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ORIGINAL ARTICLE

Detection of Iss and Bor on the surface of *Escherichia coli*

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Abstract

Aims: To confirm the presence of Iss and Bor on the outer membrane of *Escherichia coli* using Western blots of outer membrane protein (OMP) preparations and fluorescence microscopy, and explore the use of fluorescence microscopy for the detection of avian pathogenic *E. coli* (APEC) and diagnosis of avian colibacillosis.

Methods and Results: Knockout mutants of *iss* and *bor* were created using a one-step recombination of target genes with PCR-generated antibiotic resistance cassettes. Anti-Iss monoclonal antibodies (Mabs) that cross-react with Bor protein were used to study the mutants relative to the wild-type organism. These Mabs were used as reagents to study OMP preparations of the mutants with Western blotting and intact *E. coli* cells with fluorescence microscopy. Iss and Bor were detected in Western blots of OMP preparations of the wild type. Also, Iss was detected on Δ*bor* mutants, and Bor was detected on Δ*iss* mutants. Iss and Bor were also detected on the surface of the intact, wild-type cells and mutants using fluorescence microscopy.

Conclusions: These results demonstrate that Bor and Iss are exposed on *E. coli*'s outer membrane where they may be recognized by the host's immune system.

Significance and Impact of the Study: To our knowledge, this is the first report confirming Iss' location in the outer membrane of an *E. coli* isolate. Such surface exposure has implications for the use of these Mabs for APEC detection and colibacillosis control.

Introduction

Colibacillosis, caused by avian pathogenic *Escherichia coli* (APEC), is a major problem for the poultry industry in the United States, resulting in significant losses annually (Barnes *et al.* 2003). Despite efforts by researchers, the mechanisms of APEC virulence remain ill defined. One of the past problems encountered in colibacillosis control is that no single trait was known, which was characteristic of most or all APEC (Barnes *et al.* 2003). However, research has shown that resistance to serum complement may be an important contributor to APEC virulence (Ike *et al.* 1992; Nolan *et al.* 1992, 2003; Wooley *et al.* 1992, 1993) and that the increased serum survival gene or *iss* (Binns *et al.* 1979), which is associated with *E. coli* complement resistance (Binns *et al.* 1979; Chuba *et al.* 1986, 1989), occurs significantly more often in APEC than it does in *E. coli* isolates of apparently healthy birds (Pfaff-McDonough *et al.* 2000; Rodriguez-Siek *et al.* 2005). This strong association between *iss* and APEC suggested that *iss*-centric strategies might prove fruitful in studying APEC virulence and avian colibacillosis control.

The *iss* gene was first described by Binns *et al.* (1979) for its role in the complement resistance associated with a ColV plasmid of a human *E. coli* isolate. *iss* has also been localized to a large, conjugative ColV plasmid of an APEC isolate through a series of conjugation experiments using an *iss*$^+$ APEC isolate as the donor and an avirulent *E. coli* K12 strain as the recipient (Johnson *et al.* 2002). The resulting transconjugant, TC4, possessed many genotypic and phenotypic characteristics of the APEC donor, such as possession of *iss*

and an increased ability to resist the bacteriocidal effects of complement (Johnson *et al.* 2002).

Iss, the protein product of *iss*, has a signal sequence characteristic of outer membrane proteins (OMPs), suggesting that it occurs as a 10·9-kDa lipoprotein in the bacterial outer membrane (Barondess and Beckwith 1990; Horne *et al.* 2000), although this location has yet to be experimentally confirmed. The *iss* gene is thought to be a derivative of *bor*, a gene of bacteriophage λ (Chuba *et al.* 1989; Barondess and Beckwith 1990, 1995; Horne *et al.* 2000). The *bor* gene encodes Bor, a lipoprotein (10·3 kDa) of the cell envelope of *E. coli* lambda lysogens, which appears to confer complement resistance on these lysogens (Barondess and Beckwith 1995). Amino acid sequences of Iss and Bor are about 90% identical (Horne *et al.* 2000). This similarity is of concern, as it makes generation of Iss-specific monoclonal antibodies (Mabs) for use as reagents in the study of APEC and control of avian colibacillosis problematic (Foley *et al.* 2003; Lynne *et al.* 2006a). Early attempts to produce these antibodies resulted in Mabs of the IgM isotype (Foley *et al.* 2003). These were considered undesirable because of their nonspecific binding of negative controls (Lynne *et al.* 2006a). Mabs of the IgG_1 isotype were also produced, which did not bind nonspecifically to negative controls (Lynne *et al.* 2006a). However, in addition to binding Iss, both Mabs bound a recombinant Bor fusion protein (Lynne *et al.* 2006a). This cross-reactivity could limit the utility of the anti-Iss Mabs in discriminating between APEC and commensal *E. coli*, as Bor, unlike Iss, occurs widely among avian *E. coli* pathogens and commensals (T.J. Johnson, K.E. Sick and L.K. Nolan, unpublished data).

Therefore, the goal of the present work was twofold. First, we wished to determine whether Iss and Bor were located in the *E. coli* outer membrane, and if they were, to determine whether our anti-Iss Mabs would be useful in distinguishing APEC from commensal *E. coli* by their possession of Iss using two different immunological assays. Work presented here describes the use of the Mabs in detecting Iss and Bor on the outer membrane of an APEC isolate and a series of *iss* and *bor* knockout mutants with Western blotting of OMP preparations and immunofluorescent microscopy of whole cells.

Materials and methods

Bacterial strains

Strains used are summarized in Table 1 and include APEC-O2 (Johnson *et al.* 2006); *E. coli* DH5α (Chart *et al.* 2000) and *Salmonella enterica* serovar Typhimurium (NC0031977). APEC-O2 is an *iss*$^+$, *bor*$^+$ isolate implicated in avian colibacillosis (Johnson *et al.* 2006). In this isolate, *iss* is located on a 184-kb, transmissible plasmid known as pAPEC-O2-ColV (Johnson *et al.* 2006). *Escherichia coli* DH5α is a plasmid-less, K12 strain known to be *iss*$^-$, *bor*$^+$ and avirulent (Chart *et al.* 2000). When not in use, these organisms were stored at −70°C in brain heart infusion broth (Difco Laboratories, Detroit, MI, USA) with 10% glycerol.

Creation of mutants

Knockout mutants were created by deletion of *iss* from pAPEC-O2-ColV in APEC-O2, and deletions of *bor* from the chromosome of *E. coli* DH5α using the method of Datsenko and Wanner (2000) except that red-mediated recombination proteins were expressed by pSKY5000 rather than pKD46 for deletion of *iss* in APEC-O2. pSKY5000 is a derivative of pKD46, containing a chloramphenicol resistance (Chl^R) cassette. This derivative was produced by cloning a Chl^R cassette into the *Bsa*I restriction site of pKD46. This method relies on overproduction of λ-derived recombination proteins encoded by the temperature-sensitive plasmid pSKY5000 and pKD46, and PCR amplification of a kanamycin resistance (Kan^R) cassette in pKD4 or a Chl^R cassette in pKD3 flanked by 5′ and 3′ sequences of the gene targeted for deletion.

Table 1 Characteristics of isolate used

Isolate	Description	*iss*, *bor* content	Reference
APEC-O2	Pathogenic *Escherichia coli* isolate from a diseased bird; Iss is present on a transmissible 180-kb plasmid known as pAPEC-O2-ColV	*iss*, *bor*	Johnson *et al.* (2006)
APEC-O2Δ*iss*	*iss*$^-$ isogenic mutant of APEC-O2	*bor*	This study
DH5α	*E. coli* K12 strain	*bor*	Chart *et al.* (2000)
DH5α Δ*bor*	*bor*$^-$ isogenic mutant of DH5α	Neither	This study
TC4	Transconjugant	*iss*, *bor*	Johnson *et al.* (2002)
TC4 Δ*bor*	Transconjugant	*iss*	This study
TC4 Δ*iss*	Transconjugant	*bor*	This study
TC4 Δ*bor* Δ*iss*	Transconjugant	Neither	This study

Table 2 Primers used in this study

Name	Sequences (5′ to 3′)	Amplicon size	Accession no.	Reference
Primers used for creation of mutants				
for-mut-*iss*	tattcatttcccatgattctgagtacctaccaagtctgagtgtgtaggctggagctgctt		X52665	This study
rev-mut-*iss*	aaaaacaactgtagggagcccagaagtatattaatgaacacatatgaatatcctccttag			
for-mut-*bor*	atatcgatgggcaactcatgcaattatttttgagcaatacatgtgtaggctgagctgctt		X55792	This study
rev-mut-*bor*	acatacgattctgcgaacttcaaaaagcatcgggaataaccatatgaatatccttag			
Primers used to screen mutants				
iss upper	gtggcgaaaactagtaaaacagc	760	X52665	Horne *et al.* (2000)
iss lower	cgcctcggggtggataa			
bor upper	ctcgatgcaaaatacacgaaggagttagct	658	X55792	This study
bor lower	taattttctacacatacgattctgcgaact			

After electrotransformation and antibiotic selection of transformants, the expected deletions were verified by PCR protocols targeting the deleted gene and the new antibiotic resistance cassette (ABX^R) junction fragment. Primers used are summarized in Table 2.

Bacterial matings and selection of transconjugants

Various transconjugant mutants were produced by matings of mutants of donor and recipient isolates by methods reported by Nolan *et al.* (1992) (Table 3). Briefly, 0·2 ml of exponentially grown donor cells was mixed with 1·8 ml of an overnight culture of recipient cells in antibiotic medium 3 broth (Difco Laboratories). Mixtures were incubated without shaking at 37°C for 18 h. Transconjugants were selected on Luria–Bertani (LB) agar plates (Difco) containing a donor-inhibiting concentration of nalidixic acid (30 μg ml^{-1}; Amresco, Solon, OH, USA) and a recipient-inhibiting concentration of ampicillin (100 μg ml^{-1}; Amresco). Presumptive transconjugant colonies were picked from the selector plates, and absence of *iss* and/or *bor* genes was confirmed by PCR. Primers used for mutant screening are summarized in Table 1. The amplification program used was 94°C for 5 min; 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s; then 72°C for 7 min and hold at 4°C.

Outer membrane protein analysis

APEC-O2, APEC-O2 Δ*iss*, *E. coli* DH5α, and the four transconjugants (TC4, TC4 Δ*iss*, TC4 Δ*bor*, TC4 Δ*iss* Δ*bor*) were assessed for possession of Iss and Bor in their outer membranes by Western blot. OMPs were isolated by the method described by Dassouli-Mrani-Belkebir *et al.* (1988). Briefly, 25 ml of an overnight culture of each strain in LB was pelleted and resuspended in 10 ml of ice-cold Tris buffer (50 mmol l^{-1} Tris, 1 mmol l^{-1} EDTA, pH 7·8). Cell concentrations were normalized with Tris buffer. Samples were sonicated four times for 15 s at 15% continuous cycle. Cellular debris was removed by centrifugation at 1200 ***g*** for 20 min. The supernatant was centrifuged at 50 000 ***g*** for 1 h at 4°C. The pellet was resuspended in Tris–Triton buffer (10 mmol l^{-1} Tris, 5 mmol l^{-1} $MgCl_2$, 2% Triton ×100, pH 2·0), centrifuged at 50 000 ***g*** for 1 h at 4°C, and dissolved in TGS buffer (25 mmol l^{-1} Tris, 192 mmol l^{-1} glycine, 1% SDS, pH 8·6). Samples were loaded into wells of 18% polyacrylamide gel (Bio-Rad, Hercules, CA, USA), and proteins in gel were subjected to electrophoresis at 200 V using a Criterion Cell electrophoresis unit (Bio-Rad). Samples were transferred to polyvinylidine difluoride membranes with the Criterion Blotter (Bio-Rad), run at 100 V for 30 min and probed with anti-Iss Mabs (Lynne *et al.* 2006a). SDS–PAGE gel was stained with SimplyBlue SafeStain (Invitrogen, Carlsbad, CA, USA), and bound Mabs were detected with AP conjugate Substrate Kit (Bio-Rad). Densitometry analysis was performed using ImageJ Program (Abramoff *et al.* 2004).

Immunofluorescence microscopy

APEC-O2, APEC-O2 Δ*iss*, *E. coli* DH5α and the four transconjugants were assessed for possession of Iss and Bor in their outer membranes by immunofluorescent microscopy. *Salm. enterica* serovar Typhimurium was used as a negative control. Bacterial strains were assessed using methods described previously (Sheridan *et al.* 1991). Briefly, overnight cultures were serially diluted in phosphate-buffered saline (PBS) to 10^{-5}. One millilitre of

Table 3 Summary of transconjugants created

Donor	Recipient	Transconjugant	Resistances
APEC-O2	DH5α	TC4	None
APEC-O2	DH5αΔ*bor*	TC4 Δ*bor*	Chl^R
APEC-O2 Δ*iss*	DH5α	TC4 Δ*iss*	Kan^R
APEC-O2 Δ*iss*	DH5αΔ*bor*	TC4 Δ*bor* Δ*iss*	Chl^R, Kan^R

cells was immobilized on 0·6 μm polycarbonate membranes (Osmonics Inc, Minnetonka, MN, USA) using a vacuum manifold. Membranes were covered with anti-Iss Mabs (Lynne *et al.* 2006a) and incubated for 30 min at 37°C. Membranes were then washed with PBST (PBS + 0·1% Tween), covered with fluorescein-isothiocyanate-labelled anti-mouse antibody (Pierce, Rockford, IL, USA), diluted 1/50 (v/v) in a sterile 1% skim milk solution containing 0·1% Tween and incubated for 30 min at 37°C. Membranes were washed with PBST, mounted on microscope slides and examined under 60× oil immersion for fluorescent cells.

Results

To create a series of transconjugant knockout mutants, *iss* was deleted from APEC-O2, and *bor* was deleted from *E. coli* DH5α using the one-step recombination system. *iss* was replaced with a *kan* cassette in pAPEC-O2-ColV. Therefore, transconjugants resulting from a mating of APEC-O2 Δ*iss* and *E. coli* DH5α were Kan^R. *bor* in *E. coli* DH5α was replaced with a *cat* cassette, and transconjugants resulting from the matings using this strain were either Kan^R and Chl^R or Chl^R only depending on whether the APEC-O2 wild type or mutant was used as the plasmid donor strain. The original strains and the resulting knockout mutants were used to create a series of transconjugants as summarized in Table 3. Possession of *iss* and *bor* by the transconjugants was verified by PCR (Fig. 1). Transconjugants produced included (i) TC4, which was Chl^S and Kan^S; (ii) TC4 Δ*iss*, which was Chl^S and Kan^R; (iii) TC4 Δ*bor*, which is Chl^R, Kan^S and (iv) TC4 Δ*bor* Δ*iss*, which is Chl^R and Kan^R.

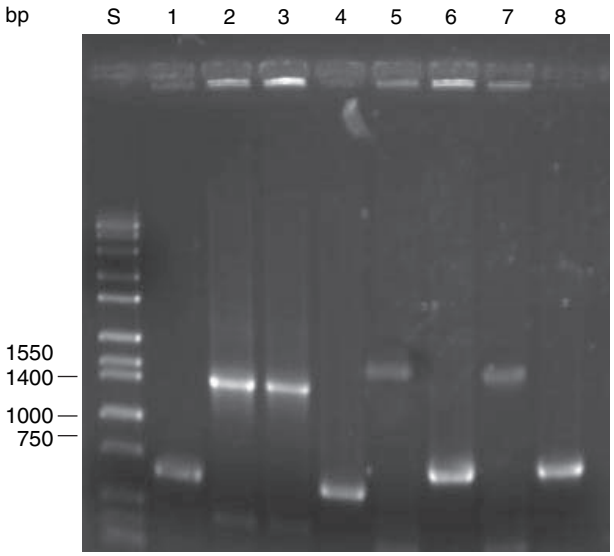

Figure 1 Agarose gel of amplicons. Screening of transconjugants for *iss* and *bor* by PCR. Lane S, molecular marker in bp; lanes 1–4, screening for possession of *bor*; lanes 5–8, screening for possession of *iss*; lanes 1 and 5, TC4 Δ*iss*; lanes 2 and 6, TC4 Δ*bor*; lanes 3 and 7, TC4 Δ*bor* Δ*iss*; lanes 4 and 8, TC4. Isolates containing *bor* will have an amplicon of 658 bp in size. In the *bor* knockout mutants, *bor* was replaced with a cat cassette with an amplicon size of 1450 bp. Isolates containing *iss* will have amplicons of 760 bp in size. In *iss* knockout mutants, *iss* was replaced with a Kan^R cassette with an amplicon size of 1800 bp. TC4 had amplicons of 658 bp for *bor* and 760 bp for *iss*. TC4 Δ*bor* had amplicons of 1450 bp for the Chl^R cassette and 760 bp for *iss*. TC4 Δ*iss* had amplicons of 658 bp for *bor* and 1800 bpfor the Kan^R cassette. TC4 Δ*bor* Δ*iss* had amplicons of 1450 bp for Chl^R cassette and 1800 bp for Kan^R cassette.

The result of the Western blot of the bacterial OMP preparations is shown in Fig. 2. Anti-Iss Mabs recognized bands corresponding to Iss and Bor. The molecular weight of Iss is 10·9 kDa, and the molecular weight of Bor is 10·3 kDa. The Mabs recognized bands corresponding in size to Iss in strains APEC-O2, TC4 and TC4 Δ*bor*. The Mabs also recognized bands corresponding in size to Bor in all strains except TC4 Δ*bor* and TC4 Δ*bor* Δ*iss* (Fig. 2). No proteins were recognized in the cytoplasmic fraction (results not shown). Comparative densitometry of the Western blot revealed a sixfold increase of Iss in APEC-O2 compared with TC4 and TC4 Δ*bor*.

Escherichia coli APEC-O2, APEC-O2 Δ*iss*, DH5α, *Salm. enterica* serovar Typhimurium, TC4 Δ*iss*, TC4 Δ*bor* and TC4 Δ*bor* Δ*iss* were assessed for the presence of Iss and Bor in the outer membrane, using fluorescence micro-

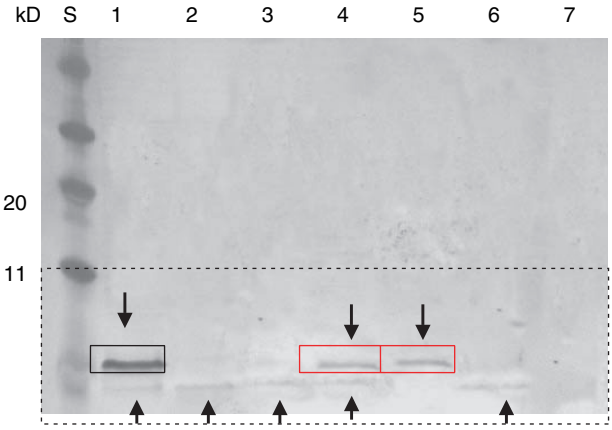

Figure 2 Western blot of OMP preparations of various organisms probed with the anti-Iss Mabs. Lane S, the molecular weight standard (Bio-Rad prestained molecular weight standard); lane 1, APEC-O2; lane 2, APEC-O2 Δ*iss*; lane 3, DH5α; lane 4, TC4; lane 5, TC4 Δ*bor*; lane 6, TC4 Δ*iss*; lane 7, TC4 Δ*bor* Δ*iss*. 'Down' arrows point to bands corresponding to Iss, while 'up' arrows point to bands corresponding to Bor. Comparative densitometry on Iss bands showed a sixfold increase of protein product in APEC-O2 (black rectangle) when compared with TC4 and TC4 Δ*bor* (red rectangle). Comparative densitometry on Bor bands showed no change in protein product between any of the *bor*$^+$ strains.

scopy. The results of the immunofluorescent microscopy assay show that the anti-Iss Mabs recognized a protein on all strains except for TC4 Δ*bor* Δ*iss* and *Salm. enterica* serovar Typhimurium (Fig. 3).

Discussion

Avian colibacillosis is a costly disease for the poultry industry, accounting for multimillion dollar losses annually (Barnes *et al.* 2003). Previous work in our lab has shown that *iss* is strongly associated with APEC but not with faecal isolates from apparently healthy birds (Pfaff-McDonough *et al.* 2000; Rodriguez-Siek *et al.* 2005), making *iss* and the protein it encodes, Iss, potential identifiers of virulent isolates. To aid in the study of complement resistance and virulence of APEC, Mabs against Iss were created (Foley *et al.* 2003; Lynne *et al.* 2006a). These antibodies recognized recombinant Iss protein but also

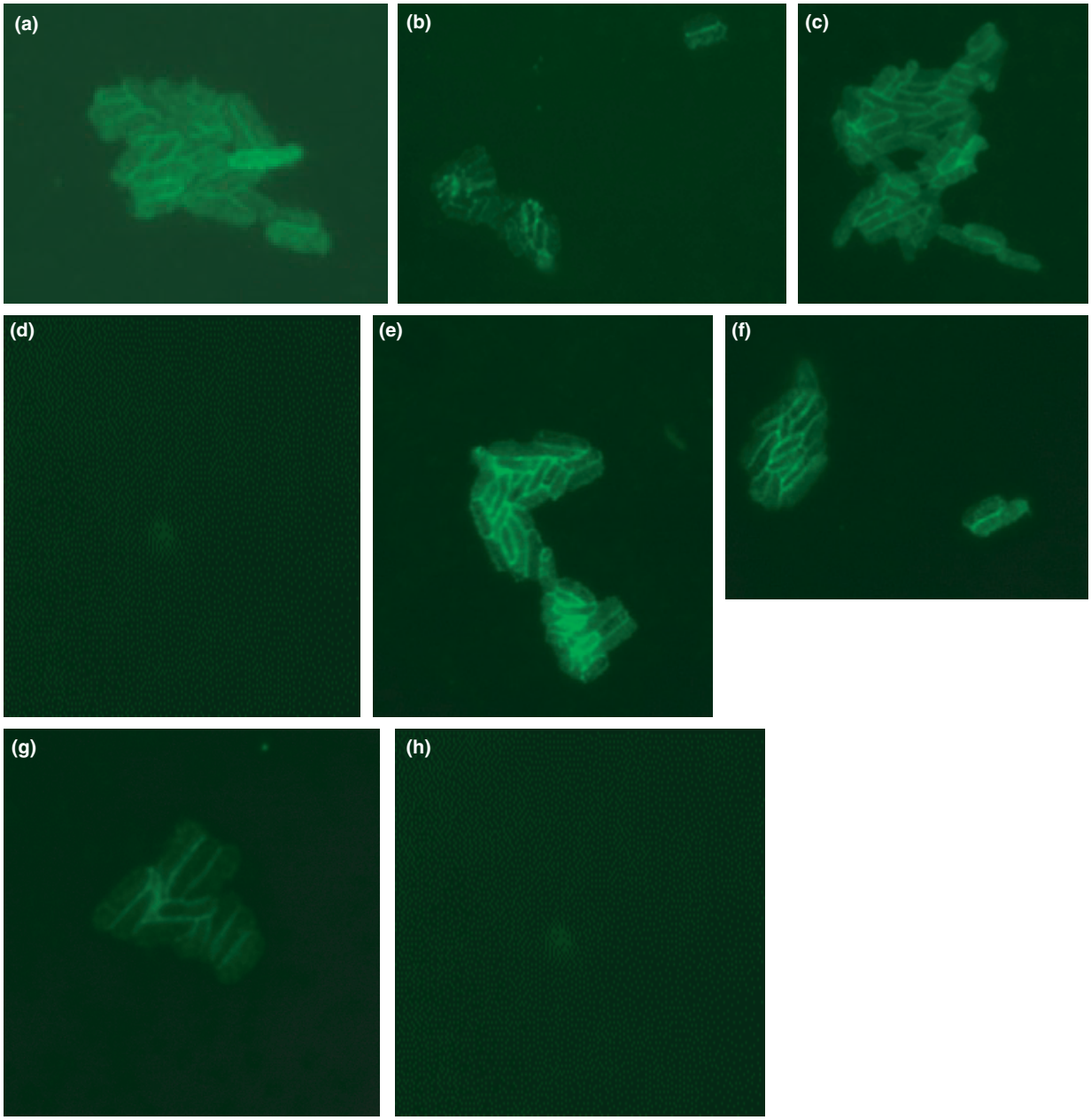

Figure 3 Images of immunofluorescent microscopy. Bacterial cells were first probed with anti-Iss Mabs, then with FITC-conjugated anti-mouse antibody. (a) APEC-O2; (b) APEC-O2 Δ*iss*; (c) DH5α; (d) *Salmonella enterica* serovar Typhimurium; (e) TC4; (f) TC4 Δ*bor*; (g) TC4 Δ*iss*; (h) TC4 Δ*bor* Δ*iss*.

bound recombinant Bor protein. Although such results are not desirable, this cross-reactivity was not surprising as Bor and Iss share about 90% homology (Horne *et al.* 2000). In the present study, the Mabs were used to detect denatured and native Iss and Bor on the outer membrane of an APEC isolate as well as various transconjugants.

The results of the Western blot of OMP preparations of the donor, recipients and various transconjugants showed that Iss is expressed in APEC-O2, but not in APEC-O2 Δ*iss* or DH5α. Also, Iss was expressed in the transconjugants, containing pAPEC-O2-ColV, an *iss*-containing plasmid. Bor was expressed in all strains tested except for strains containing *bor* deletions. According to comparative densitometry, there appears to be a higher level of expression of Iss (sixfold) in the wild-type isolate than in the resulting transconjugants, which suggests that the Mabs could prove useful in identifying APEC. That is, differential expression of Iss and Bor might be exploited to discriminate that APEC are typically Bor^+ and Iss^+, and commensal *E. coli* strains are typically Bor^+ and Iss^-. This apparent difference in expression of these two proteins may be due to an interaction of plasmid-mediated components and chromosomally mediated components present in the donor but lacking in the transconjugant that could regulate expression of Iss in wild-type strains. Future work examining *iss* expression will be needed to confirm this possibility.

Evidence has suggested that Iss would likely be surface exposed, but prior to this report, the outer membrane location of Iss had not been experimentally confirmed (Barondess and Beckwith 1990). The results of the immunofluorescent microscopy showed that Iss is surface exposed and that the Mabs were able to recognize native Iss and Bor on the outer membrane of the bacterial cell. The surface exposure of Iss offers potential benefits. First, Iss in this location on an APEC isolate would be expected to interact with the immune system of an infected host, suggesting that Iss may prove a valuable candidate as a vaccine target. In fact, in a recent study, recombinant Iss was used as a vaccine against colibacillosis (Lynne *et al.* 2006b). Birds that were immunized with Iss mounted a humoural immune response to Iss and displayed some protection against heterologous APEC challenge. Another benefit of Iss' surface exposure may be that it is accessible to Iss-directed reagents used in diagnostic protocols. As possession of Iss is strongly correlated with APEC virulence (Pfaff-McDonough *et al.* 2000; Rodriguez-Siek *et al.* 2005), the ability to detect Iss on the surface of an isolate could be used to differentiate APEC from commensal *E. coli*. Unfortunately, our Mabs also recognize Bor. Although this cross-reactivity is not surprising, considering the homology between Bor and Iss, it may diminish the utility of these Mabs in immunofluorescent diagnostic protocols. Future work to determine whether differential expression of Iss and Bor can be exploited in diagnostic protocols using the current Mabs or future work to develop Mabs that recognize Iss but not Bor would seem desirable. Strategies for doing so could include producing Mabs directed against a portion of the Iss protein not common to Bor.

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