

Adherent and invasive *Escherichia coli* are associated with persistent bovine mastitis

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Abstract

Bovine mastitis caused by *Escherichia coli* has traditionally been viewed as a transient infection. However, *E. coli* can also cause clonal persistent intramammary infection (IMI) in dairy cows. In this study, we explored the possibility that *E. coli* strains associated with persistent IMI are better able to adhere to, invade, survive and replicate in cultured mammary epithelial cells (MAC-T) than transient strains, and examined their serotype, overall genotype, phylogenetic group, and the presence of known virulence genes.

Both transient and persistent *E. coli* strains adhered to MAC-T cells, but persistent strains invaded MAC-T cells 2.6–63.5 times more than transient strains. Blocking the adhesin/invasin FimH with mannose diminished but did not eliminate adhesion and invasion of any strain. Cytoskeletal and protein kinase inhibitors cytochalasin D, colchicine, genistein and wortmannin dramatically reduced invasion of MAC-T cells by both strains. All of the persistent strains, but only one transient strain, were able to survive and replicate intracellularly in MAC-T cells over 48 h. Transient and persistent strains displayed heterogeneous serotypes and overall genotypes, but similar phylogeny (group A), and lacked virulence genes of invasive *E. coli*.

We have found that *E. coli* strains associated with persistent IMI are better able to invade and replicate within cultured mammary epithelial cells than transient strains. The invasion process involves the host cytoskeleton and signaling cascades and is not FimH dependent. Our findings suggest that the invasion of mammary epithelial cells and intracellular survival play an important role in the pathogenesis of persistent *E. coli* mastitis.

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1. Introduction

Escherichia coli is an important environmental pathogen which causes mastitis in dairy cows (Bradley

and Green, 2001; Hogan and Larry, 2003). Recent reports suggest that *E. coli* is the most common cause of clinical mastitis in well-managed dairies with low milk somatic cell counts (SCC) (Bradley and Green, 2001; Bradley, 2002). Typically, *E. coli* infections of the mammary gland are of short duration, resulting either in bacterial clearance or death of the host. However, *E. coli* can also cause clonal persistent intramammary infections (IMI) (Dopfer et al., 1999), with prevalence estimates between 5 and 24% of all mastitis cases caused by *E. coli* (Dopfer et al., 1999; Bradley and Green, 2001). The pathogenesis of persistent intramammary *E. coli* infections is still unclear.

Persistent bacterial infection generally involves adhesion, invasion and intracellular survival (Finlay and Cossart, 1997). Pathogenic *E. coli* have several fimbrial and afimbrial adhesins that mediate adhesion to host epithelial cells through cell surface compounds like proteins, glycolipids and carbohydrates (Le Bouguenec, 2005). For example, *E. coli* fimbriae type 1 pili adhesin FimH binds to mannose residues on cell surfaces and polymorphisms in FimH have been shown to influence adhesion (Hommais et al., 2003).

Invasion of cells can provide bacteria with a survival advantage, allowing them to better resist detection and clearance by both innate and adaptive immune defense mechanisms and has been described for the mammary pathogens such as *Staphylococcus aureus*, *Streptococcus uberis*, and *Streptococcus dysgalactiae* (Matthews et al., 1994; Almeida and Oliver, 1995; Lammers et al., 1999). Many invasive pathogenic bacteria have been shown to induce their own uptake into non-phagocytic cells by exploiting host cell signal transduction pathways (Finlay and Cossart, 1997). *E. coli* strains associated with other diseases often invade and persist within epithelial cells, e.g. uropathogenic *E. coli* (UPEC), meningitis/sepsis associated *E. coli* (MNEC) and adherent-invasive *E. coli* (AIEC) associated with Crohn's disease (Darfeuille-Michaud, 2002; Kaper et al., 2004). Supporting this hypothesis, a previous study showed the invasion of cultured mammary epithelial cells by mastitis-associated *E. coli* strains (Dopfer et al., 2000).

To be able to develop effective strategies for therapy and prevention, a more through understanding

of the pathobiology of persistent intramammary *E. coli* infections is required. In this study we explored the possibility that *E. coli* strains associated with persistent IMI are better able to adhere to, invade and survive within cultured mammary epithelial cells than strains associated with transient IMI.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Three *E. coli* strains associated with transient IMI, and three strains associated with persistent IMI were evaluated. The strains from transient infections, ECA-727 (synonymous with *E. coli* 727 (Hogan et al., 1999)), ECA-O157 (synonymous with *E. coli* O:157 (Lohuis et al., 1990)) and ECA-B (synonymous with *E. coli* strain B (Dopfer et al., 2000)) have been previously investigated; ECA-727 and ECA-O157 have been repeatedly shown to cause only brief transient IMI following intramammary challenge. One of the *E. coli* isolates associated with persistent IMI, strain ECC-Z (synonymous with *E. coli* strain Z (Dopfer, 2000)), has been previously investigated. Strains ECC-M and ECC-1470 were isolated from chronically infected cows in New York State and selected for this study. Presence of persistent IMI was confirmed by pulsed-field gel electrophoresis (PFGE) typing of isolates collected over time from the same infected mammary quarter according to PulseNet protocol (http://www.cdc.gov/pulsenet/protocols/ecoli_salmonella_shigella_protocols.pdf). *Salmonella typhimurium* ATCC 14028 (American Type Culture Collection, Manassas, VA) and *E. coli* DH5 α , a noninvasive *E. coli* laboratory strain were used as positive and negative controls for adhesion to and invasion of mammary epithelial cells.

Bacterial isolates were stored at -80°C , and fresh non-passaged bacteria were used for all investigations. Strains were grown in Luria–Bertani (LB) broth at 37°C overnight, without shaking. Type 1 pilus expression was confirmed by mannose-sensitive agglutination of 1% commercial baker's yeast (*Saccharomyces cerevisiae*) suspended in phosphate-buffered saline (PBS, pH 7.4). All the strains were sensitive to gentamycin.

2.2. Evaluation of adhesion and invasion by mastitis-associated *E. coli* in cultured cells

2.2.1. Tissue culture

A bovine mammary epithelial cell line, MAC-T (Huynh et al., 1991) (Nexia Biotechnologies, Ste-Anne de Bellevue, Que., Canada) was used for adhesion, invasion and survival assays. Monolayers of MAC-T cells were kept at 37 °C in 5% CO₂:95% air (v/v) using Dulbecco's Modified Eagle's Medium (DMEM, Sigma–Aldrich, St. Louis, MO) supplemented with 5% fetal bovine serum (FBS, Gemini Bio Products, Woodland, CA).

2.2.2. Adhesion assay

To quantify the total cell-associated bacteria (intracellular bacteria plus surface-adherent bacteria), we assayed adhesion as previously described (Dopfer et al., 2000) with some modifications. Briefly, stationary-phase bacteria were pelleted, washed with excess PBS, pH 7.4, and resuspended in PBS. Confluent monolayers of MAC-T cells ($\sim 3 \times 10^5$) grown in 24-well plates were infected with a multiplicity of infection (MOI), the ratio of bacteria to host cells, of 10. After 1 h of incubation at 37 °C with 5% CO₂, cells were washed three times in PBS and lysed with 1 ml of 0.1% Triton X-100 in PBS for 10 min. Lysates were serially diluted and plated on LB-agar, and colonies were enumerated following overnight incubation. Adhesion was expressed as the total number of colony-forming units (CFU)/ml recovered per well. Each assay was run in duplicate and repeated three times.

The role of type 1 pili in adhesion was investigated by mannose inhibition, and determination of *fimH* sequence. The adhesion assay was performed in the presence of 2.5% methyl α -D-mannopyranoside (Mannose, Sigma–Aldrich, St. Louis, MO). Adhesion in the presence of mannose was normalized to the no-mannose control for each strain, and reported as a percentage of the control adhesion level (100%).

2.2.3. Invasion assay

The invasive abilities of *E. coli* associated with transient and persistent IMI was evaluated quantitatively and qualitatively in cultured MAC-T cells by the gentamicin protection assay and differential staining.

2.2.3.1. Gentamicin protection assay. For the gentamicin protection assay, confluent monolayers of MAC-T cells, grown in six-well plates ($\sim 1.5 \times 10^6$), infected with *E. coli* at an MOI of 10 as described in the adhesion assay except that after the initial 1 h infection period, cells were washed three times in PBS and then incubated for another 2 h with medium containing 100 μ g/ml gentamicin (Sigma–Aldrich, St. Louis, MO) to kill any extracellular bacteria. The number of bacteria in each well was determined as described above and invasion was expressed as the total number of CFU/ml recovered per well.

2.2.3.2. Differential staining of extra- and intracellular *E. coli*. MAC-T cells were seeded and grown overnight on eight-well chamber glass slides and infected with *E. coli* strains ECA-O157 or ECC-Z at an MOI of 100. After 3 h of incubation, cells were washed three times in PBS and fixed with 3.7% formaldehyde for 15 min. Immunofluorescence staining of extracellular and intracellular bacteria was performed as described previously (Ramarao et al., 2000) with the following modifications. Polyclonal rabbit anti-*E. coli* antibody (B65001R, Biodesign, Saco, ME) was diluted 1:50 in PBS with 10% FBS and used as primary antibody. Extracellular bacteria was stained with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG antibodies (F0382, Sigma–Aldrich, St. Louis MO) and intracellular bacteria was stained with tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG antibodies (T677, Sigma–Aldrich, St. Louis MO). After staining, each slide was mounted with ProLong Antifade kit (Molecular Probes Inc., Eugene, OR.) mixed with 4',6-diamidino-2-phenylindole (DAPI) and sealed with nail varnish. Cells were viewed with an Axioskop 2 plus epifluorescence microscope and images were captured with AxioCam and AxioVision (Carl Zeiss Inc., Thornwood, NY). Digital images were superimposed and assembled in Adobe Photoshop (Adobe Systems, Inc., San Jose, CA).

2.2.4. Invasion inhibition studies

To determine the role of FimH on invasion, 2.5% mannose was added to culture media and the invasion assay was performed as described above. Invasion in the presence of mannose was normalized to the no-

mannose control for each strain, and reported as a percentage of the control invasion level (100%).

To test effects of specific inhibitors on bacterial invasion, we examined the effect of the microfilament inhibitor cytochalasin D, the microtubule inhibitor colchicine, tyrosine protein kinase (TPK) inhibitor genistein and phosphoinositide 3-kinase (PI 3-kinase) inhibitor wortmannin on the ability of *E. coli* strains, ECA-0157 and ECC-Z, or *S. typhimurium* to invade MAC-T cells. All inhibitors were purchased from Sigma–Aldrich. Stock solutions were prepared at the following concentrations in dimethyl sulfoxide, except colchicine which was prepared in dH₂O: cytochalasin D, 1 mg/ml; colchicine, 1 mg/ml; genistein, 100 mM; wortmannin, 0.1 mM. Prior to use, inhibitors were diluted at various concentrations in DMEM supplemented with 5% FBS. Cytochalasin D (0.31, 0.62 and 1.25 µg/ml) and colchicine (1.25, 2.5, 5 and 10 µg/ml) were added 1 h prior to addition of bacteria, genistein (150 and 250 µM) was added 30 min prior and wortmannin (100 and 200 nM) was added 10 min prior to infection. As a control, DMSO was added to cells at a final concentration of 0.1%. Cells were infected at an MOI of 100. All inhibitors and DMSO were maintained throughout the 1 h infection period. Following the 1 h infection and 2 h gentamicin killing period, the number of intracellular bacteria was determined by plating serial dilutions as described above. Invasion in the presence of each inhibitor was normalized to the no-inhibitor control for each strain, and reported as a percentage of the control invasion level (100%). Viability of MAC-T cells was assessed by trypan blue exclusion. None of the inhibitors or DMSO alone had any effect on bacterial growth, bacterial adhesion or host cell viability during the course of experiment (data not shown).

2.3. Intracellular survival assay

To determine whether *E. coli* could survive or multiply within MAC-T cells, the standard invasion assay was modified by further incubation of infected monolayers for up to 48 h. After the 1 h invasion and 2 h incubation with 100 µg/ml of gentamicin, cells were washed once in PBS and fresh medium containing 15 µg/ml of gentamicin was added to cells. At 2, 24 and 48 h, the number of intracellular bacteria was determined as described above. Survival

was expressed as the percentage of bacteria present within cells at 24 and 48 h compared to the number internalized at 2 h (100%).

2.4. Fluorescence in situ hybridization (FISH) for in vivo localization of mastitis associated *E. coli* in mammary biopsies

2.4.1. Cow and mammary tissue sampling

Biopsy samples of the mammary epithelia were obtained from a Holstein-Friesian cow, naturally infected with persistent *E. coli* strain ECC-M. The cow was 9 months into her third lactation. Her first clinical mastitis was noted at d17 of lactation and she suffered several recurrences in subsequent months. The cow was purchased from the farmer and follow-up milk samples were taken from the affected left hind quarter for 132 consecutive days. *E. coli* counts and SCC of the samples were determined as described previously (Grohn et al., 2004), and selected *E. coli* isolates were examined by PFGE.

For in vivo localization of *E. coli*, mammary tissues were taken from the left hind quarter, using ultrasound guide to a 16 g percutaneous biopsy gun (C.R. Bard, Covington, GA). Control samples were obtained from the right hind quarter. Biopsy samples were fixed in 10% buffered formalin, embedded in paraffin and sectioned at 4–6 µm.

Cornell University operates under an approved Animal Welfare Assurance (A3347-01, A-3125-01) and is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. The Institutional Animal Care and Use committees at Cornell University approved the project.

2.4.2. FISH

Preparation of tissue sections and in situ hybridization of bacteria on glass slides were performed as described previously (Priestnall et al., 2004) with the following modifications. The universal eubacterial oligonucleotide probe EUB-338 [GCT GCC TCC CGT AGG AGT], and the irrelevant control probe nonEUB-338 [ACT CCT ACG GGA GGC AGC] complementary to EUB-338, both synthesized and 5'-labelled (IDT, Coralville, IA) with the fluorochrome Cy3 were used. Tissue slides were incubated with 150 ng of the appropriate probe in 30 µL hybridization

buffer (20 mM TRIS, 0.9 M NaCl, 0.1% SDS, pH 7.2) overnight at 46 °C, rinsed in warm wash solution (20 mM TRIS, 0.9 M NaCl, pH 7.2) and incubated in wash solution at 48 °C for 30 min. Slides were then rinsed with sterile water and dried in an upright rack at 46 °C for 10 min and mounted with ProLong Antifade kit (Molecular Probes Inc., Eugene, OR) mixed with DAPI. Sections were examined with an Olympus BX51 plus epifluorescence microscope and images were captured with an Olympus DP70 Camera (Olympus America Inc., Melville, NY).

Tissue sections with evidence of bacterial invasion on eubacterial FISH were subsequently evaluated with an *E. coli/Shigella* (*E. coli* 16srRNA: GCA AAG GTA TTA ACT TTA CTC CC) FISH probe (Jansen et al., 2000).

2.5. Molecular characterization of *E. coli* strains

The genetic diversity of *E. coli* isolated from transient and persistent IMI, was evaluated by Random Amplified Polymorphic DNA (RAPD)-PCR with informative primer 1283 as previously described (Wang et al., 1993). The major *E. coli* phylogenetic groups (A, B1, B2, and D) (Herzer et al., 1990; Clermont et al., 2000) were determined by triplex PCR as described previously (Clermont et al., 2000) with *E. coli* strains ECOR-03, 25, 26, 34, 48, 50, 62 and 64 (ECOR collection: <http://foodsafety.msu.edu/whitam/ecor/index.html>) used as controls. *E. coli* isolates were serotyped for OH antigens and screened by PCR for the presence of genes encoding K99, F1845 and CS31A fimbriae; heat labile toxin (LT); heat stable toxins, STa and STb; shiga like toxin types I and II, SLTI and SLTII; cytotoxic necrotizing factors 1 and 2 (*cnf1* and *cnf2*); and intimin-gamma (*eae*) at the *E. coli* Reference Center at Penn State University (DebRoy and Maddox, 2001). Additional PCR was performed to determine the presence of genes encoding P fimbriae (*pap*), S fimbriae (*sfa*) afimbrial adhesin I (*afal*), and hemolysin (*hly*) which were previously identified in UPEC (Yamamoto et al., 1995). The presence of the *ipaH* gene (a marker for *Shigella* and enteroinvasive *E. coli*) was also determined by PCR as previously described (Martin et al., 2004).

Because FimH polymorphism can affect adhesion (Hommais et al., 2003), FimH sequences were determined by PCR and sequencing. Oligonucleotide

primers were designed based on the published sequence of *fimH* gene in *E. coli* K-12 strain MG1655 (GenBank accession number U00096) using the Primer Select (DNA Star, Madison, WI) (*fimH*-F, 5'-CAG GGA ACC ATT CAG GCA GTG ATT AGC ATC-3'; *fimH*-R, 5'-AAT ATT GCG TAC CAG CAT TAG C-3'). PCR was performed in a BioRad MyCycler automatic thermal cycler (BioRad Laboratories, Hercules, CA) with denaturation at 96 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min, for a total of 40 cycles. PCR products were cloned, into pGEM-T Easy (Promega, Madison, WI) according to the manufacturer's instructions. Sequencing was performed at Cornell University's BioResource Center using M13 primers and an ABI 3700 automated sequencer (Perkin-Elmer Biosystems, Foster City, CA). DNA sequence data obtained with both forward and reverse primers were proofread and assembled with the Seqman (DNA Star, Madison WI) to yield the final *fimH* sequences used for analyses. Sequences were aligned using the Clustal W algorithm in MegAlign (DNA Star, Madison WI).

2.6. Statistical analysis

All assay data were summarized using the mean of three or more replicates per experiment. Mean values from each experiment (at least three) were used as input for the statistical analysis. If necessary, data were normalized by logarithmic transformation prior to one-way-analysis-of-variance (one-way ANOVA), where the strain type was used as the 'treatment' variable. Survival assay data was analyzed by repeated measures of ANOVA, where "time" was used as the treatment variable. Post hoc comparisons were performed with LSD and Tukey HSD tests. Statistical significance was defined at $P < 0.05$. All analyses were done in the statistical program Statistix[®] (Analytical Software, Tallahassee, FL).

3. Results

3.1. Adhesion to and invasion of cultured bovine mammary epithelial cells

All strains of *E. coli* isolated from both transient and persistent IMI adhered to MAC-T cells similarly

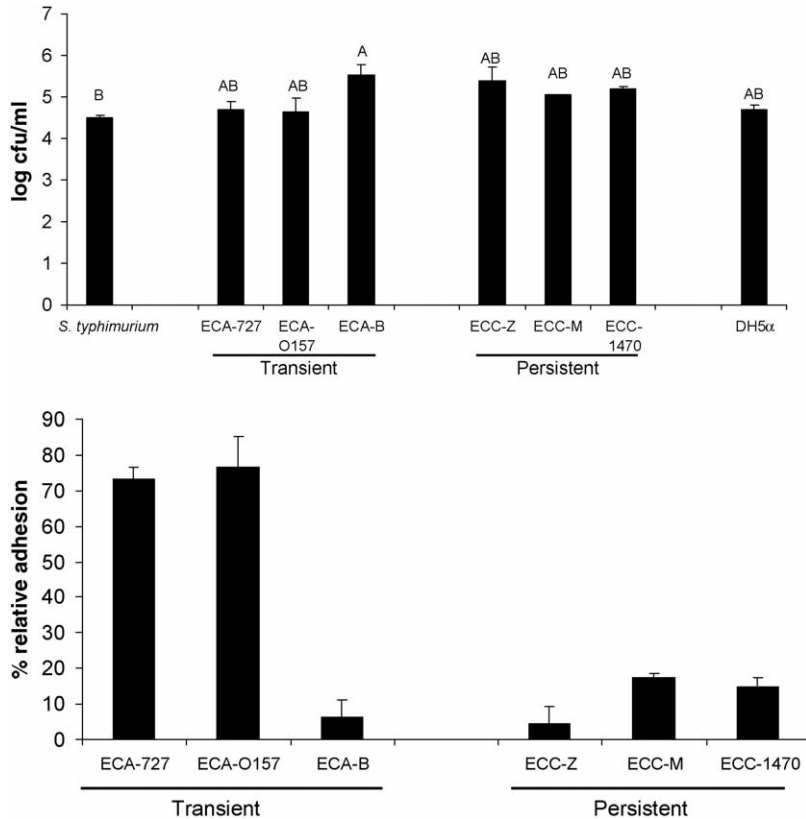


Fig. 1. Top panel: adhesion of *E. coli* and *S. typhimurium* to mammary epithelial cells. *E. coli* strains were allowed to adhere to MAC-T mammary epithelial cells for 1 h. The results of statistical analysis are indicated by the letters A and B. Strains with different letters are significantly different from each other ($P < 0.05$). Bottom panel: adhesion in the presence of 2.5% mannose. Results were expressed as percentage adhesion of the control (no-mannose added). The values indicate means of at least three separate experiments \pm S.D.

to DH5 α and better than *S. typhimurium* (Fig. 1). Neither the two pathogroups, nor individual strains within a group, differed in their ability to adhere to cells ($P > 0.05$).

Blocking type 1 fimbria-mediated binding by mannose decreased adhesion by 23–95% (Fig. 1). The two most adherent strains (ECA-B and ECC-Z) demonstrated the highest levels of inhibition (94 and 95%, respectively). Two transient strains (ECA-727 and ECA-O157) demonstrated the lowest levels of inhibition.

The gentamicin protection assay showed the group of persistent *E. coli* strains was 10.6 and 51.8 times more invasive than the group of transient *E. coli* strains and the DH5 α negative control strain, respectively ($P < 0.05$) (Fig. 2). Comparison of

individual strains showed a wide range of differences in invasion between transient and persistent *E. coli* strains (2.6–63.5 times). The most invasive persistent *E. coli* strain (ECC-Z) invaded 63.5 and 128.7 times more frequently than the least invasive transient strain (ECA-O157) and DH5 α , respectively (Fig. 2). One transient strain (ECA-B) invaded as well as persistent strains ECC-M and ECC-1470. Blocking FimH with 2.5% mannose decreased invasion by 65–76% (Fig. 2), but did not significantly impact the relative differences in invasion between transient and persistent strains—the group of persistent strains invaded 13.4 times more than the group of transient strains. No cytotoxicity was detected with the bacterial concentration used for adhesion and invasion studies.

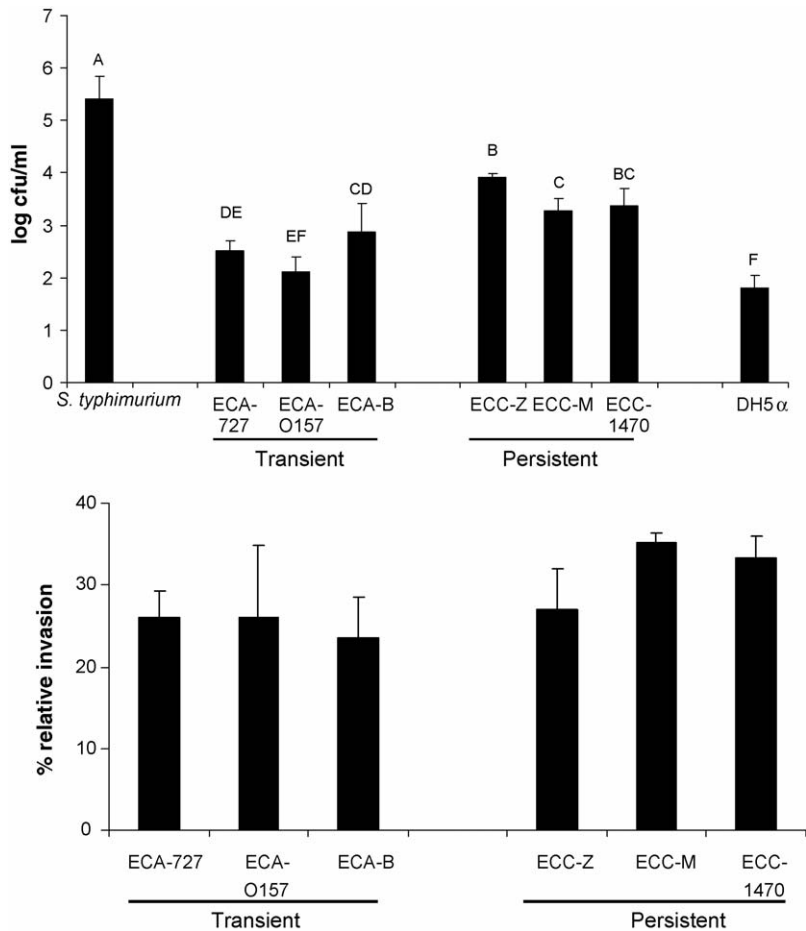


Fig. 2. Top panel: invasion of MAC-T mammary epithelial cells. MAC-T mammary epithelial cells were infected at an MOI of 10 for 1 h and gentamicin treated for 2 h. The results of statistical analysis are indicated by the letters A–F. Strains with different letters are significantly different from each other ($P < 0.05$). Bottom panel: invasion of MAC-T mammary epithelial cells by *E. coli* in the presence of 2.5% mannose. Results were expressed as a percentage invasion of the control (no-mannose added). The values indicate means of at least three separate experiments \pm S.D.

Invasion of MAC-T cells by *E. coli*, examined by double immunofluorescence labeling, confirmed that persistent strain ECC-Z was substantially more invasive than transient strain ECA-O157 (Fig. 3A and B).

3.2. Effects of cytoskeletal disrupting compounds and protein kinase inhibitors on bacterial invasion

The cytoskeletal disrupting compounds, cytochalasin D and colchicine, reduced invasion of MAC-T cells by both transient and persistent *E. coli* strains by approximately 90 and 75%, respectively (Fig. 4). Consistent with previous reports, cytochalasin D, but

not colchicine, inhibited invasion of control *S. typhimurium* strain (Finlay et al., 1991; Finlay and Cossart, 1997).

The protein kinase inhibitors, genistein (150 and 250 μ M) and wortmannin (100 and 200 nM), inhibited internalization of both transient and persistent strains by approximately 55–80%, respectively. Genistein also inhibited the invasion of *S. typhimurium* 37%, while wortmannin inhibited *S. typhimurium* invasion only slightly (6 and 2% at 100 and 200 nM, respectively).

Despite similar proportional reduction in invasion with both transient and persistent *E. coli* strains, the

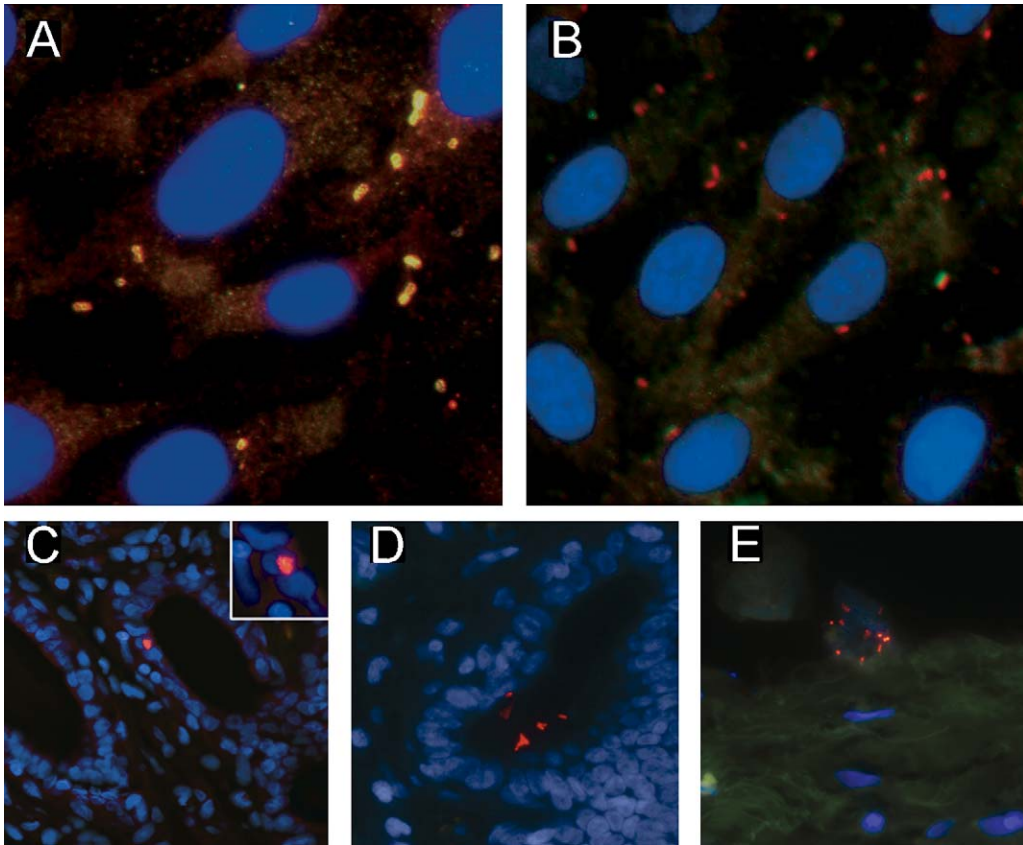


Fig. 3. In situ localization of *E. coli* in cultured mammary epithelial cells and mammary tissue from a cow with persistent coliform mastitis. Monolayers infected with transient strain ECA-O157 (A) and persistent strain ECC-Z (B) were incubated with differently labeled secondary antibodies before (FITC-green) and after (TRITC-red) permeabilization. Intracellular bacteria are red, and extracellular bacteria are yellow. Mammary tissue from a cow persistently infected with strain ECC-M was examined by FISH with probes EUB-338 (C and E) and *E. coli* (D). Bacteria are seen in red, and DAPI stained nuclei are blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

absolute number of invasive bacteria *after* inhibition was still approximately 60 times greater with the persistent strain, ECC-Z, than ECA-O157, maintaining the relative differences observed before inhibition.

3.3. Intracellular survival in MAC-T cells

During 48 h period, the number of bacteria of all persistent *E. coli* strains and one transient strain almost doubled (Fig. 5), indicating that organisms were replicating intracellularly. The most adherent and invasive transient strain, ECA-B, and DH5 α did not survive and their numbers decreased to 30 and 66%, respectively, at 48 h. There was no evidence of

damage to the MAC-T cells by light microscopy at any of the time points evaluated.

3.4. In vivo localization of *E. coli*

Biopsy samples of the mammary epithelia were obtained from a cow, naturally infected with persistent *E. coli* strain ECC-M. SCC and *E. coli* counts of the milk samples obtained from the cow over 132 days, and PFGE analysis of the selected *E. coli* isolates confirmed the presence of persistent *E. coli* IMI (Fig. 6).

Evaluation of biopsy samples from the cow with FISH revealed the presence of bacteria within

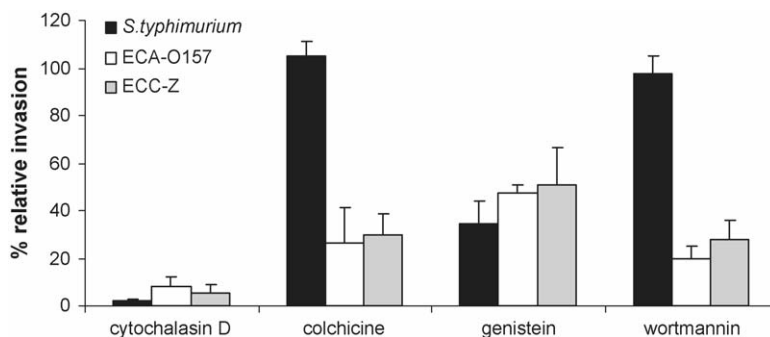


Fig. 4. Effects of eukaryotic cell inhibitors on invasion of MAC-T mammary epithelial cells by transient strain ECA-O157, persistent strain ECC-Z, and *S. typhimurium*. Cells were pretreated with cytochalasin D (1.25 $\mu\text{g}/\text{ml}$, 1 h), colchicine (1.25 $\mu\text{g}/\text{ml}$, 1 h), genistein (150 μM , 30 min) or wortmannin (200 nM, 10 min). Infections were performed at an MOI of 100 for 1 h in the presence of the inhibitors. Intracellular bacteria were determined after 2 h gentamicin treatment. Results are expressed as the percent of intracellular bacteria relative to invasion without inhibitor. The values indicate means of at least three separate experiments \pm S.D.

mammary epithelial cells and mammary duct (Fig. 3C–E).

3.5. Characterization of *E. coli* strains

Evaluation of O:H antigens indicated that no common serotype was associated with either transient or persistent IMI isolates (Table 1). The results of RAPD-PCR were consistent with diversity in overall genotype (Fig. 7). Despite their diversity in serotype, genotype, and disease association both transient and persistent strains belonged to phylogenetic group A.

A PCR based screen failed to detect virulence genes encoding K99, F1845 and CS31A fimbriae; heat labile toxin (LT); heat stable toxins, STa and STb; shiga toxin types 1 and 2 (*stx1* and *stx2*); cytotoxic necrotizing factors 1 and 2 (*cnf1* and *cnf2*), intimin (*eae*), P fimbriae (*pap*), S fimbriae (*sfa*), afimbrial adhesion I (*afaI*), hemolysin (*hly*) and *ipaH* that are typically associated with cell invasion by diarrheagenic and extraintestinal *E. coli*. All mastitis associated strains and DH5 α , were positive for *fimH*, the adhesion-encoding gene associated with epithelial cell invasion in uropathogenic *E. coli*. Sequencing to detect potentially pathoadaptive *fimH* alleles showed

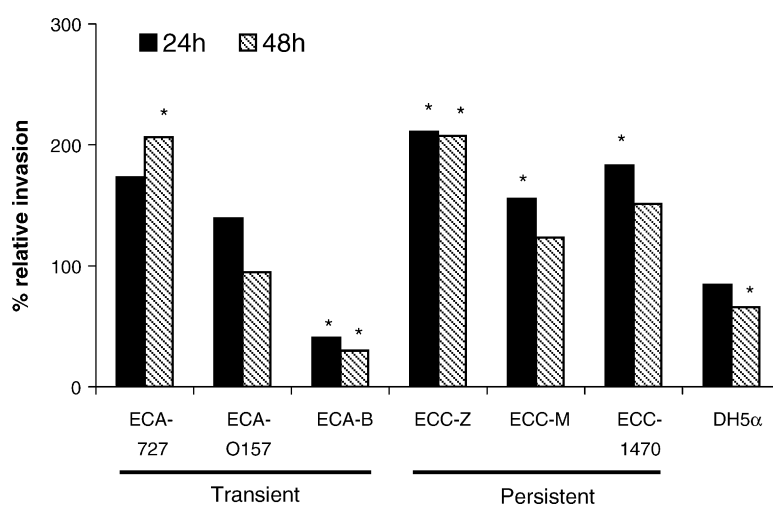


Fig. 5. Survival of transient and persistent *E. coli* strains in MAC-T cells over 48 h. Results are expressed as a percentage of bacteria recovered at 2 h. Values greater than 100% are consistent with replication. *Significantly different from 2 h within a strain ($P < 0.05$).

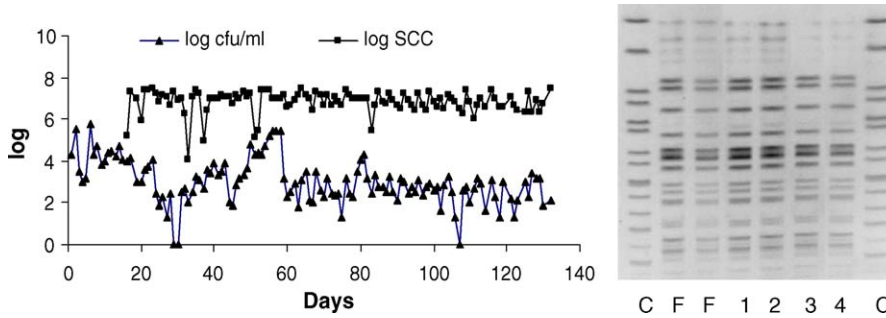


Fig. 6. Somatic cell counts and coliform counts of the milk samples from a cow with persistent *E. coli* infection obtained on 132 consecutive days, and pulsed-field gel electrophoresis (PFGE) of selected *E. coli* isolates; C: standard control strain; F: *E. coli* isolates obtained from the cow on farm; 1–4: selected *E. coli* isolates obtained during 132 days of sampling period.

Table 1
Characterization of *E. coli* strains isolated from dairy cows with transient and persistent intramammary infections

Source	Strain	Reference	Serotype	Phylogroup	Virulence genes*	FimH**				
Control	K12 MG1655***			A	–	–16	–12	12	27	119
Transient IMI	ECA-727	[25]	O–:H27	A	–	N	V	P	V	A
Transient IMI	ECA-O157	[32]	O157:H27	A	–	T	V	P	A	V
Transient IMI	ECA-B	[13]	O8:H19	A	–	T	V	P	V	A
Persistent IMI	ECC-Z	[12]	O74:H39	A	–	T	V	S	V	A
Persistent IMI	ECC-M	This study	O–:H44	A	–	T	V	P	A	A
Persistent IMI	ECC-1470	This study	OX18:H–	A	–	T	V	P	A	A

* PCR was performed for the genes encoding for: K99, F1845 and CS31A fimbriae; heat labile toxin (LT); heat stable toxins, STa and STb; shiga toxin types 1 and 2 (*stx1* and *stx2*); cytotoxic necrotizing factors 1 and 2 (*cnf1* and *cnf2*), intimin (*eae*), P fimbriae (*pap*), S fimbriae (*sfa*), afimbrial adhesin I (*afaI*), hemolysin (*hly*) and *ipaH*.

** FimH sequence polymorphisms. Only polymorphic residues are shown.

*** GenBank accession no. U00096.

the FimH sequence of strain ECA-B was identical to that of K-12 MG1655. Strain ECC-Z had a P12S mutation. Strains ECA-B and ECC-Z had an A27V mutation, also found in *E. coli* K-12 strain MG1655. Strains ECA-727 and ECA-O157 had A119V mutation.

4. Discussion

This study explored the possibility that *E. coli* strains associated with persistent IMI are better able to adhere to, invade, survive and replicate within cultured mammary epithelial cells than strains associated with transient IMI. We have clearly demonstrated that *E. coli* strains associated with persistent IMI adhere similarly to, but invade, survive and replicate in

mammary cell culture to a much greater extent than strains associated with transient IMI.

Although enhanced ability to invade often depends on enhanced ability to adhere (Finlay and Cossart, 1997; Martinez et al., 2000), the enhanced invasiveness of the persistent *E. coli* strains cannot be attributed to differences in adhesion, as both transient and persistent *E. coli* strains, adhered similarly to MAC-T cells. Blocking FimH with mannose decreased adhesion and invasion in both transient and persistent strains, but differences in the absolute numbers of invasive bacteria between transient and persistent strains were maintained. Thus, FimH functions as more as an adhesin than an invasin in these strains, and FimH-independent mechanisms must be utilized for invasion by persistent *E. coli* strains. While neither transient nor persistent *E. coli*

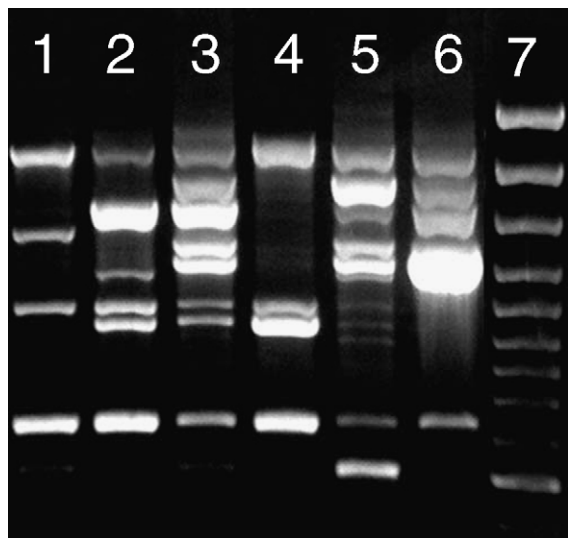


Fig. 7. Genetic diversity of mastitis associated *E. coli* strains. 1.5% agarose gel electrophoretogram of RAPD-PCR of genomic DNA extracted from transient strains (ECA-727, ECA-O157, ECA-B, lanes 1–3) and persistent strains (ECC-Z, ECC-M, ECC-1470, lanes 3–6). Lane 7 = molecular weight marker.

strains expressed consistent *fimH* polymorphisms (Table 1), and did not differ in their abilities to adhere to MAC-T cells, the two strains demonstrating the best adhesion (ECA-B and ECC-Z) both contained the A27V polymorphism, which has been reported to confer a pathoadaptive advantage in group B2 uropathogenic *E. coli* isolates (Hommals et al., 2003). ECA-B and ECC-Z strains also showed a greater inhibition of adhesion by mannose than the other *E. coli* strains. Thus, FimH polymorphism does not appear to confer a pathoadaptive advantage to *E. coli* associated with persistent coliform mastitis (since strains without this phenotype still cause disease), although in specific strains, it might confer increased reliance on FimH for adhesion.

Our results suggest that host cell cytoskeletal rearrangement and phosphorylation-mediated signaling cascades facilitate invasion of MAC-T cells by *E. coli*. Cytochalasin D and colchicine inhibited invasion of MAC-T cells, suggesting that invasion depends on the usurpation of cellular actin filaments and microtubules, similar to previous reports of microfilament and microtubule dependent invasion with AIEC associated with Crohn's disease, *Klebsiella pneumoniae* and *Campylobacter jejuni* (Oelschlaeger and Tall,

1997; Boudeau et al., 1999; Biswas et al., 2003). Genistein (a specific tyrosine kinase inhibitor) and wortmannin (a PI 3-kinase inhibitor) also inhibited invasion of MAC-T cells, suggesting that the invasion of persistent *E. coli* strain is dependent on tyrosine protein kinase and PI 3-kinase activation. These findings mirror those demonstrating that invasion of bladder epithelial cells by UPEC, and intestinal epithelial cells by *E. coli* associated with Crohn's disease (AIEC) requires host protein tyrosine phosphorylation and also PI 3-kinase activation (Boudeau et al., 1999; Martinez et al., 2000).

Intracellular survival and replication is an important attribute for the maintenance of an intracellular infection. The number of all persistent *E. coli* strains and one transient strain almost doubled during 48 h. In contrast, one of the remaining transient *E. coli* strains (ECA-B, which showed the best adhesion and invasion) markedly decreased over 48 h. Similarly, the number of non-pathogenic *E. coli* strain DH5 α decreased steadily over 48 h. These findings of intracellular survival and replication by the persistent strains parallel those examining UPEC and AIEC survival in cultured epithelial cells (Boudeau et al., 1999; Mulvey et al., 2001).

We subsequently explored the relevance of these in vitro observations to mastitis by applying FISH to mammary gland biopsies obtained from the cow that was persistently infected with strain ECC-M. We were able to demonstrate, for the first time, the presence of *E. coli* within mammary epithelium in a cow with naturally occurring *E. coli* mastitis, and provide clear evidence to support an intracellular niche in persistent *E. coli* mastitis.

To investigate the bacterial basis for adhesion, invasion and survival we performed serotyping, RAPD-PCR, phylogenetic grouping and PCR for virulence genes associated with diarrheagenic and extraintestinal *E. coli*. The diverse serotypes and RAPD patterns do not support the association of either the transient or persistent groups of strains with a single serotype or clonal group, although all the mastitis associated strains we investigated appear to cluster in phylogenetic group A which contains most commensal (non-pathogenic) strains of *E. coli* (Picard et al., 1999).

We found no evidence of the K99, F1845, CS31A, LT, STa, STb (associated with ETEC), *stx1* and *stx2* (associated with EHEC), *cnf1* and *cnf2* (associated

with NTEC and UPEC), *pap*, *hly*, *sfa* and *afaI* (associated with UPEC), *eae* (associated with EPEC and EHEC), and *ipaH* (associated with EIEC and *Shigella*) virulence genes typically associated with invasion of diarrheagenic and uropathogenic pathogenic *E. coli* to account for the invasive behavior of mastitis associated *E. coli* strains. Our findings are consistent with the absence of virulence genes *stx1*, *stx2*, *cnf1*, *cnf2*, *eae* (Bean et al., 2004) in the majority of mastitis associated *E. coli*.

In conclusion, this study has demonstrated that *E. coli* strains associated with persistent coliform mastitis invade and survive in cultured mammary epithelial cells more effectively than strains associated with transient infection. Invasion of cultured mammary epithelial cells involves host cell cytoskeletal rearrangement, intracellular signaling cascades and is not dependent on FimH. The bacterial determinants of invasion are unclear, as invasive strains are diverse in serotype and overall genotype, though they cluster in group A, and lack virulence factors associated with invasive diarrheagenic and extraintestinal pathogenic *E. coli*. Our observations suggest that persistent coliform mastitis is a consequence of infection with *E. coli* strains that are able to invade and survive within mammary epithelial cells, avoiding host defenses by as-yet undetermined means.

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