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Original article

Quantification of bacterial internalization by host cells using a β-lactamase reporter strain: *Neisseria gonorrhoeae* invasion into cervical epithelial cells requires bacterial viability

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Abstract

Neisseria gonorrhoeae can invade into cervical epithelial cells to overcome this host defense barrier. We developed a β-lactamase reporter system that allowed us to quantify at the single cell level if a host cell internalized a viable or nonviable microorganism. We autodisplayed β-lactamase on the surface of FA1090 [FA1090Φ(*bla-iga′*)] and demonstrated by confocal fluorescence microscopy and flow cytometry that FA1090Φ(*bla-iga′*) cleaved the β-lactamase substrate CCF2-AM loaded into host cells only when gonococci were internalized by these host cells. While FA1090Φ(*bla-iga′*) adhered to almost all ME180 cells, viable *N. gonorrhoeae* were internalized by only a subset of cells during infection. Nonviable gonococci adhered to, but were not internalized by ME180 cells, and failed to recruit F-actin to sites of adherent bacteria. Overall, we show that epithelial cell invasion is a dynamic process that requires viable *N. gonorrhoeae*. We demonstrate the advantages of the β-lactamase reporter system over the gentamicin protection assay in quantifying bacterial invasion. The reporter system that we have developed can be adapted to studying the internalization of any bacterial species into any host cell.

Keywords: Autodisplay; β-lactamase; CCF2-AM; F-actin; Invasion/internalization; *Neisseria gonorrhoeae*

1. Introduction

Neisseria gonorrhoeae (gonococci or GC), the causative agent of the sexually transmitted disease gonorrhea, colonize mucosal epithelia and endothelia and trigger an intense inflammatory response characterized by neutrophil influx. GC may cross the mucosal barrier by triggering their own internalization into cervical endothelial cells (for review, see [1]). Internalization requires both bacterial and host cell factors [2]. Pili, in conjunction with one or more gonococcal invasins (opacity proteins (Opa), lipooligosaccharide (LOS), and/or porin) or iC3b surface deposition, can induce changes in host cell signaling to drive host filamentous actin (F-actin) polymerization beneath adherent GC, triggering microvilli elongations that promote internalization [3–8]. Scanning electron micrographs revealed that nonviable GC fail to induce microvilli elongation in Hec1B cervical epithelial cells, and visual internalization of dead GC by these cells was not seen [3]. The F-actin rearrangements normally observed in epithelial cells during the internalization of viable GC into epithelial cells are not seen during the interaction of dead GC with various epithelial cell lines [9].

Current methods to measure GC internalization into host epithelial cells rely on the quantification of intracellular bacteria through adaptations of the gentamicin protection assay [10]. Gonococci are deemed to be internalized if they survive after gentamicin is added to infected host cells [10,11]. Quantifying GC internalization using the gentamicin protection assay possesses inherent limitations: it does not measure the number of host cells that contain internalized GC; it does not measure the frequency by which GC are internalized by host cells; it cannot determine if nonviable GC are capable of

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entering into host cells; and it underestimates the number of intracellular GC if the internalized bacteria aggregate within host cells or overestimates the number of internalized GC if not all extracellular GC are killed during gentamicin treatment.

In the present study, we established a gonococcal reporter strain that expresses a β-lactamase ((Bla)-IgA protease β-domain (IgAβ) fusion protein) in an FA1090 background using the strategy of autotransporter-mediated surface display (autodisplay). This strategy allows heterologous passenger domains, such as Bla, to be expressed on the outer surface of bacteria when fused with an autotransporter protein. Using the FA1090Φ(*bla-iga′*) strain, we demonstrate that only a subpopulation of human cervical epithelial cells (ME180) internalize viable GC and show that gonococcal viability is required for GC internalization into host cells.

2. Materials and methods

2.1. Bacterial strains, cell lines, plasmids, and reagents

GC strain FA1090 was obtained from Dr. W.M. Shafer (Emory University, Atlanta, GA) and maintained on gonococcal media base (GCK) (Becton Dickinson, Sparks, MD) containing 5 g/l agar and 1% Kellogg's supplement [12]. Penicillin G (Pen) was added to GCK agar plates as indicated. GC were killed with gentamicin sulfate (BioWhittaker/Cambrex Biosciences, Walkersville, MD) as described previously [13]. Piliated (Pil^+), Opa-expressing (Opa^+) GC strains were used in all experiments and colonies exhibiting these phenotypes were selected based on the light refraction properties of GC colonies on agar plates using a dissecting light microscope. *Escherichia coli* strain DH5α *mcr*$^-$ was obtained from New England Biolabs (Beverly, MA). The chimeric Bla plasmids pFT180 [14] and pK18UP [15] have been described previously. ME180 cells, a human cervical epidermal carcinoma cell line (HTB-33; American Type Culture Collection, Manassas, VA), were maintained in RPMI 1640 (Gibco/Invitrogen, Grand Island, NY) supplemented with 5% fetal bovine serum (FBS) (HyClone, Logan, UT), 1% Pen (1000 U/ml stock)–streptomycin (10 mg/ml stock) solution (Gibco/Invitrogen), and 10 μM Hepes (Mediatech, Herndon, VA). Chemicals used in this study were reagent grade or better and were obtained from Sigma Aldrich (St. Louis, MO) unless otherwise specified.

2.2. Establishing the reporter strain, FA1090Φ(bla-iga′)

The Bla-IgAβ fusion protein was constructed using a gene replacement strategy (Fig. 1A) and was expressed in FA1090 to yield strain FA1090Φ(*bla-iga′*). FA1090 chromosomal DNA was purified with a kit from Promega (Madison, WI). Transformants were selected on GCK agar containing 10 ng/ml Pen (GCK-Pen). All primers used in this study are described in Table 1.

2.3. Nitrocefin hydrolysis assay

Bla activity was determined using a modified version of a spectrophotometric assay established by O'Callaghan, *et al.* [16]. Briefly, GC were grown overnight in broth at 37 °C, resuspended to a turbidity of 100 Klett units (green filter) as measured by a Klett–Summerson colorimeter, and collected by centrifugation. Pellets were resuspended in 0.1 M phosphate buffer, 1 mM EDTA, pH 7.0 and supernatants were diluted 10-fold in the same buffer solution. A 500 μg/ml stock solution of nitrocefin (Calbiochem, LaJolla, CA) was prepared according to the manufacturer's instructions. Dilutions of bacteria, culture supernatants, or buffer controls were incubated with nitrocefin (fc 50 μg/ml) (RT, 30 min). The absorbance at 520 nm was measured. The Bla activity of 100 arbitrary units (aU) is defined as the amount of nitrocefin hydrolyzed in 30 min by 1×10^9 FA1090Φ(*bla-iga′*) in a 1-ml reaction. Enzymatic activity in all other samples was normalized to this reading.

2.4. Gentamicin protection assay

ME180 cervical epithelial cells were seeded in 24-well tissue culture plates at 1×10^5 cells/well and incubated overnight at 37 °C in 5% CO_2. Prior to use, cells were viewed under a light microscope to ensure they had reached ~80% confluency in the well. Bacteria were also grown overnight on GCK agar, resuspended to a turbidity of 100 Klett units, added to ME180 cells at an MOI of 10, and incubated in internalization media (IM) (RPMI, 5% FBS, 0.5% Kellogg's supplement, and 10 mM Hepes) for 6 h. Cells were washed four times with IM. To quantify the total number of host cell-associated GC, including adherent and internalized GC, cells were treated with 1% saponin (15 min) and aliquots plated on GCK agar. To quantify the number of internalized GC, gentamicin (200 μg/ml) was added 2 h prior to the preparation of ME180 cell lysates. The number of CFU arising on GCK agar was determined after 36–48 h incubation.

2.5. Immunofluorescence microscopy analysis

ME180 cells were seeded on coverslips in 24-well plates overnight, incubated with GC for various lengths of time, washed three times with IM and once with phosphate-buffered saline (PBS), fixed (20 min) with 4% paraformaldehyde (Electron Microscopy Sciences, Ft. Washington, PA) and treated with blocking solution (10% FBS and 10 μM glycine in PBS, 5 min). Extracellular, adherent GC were stained with an Oregon green conjugated goat anti-GC antibody (Ab) (4 °C, 60 min). Cells were washed three times with PBS and treated with a permeabilization solution (0.1% Triton X-100, 10% FBS, 10 mM glycine in PBS) for 5 min at RT. F-actin was stained with Alexa Fluor 546-labeled phalloidin (4 U/ml) (Molecular Probes/Invitrogen) in the permeabilization solution (4 °C, 60 min). Cells were washed with PBS, fixed with 2% paraformaldehyde, mounted on glass slides and analyzed using a Zeiss LSM 510 laser scanning confocal microscope.

2.6. Cellular distribution of CCF2-AM

ME180 cells were seeded in 24-well plates as described above and washed with RPMI 1640 containing 10 μM Hepes.