (5). Both lines constitutively expressed defensins. When related to the quantity of total RNA used for qRT-PCR, twice the amount of IDE12 RNA was used to attain  $C_t$  values similar to those for DAE15 defensin, indicating DAE15 cells contained more defensin transcript than IDE12 cells. We did not observe significant differences in defensin transcription between unstimulated and *R. peacockii*-infected IDE12 cultures ( $t = 1.815$ ,  $df = 4$ ,  $P = 0.144$ ) (Fig. 5). Bacterial stimulation significantly upregulated defensin transcription in *R. peacockii*-infected ( $t = 4.863$ ,  $df = 4$ ,  $P = 0.008$ ) and uninfected IDE12 cultures ( $t = 8.193$ ,  $df = 4$ ,  $P < 0.001$ ) by 8- and 12-fold, respectively, when compared to unstimulated IDE12 cultures (Fig. 5). Transcription of defensin in stimulated DAE15 cells was not uniform between replicate cultures, and was not significantly different between control, *R. peacockii* infected, and stimulated *R. peacockii*-infected cultures. Downregulation of defensin by stimulated DAE15 cells was statistically significant (t-test,  $t = 4.456$ ,  $dof = 4$ ,  $P = 0.011$ ), but transcript abundance in stimulated *R. peacockii* infected cells was unchanged from controls, suggesting defensin downregulation is not caused by *R. peacockii* but is a response to *E. coli* and *M. luteus* that is specific to this cell line.



## Discussion

The effects of rickettsia-like endosymbionts on tick immune responses to bacteria are unknown. We used tick cell culture to examine whether two tick cell lines that can phagocytose *B. burgdorferi* (18) also produce soluble antibacterial factors. Additionally, we investigated whether infection by *R. peacockii*, an endosymbiont of *D. andersoni*, elicited an immune response, or modified responses to heat inactivated *E. coli* and *M. luteus*. We found the IDE12 and DAE15 cell lines constitutively expressed antibacterial factors including a protein with immunologic identity to lysozyme as well as defensin-like transcripts. IDE12 cells secreted most of their antibacterial factors whereas DAE15 cells retained most of their antibacterial factors. Both lines upregulated expression of lysozyme when stimulated, but expression was unaffected by rickettsial infection. Transcripts of defensin-like genes were found in both cell lines and bacterial stimulation upregulated expression of defensin by uninfected and *R. peacockii* infected IDE12 cultures. In comparison, defensin expression by bacteria-stimulated DAE15 cultures was downregulated slightly, while transcript abundance in unstimulated cultures infected with *R. peacockii*, and *R. peacockii* infected cultures stimulated with *E. coli* and *M. luteus* was unchanged from uninfected controls. While we focused on two factors, lysozyme and defensin, that are known to have immune functions in ticks, we expect other less characterized antimicrobial factors may be present and may have been important in the immune response. Moreover, the tissues represented by cells in tick cell lines are unknown and tick cell lines may not generate the global immune response to rickettsial infection in the same way as in immunocompetent tick tissues. Given this caveat, we view these cell lines as models of generalized humoral responses, without specific focus on immunocompetent tick cells such as hemocytes or midgut cells.

There are few reports examining the effects of endosymbiotic bacteria on host immune responses despite their prevalence in arthropods. Bourtzis et al (28) found that *Wolbachia*, an endosymbiotic bacterium that infects a wide range of arthropods, did not appear to induce or suppress defensin expression in *Drosophila simulans* or *Aedes albopictus*. It is not known how *Wolbachia* avoids eliciting immune responses, but living inside cells may be an important adaptation that hides *Wolbachia* from arthropod immune systems (29). In nature, *R. peacockii* inhabits the ovaries of female *D. andersoni* and is rarely found in hemolymph (16). The ovaries of *D. variabilis*, a tick closely related to *D. andersoni*, contain little lysozyme transcript relative to the abundant lysozyme transcript found in *D. variabilis* hemocytes (9), suggesting *R. peacockii* inhabits lysozyme-poor tissues as part of a strategy of avoiding contact with more immunoresponsive tick tissues. Furthermore, the presence of a disrupted rickA gene (30), involved with rickettsial motility, would restrict the rickettsiae to the ovarian tissues and sequestered away from the immune responses found in the gut and hemocytes.

Although *R. peacockii* is an obligate intracellular bacterium, rickettsiae are exocytosed by cells (17), and cell-free rickettsiae were present in the medium of infected IDE12 and DAE15 cell cultures. These cell-free rickettsiae did not induce upregulation of lysozyme in IDE12 or DAE15 cells, or defensin in IDE12 cells. Furthermore, the lack of responses by the *I. scapularis* line IDE12 against an endosymbiont from a phylogenetically distant tick, suggests coevolution between the tick host and endosymbiont is not responsible for this lack of activity against endosymbionts. This suggests an uncharacterized aspect of *R. peacockii*'s biology enables it to go unnoticed by immunoresponsive tick cells, thus not stimulating an immune response.

Bacterial pathogens can limit immune responses in host cells by interfering with intracellular signaling cascades that induce transcription and synthesis of antimicrobial peptides (31). It did not appear that *R. peacockii* constrained lysozyme or defensin expression by infected tick cells, as infection did not influence expression by infected but otherwise unstimulated cells or

decrease the responsiveness of IDE12 or DAE15 cells to bacterial stimulation. Similarly, *Spiroplasma* infecting *D. melanogaster* did not upregulate expression of antimicrobial peptides, or prevent upregulation of antimicrobial peptides after stimulation by inactivated *E. coli* and spores of the entomopathogenic fungus *Beauveria bassiana* (29). The tick-endosymbiont relationship may be synonymous to the *Drosophila* response to *Spiroplasma*. Exocytosed *R. peacockii* were present in the culture medium without inducing upregulation of defensin or lysozyme from host cells, but not preventing host cells from responding to the stimuli presented by *E. coli* and *M. luteus*. *R. peacockii* is less susceptible to killing by antimicrobial peptides than are some free-living bacteria (13), and may rely on this intrinsic property to protect themselves against host cell immune responses rather than interfering with host cell responses.

Rickettsial mechanisms of immune evasion and microbial recognition systems used by ticks are uncharacterized. Eukaryotic cells recognize extracellular and intracellular bacteria by detecting highly conserved surface antigens, such as peptidoglycan, with pattern recognition receptors (PRRs) (32,33). The lack of responses against *R. peacockii* may indicate rickettsial surfaces lack molecular determinants present on the surfaces of *E. coli* and *M. luteus*, as was likely to be the case with the *Spiroplasma* infecting *Drosophila* (29). Rickettsial surface antigens are poorly characterized, although rickettsial peptidoglycan resembles the peptidoglycan from other gram-negative bacteria (34), and would be predicted to stimulate immune responses. Alternatively, rickettsial avoidance of the arthropod immune system may rely on a coating of host proteins to prevent recognition by host PRRs. A similar behavior may occur in *Wolbachia*, where these bacteria closely associate with proteins from the host cell (35). Taken together, it appears that molecular mimicry may hide *R. peacockii* from detection by PRRs on the tick cells, leaving the host cells capable of mounting normal immune responses when stimulated by other bacterial challenges.

Tick-endosymbiont interactions are frequently overlooked aspects of tick biology. A better understanding of these interactions may clarify how bacteria pathogenic to vertebrates evade the tick immune system. The results presented here used tick cell culture to characterize an aspect of the tick response to rickettsial endosymbiosis.

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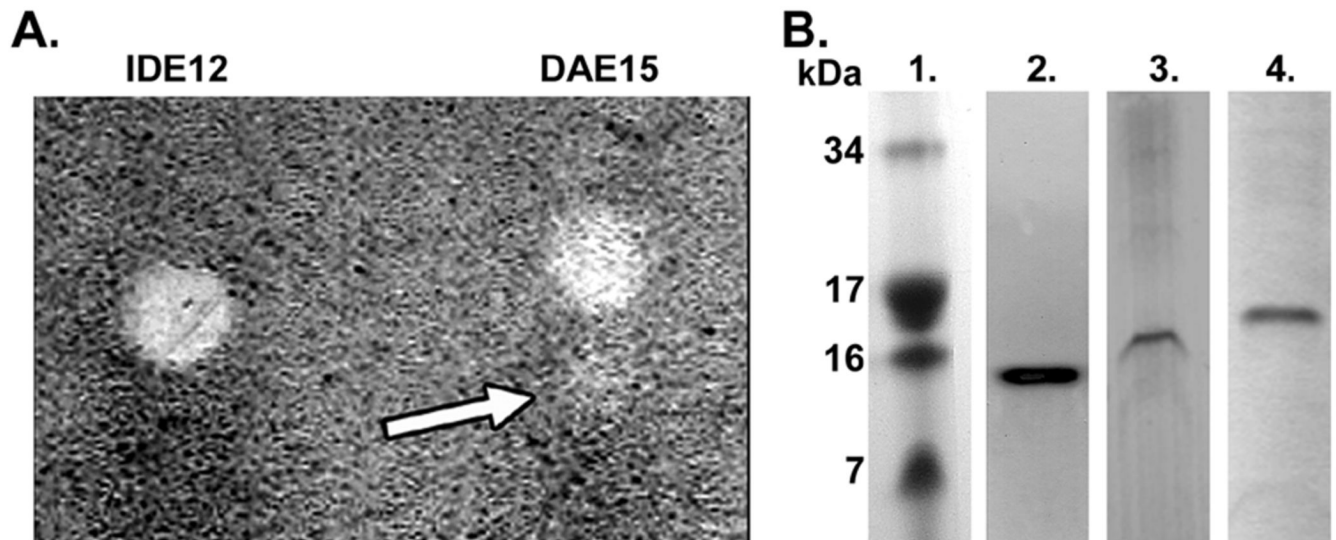
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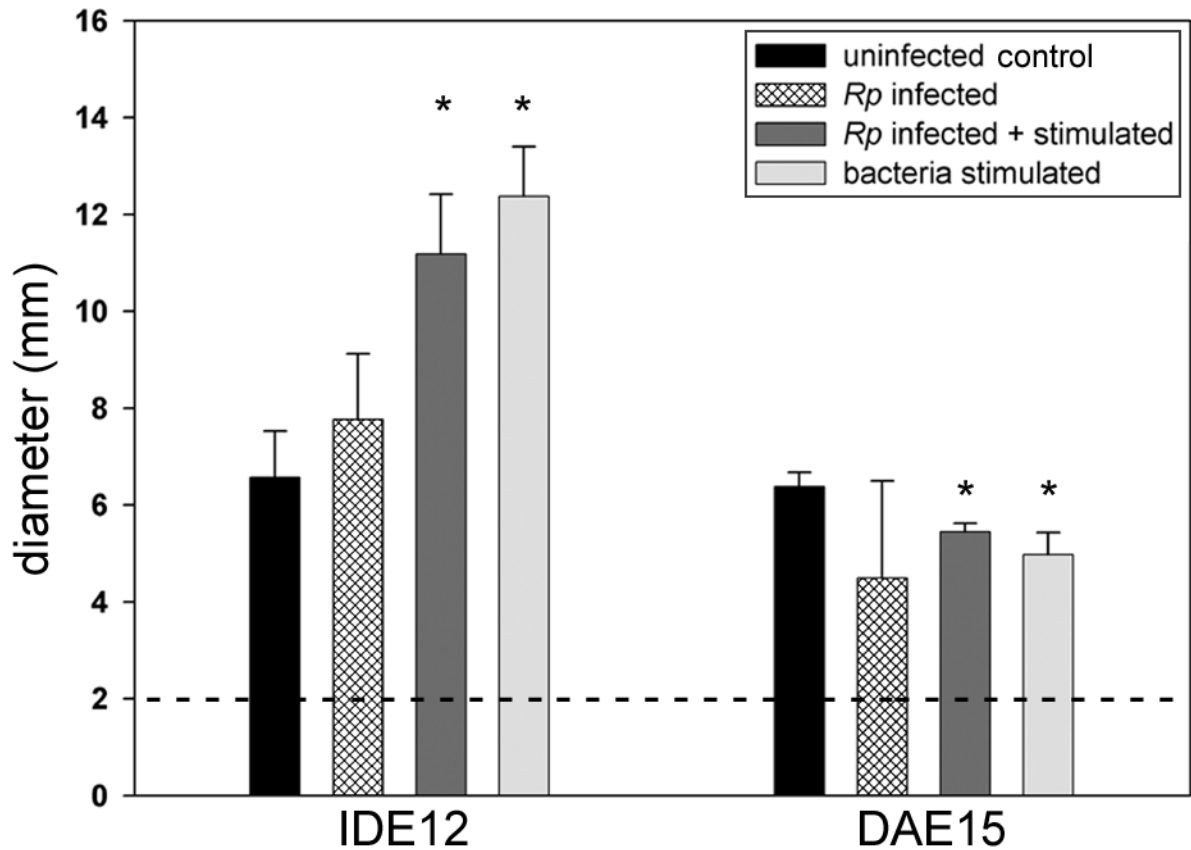
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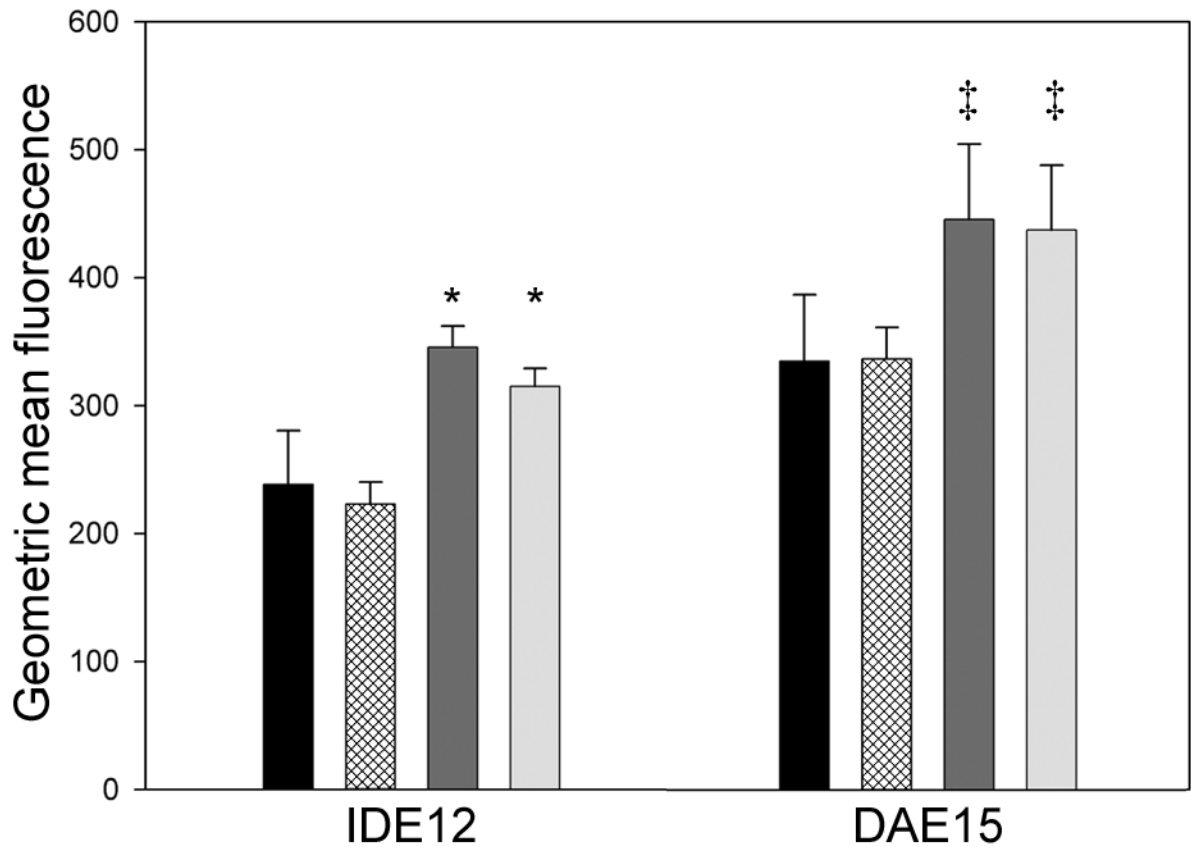
**Figure 1. Lysate from IDE12 and DAE15 cells contains antimicrobial activity and lysozyme-like immunoreactivity**

A. Lysate from IDE12 and DAE15 cells were electrophoresed under nonreducing conditions on a polyacrylamide gel with *M. luteus* overlay. Proteins with antibacterial activity produced bacteria-free zones in both lines that can be seen as clear zones in the bacterial lawn. The image contrast has been inverted to enhance the visibility of the small area of bacteriolytic activity located in the DAE15 lane (arrow). B. Western blotting of IDE12 and DAE15 lysate using polyclonal anti-lysozyme antibodies shows an immunoreactive protein at 14-16 kDa. Images are from independent, representative experiments. Lane 1. Coomassie Blue stained SeeBlue Plus2 molecular weight marker in polyacrylamide gel. Lane 2. Chicken egg lysozyme. Lane 3. IDE12 cell lysate. Lane 4. DAE15 cell lysate.



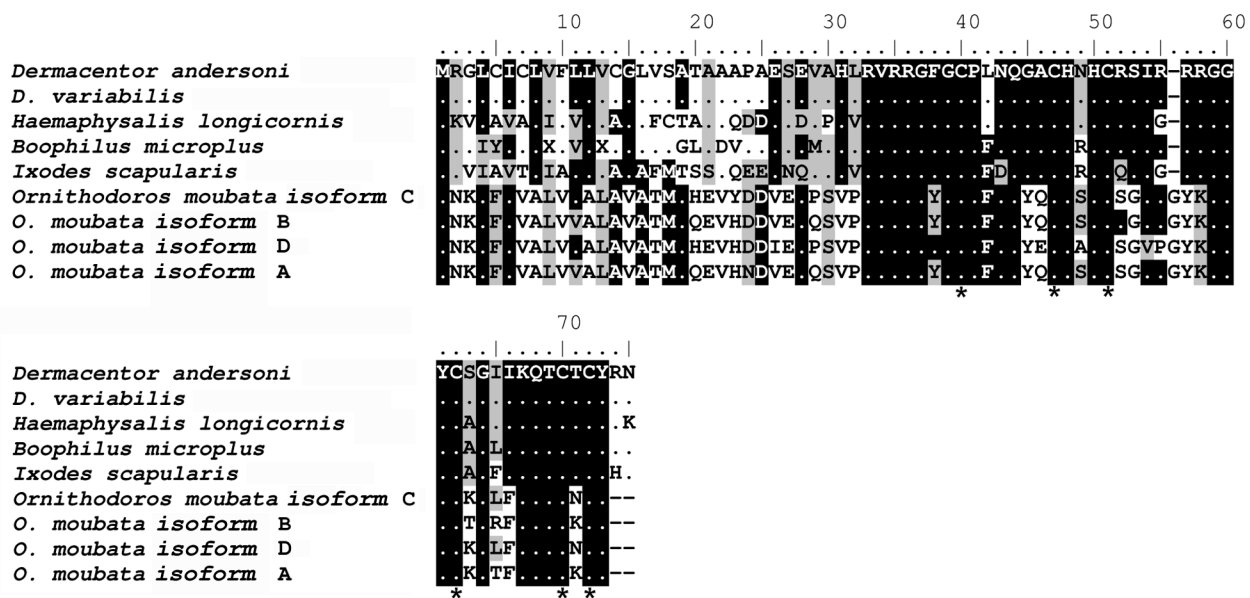


**Figure 2. Antibacterial activity of secreted antimicrobial factors from IDE12 and DAE15 cultures**  
 Samples of concentrated medium from each line were loaded into the same dish to minimize inter-plate variation. The diameter of the cannula (2 mm) is indicated by the dotted line. The diameter of the inhibited *M. luteus* growth was determined from three replicate cultures and expressed as the mean diameter  $\pm$  standard deviation. Treatments showing statistically significant differences from uninfected controls are indicated with asterisks.



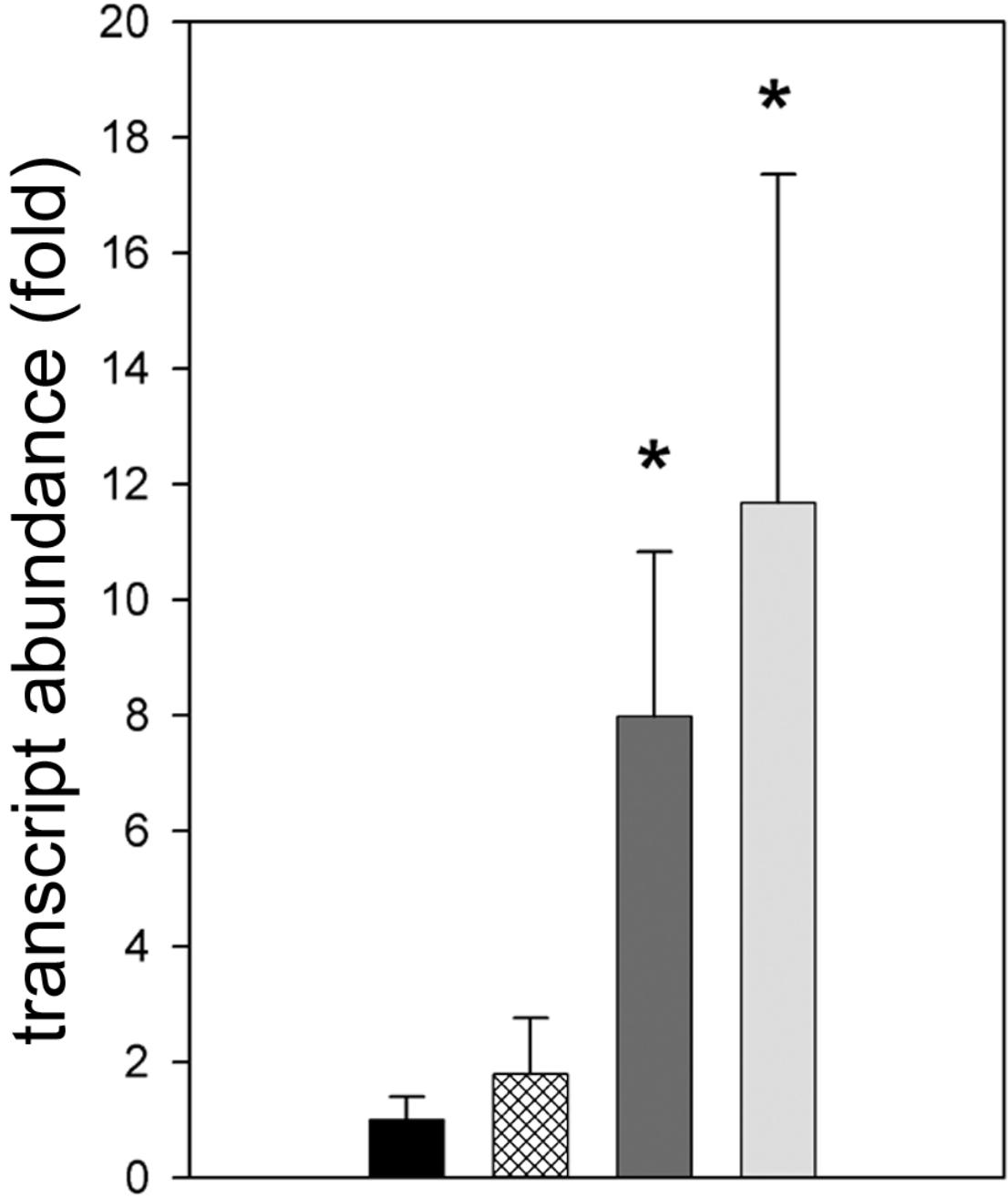
**Figure 3. Intracellular lysozyme content in IDE12 and DAE15 cells**

Bars represent the geometric mean fluorescence (arbitrary units) of cells from three replicate cultures  $\pm$  standard deviation. Treatments are indicated as in the legend of Fig. 2. Treatments showing statistically significant differences from the uninfected IDE12 controls are indicated by asterisks. Statistically significant differences were not observed between uninfected and *R. peacockii* infected cells in either line in the absence of bacterial stimulation. Double daggers (‡) indicate statistically significant differences between unstimulated, *R. peacockii* infected DAE15 cells and *R. peacockii*-infected and uninfected DAE15 cells stimulated with bacteria.



**Figure 4. Alignment of the translated amino acid sequence of the *D. andersoni* defensin with other tick defensins**

The defensin from *D. andersoni* was aligned against the sequences of *D. variabilis* preprodefensin (AAO24323), *Haemaphysalis longicornis* defensin (BAD93183), *Boophilus microplus* defensin (AAO48943), *I. scapularis* preprodefensin (AAV74387), and the *Ornithodoros moubata* defensin isoforms D (BAC22073), B (BAB41027), C (BAC22074), and A (BAB41028). GenBank accession numbers are indicated in parentheses. Similar residues are indicated with grey shading and identical residues indicated with black shading. The positions of conserved cysteine residues are indicated by asterisks.



**Figure 5. Defensin transcript abundance in IDE12 cells increases following bacterial stimulation**  
Bars represent the fold change in defensin transcript relative to defensin transcript abundance in uninfected, unstimulated controls  $\pm$  the standard deviation. Treatments are indicated as in the legend of Fig. 2. Statistically significant differences from uninfected control cultures are indicated with asterisks.

**Table 1**  
Zone diameters and ratio of concentrated lysate and media from IDE12 and DAE15 cultures.

Sample	IDE12			DAE15		
	lysate, <sup>a,b</sup>	medium, <sup>a,c</sup>	ratio	lysate, <sup>a,b</sup>	medium, <sup>a,c</sup>	ratio
Control	3.15	6.57 ± 0.95	0.48	8.49	6.38 ± 0.29	1.33
Rp infected	3.15	7.77 ± 1.35	0.41	7.65	4.49 ± 2.00	1.70
Rp infected, stimulated	3.09	11.18 ± 1.24	0.28	8.02	5.44 ± 0.18	1.47
Stimulated	6.83	12.37 ± 1.02	0.55	7.37	4.97 ± 0.47	1.48

<sup>a</sup> Zone diameters are given in millimeters.

<sup>b</sup> Lysate zones were produced by lysate from three replicates that had been pooled.

<sup>c</sup> Media-produced zones diameters include the standard deviation of three replicates.



**Table 2**

Primers used for sequencing and qRT-PCR.

Primer name	Primer (5' - 3') <sup>a</sup>	Product size (bp)
Var157 fw	TCT GGC ATC ATC AAG CAG AC	157
Var157 rev	CTG CAA GTA TTC CGG GGT TA	
DvDef fw	TGA GAC GAC AAA GAA GGT CGG CCA	543
DvDef rev	AAC ATG GAG TCG CAG TAG CGT TGT	
DaDefInt fw	CTT GTT TGC GGT CTT GTA TC	---
DaDefInt Rev	TCG ACG AAC ACG GAG GTG AG	---
DaDef rev2	GGC AGA TGC AAA GTC CAC GCA TTT	---
DaDef_85RL	CGC ACC AAG GCA GAT GCA AAG TC[JOE] G	55
DaDef_85RL/74FU	TTT GGG ACT GAG TGA AAC TTG C	
IscapAct_25FL	CGA ACG ATG ACC CAG ATC ATG TTC[FAM] G	87
IscapAct_25FL/67RU	GGA GGC GTA CAG GGA GAG CAC	
IsDef_101FL	CGA TCA TCA TTG CTG TTA CCT TGA TC[JOE] G	70
IsDef_101FL/124RU	CTC TTG TGC GCT GGA AGT CAT A	

<sup>a</sup>The fluorescent dye on the fluorophore-labeled LUX primer, either carboxyfluorescein (FAM) or 6-carboxy-4', 5'-dichloro-2', 7'-dimethoxyfluorescein (JOE), is listed inside brackets.