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Abstract

Colibacillosis, caused by avian pathogenic *Escherichia coli* (APEC) is a major problem for the poultry industry resulting in significant losses annually. Previous work in our lab and by others has shown that the increased serum survival gene (*iss*) is a common trait associated with the virulence of APEC. This gene was first described for its contributions to *E. coli* serum resistance. However, recently published research has called the contribution of *iss* to this trait into question. In the present study, the level of serum resistance conferred on an *E. coli* isolate by *iss* is examined. Additionally, the contribution of λ *bor* gene to *E. coli* serum resistance is studied, as *iss* is thought to be derived from *bor* and *bor* occurs commonly among *E. coli*. To better understand the *iss* and *bor* contributions to serum resistance, a series of *iss* and *bor* mutants was generated. An *iss* deletion (*iss*⁻) mutant showed a significant drop in its resistance to serum. Similarly, a *bor* mutant showed a drop in serum resistance but not as drastic as that observed with the *iss* mutant, suggesting that *iss* contributes more to serum resistance than *bor* in this *E. coli* strain. Also, when *iss* was reintroduced into the *iss*⁻ mutant the wild-type level of serum resistance was restored, confirming that the deletion of *iss* was responsible for the change in resistance seen in the mutant.

Keywords

iss, *bor*, *Escherichia coli* virulence, avian colibacillosis, *Escherichia coli*, APEC, Bor, complement resistance, *Iss*, serum resistance

Disciplines

Genomics | Veterinary Microbiology and Immunobiology | Veterinary Preventive Medicine, Epidemiology, and Public Health

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Research Note—

Characterization of a Series of Transconjugant Mutants of an Avian Pathogenic *Escherichia coli* Isolate for Resistance to Serum Complement

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SUMMARY. Colibacillosis, caused by avian pathogenic *Escherichia coli* (APEC) is a major problem for the poultry industry resulting in significant losses annually. Previous work in our lab and by others has shown that the increased serum survival gene (*iss*) is a common trait associated with the virulence of APEC. This gene was first described for its contributions to *E. coli* serum resistance. However, recently published research has called the contribution of *iss* to this trait into question. In the present study, the level of serum resistance conferred on an *E. coli* isolate by *iss* is examined. Additionally, the contribution of λ *bor* gene to *E. coli* serum resistance is studied, as *iss* is thought to be derived from *bor* and *bor* occurs commonly among *E. coli*. To better understand the *iss* and *bor* contributions to serum resistance, a series of *iss* and *bor* mutants was generated. An *iss* deletion (*iss*⁻) mutant showed a significant drop in its resistance to serum. Similarly, a *bor* mutant showed a drop in serum resistance but not as drastic as that observed with the *iss* mutant, suggesting that *iss* contributes more to serum resistance than *bor* in this *E. coli* strain. Also, when *iss* was reintroduced into the *iss*⁻ mutant the wild-type level of serum resistance was restored, confirming that the deletion of *iss* was responsible for the change in resistance seen in the mutant.

RESUMEN. *Nota de Investigación*—Caracterización de una serie de mutantes transconjugadas de un aislamiento de *Escherichia coli* para su resistencia al complemento del suero.

La colibacilosis causada por cepas patógenas de *Escherichia coli* es un problema importante para la industria avícola, resultando anualmente en pérdidas significativas. Trabajos previos en nuestro laboratorio y en otros han demostrado que el gen de resistencia aumentada (por sus siglas en Inglés *iss*) es una característica comúnmente asociada con la virulencia de cepas patógenas de *Escherichia coli*. Este gen fue descrito en un principio por su contribución a la resistencia de *E. coli* en el suero. Sin embargo, investigaciones publicadas recientemente ponen en duda el aporte del gen *iss* a la resistencia de *E. coli* en el suero. En el presente estudio, se examina el nivel de resistencia al suero (complemento) conferido por *iss* a un aislamiento de *E. coli*. Adicionalmente se estudia la contribución del gen λ *bor* a la resistencia contra el suero, esto debido a que se asume que el gen *iss* proviene del gen *bor* y que el gen *bor* esta comúnmente presente en *E. coli*. Con la finalidad de entender mejor la contribución de los genes *iss* y *bor* a la resistencia a suero, se generaron una serie de mutantes de *iss* y *bor*. Una mutante por delección de *iss* (*iss*⁻) mostró una disminución significativa en su resistencia al suero. Similarmente, una mutante de *bor* mostró una disminución en su resistencia al suero, aunque no tan drástica como la observada en la mutante *iss*, lo que sugiere que *iss* contribuye más a la resistencia en suero en esta cepa de *E. coli*. Además, cuando *iss* se reintrodujo en la mutante *iss*⁻ se reestableció el nivel previo de resistencia al suero, confirmando que la delección del gen *iss* fue responsable del cambio en resistencia observado en la mutante.

Key words: *iss*, *bor*, *Escherichia coli* virulence, avian colibacillosis, *Escherichia coli*, APEC, Bor, complement resistance, Iss, serum resistance

Abbreviations: APEC = avian pathogenic *Escherichia coli*; LB = Luria Bertani; PCR = polymerase chain reaction; PG = peptone glucose

Colibacillosis accounts for multimillion dollar losses in the poultry industry annually (1). Despite this fact, the basic mechanisms of virulence used by avian pathogenic *Escherichia coli* (APEC) to cause disease in poultry remain ill defined. However, the ability of avian *E. coli* to resist the bactericidal effects of serum complement appears to play a significant role in the development of colibacillosis in poultry (13,16,25,26,27,28,33,36,37). Serum resistance in *E. coli* has been related to several structural factors including a K1 antigenic capsule (10,22), a smooth lipopolysaccharide layer (11,14), and certain outer membrane proteins, including TraT, Iss, and OmpA (3,5,8,9,34). Recent work in our laboratory

has shown that the presence of *iss* is strongly correlated with an APEC isolate's ability to cause disease in poultry (29,30).

The increased serum survival gene, *iss*, first described for its role in the serum resistance associated with a ColV plasmid in a human *E. coli* isolate, increases the virulence of an *E. coli* isolate for day-old chicks 100-fold and its complement resistance over 20-fold (2,4,8,9). Iss, the protein product of *iss*, is thought to occur as a 10 to 11 kD lipoprotein in the bacterial outer membrane (3,15). *iss* may be a derivative of *bor*, a gene of bacteriophage λ (2,3,8,15). Bor, a lipoprotein of the cell envelope of *E. coli* λ lysogens, appears to confer complement resistance on these lysogens (2). Amino acid sequences of Iss and Bor are about 90% identical (15).

The *iss* gene has recently been localized to large plasmids that typify the APEC pathotype (17,20). To date, it is known that these *iss*-containing APEC plasmids may encode ColV or ColBM. Regardless

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Table 1. Characteristics of strains used.

Isolate	Description	<i>iss</i> , <i>bor</i> content	Reference
APEC-O2	Pathogenic <i>E. coli</i> isolate from a diseased bird; <i>iss</i> is present on a transmissible 180-kb plasmid known as pAPEC-O2-ColV	<i>iss</i> , <i>bor</i>	20
DH5 α	<i>E. coli</i> K12 strain	<i>bor</i>	7
DH5 α Δ <i>bor</i>	<i>bor</i> ⁻ isogenic mutant of DH5 α	none	23
TC4	Transconjugant	<i>iss</i> , <i>bor</i>	23
TC4 Δ <i>bor</i>	Transconjugant mutant	<i>iss</i>	23
TC4 Δ <i>iss</i>	Transconjugant mutant	<i>bor</i>	23
TC4 Δ <i>bor</i> Δ <i>iss</i>	Transconjugant mutant	none	23
TC4 Δ <i>iss</i> + pZCiss	Transconjugant mutant complemented with <i>iss</i>	<i>iss</i> , <i>bor</i>	This study
TC4 Δ <i>bor</i> Δ <i>iss</i> + pZCiss	Transconjugant mutant complemented with <i>iss</i>	<i>iss</i>	This study

of the colicin encoded, these plasmids contain a pathogenicity island characterized by possession of *iss*, several iron acquisition operons, and other genes (17). These plasmids are often conjugative and confer upon the recipient strain such traits as colicin production, aerobactin production, and increased serum resistance (17).

Recently, Mellata *et al.* (24) questioned the role of *iss*/Iss in the ability of an APEC isolate to resist serum complement. In their study, strains that contained *iss* and *traT* genes but had lost their K1 or O serotype were not protected against the bactericidal effect of serum. However, these strains were less sensitive to serum than *iss* and *traT* negative control strains. Based on these results, the authors concluded that Iss plays a limited role in the serum resistance of the APEC strain they studied.

In an effort to clarify the role of Iss in *E. coli* complement resistance and to better understand the mechanisms of APEC virulence, a series of transconjugant *iss* and *bor* mutants of an APEC isolate were studied (23).

MATERIALS AND METHODS

Bacterial strains. Strains used in this study are summarized in Table 1 and include APEC-O2, an *iss*⁺ virulent APEC strain isolated from the joint of a chicken with colibacillosis. APEC-O2 possesses pAPEC-O2-R, a 101-kb, multidrug-resistance-encoding plasmid (19),

and pAPEC-O2-ColV, a 180-kb, ColV-encoding plasmid (20). pAPEC-O2-ColV contains a 93-kb cluster of putative virulence genes, which includes *iss* (20). Both plasmids are transmissible by conjugation (19,20). Also, *E. coli* DH5 α (7), a plasmidless, avirulent K12 strain that is *iss*⁻ and *bor*⁺, and a series of mutants previously created (23) were used in this study. 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* DH5 α by a one-step inactivation method using λ -derived recombination proteins and polymerase chain reaction (PCR) products. Transconjugants were generated using an APEC-O2 or APEC-O2 Δ *iss* as the plasmid donor and *E. coli* DH5 α or DH5 α Δ *bor* as the plasmid recipients (23). When not in use, these organisms were stored at -70 C in brain heart infusion broth (Difco Laboratories, Detroit, MI) with 10% glycerol. When in use, these organisms were grown on either MacConkey or Luria Bertani (LB) agar (Difco) overnight at 37 C.

Molecular cloning. For creation of pZCiss, the *iss* gene was amplified using primers previously described (15). The amplified *iss* gene was prepared for ligation using the Wizard PCR preps DNA purification system (Promega, Madison, WI). The *iss* amplicon was ligated into pGEM-T Easy, and the recombinant plasmid DNA was used to transform *E. coli* JM109 competent cells according to the manufacturer's protocol (Promega). Plasmid DNA was purified from these transformants using the Wizard Plus Minipreps DNA Purification System (Promega) and digested with *Nsi*I and *Sac*II for 2 hr at 37 C. Digests were run in 1% low melt agarose gel, stained with ethidium bromide,

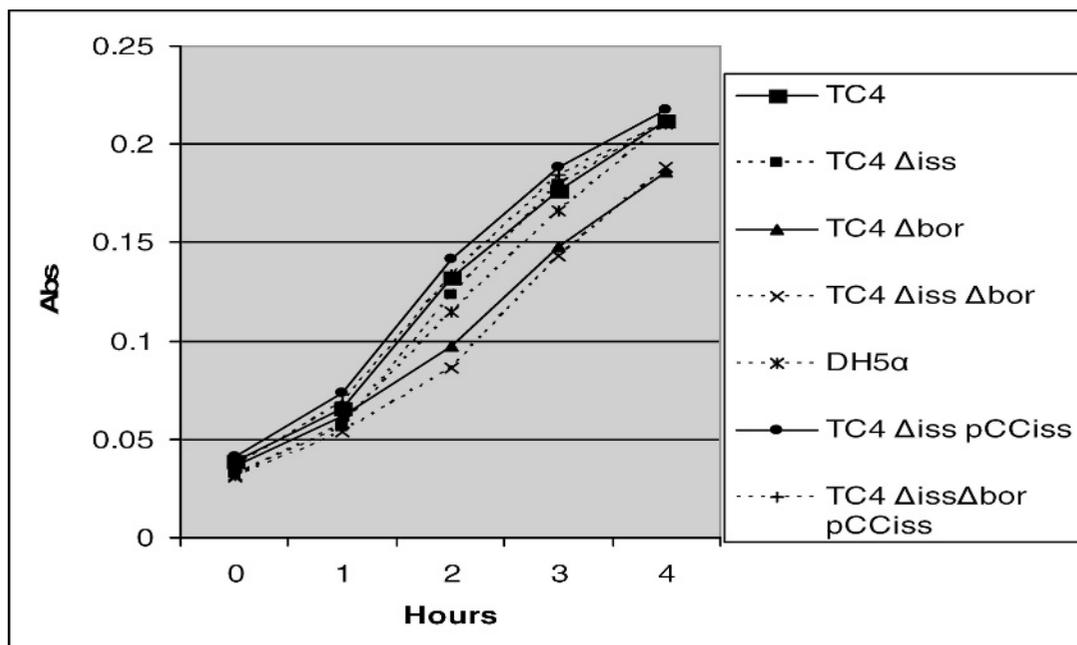


Fig. 1. Growth in PG broth.

Table 2. Growth in normal serum using microtiter method.

Isolate	Growth rate mean	Growth rate class ^{AA}
TC4	0.052	A
TC4 Δiss + pZCiss	0.049	A
TC4 Δbor	0.030	B
TC4 $\Delta bor \Delta iss$ + pZCiss	0.033	B
TC4 Δiss	0.004	C
DH5 α	0.000	C
TC4 $\Delta bor \Delta iss$	0.000	C

^AGrowth rates with the same letter are not significantly different ($P > .05$), calculated using a one-way ANOVA.

and viewed under UV light. A 720-bp band, corresponding in size to the predicted *iss* amplicon, was excised from the gel and purified from the agarose using MinElute Gel Extraction Kit (Qiagen, Valencia, CA). *Nsi*I- and *Sac*II-digested *iss* was ligated into pZC320 using standard procedures (32) to form pZCiss. Ligations were transformed in *E. coli* BL21 (DE3) using the calcium chloride method (31). Transformants were selected on LB agar containing ampicillin (100 μ g/ml).

Serum resistance assay by microtiter method. Strains were assessed for their ability to resist the bactericidal effects of complement by a microtiter plate method previously described (21). Briefly, 2-hr cultures of isolates grown in LB broth were diluted to 10^5 colony-forming units (CFU)/ml in peptone glucose (PG) broth. One hundred microliters of these cultures was inoculated into flat-bottomed 96-well microtiter plates. A 100- μ l amount of 50% chicken serum diluted in phosphate-buffered saline (35) was added to each well. Medium and culture controls were also included. Plates were monitored for growth using a microplate reader set at 490 nm. Plates were incubated at 37 C and read every 30 min for 4 hr.

Serum resistance assay by plate count method. Strains were assessed for their ability to resist the bactericidal effects of complement by a plate count method previously described (21) in order to verify the results of the microtiter method. The strains were grown overnight in a 2-ml culture of LB broth. The next day, cell densities were calculated by spectrophotometry, and cultures were diluted in LB with 25% chicken sera to a starting concentration of 10^3 CFU/ml, which was confirmed by viable counts. Mixtures were incubated at 37 C with shaking. Samples were

taken every hour for 6 hr and plated on MacConkey agar to determine viable counts. Results shown are the average of three trials. The experiment was repeated using LB with no serum as a medium control.

Biostatistics. All growth rate data for the strains were analyzed using linear regression analysis (Systat, Evanston, IL). The growth rates of each *E. coli* strain were determined as previously described by Broughall *et al.* (6). Briefly, all data were included from the point at which the cell concentration had increased to 150% of the inoculated concentration to the point where the population density ceased to increase. Specific growth rates were calculated from the slopes of the generated regression lines as described by Duffy *et al.* (12). A one-way ANOVA was used to test the null hypothesis of equal mean growth rates among the strains. A *post hoc* test, Fisher LSD, was used to identify differences between specific strains with a Type I error rate of 5% ($\alpha = .05$) for all tests.

RESULTS

Previously, a series of *iss* and *bor* transconjugant mutants were created (23). In order to complement *iss*⁻ transconjugant mutants, pZCiss was produced. The *iss* gene was first amplified and cloned into pGEM-T Easy cloning vector. *iss* was then cleaved from pGEM-T Easy and successfully cloned into pZC320. The construct was termed pZCiss. This construct was successfully transformed into *iss*⁻ transconjugant mutants and selected on ampicillin-containing LB agar.

Growth rates amongst all the strains for the microtiter test, including wild types, mutants, and complemented mutants, were not statistically different in PG broth (Fig. 1). However, differences were observed when strains were grown in serum. Deletion of *iss* from pAPEC-O2-ColV significantly decreased the growth of the transconjugants in chicken serum (Table 2, Fig. 2). Deletion of *bor* from the chromosome of the transconjugants also significantly decreased their growth in chicken serum (Table 2, Fig. 2). TC4 Δbor had a growth rate in chicken serum significantly higher than DH5 α , TC4 Δiss , and TC4 $\Delta bor \Delta iss$ ($p < .05$ for all) but did not attain the growth rate of TC4. TC4 Δiss grew at a rate statistically indistinguishable from TC4 $\Delta bor \Delta iss$. Transconjugants with *iss* deletions that were complemented with pZCiss attained wild-type

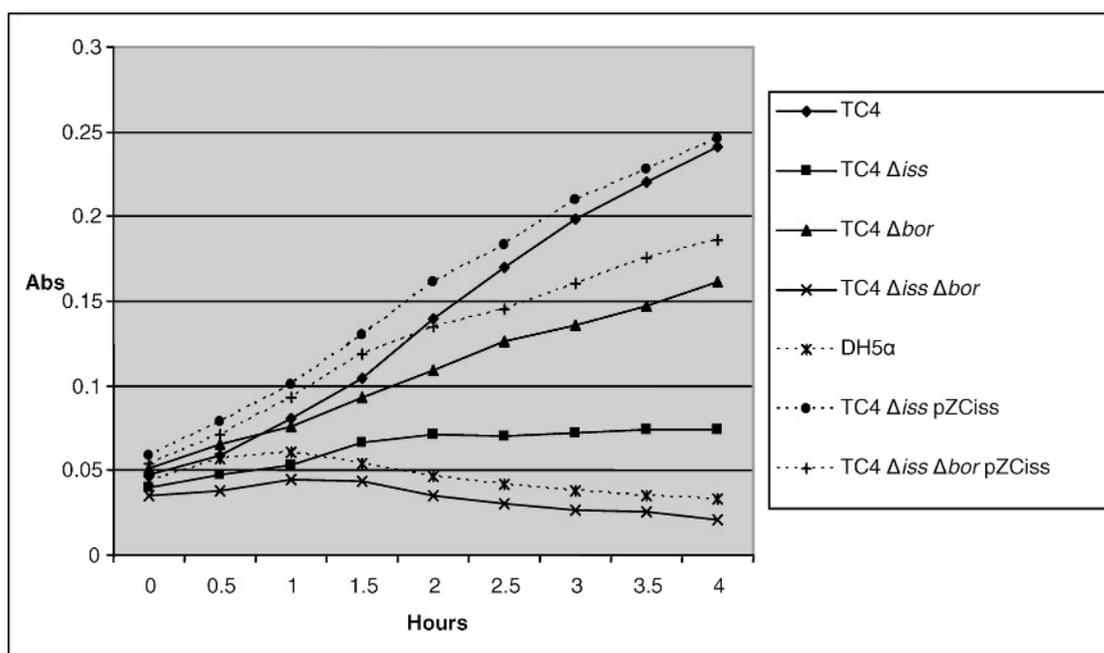


Fig. 2. Growth in chicken serum (microtiter method).

Table 3. Growth in normal serum using plate count method.

Isolate	Growth rate mean	Growth rate class ^{AA}
TC4	1.09	A
TC4 Δ <i>iss</i> + pZCiss	1.04	A
TC4 Δ <i>bor</i>	0.87	B
TC4 Δ <i>bor</i> Δ <i>iss</i> + pZCiss	0.85	B
TC4 Δ <i>iss</i>	0.61	C
DH5 α	0.54	C
TC4 Δ <i>bor</i> Δ <i>iss</i>	0.55	C

^AGrowth rates with the same letter are not significantly different ($P > .05$), calculated using a one-way ANOVA.

levels of serum resistance. That is, TC4 Δ *iss* + pZCiss grew at a rate statistically indistinguishable from TC4, and TC4 Δ *bor* Δ *iss* + pZCiss grew at a rate statistically indistinguishable from TC4 Δ *bor*.

Growth rates of the strains using the plate count method showed similar results. Growth rates for all strains was not significantly different in LB broth (results not shown), while differences in growth rates were seen when grown in serum (Table 3, Fig. 3).

DISCUSSION

Previously we have found that the acquisition of pAPEC-O2-ColV by *E. coli* DH5 α increased its serum resistance, but the gene or genes on the ColV plasmid responsible for the serum resistance phenotype were not identified. Since *iss*, which has been associated with serum resistance (4,5,8,9), has been localized to pAPEC-O2-ColV (17) and pAPEC-O1-ColBM (18), and since these plasmids typify the APEC pathotype (30), it seems likely that *iss* would contribute to the increased serum resistance conferred by the ColV plasmid. However, since genes other than *iss*, such as *traT*, which is

thought to contribute to serum resistance, occur on ColV plasmids, we cannot be sure if increased serum resistance is due to the possession of *iss* alone. Therefore, this study sought to determine the level of serum resistance conferred on an isolate by possession of *iss* using a series of previously created isogenic mutants (23). Also, since *iss* and *bor* share about 90% homology (15), we wished to compare the levels of serum resistance conferred on an *E. coli* strain by the possession of *bor* and *iss*.

Transconjugants containing pAPEC-O2-ColV Δ *iss* were significantly less resistant to serum than the wild-type transconjugant. Indeed, the mutant's ability to grow in serum was so impaired that there was almost no growth at all. When *bor* was deleted from the chromosome of DH5 α , a significant decrease in serum resistance, as compared to the wild-type transconjugant, was observed. However, this decrease was not as large as that seen in the *iss*⁻ mutant, suggesting that *iss* contributes more to the serum resistance of TC4 than does *bor*. When *iss* was reintroduced into the *iss*⁻ mutants, the serum resistance of the mutant was restored to the wild-type transconjugant level, confirming that the mutation of *iss* was responsible for the change in the mutant's complement sensitivity. These results are interesting and seem to confirm the role of *iss* in the complement resistance associated with ColV plasmids. Considering that *iss* is found significantly more often in APEC isolates than in avian commensal *E. coli* isolates (29,30), whereas *bor* is found in the chromosome of most *E. coli* isolates. Since *Iss* and *Bor* both occur on the outer membrane of the bacterium (23), are involved in serum resistance (3,4,5,8,9), and have about 90% amino acid homology (15), the difference in their contribution to serum resistance could be due to the level of expression of the genes, with *iss* being up-regulated over *bor*, or due to difference in protein structure. Currently, research in our lab is focused on determining the levels of gene expression by reverse transcriptase PCR to help answer this question.

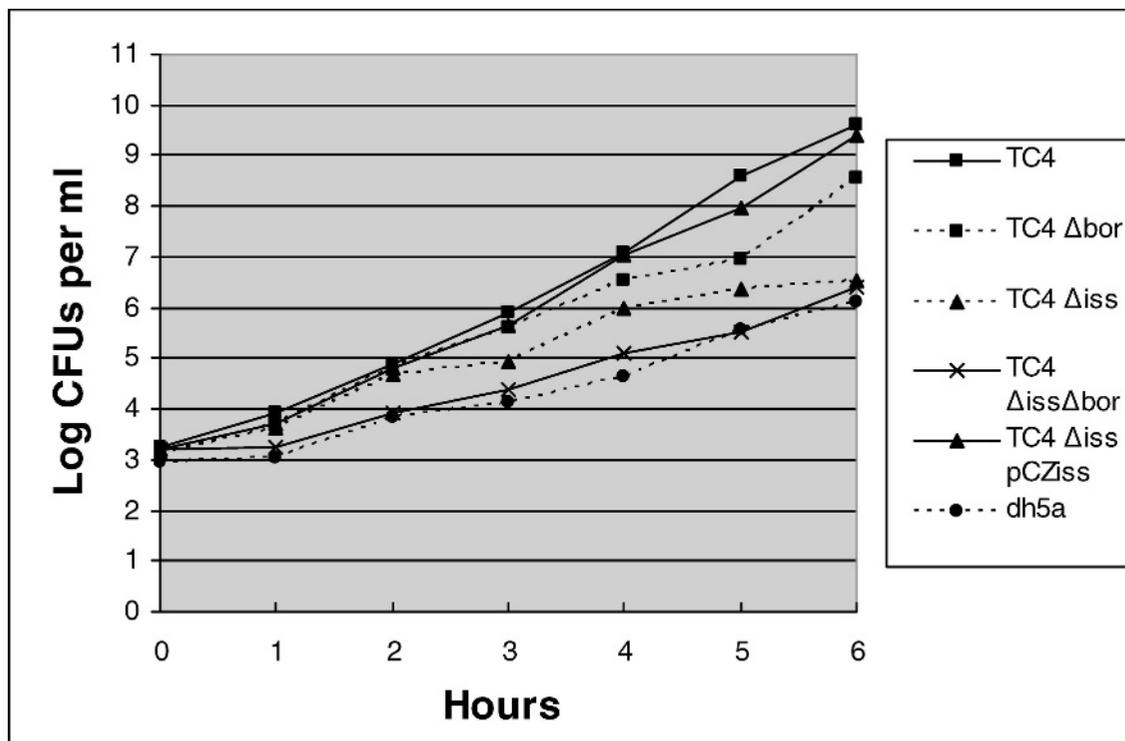


Fig. 3. Growth in chicken serum (plate count method). Growth in chicken serum of DH5 α , the transconjugant and corresponding mutants, and the *iss*-complemented mutants. Growth curves were determined by the plate count method and reflect an average of three trials for each strain.

The results from the plate count method verify the results of the microtiter method. That is, we were able to arrange strains in groups whose growth rates were not statistically different from each other. It is interesting to note that while the groupings were the same between the two methods, the growth rates were different using the two methods. Using the microtiter method, strains TC4 Δiss , DH5 α , and TC4 $\Delta bor \Delta iss$ showed little to no growth, but showed considerable growth using the plate count method. While the microtiter method is a fast and easy method to determine growth rates, it may not be as accurate as a plate count method.

Recently, Mellata *et al.* (24) stated that *Iss* and *TraT* played a limited role in complement resistance due to the fact that strains containing *iss* and *traT* genes, but had lost the K1 or O serotype were not protected against the bactericidal effect of serum. However, these strains were less sensitive than *iss*⁻ and *traT*⁻ control strains. While certainly there may be other chromosomal factors that contribute to serum resistance, *iss* appears to play the major role in the serum resistance associated with pAPEC-O2-ColV.

In summary, a series of *iss* and *bor* mutants were generated to study the contributions of each gene to serum resistance. Deletion of *iss* in a strain conferred a significant drop in its resistance to serum. Similarly, a *bor* mutant showed a drop in serum resistance but not as drastic as that observed with the *iss* mutant. Also, when *iss* is reintroduced into the *iss*⁻ mutant, the wild-type level of serum resistance was restored, confirming that the deletion of *iss* was responsible for the change in resistance seen in the mutant.

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