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MOLECULAR PATHOGENESIS

The Periplasmic Trehalase Affects Type 1 Fimbria Production and Virulence of Extraintestinal Pathogenic *Escherichia coli* Strain MT78

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ABSTRACT Extraintestinal pathogenic *Escherichia coli* (ExPEC) is responsible for various infections outside the gastrointestinal tract in humans and other animals. ExPEC strain MT78 is invasive to various nonphagocytic cells and highly virulent *in vivo*. To identify genes required for invasion of nonphagocytic cells by this strain, we applied signature-tagged mutagenesis to generate a library of mutants and tested them for invasion of avian fibroblasts. Mutants showing reduced cellular invasion included those with insertions in the *fim* operon, encoding type 1 fimbriae. Another attenuated mutant showed a disruption in the *treA* gene, which encodes a periplasmic trehalase. The substrate of TreA, trehalose, can be metabolized and used as a carbon source or can serve as an osmoprotectant under conditions of osmotic stress in *E. coli* K-12. We generated and characterized mutant MT78Δ*treA*. In contrast to the wild type, MT78Δ*treA* was able to grow under osmotic stress caused by 0.6 M urea but not in minimal M9 medium with trehalose as the only carbon source. It presented decreased association and invasion of avian fibroblasts, decreased yeast agglutination titer, and impaired type 1 fimbria production. In a murine model of urinary tract infection, MT78Δ*treA* was less able to colonize the bladder. All phenotypes were rescued in the complemented mutant. Our results show that the *treA* gene is needed for optimal production of type 1 fimbriae in ExPEC strain MT78 and that loss of *treA* significantly reduces its cell invasion capacity and colonization of the bladder in a murine model of urinary tract infection.

KEYWORDS ExPEC, extraintestinal pathogenic *E. coli*, MT78, TreA, avian fibroblasts, signature-tagged mutagenesis, trehalase, trehalose, type 1 fimbriae, urinary tract infection

Extraintestinal pathogenic *Escherichia coli* (ExPEC) is responsible for various infections outside the gastrointestinal tract in humans and other animals, such as dogs, cats, and farm animals (1). Urinary tract infections (UTIs) are among the most common infections in humans, being caused mainly by ExPEC (2). ExPEC is also an important cause of neonatal meningitis among newborns with very low body weight (3, 4). In the poultry industry, the various forms of colibacillosis, comprising either localized or systemic infections caused by ExPEC, are of great economic importance worldwide (5). Importantly, certain phylogenetic or clonal groups of ExPEC have also been shown to be commonly associated with infections in poultry and in humans, suggesting that poultry may provide a potential reservoir for ExPEC strains in humans (6, 7).

ExPEC strain MT78 (O2:H$^+$:K1, sequence type 95 [ST]95), also known as BEN2908, was isolated from the trachea of a diseased chicken in France in 1982 (8). Since then, this strain has been well characterized: MT78 is an efficient colonizer of the chicken

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intestine (9, 10), is highly virulent *in vivo* (11, 12), strongly interacts with and resists killing by avian macrophages and heterophils (12, 13), and causes apoptosis in macrophages *in vitro* (12). The most remarkable capacity of this strain is that it is highly invasive to nonphagocytic cells, such as avian fibroblasts (CEC-32) (14) and hepatocytes (LHM), human type II pneumocytes (A549) (15), and brain microvascular endothelial cells (16). Other ExPEC strains can be as virulent as MT78, but they are less able to invade eukaryotic cells (12, 14). The specific mechanisms underlying the invasion capacity of strain MT78, however, have not been fully elucidated.

In order to identify genes required for invasion of nonphagocytic cells by ExPEC MT78, we applied a signature-tagged mutagenesis (STM) approach to generate a library of mutants and tested them *in vitro* for invasion of avian fibroblasts. One of the mutants attenuated for cellular interaction and invasion contained a transposon insertion in *treA* that encodes a periplasmic trehalase, TreA, involved in catabolism of trehalose in the periplasm (17). Trehalose is an $\alpha(1\rightarrow1)$-dimer of glucose that plays a dual role in *E. coli* and many other bacterial species. It can be metabolized and used directly as a carbon source, or, under conditions of osmotic stress, it can serve as an osmoprotective agent (18). Although the role of TreA in trehalose metabolism and osmoprotection was demonstrated in the 1990s, to the best of our knowledge, there is no reported role for TreA in the virulence or fitness of *E. coli*.

The aim of this work was to characterize the *treA* mutant of the ExPEC strain MT78 in order to better understand the role of the periplasmic trehalase in the virulence and fitness of this strain. To achieve that, we tested the mutant for its growth under different conditions, its capacity of interaction with eukaryotic cells, its production of type 1 fimbriae, and its virulence in a mouse urinary tract infection model.

RESULTS

Individual mutants from the STM library showed reduced adhesion and invasion of avian fibroblasts. Nineteen pools of 90 STM mutants each were tested for invasion in CEC-32 fibroblasts, and 68 mutants showed a reduced hybridization signal when recovered from inside the cells. Since only a small amount of the inoculum actually invades the fibroblasts, it is possible that not all 90 mutants within the pool may invade cells, regardless of whether or not the transposon insertion disrupted a gene involved in adherence/invasion. Some of these signal-attenuated mutants were therefore tested individually for adhesion to and invasion of avian fibroblasts. Figure 1 shows the results for four mutants, 8G4, 8E12, 7A2, and 8C6, tested individually. The transposon insertion regions of these mutants were determined. The two nonadherent and noninvasive mutants, 7A2 and 8C6, had transposon insertions within the *fim* operon, in *fimA* and *fimI*, respectively. Type 1 fimbriae, encoded by the *fim* operon, are the major fimbrial structures in many *E. coli* strains and play an important role in ExPEC adhesion and cellular invasion (19, 20). As such, it was expected that disruption of the *fim* operon could reduce the capacity of these mutants to adhere to and invade eukaryotic cells. Mutant 8E12, however, was able to adhere to and invade cells but to a lesser extent than wild-type (WT) strain MT78. 8E12 was found to contain a transposon insertion within *ibeR*, a regulator of the *ibeA* gene, which contributes to adhesion/invasion in some *E. coli* strains by promoting or regulating the expression of type 1 fimbriae (20). These results validated the STM screening procedure. Mutant 8G4 displayed decreased cell adhesion and invasion. This mutant contained an insertion in the *treA* gene, which codes for a periplasmic trehalase. TreA has not previously been identified to play a role in *E. coli* adhesion or invasion of host cells.

The *treA* mutant is able to grow on LB agar with 0.6 M urea. Trehalose metabolism is one of the systems that respond to osmotic stress and has been characterized in *E. coli* laboratory strain K-12 (17, 18, 21). We therefore tested the growth of the wild type, mutant MT78Δ*treA*, and a complemented mutant on modified LB agar containing different concentrations of NaCl or urea. All three strains were able to grow on LB agar with 0.5 M NaCl (Fig. 2A) and with 0.3 M urea (Fig. 2B and D), and no strain could grow on LB agar with 1 M NaCl (results not shown). In contrast, on LB

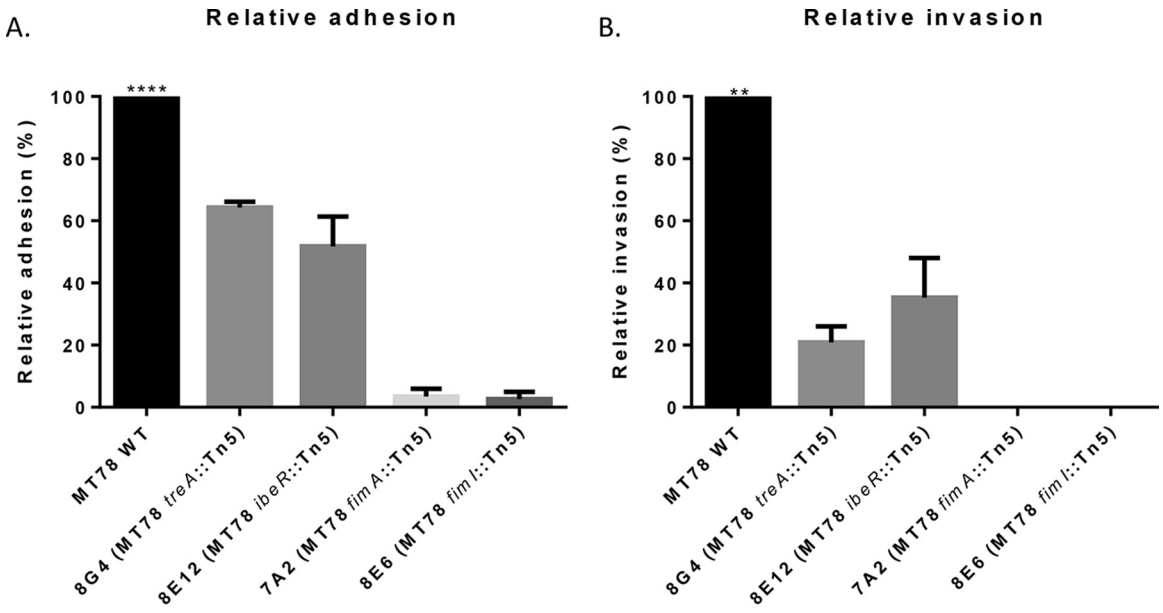

FIG 1 Adherence and invasion assays of individual transposon mutants obtained from the STM screen. Cells were infected at an MOI of 200 CFU/cell. Bars represent normalized percentages + standard error of recovered bacteria at 1 h after infection (A) and at 4 h after infection, the first hour without antibiotic, followed by washes, and the last 3 h in the presence of gentamicin 50 μg/ml (B). (Adhesion of the WT ranged from 22% to 35% of the inoculum; invasion ranged from 0.8% to 1.5%.) **, $P < 0.01$; ****, $P < 0.001$ (Kruskal-Wallis test).

agar with 0.6 M urea, while the WT and the complemented mutant were virtually unable to grow, the mutant MT78Δ*treA* grew as well as it did in the absence of urea (Fig. 2C and E). This result suggests that an accumulation of trehalose in the periplasm, due to the absence of the enzyme TreA, could possibly protect the MT78Δ*treA* mutant against the osmotic stress caused by the addition of 0.6 M urea on LB agar.

The *treA* mutant did not grow in M9 minimal medium with trehalose as the only carbon source. We also tested the growth of MT78Δ*treA* in M9 minimal medium with trehalose as the only carbon source, under the same conditions as described for growth in LB. While the WT strain was able to grow, in agreement with previous data (22), the *treA* mutant did not grow, suggesting that the periplasmic TreA enzyme is required for catabolism of trehalose in MT78, in contrast to what has been described for *E. coli* K-12 (17).

The *treA* mutant is less adherent and invasive for avian cells. ExPEC strain MT78 is a highly adherent and invasive strain in different cell models (14–16). The adhesion and invasion capacities of the *treA* mutant were tested using the avian fibroblast CEC-32 cell line. The results are presented in Fig. 3. Whereas 28% of the initial inoculum from the wild-type strain was recovered after 1 h of interaction with avian fibroblasts, only 16% of the *treA* mutant inoculum was recovered. The adhesive capacity of the *treA* mutant was successfully rescued by complementation through the introduction of a single copy of the *treA* gene (Fig. 3A). Inclusion of D-mannose or D-mannopyranose (3%) in cultures to block the effect of type 1 fimbriae greatly reduced cell association of all strains tested in the cell association assay (Fig. 3B). The effect of the *treA* deletion was even more pronounced in the capacity of the mutant to invade avian fibroblasts: compared to the WT parent, the level of invasion by the *treA* mutant decreased by 70%, from a mean invasion of 1.4% of the initial inoculum to only 0.4%. Further, the invasive capacity of the *treA* mutant was regained in the complemented mutant (Fig. 3C).

Deletion of *treA* reduces the yeast agglutination titer. To determine if deletion of *treA* reduces surface levels of type 1 fimbriae, we performed yeast agglutination experiments with the WT MT78 strain, the MT78Δ*treA* mutant, and the complemented mutants grown in different media and under different conditions. Yeast agglutination is a well-established assay to test the production of type 1 fimbriae. After stationary

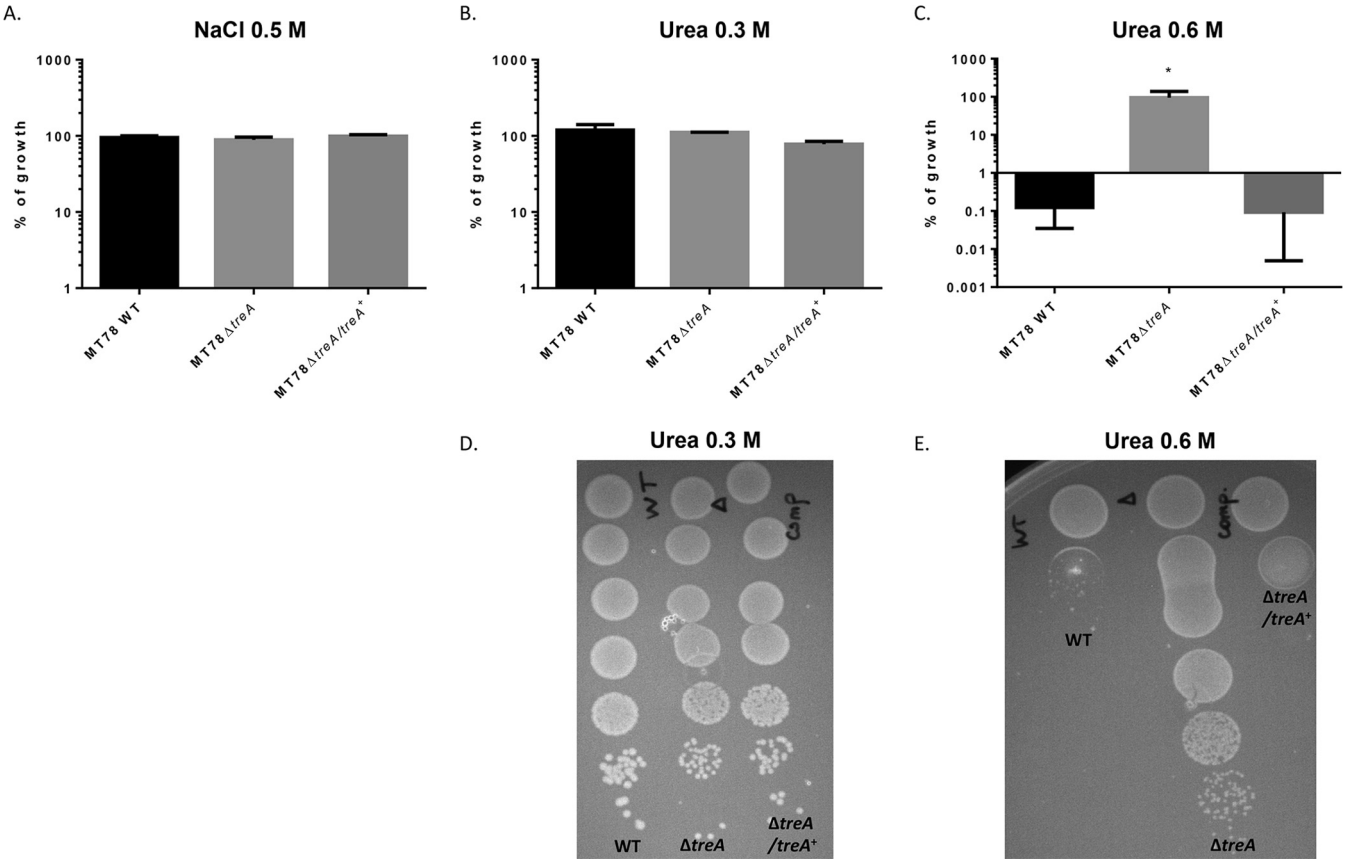

FIG 2 Growth under conditions of osmotic stress. Strains were grown with shaking in LB medium until mid-log phase (OD_{600}, 0.6) and plated on LB agar (taken as 100% growth) and LB agar with 0.5 M NaCl (A), LB agar with 0.3 M urea (B), and LB agar with 0.6 M urea (C). Graphs show the mean growth relative to that in LB with no additions, plus standard error. Assays were performed three times in duplicate. *, $P < 0.05$ (Kruskal-Wallis test). Plates showing growth on LB agar with 0.3 M (D) and 0.6 M (E) urea. Each drop represents a 1/10 dilution, from the most concentrated (top) to the most diluted (bottom).

growth in LB, mid-log growth with shaking in LB, and overnight growth in human urine with shaking, MT78Δ*treA* agglutination titers were reduced by 22%, 36%, and 45%, respectively, compared to that of the WT strain (Fig. 4). Agglutination titers of both chromosomal and plasmid-complemented mutants regained levels similar to that of the WT strain (Fig. 4). To verify if type 1 fimbriae were reduced in MT78Δ*treA*, we performed Western blotting, which confirmed that type 1 fimbriae were indeed reduced in the mutant compared to the wild type and the plasmid-complemented mutant (Fig. 5). To determine if reduction of type 1 fimbriae was due to the position of the invertible promoter, we tested the switch of the *fimS* promoter region, from the ON to the OFF position. We did not observe any difference between the wild-type strain and the mutant under any of the conditions tested (Fig. 6).

The *treA* mutant is less able to colonize the bladder in the murine urinary tract infection model. Type 1 fimbriae are important structures for ExPEC colonization of the bladder in UTIs (1). Since the *treA* mutant demonstrated decreased yeast agglutination associated with type 1 fimbria production, we tested its capacity to cause urinary tract infection in the CBA/J mouse model. Forty-eight hours after urethral inoculation of 2×10^9 CFU, bladders and kidneys were collected, homogenized, diluted, and plated for bacterial counting. The *treA* mutant colonized the bladder 10-fold less than the wild-type strain ($P < 0.0001$) (Fig. 7). In an independent assay, the complemented mutant regained the capacity of colonization ($P < 0.01$). This reduction in colonization may be due to reduced production of type 1 fimbriae. In contrast to the colonization of the bladder, there was no significant difference in bacterial numbers in the kidneys.

The FimH adhesin of strain MT78 and that of noninvasive ExPEC are nearly identical. A possible explanation for the invasive phenotype of MT78 may be the

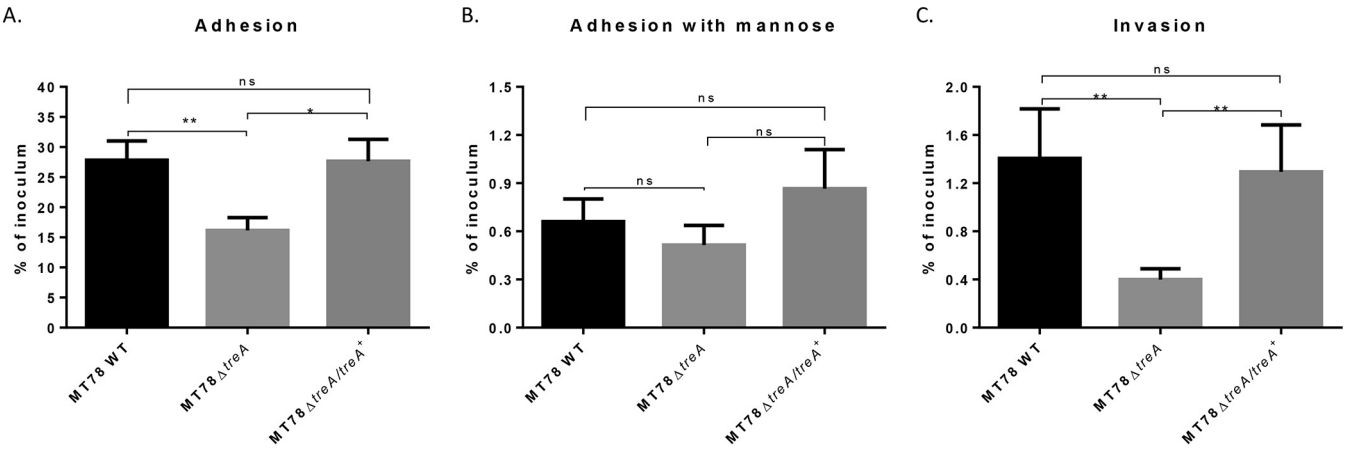

FIG 3 Adhesion to and invasion of CEC-32 avian fibroblasts. Cells were infected at an MOI of 10 CFU/cell, as described in Materials and Methods. Bars represent the mean percentage + standard error of recovered CFU compared to that of inocula 1 h after infection (A), 1 h after infection in medium with 3% D-mannose or D-mannopyranose (note that the scale *y* axis is reduced) (B), and 4 h after infection, the first hour without gentamicin, followed by washes and reincubation in the presence of gentamicin at 50 μg/ml for a further 3 h (C). Assays were performed at least four times in triplicate for each condition. ns, statistically nonsignificant; *, $P < 0.05$; **, $P < 0.01$ (Mann-Whitney test).

differences in sequence of the FimH fimbrial adhesive tip. We thus aligned the amino acid sequences of FimH from MT78, uropathogenic *E. coli* (UPEC) CFT073, and phylogenetically similar avian pathogenic *E. coli* (APEC) IMT5155, since these last two strains are noninvasive in the CEC-32 model (14; Daniel Pavanelo, unpublished results). There are only two amino acid differences among the strains, a G180-to-S180 substitution in MT78, in comparison to the sequences of CFT073 and IMT5155, and an A184-to-V184 substitution compared to the sequence of CFT073. These two differences are conservative substitutions, since glycine and serine are polar and alanine and valine are nonpolar amino acids. It is thus unlikely that the differences observed among these strains concerning interaction with cells could be attributed solely to specific amino acid sequence differences in FimH proteins.

DISCUSSION

Several research groups have successfully used STM to identify virulence-associated genes in a series of bacterial pathogens and models (23–27). Our STM library screening resulted in the identification of mutants that were attenuated in cellular invasion of fibroblasts with transposon insertions in genes of the *fim* operon and in *treA*, which

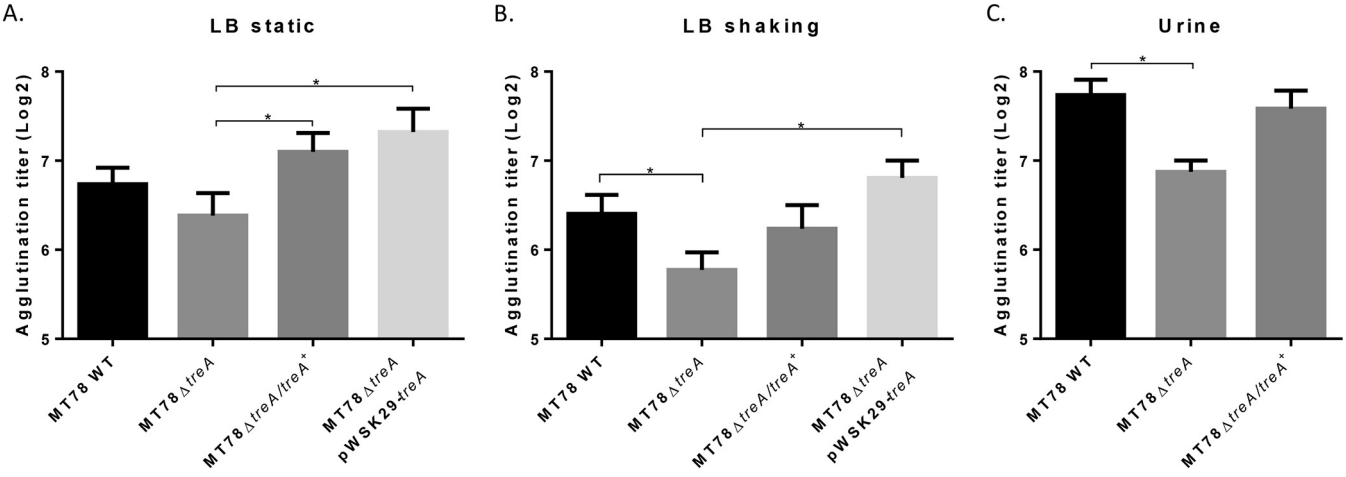

FIG 4 Yeast agglutination. Yeast agglutination, which correlates with the level of type 1 fimbria functionality, was measured under three different conditions: after static growth overnight in LB (A), after mid-log growth with shaking in LB (B), and after overnight growth with shaking in human urine (C). *, $P < 0.05$ (Mann-Whitney test).

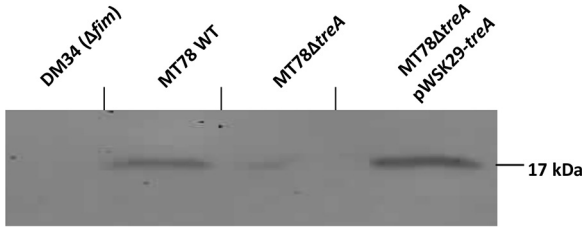

FIG 5 Western blot analysis. Western blotting of fimbrial extracts of strains cultured to the mid-log phase of growth in LB medium.

encodes a periplasmic trehalase. In *E. coli* K-12, the final cytosolic concentration of trehalose is regulated by a futile cycle: cytosolic enzymes OtsA and OtsB synthesize trehalose from glucose in the cytoplasm (28), which is translocated to the periplasm and degraded by TreA into two molecules of glucose (17). Under osmotic stress, the final concentration of the osmoprotectant trehalose is increased due to higher activity of both cytosolic OtsA and OtsB, despite a concomitant increase in periplasmic TreA activity (21). Therefore, one would expect a *treA* mutant to produce higher levels of trehalose and be potentially more resistant to osmotic stress. Although there was no difference between the wild type and mutant concerning resistance to osmotic stress caused by NaCl, the *treA* mutation did confer increased resistance to stress caused by 0.6 M urea, a condition under which growth of the wild-type strain was impaired.

In a recent report, TreA has been shown to be involved in the virulence of *Burkholderia pseudomallei* (29). Because the MT78Δ*treA* mutant displayed an attenuated capacity to invade eukaryotic cells (Fig. 1 and 3), we performed yeast agglutination assays and Western blotting against type 1 fimbriae to investigate any effect on type 1 fimbria production. As shown in Fig. 4 and 5, in comparison to the WT strain, the mutant had a reduced production of type 1 fimbriae and lowered yeast agglutination. This reduction in type 1 fimbriae cannot be explained by the *fimS* switch, since we did not observe any differences in orientation of the *fimS* switch in these strains (Fig. 6). Type 1 fimbria expression of ExPEC is regulated by several mechanisms, such as global regulators H-NS, Lrp, IHF, and FNR (30, 31), recombinases FimB and FimE, some virulence-associated genes, such as *ibeA* and *ibeT* (20) in certain strains, and a phosphate metabolism operon, *pst* (19), among others. Since the position of the promoter suggests that the expression of type 1 fimbriae is not reduced, the effect of deletion of *treA* on type 1 fimbria production is likely a posttranscriptional or posttranslational event.

Our results showed a somewhat more pronounced reduction of yeast agglutination when bacteria were grown under agitation until the mid-log phase instead of when bacteria were grown statically (Fig. 4). In cell assays, the reduction in adhesion and invasion capacities was also more marked when bacteria were grown under agitation until the mid-log phase (results not shown). Similarly, other mutations in genes such as

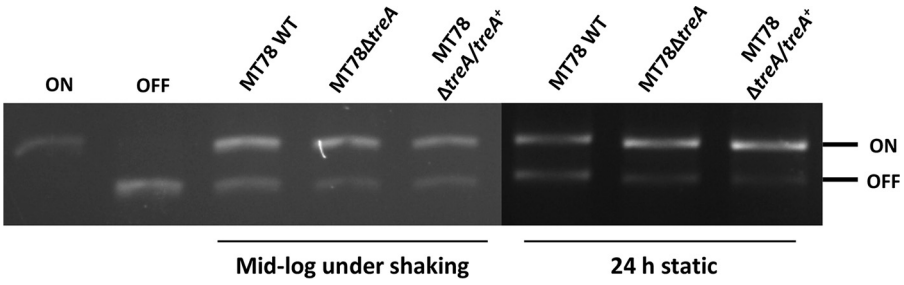

FIG 6 Verification of *fimS* orientation. The *fimS* region was PCR amplified, and the product was digested with HinfI. Fragments of different sizes indicate the ON or OFF orientation. Only the top half of the gel is shown.

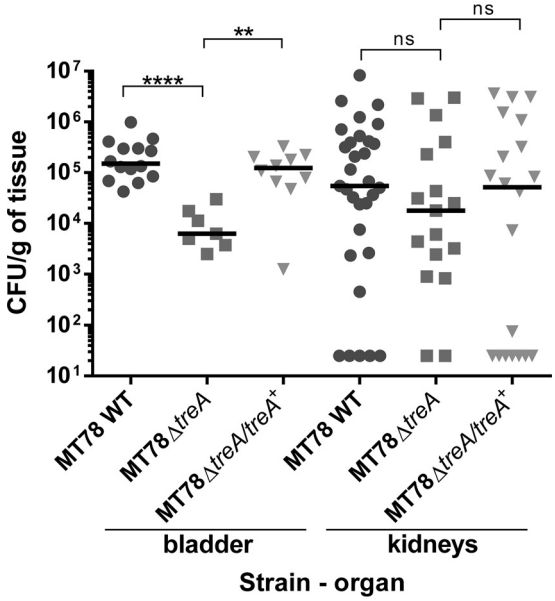

FIG 7 *In vivo* urinary tract infection in mice. CBA/J mice were infected transurethrally, the animals were euthanized, and organs were collected 48 h postinfection. Each data point represents a sample from an individual mouse, and horizontal bars indicate the medians. Two independent monoinfections were performed: with MT78 WT and MT78Δ*treA* and with MT78 WT and MT78Δ*treA/treA*$^+$. Results for MT78 WT were pooled. Each kidney was sampled separately. ns, statistically nonsignificant; **, $P < 0.01$; ****, $P < 0.0001$ (Mann-Whitney test).

the *pst* system also resulted in a more marked change in production of type 1 fimbriae during growth to mid-log phase than during overnight static growth (19).

It is not the first time that a gene related to carbohydrate metabolism is reported to be involved in the virulence of ExPEC strain MT78. A *selC*-associated genomic island, named AGI-3, was found in the MT78 genome, with three genes coding for sugar metabolism: a transcriptional regulator of the LacI family, a hexuronate transporter, and an α-glycosidase. Without these genes, MT78 was impaired in its ability to metabolize carbohydrates, including trehalose, and was less able to cause bacteremia and colonize the liver of 3-week-old white Leghorn chickens 24 h and 48 h postinfection (22). The metabolic operon *frz* was also found in the MT78 genome, and when this operon was deleted, the strain was less able to interact with lung (A549), liver (LMH), and intestinal (Caco-2) cells, to colonize the intestine of axenic white Leghorn chicks, and, as in our work, to express type 1 fimbriae (32). The mechanism by which a loss of sugar metabolism genes affects the expression of type 1 fimbriae, however, remains to be elucidated.

Some studies relating osmotic stress and type 1 fimbria expression in UPEC strains have shown contradictory results and are so far inconclusive. In 2002, Schwan and colleagues showed that a decrease in type 1 fimbria expression occurred under osmotic stress caused by NaCl and low pH in the UPEC strain NU149 (33). In 2004, Snyder and colleagues performed a transcriptome analysis of the UPEC strain CFT073 during UTI, and type 1 fimbria expression was upregulated together with genes that are regulated by the osmotic stress response (34). In 2013, Withman and colleagues showed, also for CFT073, that type 1 fimbriae were more expressed under osmotic stress caused by an increase in urea but did not respond to an increase in NaCl (35). Finally, in 2015, Greene and colleagues concluded that for UPEC strain UTI89, type 1 fimbriae are less expressed in human urine (36). According to our results, MT78 showed a higher yeast agglutination titer in human urine than in LB (Fig. 4), but urine composition is widely variable, which could explain this difference. In *E. coli* K-12, *treA* expression has been involved in the osmotic stress response, whereas we found a reduction in type 1 fimbria production in the MT78Δ*treA* mutant. We cannot strictly define the specific roles of osmotic stress

or its regulation in type 1 fimbriae in *E. coli* based on our results and other reports, which have included investigations with a number of different *E. coli* strains, each of which may have distinct mechanisms of regulation and production of type 1 fimbriae. However, from our data, it is clear that TreA plays a role mediating cell invasion by strain MT78 and its level of production of type 1 fimbriae.

FimH of MT78 is almost identical to the adhesin tip of UPEC CFT073 and APEC IMT5155. Despite these three strains behaving differentially in contact with cells (12, 14), they would all have a high-affinity FimH, because they harbor an aspartate residue at position 188. This particular residue has been shown to increase the affinity of FimH to mannose, and strains with this characteristic tend to be more focally adherent and to spread less throughout the host (37). Mutations at positions 27, 62, 70, 78, and 128 could also explain differences in FimH-mannose affinity (37); however, strains MT78, CFT073, and IMT5155 show the same residues at these sites, which are frequently variable. Since type 1 fimbriae are an important adhesin and the FimH adhesin is highly similar in these three *E. coli* strains, despite their demonstrating differences in cell adherence/invasion, other differences among these strains or distinct regulation of type 1 fimbriae may contribute to the capacity of strain MT78 to invade a wide variety of eukaryotic cells.

E. coli is a natural colonizer of warm-blooded animals, but some clones can cause disease in a wide variety of niches. ExPEC strains cause diseases in humans and other animals and have a zoonotic potential. Neonatal meningitis *E. coli* (NMEC) strains have been shown to successfully colonize chickens in a chick colisepticemia model, and APEC strains were able to cause meningitis in a rat neonatal meningitis model (38). In addition, Skyberg and colleagues have shown that the introduction of a plasmid from an APEC strain into a commensal *E. coli* strain from poultry conferred an increased capacity to colonize the urinary tract of mice (39). In our current report, APEC strain MT78, which belongs to ST95, a common ExPEC sequence type, was shown to cause urinary tract infection in mice at levels comparable to those caused by human UPEC strains such as CFT073. This result also reinforces the zoonotic potential of certain ExPEC isolates.

In summary, we generated a mutant library using signature-tagged mutagenesis to better understand the invasive phenotype of MT78 in host cells. Attenuated mutants showed transposon insertions in genes from the *fim* operon or in genes that affect type 1 fimbria expression. Our data strongly suggest a critical role of type 1 fimbriae in MT78 invasion of eukaryotic cells. Specifically, our results demonstrate that the *treA* gene is needed for the optimal production of type 1 fimbriae in this particular ExPEC strain and that the loss of *treA* significantly reduces cell invasion and colonization of the bladder in a murine urinary tract infection model.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. All strains and plasmids used in this study are listed in Table 1. Strains and mutants were grown in LB, LB with different amounts of NaCl or urea, M9 with trehalose as the sole carbon source, human urine, or brain heart infusion (BHI) medium. Stock cultures were maintained in glycerol stocks at −80°C.

Construction and analysis of the STM library. Random mutants were generated as described previously (25). A total of 19 pools with 90 mutants each ($n = 1,710$) were screened in cell invasion assays (described below). Identification of mutants with reduced invasion was determined as described previously, using input and output pools from the cell assays (25). Attenuated mutants had the transposon insertion region amplified by nested PCR, and unique bands were selected, gel purified, and sequenced by Sanger sequencing (25) at ATCGene (Porto Alegre, Brazil) or the Genome Canada facility (McGill University, Montreal, Canada). Primers used are listed in Table 2.

Construction of specific mutants and the complemented strain. Specific mutants were generated by the procedure described by Datsenko and Wanner, using plasmid pKD4 as the template for the kanamycin resistance cassette (42). Primers used are listed in Table 2.

The *treA* mutant was complemented with a low-copy-number plasmid and with a single chromosomal gene copy. For the plasmid complementation, MT78 *treA* was PCR amplified with primers QFtreApWSK29compl_F and QFtreApWSK29compl_R (Table 2) and cloned into a pWSK29 low-copy-number plasmid (44). The plasmid pWSK29-*treA* was used to transform, by electroporation, the mutant MT78Δ*treA*.

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Characteristic(s)	Resistance	Reference or source
Strains			
MT78 (O2:H$^+$:K1)	WT ExPEC strain	Nalidixic acid	8, 14
S17-1 λpir	Conjugative strain used for creation of STM library	None	25
DH5α	Vector strain	None	Bethesda Laboratories
MT78Δ*treA*	*treA*::Km	Nalidixic acid, kanamycin	This study
MT78Δ*treA*/*treA*$^+$	*treA*::Km, *treA*+cat	Nalidixic acid, kanamycin, chloramphenicol	This study
MT78Δ*treA*/pWSK29-*treA*	*treA*::Km, pWSK29-*treA*	Nalidixic acid, kanamycin, ampicillin	This study
7A2 (STM mutant)	MT78 *fimA*::Tn*5*	Nalidixic acid, kanamycin	This study
8C6 (STM mutant)	MT78 *fimI*::Tn*5*	Nalidixic acid, kanamycin	This study
8E12 (STM mutant)	MT78 *ibeR*::Tn*5*	Nalidixic acid, kanamycin	This study
8G4 (STM mutant)	MT78 *treA*::Tn*5*	Nalidixic acid, kanamycin	This study
DM34	MT78Δ*fim*	Nalidixic acid	40
Plasmids			
pUT-mini-Tn*5*km2	Plasmid used for creation of STM library (90 different tags within the transposon)	Ampicillin, kanamycin	23, 25, 41
pKD46	Plasmid used for nonpolar mutation	Ampicillin	42
pKD4	Plasmid used as template for kanamycin cassette amplification	Kanamycin	42
pGPTn7-Cm	Plasmid used for complementation	Ampicillin, chloramphenicol	43
pWSK29	Plasmid used for complementation	Ampicillin	44

For the chromosomal complementation, the *treA* gene with its promoter was cloned into a pGPTn7-Cm plasmid upstream of a chloramphenicol cassette (43). The entire region comprising *treA* plus cassette was PCR amplified and used to complement the *treA* mutant by the same method with which the mutation was generated. Primers used in this reaction are listed in Table 2. The original *treA* promoter region was replaced by this *treA* allele by homologous recombination. The complemented strain contains both chloramphenicol and kanamycin cassettes inserted downstream of the *treA* gene.

Growth under conditions of osmotic stress. Strains were tested for the capacity to grow under conditions of osmotic stress caused by NaCl or urea. Strains were inoculated 1:100 from an overnight preinoculum in LB medium and grown until mid-log phase with shaking. They were serially diluted and plated on LB agar alone and LB agar with 0.5 M NaCl, 1 M NaCl, 0.3 M urea, and 0.6 M urea. Colonies were enumerated, and growth under each condition was compared to growth on LB agar.

Cell association and invasion assays. The eukaryotic cell assays were performed with CEC-32 avian fibroblasts (45) as described previously (14), with some modifications. Briefly, 5×10^4 cells/well were distributed in 96-well plates, or 2×10^5 cells/well were cultured in 24-well plates, in high-glucose Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (infection medium). Immediately before infection, cultures were washed once with phosphate-buffered saline (PBS) to remove dead cells, returned to the infection medium, and infected at an estimated multiplicity of infection (MOI) of 10, 20, or 200 CFU per cell. Bacterial strains were grown in LB medium with shaking (240 rpm) until mid-log phase. For STM library screening, a set of 90 mutants were pooled from overnight cultures (10 μl each) and inoculated in 50 ml of LB with shaking until mid-log phase. Aliquots of the inocula used for infections were diluted and plated to confirm the MOI, in single infections. For STM screening, samples were collected and used as input pools. After 1 h of incubation at 37°C, the medium was removed, and the cells were washed three times with PBS. For the association assays, the cells were then lysed with 1% (vol/vol) Triton X-100 in PBS for 5 min. The lysates were serially diluted and plated on LB agar for CFU counting. For the invasion assays, the cells were further incubated in culture medium supplemented with 50 μg/ml gentamicin for 3 h, after which they were lysed, and the lysates were diluted and plated. For STM screening, only invasion assays were performed. Recovered bacteria were used as the output pools.

To verify type 1 fimbria-specific adherence, all assays were performed in the presence or absence of 3% α-D-mannose or D-mannopyranose.

Yeast agglutination. In order to detect differences in type 1 fimbria production, strains were tested for yeast agglutination as described previously (19, 46). Bacterial strains were incubated in LB medium or human urine at 37°C until mid-log or stationary phase with or without agitation. A suspension of approximately 2×10^9 bacterial cells in PBS was serially diluted 1:2 to a dilution of 2^{-10} bacterial cells in microtiter plates. In each well, an equal volume of a 1.5% commercially available yeast suspension was added. After 30 min of incubation on ice, agglutination was visually monitored and the agglutination titer was determined using the most diluted well in which agglutination was observed.

Preparation of fimbrial extracts and Western blotting. Preparation of fimbrial extracts was performed as described previously (47), with slight modifications. Briefly, bacteria were grown in 100 ml LB medium with shaking; after reaching mid-log phase (optical density at 600 nm [OD_{600}], 0.6), cells were collected by centrifugation at 1,000 × *g* for 15 min and resuspended in 1 ml of 150 mM NaCl–50 mM Tris-HCl, pH 7.8. Cell suspensions were incubated at 56°C for 60 min and vortexed at maximum speed for 2 min before being pelleted by centrifugation (maximum speed) in a microcentrifuge for 2 min. Proteins were precipitated with 100% trichloroacetic acid (TCA) at a final ratio of 1:10. The tube was incubated for

TABLE 2 Oligonucleotides used in this study

Primer	Sequence (5′–3′)	Function	Reference
P6	CCT AGG CGG CCA GAT CTG AT	Amplification of transposon insertion region	25
P9	CGC AGG GCT TTA TTG ATT C	Amplification of transposon insertion region	25
Arbi 1	GGC CAC GCG TCG ACT AGT ACN NNN NNN NNN GAT AT	Amplification of transposon insertion region	25
Arbi 2	GGC CAC GCG TCG ACT AGT AC	Amplification of transposon insertion region	25
Arbi 3	GGC CAC GCG TCG ACT AGT ACN NNN NNN NNN TGA CG	Amplification of transposon insertion region	25
Arbi 4	GGC CAC GCG TCG ACT AGT ACN NNN NNN NNN ACG CC	Amplification of transposon insertion region	25
Arbi 5	GGC CAC GCG TCG ACT AGT ACN NNN NNN NNN TAC NG	Amplification of transposon insertion region	25
treA_F	CGT AAT CGG CAT ACC AGC CT	Verification of *treA* presence	This study
treA_R	TGT TGA AAA CCT GCA AGC CG	Verification of *treA* presence and of *treA* chromosomal complementation	This study
treAKO_F	ATG AAA TCC CCC GCA CCT TCT CGC CCG CAA AAA ATG GCG TTA ATT CCA GCG TGT AGG CTG GAG C TG CTT C	Mutation of *treA*	This study
treAKO_R	GGA CGC GTC GCC GGA ACA TTG TCA CAC GGT TGC TCT TTC GGG CAG ATC AAA TGG GAA TTA GCC ATG GTC C	Mutation of *treA*	This study
QFtreApWSK29compl_F	GTA CCG GGC CCC CCC TCG AGT CCG GCA ATT TAC TCT GCA CT	Cloning of *treA* into pWSK29 plasmid	This study
QFtreApWSK29compl_R	TCC CCC GGG CTG CAG GAA TTC AGG GAG AAT GGG GAG TGG GGG	Cloning of *treA* into pWSK29 plasmid	This study
QFtreAcompl_F	CCG GGC CCA AGC TTC TCG AGT CCG GCA ATT TAC TCT GCA CT	Cloning of *treA* into pGPTn7Cm plasmid and amplification of *treA-cat* fragment for complementation	This study
QFtreATn7compl_R	CCC CGG GCT GCA GGA ATT CAG GGA GAA TGG GGA GTG GGG G	Cloning of *treA* into pGPTn7Cm plasmid	This study
Dat_treA_compl_R	CGG CGA AAT AGA GTG ATA AAA TAA CAT CTG TTT ATT AGT CAG CCA GCG ACA AGC TTT CAG AAC GCT CGG TTG CCG C	Amplification of *treA-cat* fragment for chromosomal complementation	This study
screening_treAKO_F	ACC GTT CGG ATG GCA TCA TT	Checking of *treA* mutation	This study
K1	CAG TCA TAG CCG AAT AGC CT	Checking of *treA* mutation	This study
Pri3	TCG TTT TGC CGG ATT ATG GG	Verification of *fimS* orientation	19
Pri4	AGT GAA CGG TCC CAC CAT TAA CC	Verification of *fimS* orientation	19

20 min on ice, followed by centrifugation at 20,000 × *g* for 15 min at 4°C. The protein pellet was washed twice with 50 mM Tris-EDTA (pH 12.0) and resuspended in 50 mM Tris-EDTA, pH 8.0, at 1/10 of the supernatant's initial volume. Western blotting was performed as described previously (46).

Detection of the ON/OFF state of the *fimS* region. Detection of the orientation of the *fimS* region was performed as described previously (19). Strains were cultured statically in LB for 24 h to enhance the ON orientation in the bacterial population and with shaking until mid-log phase to observe the orientation at the time point of cell assays and yeast agglutination. As a control, the wild-type strain was statically grown for 96 h to select for increased switching toward the ON orientation and repetitively on agar plates to select for an increased switching toward the OFF orientation.

Experimental UTIs in CBA/J mice. Experimental urinary tract infections in CBA/J mice were performed as previously described (48). Briefly, 5-week-old female mice were infected with 20 μl (2×10^9 CFU) of bacteria through a catheter inserted in the urethra. At 48 h postinfection, the mice were euthanized. Bladders and kidneys were then collected, homogenized, diluted, and plated on MacConkey agar plates to enumerate bacterial CFU.

Alignment of FimH amino acid sequences. Amino acid sequences of the FimH protein from CFT073 (GenBank accession no. AE014075.1), IMT5155 (GenBank accession no. CP005930.1), and MT78 (8, 14; sequence not public) were aligned using the Clustal Omega Multiple Sequence Alignment server (49).

Statistical analysis. The Mann-Whitney test was used to compare the samples by pairs, and the Kruskal-Wallis test was used to compare groups, since the distribution of results was nonparametric. Tests were performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA, USA).

Ethics statement. The experimental protocol for handling animals was approved by the Ethical Committee of INRS (protocol number 1206-03).

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