Veterinary and Animal Science 3 (2017) 10–17

Contents lists available at ScienceDirect

Veterinary and Animal Science

journal homepage: www.elsevier.com/locate/vas

The *GadX* regulon affects virulence gene expression and adhesion of porcine enteropathogenic *Escherichia coli in vitro*

Hannah-Sophie Braun[a,*], Gerhard Sponder[a], Jörg R. Aschenbach[a], Katharina Kerner[b], Rolf Bauerfeind[b], Carolin Deiner[a]

[a] Institute of Veterinary Physiology, Freie Universität Berlin, Oertzenweg 19b, 14163 Berlin, Germany
[b] Institute of Hygiene and Infectious Diseases of Animals, Justus Liebig University, Frankfurter Strasse 85-89, 35392 Gießen, Germany

ARTICLE INFO

Keywords:
EPEC
GadX
GABA
Adhesion
Bacteria-host interaction
Diarrhea

ABSTRACT

The ability of enteropathogenic *Escherichia coli* (EPEC) to express virulence factor genes and develop attaching and effacing (AE) lesions is inhibited in acidic environmental conditions. This inhibition is due to the activation of transcription factor GadX, which upregulates expression of glutamic acid decarboxylase (Gad). Gad, in turn, produces γ-aminobutyric acid (GABA), which was recently shown to have a beneficial effect on the jejunal epithelium *in vitro* due to increased mucin-1 levels.

In the present study, we sought to test whether forced GadX activation/overexpression abolishes virulence associated features of EPEC and provokes increased GABA production. EPEC strains were isolated from diarrheic pigs and submitted to activation of GadX by acidification as well as *gadX* overexpression via an inducible expression vector plasmid. GABA concentrations in the growth medium, ability for adhesion to porcine intestinal epithelial cells (IPEC-J2) and virulence gene expression were determined.

Growth in acidified media led to increased GABA levels, upregulated *gadA/B* expression and downregulated mRNA synthesis of the bacterial adhesin *intimin*. EPEC strains transformed with the *gadX* gene produced 2.1–3.4-fold higher GABA levels than empty-vector controls and completely lost their ability to adhere to IPEC-J2 cells and to induce actin accumulation.

We conclude that intensified *gadX* activation can abolish the ability of EPEC to adhere to the intestinal epithelium by reducing the expression of major virulence genes.

1. Introduction

Piglets around weaning are known to be exceedingly susceptible for gut infections, since they undergo environmental and psychological stress as well as nutritional changes. The postweaning diarrhea, starting around 3–10 days after the piglets are weaned, has been a focus of research due to the high economic losses it entails in pig production (Fairbrother, Nadeau, & Gyles, 2005; Hampson, Woodward, & Connaughton, 1993; Tsiloyiannis, Kyriakis, Vlemmas, & Sarris, 2001).

These losses are not only caused by severe diarrhea and the resulting higher mortality but also by decreased growth performance and reduced weight gain. A characteristic pathomechanism of enteropathogenic *Escherichia coli* (EPEC) to cause diarrhea in humans and animals is the ability to provoke attaching and effacing lesions (AE lesions) (Bruant et al., 2009; DebRoy & Maddox, 2001; Girard, Batisson, Frankel, Harel, & Fairbrother, 2005; Kim, Kim, Hur, & Lee, 2010; Nataro & Kaper, 1998). AE lesions are histopathological alterations in epithelial cells of the intestine. They are characterized by the effacement of microvilli through rearrangement of the epithelial cell cytoskeleton (Kaper, McDaniel, Jarvis, & Gomez-Duarte, 1997), leading to a pedestal-forming actin accumulation directly beneath the adherent bacteria (Kalman et al., 1999; Kaper et al., 1997; Kaper, Nataro, & Mobley, 2004). The concurrent destruction of the enteric brush border results in enteric malfunction and diarrhea.

Since infections with EPEC usually occur orally, EPEC, as well as many other bacteria, have evolved a system to cope with low pH conditions using the enzyme glutamic acid decarboxylase (Gad), which converts glutamic acid into γ-aminobutyric acid (GABA). The subsequent release of GABA via a GABA-glutamate antiporter represents the factual extrusion of protons and stabilizes the bacteria's inner pH milieu (Foster, 2004; Hersh, Farooq, Barstad, Blankenhorn, & Slonczewski, 1996; Richard & Foster, 2003). In a previous study, we have shown that luminal GABA has significant local effects in the small intestine, namely a selective upregulation of mucin

* Corresponding author.
E-mail addresses: hannah-sophie.braun@fu-berlin.de (H.-S. Braun), gerhard.sponder@fu-berlin.de (G. Sponder), joerg.aschenbach@fu-berlin.de (J.R. Aschenbach), katharina.kerner@vetmed.uni-giessen.de (K. Kerner), rolf.bauerfeind@vetmed.uni-giessen.de (R. Bauerfeind), carolin.deiner@fu-berlin.de (C. Deiner).

http://dx.doi.org/10.1016/j.vas.2017.04.001
Received 27 November 2016; Received in revised form 19 February 2017; Accepted 26 April 2017
Available online 27 April 2017

1 (MUC1) (Braun, Sponder, Pieper, Aschenbach, & Deiner, 2015). The MUC1 protein is the best characterized transmembrane mucin and is an important player of intestinal defense. This implies that GABA ingested with vegetable food or produced by bacteria can have a direct stimulating effect on a key component of the mucosal barrier function.

In *E. coli* two functionally undistinguishable isoforms of Gad are known: GadA and GadB (De Biase, Tramonti, John, & Bossa, 1996; Smith, Kassam, Singh, & Elliott, 1992). Gene expression of *gadA* and *gadB* is regulated by the transcription factor *gadX*, located downstream of *gadA*. In case of acidic environmental conditions, *gadX* expression is increased, thereby leading to an upregulation of GadA/B production (Tramonti, Visca, De Canio, Falconi, & De Biase, 2002). As the pH optimum of bacterial Gad activity is discussed to be between 3.8 and 5 (De Biase, Tramonti, Bossa, & Visca, 1999; Shukuya & Schwert, 1960), it can be speculated that the more acidic the environment (e.g. stomach, cecum), the more GABA is produced, thereby solidifying the mucosal barrier. However, growth circumstances have to be considered because the presence of the *gadA/B* gene transcripts did not necessarily correlate with Gad enzyme activity, especially when cells were grown under acidic conditions (Castanie-Cornet, Penfound, Smith, Elliott, & Foster, 1999).

In addition to its upregulating effect on the Gad system, GadX has also been shown to downregulate the expression of genes of the locus of enterocyte effacement (LEE) (Shin et al., 2001). It is widely accepted that LEE is largely responsible for the pathogenicity of EPEC as it includes almost all virulence genes necessary for the formation of AE lesions (An et al., 2000; Elliott et al., 1998; McDaniel & Kaper, 1997; McDaniel, Jarvis, Donnenberg, & Kaper, 1995; Shaw, Cleary, Murphy, Frankel, & Knutton, 2005). LEE comprises EscU, a component of the type III secretion system (T3SS), a needle-forming multiprotein complex, which spans through the inner and outer bacterial membrane and facilitates the translocation of bacterial effector molecules directly from the bacterial into the host cell cytoplasm (Jarvis et al., 1995).

Further gene loci on LEE encode for the translocated intimin receptor (Tir) and a Tir-specific chaperone (CesT). Upon its translocation into the host cell cytoplasm via the T3SS, Tir is inserted into the host cell membrane and serves as a receptor for the bacterial adhesin intimin (Kenny et al., 1997; Kenny, 2002). Intimin, an outer membrane protein, is known as a major virulence factor of EPEC as it is required for the intimate attachment of the bacterium to the host cell. Intimin is also encoded by a gene, *eae,* localized within the LEE. The *eae* gene is commonly used in diagnostic PCR procedures for the identification of EPEC (An et al., 2000; Gomez-Duarte & Kaper, 1995; Jerse, Yu, Tall, & Kaper, 1990).

The LEE-encoded regulator (Ler) activates the expression of all virulence genes of LEE. Ler itself is positively regulated by the plasmid encoded regulator (PER) (Mellies, Elliott, Sperandio, Donnenberg, & Kaper, 1999). The *per* gene, in turn, is a region located on a 90 kb plasmid, which is inhibited by GadX (Shin et al., 2001). Hence, it can be speculated that the more acidic the environment, the more GadX is activated and the more PER is inhibited. This prevents EPEC from unnecessary expression of virulence factors in adverse environments, e.g. during their passage through the stomach.

Acidic conditions were observed to influence Gad activity (Castanie-Cornet et al., 1999). However, in the laboratory setting, acidification may irritate the hosting cell layer. Therefore, we have used acidification only as an initial proof of concept and then switched to a *gadX* overexpression model at neutral pH values. Accordingly, we used different EPEC strains isolated from diarrheic pigs, introduced an inducible expression vector plasmid containing the *gadX* gene and subsequently examined GABA production, adhesion ability *in vitro* and virulence gene expression on the mRNA level.

2. Methods

2.1. Experimental design

2.1.1. Proof of concept: influence of acidification on GABA production, gadA/B and eae expression

One EPEC strain was grown at either pH 5.0 or pH 7.0. RNA was isolated, reversely transcribed and analyzed for the expression levels of *gad A/B* and *eae*. GABA concentrations in the medium were measured. The experiment was repeated on four consecutive days to a total of four replicates per sample.

2.1.2. Influence of gadX overexpression on GABA production

One EPEC strain was transformed with either an inducible *gadX* plasmid or an empty plasmid. Two, three and four hours after induction with isopropyl-β-D-thiogalactopyranosid (IPTG), GABA concentration was measured in the culture medium (pH 7.0). According to the results, two additional EPEC strains were transformed and GABA levels measured only after two hours of induction with IPTG. The experiment was repeated on three consecutive days to a total of three replicates per sample.

2.1.3. Influence of gadX overexpression on virulence gene expression

RNA of transformed porcine EPEC strains was isolated 2 h after induction of the plasmid and analyzed for gene expression changes of *gadX* and *gadA/B* as well as of the major virulence factors intimin, *tir*, *cesT* and *escU*. Duplicates of the experiment were repeated on four consecutive days to a total of eight replicates per sample.

2.1.4. Influence of gadX overexpression on adhesion ability

The ability to adhere and cause AE lesions was tested *in vitro* on intestinal porcine epithelial cells (IPEC-J2) using the fluorescent actin staining (FAS) test and a regular adhesion test with the three transformed porcine EPEC strains and their respective wild type strains (one replicate per strain).

2.1.5. pH measurements in the gastrointestinal tract

In order to evaluate the physiological pH levels in different segments of the gastrointestinal tract, digesta samples of six pigs were analyzed using a pH meter.

2.2. Method details

2.2.1. Bacterial strains, culture conditions and transformation

All EPEC used in the present study (Table 1) had been isolated from either diarrheic piglets or porkers, and had been checked for the presence of the intimin gene *eae*, their ability to cause actin accumulation in intestinal porcine epithelial (IPEC-J2) cells (FAS test) and their growth performance in M9 minimal medium. According to the presence or absence of the *bfpA* plasmid (Nataro & Kaper, 1998), strains belonged to the group of typical or atypical EPEC, the latter being more common in pigs (Bruant et al., 2009; Frohlicher, Krause, Zweifel, Beutin, & Stephan, 2008). Strains P185/04-2, and 4181/05-2 were characterized as atypical EPEC lacking the *bfpA* plasmid but harboring several other virulence-associated genes (e.g. non LEE virulence genes

Table 1
Main characteristics of the used EPEC strains (isolated from diarrheic pigs).

Strain designation	Serotype	*eae* type	*bfpA* plasmid	FAS intensity[a]
P185/04–2	O76:H7	g2/q	No	1
P6414/05–1	O40:H10	e1	Yes	2
4181/05–2	Ont: H-	k	No	2

[a] FAS intensity was scored from 0 to 3 (0 = 0% of all cells positive, 1: < 30% of all cells positive; 2: 30–60% of cells positive; 3: > 60% of cells positive).

as *astA*, *irp2*, *fyuA*). Strain P6414/05-1 was the only typical EPEC available.

The standard culture medium to grow or transform bacteria was LB medium supplemented with ampicillin (0.1 g/l) at pH 7.3 and 37 °C. Bacteria were transferred to M9 minimal salt medium for experiments because standard LB medium was found to contain GABA (M9 minimal salt medium: Na_2HPO_4 15.1 mmol/l, KH_2PO_5 6.6 mmol/l, NH_4Cl 18.6 mmol/l, NaCl 8.6 mmol/l, MES glucose 22.0 mmol/l, glutamate 2.0 mmol/l, $MgSO_4$ 2 mmol/l, $CaCl_2$ 0.1 mmol/l, ampicillin 0.1 g/l; pH 7.0; 37 °C). For the proof of concept, the pH of M9 was adjusted to 5.0 or 7.0.

The expression vector plasmid pQE-80 L (4751 bp; Ampr; Qiagen, Hilden, Germany) was used as it includes a cis-*lacIq* gene that over-expresses the Lac repressor, strongly suppressing protein expression from the Lac promoter unless induced with IPTG. The *gadX* encoding DNA (EcoGene Accession Number EG12243) was synthetically manufactured (Life Technologies, Darmstadt, Germany) with restriction sites for BamH1 and HindIII. T4 Ligase (Fermentas, St. Leon-Rot, Germany) was used to ligate the insert into the linearized plasmid resulting in the recombinant plasmid pQE-80L:*gadX*. Porcine EPEC were treated with 75 mM $CaCl_2$ to produce chemically competent cells, transformation was accomplished by heat shock (Sambrook & Russell, 2006). The untreated plasmid pQE-80L was used to generate strain-specific empty plasmid transformants, accordingly.

2.2.2. GABA measurements

For GABA measurements, the OD_{600} of the respective overnight culture was determined and the volume necessary for a final culture volume of 10 ml with an OD_{600} of 0.1 was added to prewarmed M9 medium. IPTG was added. For each time point, 1 ml of the culture was taken, OD_{600} was determined for later normalization, samples were centrifuged for 15 min (at 14,000 rpm, 0 °C) and 200 µl of the supernatant were precipitated with 200 µl of 0.2 M perchloric acid. Analysis of GABA was performed by Knauer GmbH (Berlin, Germany) using ultra-high performance liquid chromatography with electrospray ionization-mass spectrometry detection (Cohen & Michaud, 1993).

2.2.3. Bacterial RNA isolation and cDNA synthesis

A volume equivalent to 1 ml with an OD_{600} of 0.2 was collected from the respective bacterial overnight culture two hours after induction with IPTG or acidification, respectively. After centrifugation for 15 min (14,000 rpm, 0 °C) the supernatant was discarded and the pellet was resuspended in lysozyme according to the manufacturer's instructions for Gram-negative bacteria of the peqGOLD Bacterial RNA Kit (Peqlab Biotechnologie GmbH, Erlangen, Germany). Notwithstanding the peqGOLD protocol, lysis of cell membranes was facilitated by incubating the pellet in liquid nitrogen for 15 min before resuspension; thereafter, it was proceeded with the peqGOLD protocol including DNAse treatment.

RNA integrities and concentrations were examined by use of a lab-on-a-chip system (Prokaryote Total RNA Nano, Agilent Technologies, Waldbronn, Germany) showing RINs around 9.6. Only RNAs with RINs > 7 were used for cDNA synthesis, assuming that lower quality could affect results. RNA yields ranged from 6 to 100 ng/µl.

One hundred ng of RNA were transcribed to the reverse strand using the iScript cDNA synthesis kit (Bio-Rad Laboratories GmbH, Munich, Germany) in accordance with manufacturer's instructions. The reaction product was diluted 1:10 and stored at −20 °C until further processing.

2.2.4. Reverse transcription-quantitative PCR (RT-qPCR)

Primers were designed to detect the target genes *gadX*, *gadA* and *gadB* (genes encoding glutamic acid decarboxylases A and B, respectively), *eae*, *tir*, *cesT* and *escU* using Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/primer3, for primer sequences see Table 2). The primer set for detection of *gadA* and *gadB* was designed to detect a highly conserved region in both isoforms, hence, it is referred to as *gadA/B*. Specificity of established RT-qPCR assays was assured by electrophoresis of PCR products through 1.5% agarose gels and sequencing of amplicons (GATC Biotech, Konstanz, Germany). For normalization, reference genes *cysG* and *gapdh* (primer sequence published by Carey, Kostrzynska, & Thompson (2009) were used. According to geNorm (Biogazelle NV, Zwijnaarde, Belgium), neither reference gene *rrsA* showed a sufficiently stable expression level, nor previously tested genes *hcaT* and *idnT* (Zhou et al., 2011).

Relative quantification of specific mRNA was conducted on a ViiA7 Cycler (Life Technologies, Darmstadt, Germany) according to the following protocol: 95 °C for 10 min, subsequent 40 cycles at 95 °C for 15 s and 1 min at 60 °C, followed by a melting curve analysis at 95 °C for 15 s, cooling down to 60 °C for 1 min and heating up slowly to 95 °C for 15 s while monitoring fluorescence.

Every sample reaction was carried out in triplicates on a 384-well plate. Each 10 µl reaction contained 5 µl of cDNA, 4.64 µl of iQ™ SYBR® Green Supermix (Bio-Rad Laboratories GmbH, Munich, Germany) and 0.18 µl of forward and reverse primers (20 µmol/l). No template controls (NTC) and negative RT samples (reverse transcription omitted) were used for every target gene. Samples were excluded from evaluation if Cq difference between sample and respective NTC was < 5 or if the negative control RT was positive.

Resulting Cq values and dilution series-based gene specific amplification efficiencies (which were at least 96% for all primer pairs) were entered into the software qbasePLUS (Biogazelle NV, Zwijnaarde, Belgium) for normalization with the two reference genes and subsequent expression analysis. Results were exported as calibrated normalized relative quantity (CNRQ) values. For every gene of interest, the mean CNRQ value of each empty pQE-80L group (one group per strain) was calculated and used to set all values of the group to 1. CNRQ values of the pQE-80L::*gadX* groups were related to their respective empty pQE-80L expression level (proof of concept: results of experiments at pH 5.0 were related to those at pH 7.0).

2.2.5. In vitro adhesion test

IPEC-J2 cells were seeded at a concentration of 3×10^5 cells per well in 24-well culture plates and grown for two days in DMEM/Ham's F12 medium (Biochrom, Berlin, Germany, pH 7.3) with 5% fetal calf serum (PAA Laboratories GmbH, Cölbe, Germany). Penicillin/streptomycin solution (1%, PAA Laboratories GmbH, Cölbe, Germany) was added for the first day only. Immediately before the adhesion test, IPEC-cells were washed twice with HEPES buffer.

Transformed bacteria were cultivated overnight in LB medium with pH 7.2 containing 50 µg/ml of carbenicillin (PAA Laboratories GmbH, Cölbe, Germany) at 37 °C, then diluted in LB medium 1:100 and induced with 0.2 mM IPTG for two hours. Wild type bacteria were treated accordingly but grown without carbenicillin. Then bacterial suspensions were diluted to a concentration of 1×10^8 cfu per ml DMEM/HAM's F12 (5% fetal calf serum, 1% mannose, 0.2 mM IPTG, w or w/o carbenicillin, respectively) and one ml bacterial suspension was given to each well of washed IPEC-J2 cells.

After 3 h of incubation, IPEC-J2-cells were washed 6 times with PBS to eliminate non-adherent bacteria. For lysis of IPEC-J2 cells, 1 ml of Triton-x-100 (1%) was added. The whole content of each well was transferred to a tube and incubated on ice for 1 h. Quantification of the adherent bacteria was carried out by plating log5-dilution series on LB agar (w/ or w/o carbenicillin; 100 µl/plate) followed by overnight culture at 37 °C. Dilutions containing less than 300 CFUs/100 µl were used to calculate the number of adherent bacteria of each well.

2.2.6. Fluorescent-actin staining (FAS) test

Wild type and transformed porcine EPEC used in the *in vitro* adhesion tests were also examined for their ability to accumulate microfilaments in the apical cytoplasm of IPEC-J2 beneath attached bacteria in a FAS test according to Knutton et al. (1991). The degree of microfilament accumulation in the FAS test is representative of a bacterium's

Table 2
Sequences (5´ to 3´) of primers used for RT-qPCR and resulting amplicon sizes.

Gene	Forward primer	Reverse primer	Amplicon Size [bp]
gadX	ATGACGCCCACAGAGTATCAG	GGTCAGTGCCGTAAAAATTCCC	101
gadA/B	TTACCAGGTTGCCGCTTATC	ACGCAGACGTTCAGAGAGGT	163
eae	ACCGTCATATCCGGCATTAG	ACCATGACGGTAATCGATCC	169
tir	TCAGGGGAGAAAACAATGAACG	GCCACTACCTTCACAAACAGAC	101
cesT	CGACAGATAACCCTAACTTCGC	CCATCGACTTAACGACGACTTC	186
escU	GGGCGATGTAACAAAAAGTGA	CCGCACCTAATATTTCACGA	176
cysG	TTTCTGGGAGAAATTGTTCG	TTGCAGTCCTTTCAGTGTCA	181
gapdh	TCCGTGCTGCTCAGAAACG	CACTTTCTTCGCACCAGCG	299

ability to cause attaching and effacing lesions.

IPEC-J2 cells were grown to confluence on 12 mm-cover slips (Hecht-Assistant, Sontheim v.d. Rhön, Germany) and infected with 1×10^7 cfu of bacteria in 1 ml of DMEM/HAM's F12 per cover slip. After incubation with the bacterial suspension for 3 h, cells were washed 3 times with HEPES buffer and incubated for additional 3 h in fresh DMEM/HAM's F12. Subsequently, cells were washed three times with PBS-buffer and fixed with 2% paraformaldehyde and permeabilized with 0.005% digitonin (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany). After 50 µl of a 5 µg/ml fluorescein isothiocyanate (FITC)-phalloidin solution (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) had been added to each well, plates were incubated in the dark for 30 min in a humidified chamber. Then, nuclei and bacterial cells were counterstained with 10 µg/ml of propidium iodide (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) for 2 min. Finally, cover slips were mounted on glass slides using Mowiol mounting medium (Clariant, Sulzbach am Taunus, Germany) and evaluated under a fluorescence microscope (DM RB, Leica, Wetzlar, Germany).

2.2.7. pH measurements in the gastrointestinal tract

In order to determine the physiological pH levels in the different segments of the gastrointestinal tract, digesta samples of stomach, duodenum, mid jejunum, ileum, cecum and mid colon of six healthy pigs were collected from the slaughterhouse. The samples were taken immediately after slaughter and pH levels were determined using a portable pH meter (pH-Meter 1140, Mettler Toledo, Gießen, Germany) calibrated at 37 °C with standard buffer (pH 4 and 7) before use. Pigs had been fed a standard fattening diet.

2.2.8. Statistics

Summarized data of multiple measurements are presented as arithmetic mean and standard error of means (SEM). For statistical analyses Sigma Plot 11.0 (Systat Software Inc., San José, CA, USA) was used. ANOVA or a Student´s *t*-test was performed and if normality test failed, the Wilcoxon-Mann-Whitney test was applied.

3. Results

3.1. Proof of concept: influence of acidification on GABA production, gadA/B and eae transcription

The EPEC wild type strain P185/04-2 produced GABA only at low pH conditions: at pH 5.0 an average GABA concentration of 81.2 ± 10.3 µmol/l was found in the M9 minimum salt medium (Table 3), whereas at pH 7.0 no GABA was detected (detection limit 5 µmol/l). Hence, normalization to the respective OD_{600} values, as performed in all later GABA concentration measurements, was not feasible and conclusions regarding the rate of increase could not be drawn. However, the increased level of *gadA/B* mRNA was measurable, which was 7.2-fold higher at pH 5.0 compared to pH 7.0 ($p < 0.01$). Growth in acidified medium reduced *eae* transcription by 54% showing a final *eae* mRNA level of 0.46 ± 0.13 compared to the level at pH 7.0 ($p = 0.2$).

3.2. Influence of gadX overexpression on GABA production

Two, three and four hours after induction with IPTG, GABA concentrations were measured in the culture supernatant of EPEC strain P185/04-2 transformed with either pQE-80L:*gadX* or the empty vector pQE-80L. Values were normalized to the respective culture density (OD_{600} value). GABA concentrations in cultures of pQE-80L:*gadX* transformants were at least twofold higher than in cultures of empty vector transformants (Table 3). Concentrations determined after two, three and four hours of incubation did not differ from each other significantly. Therefore, GABA concentrations were determined only after two hours for EPEC strains P6414/05-1 and 4181/05-2. GABA concentrations were distinctly lower in these strains, but so were OD_{600} values, hence, after normalization these strains showed GABA differences between empty vector controls and *gadX* transformants comparable to those seen in P185/04-2 with up to 3.4-fold higher values in cultures of pQE-80L:*gadX* transformants.

3.3. Influence of gadX overexpression on virulence gene expression

Upon induction with IPTG, *gadX* mRNA levels rose significantly (200 to 1100-fold) in all *gadX* transformants compared to the respective empty plasmid transformants which were set to 1.0 (Fig. 1, $p < 0.01$). In accordance with these results, transcription levels of *gadA/B* were also markedly higher 14–24-fold) in each *gadX* transformant (Fig. 2, $p < 0.05$). To test whether these increased mRNA levels coincided with decreased expression of LEE-encoded virulence genes, transcription of *eae*, *tir*, *cesT* and *escU* were investigated. Interestingly, basal transcription levels of these virulence genes strongly depended on the EPEC strain tested (e.g. only low expression of *eae* in P185/04-2 and almost no *tir* expression in P6414/05-1; data not shown). However, *gadX* overexpression reduced *eae*, *tir*, *escU* and *cesT* transcription in all three strains. This was significant for *eae* in strains P185/04-2 and P6414/05-1 (Fig. 3A), for *tir* in strain P185/04-2 (Fig. 3B) and for *escU* in strain P6414/05-1 (Fig. 3C). As CNRQ data showed large variances between the eight experiments, other values failed to be statistically significant ($p \geq 0.05$).

3.4. Influence of gadX overexpression on adhesion ability in vitro

Adhesion and FAS tests were performed to test for the functional consequences of decreased virulence factor expression following *gadX* overexpression. Wild type porcine EPEC and those transformed with empty pQE-80L adhered to IPEC-J2 cells distinctively, whereas bacteria transformed with pQE-80:*gadX* did not show any adhesion (Fig. 4). Noticeably, not a single colony forming unit was detected to be adherent in any tested EPEC strain after transformation with pQE-80L:*gadX*. The latter finding suggests that *gadX* overexpression leads to functionally relevant downregulation of virulence genes in porcine EPEC.

In agreement with the results of the *in vitro* adhesion test, transformation of porcine EPEC with pQE-80L::*gadX* completely abolished characteristic microfilament accumulation in IPEC-J2 cells that usually

Table 3
Normalized GABA concentrations in culture supernatants of EPEC.

Strain	Plasmid	Hours of IPTG induction	GABA* [µmol/l]	Mean OD_{600}	Normalized GABA* [µmol/l /OD_{600}]	Significance**
P185/04–2	Wild type, pH 7.0	–	< 5.0	0.540	–	
	Wild type, pH 5.0	–	81.2 ± 10.3	0.524	156.5 ± 26.2	a
	Empty pQE-80L	2	181.1 ± 13.1	0.659	274.7 ± 19.6	b
		3	192.8 ± 40.9	0.665	290.7 ± 63.9	a, b
		4	173.5 ± 39.0	0.674	260.5 ± 65.5	ab
	pQE-80L:*gadX*	2	398.2 ± 18.6	0.634	628.3 ± 28.1	c
		3	417.3 ± 14.0	0.669	624.1 ± 25.5	c
		4	366.6 ± 42.4	0.681	537.2 ± 58.7	c
P6414/05–1	Empty pQE-80L	2	16.5 ± 0.7	0.308	53.7 ± 3.7	d
	pQE-80L:*gadX*	2	53.2 ± 5.4	0.363	146.8 ± 13.8	a, e
4181/05–2	Empty pQE-80L	2	19.6 ± 1.6	0.408	48.3 ± 5.0	d
	pQE-80L:*gadX*	2	67.7 ± 5.5	0.413	164.4 ± 13.9	a, e***

* data are given as arithmetic means and standard error of the mean of three replicates (except for wild type P185/04-2 where four replicates where used).
** supernatants that do not share the same letter, differ significantly ($p < 0.05$).
*** if normality test failed, a Wilcoxon-Mann-Whitney test was performed instead of Student´s *t*-test.

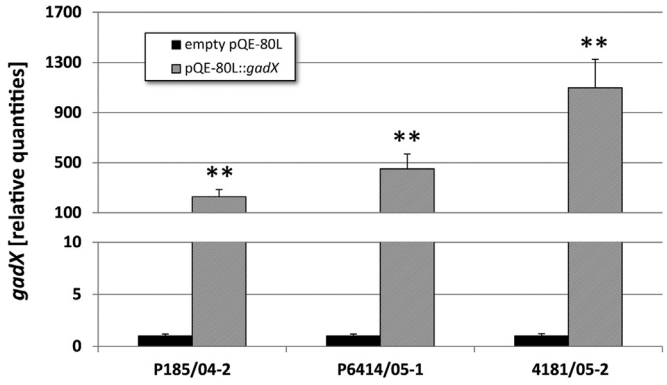

Fig. 1. Transcription of *gadX* in transformed EPEC strains. Plasmid (pQE-80 L)-encoded *gadX* transcription was induced in two atypical (P185/04-2 and 4181/05-2) and one typical EPEC strain (6414/05-1) with IPTG for two hours. Analysis by RT-qPCR revealed that *gadX* mRNA was significantly increased in *gadX* transformants (hatched bars) compared with respective transformants harboring the empty plasmid pQE-80L only (black bars, mRNA level set to 1.0). Data are given as arithmetic means and standard errors of means of eight replicates (duplicates tested on four consecutive days); **$p < 0.01$.

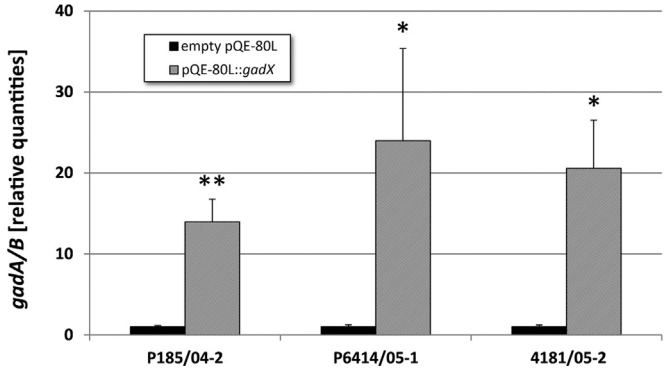

Fig. 2. Transcription of *gadA/B* in transformed EPEC strains. Upon IPTG induction, those transformants harboring pQE-80L:*gadX* showed significantly higher *gadA/B* transcription (hatched bars) than the respective transformants harboring the empty plasmid pQE-80L only (black bars, mRNA level set to 1.0). Data are given as arithmetic means and standard errors of means of eight replicates (duplicates tested on four consecutive days); **$p < 0.01$, *$p < 0.05$.

occurs upon exposure to LEE-encoding *E. coli* such as EPEC and some EHEC. Thus, adhering bacteria and actin accumulation beneath these bacteria were only found in IPEC-J2 cells cultured with wild type EPEC or their derivatives transformed with empty pQE-80L but not after incubation with pQE-80L:*gadX* transformants (Fig. 5).

3.5. pH measurements in the gastrointestinal tract

As expected, the lowest pH was found in the digesta fluid of the stomach (4.1 ± 0.3, average pH of N = 6), whereas the jejunum appeared to have the highest pH (6.5 ± 0.1), decreasing again in the cecum (6.0 ± 0.2, see additional file 1). These findings are very much in line with the results of our previous study (Braun et al., 2015): highest concentrations of GABA were found in the stomach whereas the lowest GABA concentration was detected in the jejunum, increasing again towards the hindgut.

4. Discussion

EPEC have been intensely studied due to their causative role in endemic infant diarrhea in developing countries (Levine & Edelman, 1984; Rowe, 1979; Trabulsi, Keller, & Tardelli Gomes, 2002) and they were even regarded to be the most common bacterial pathogens in infants (Gomes et al., 1991; Katouli, Jaafari, Farhoudi-Moghaddam, & Ketabi, 1990; Khan, Iqbal, Ghafoor, & Burney, 1988; Mubashir et al., 1990). Animals have been considered a significant source for atypical EPEC infections in humans (Chandran & Mazumder, 2013), whereas typical EPEC have mainly been isolated from humans and only infrequently from dogs or pigs (Beaudry, Zhu, Fairbrother, & Harel, 1996; Kaufmann et al., 2006). Thus, EPEC infections are still of importance, requiring further elucidation of the interplay of EPEC metabolism and the hosts immune system. The fact that crucial functions of pathogenicity – as for the induction of LEE genes by gadX (Branchu et al., 2014)- have also been found to be present in other pathogens like EHEC- might imply a viable approach for new prevention and treatment strategies.

In the present study, we could show that acidification of the growth medium of a wild type EPEC strain can upregulate *gadA/B* expression, lead to measurable concentrations of GABA, and suppress *eae* expression. Plasmid-driven *gadX* overexpression in a pH neutral environment can lead to a complete loss of EPEC's ability to attach to and interact with IPEC-J2 cells due to downregulation of LEE virulence genes. Additionally, *gadX* overexpression in a pH neutral environment also increased GABA production. These results clearly demonstrate that GadX is important for GABA production and a crucial factor of the regulation of AE-promoting EPEC virulence genes.

Regarding Gad expression after acidification, we found a 7.2-fold increased *gadA/B* expression at pH 5.0. Accordingly, we found an average production of 81.2 µmol/l GABA of wild type EPECs at pH 5.0, whereas at pH 7.0 GABA amounts appeared to be below the detection limit of 5 µmol/l. This is not astonishing, as both Gad expression and decarboxylation of glutamic acid are usually stimulated by the presence

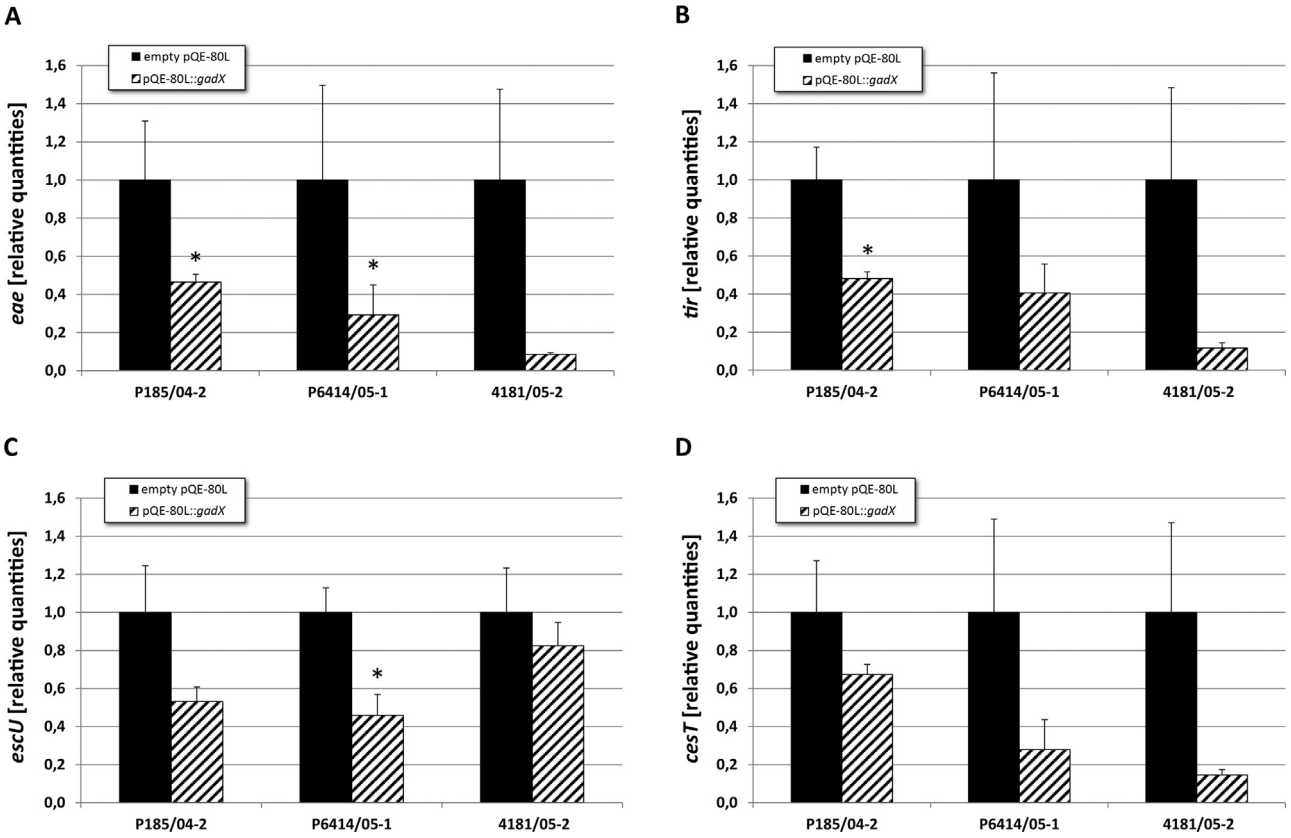

Fig. 3. Transcription of virulence genes in transformed EPEC strains. Transcriptions of *eae* (A), *tir* (B), *escU* (C) and *cesT* (D) in IPTG-treated pQE-80L::*gadX* transformants (hatched bars) were compared to those of the respective transformants harboring the empty plasmid pQE-80L only (black bars, mRNA level set to 1.0). Despite a high variability in baseline expression (data not shown), all virulence factors were numerically or significantly down-regulated in all EPEC transformants that had shown *gadX* overexpression (see Fig. 1). Data are given as arithmetic means and standard errors of means of eight replicates (duplicates tested on four consecutive days); *p < 0.05.

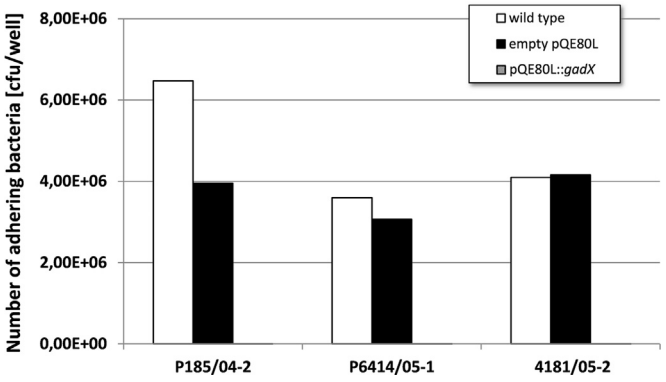

Fig. 4. Adhesion of wild type and transformed EPEC strains to IPEC-J2 cells. After transformation with pQE-80L:*gadX*, all three strains lost their ability to adhere to jejunal porcine epithelial cells (IPEC-J2) *in vitro* (hatched bars; not visible as the number of adhering bacteria was below the detection limit of the assay which was 10 cfu/well). In contrast, all wild type strains were able to adhere, as well as the respective transformants harboring empty pQE-80L. (one replicate per strain).

of excess protons (Castanie-Cornet et al., 1999). Nevertheless, this does not explain why GABA production of the wild type strain at pH 7.0 was below the detection limit, whereas the same strain containing an empty plasmid produced 174–193 µmol/l GABA at pH 7.0. Even though this effect might have been induced by the transformation with the empty plasmid, or by the use of IPTG, we should also consider that the variance between experiments might have been caused by other factors. When looking closer into our data, differences were primarily due to considerable variance in growth performance (expressed as OD_{600}) and baseline expression of the selected LEE genes between strains (e.g., very low expression levels of all factors in strain 4181/05-2), between virulence factors (e.g., generally low expression of *cesT* in all strains) and even between experiments performed under apparently identical experimental conditions.

The importance of growth performance on bacterial gene expression and metabolism is already known from previous studies. For example, Castanie-Cornet et al. (1999) and De Biase et al. (1999) showed that *gadA/B* gene activity depends on whether bacteria are in the log phase or in the stationary phase. Shin et al. (2001) found large variances in virulence gene expression related to slight changes of growth conditions. Also Yokoigawa, Takikawa, Okubo, and Umesako (2003) showed that growth phase and temperature can influence the expression of Gad genes. Although the experimental setup was always the same, we cannot tell whether the growth phase of all strains and experiments was always identical at the time of sampling. As there was no difference seen between the three time points tested with strain P185/04-2, neither in terms of OD_{600} nor in terms of GABA production, we assumed that after two hours the stationary phase had been reached. Hence, all further experiments (GABA measurements and RNA isolations) were conducted after two hours of incubation. Although GABA concentrations were distinctly lower in strains P6414/05-1 and 4181/05-2, using OD_{600} to normalize GABA levels resulted in GABA differences between empty vector controls and pQE-80L::*gadX* transformants comparable to those seen in P185/04-2 with 2.7–3.4-fold higher values.

Our results partially conflict with those of Shin et al. (2001), who tested *gadX*-transformed typical EPEC positive in FAS test despite lower protein expression levels for intimin, Tir, CesT and even slightly decreased *BfpA* expression. They concluded that the production of virulence proteins – although reduced – was still sufficient to induce actin cytoskeletal rearrangements in HeLa cells. Since these authors did not use a vector providing overexpression driven by the *taq* promoter, *gadX* overexpression in transformed EPEC could have been lower compared

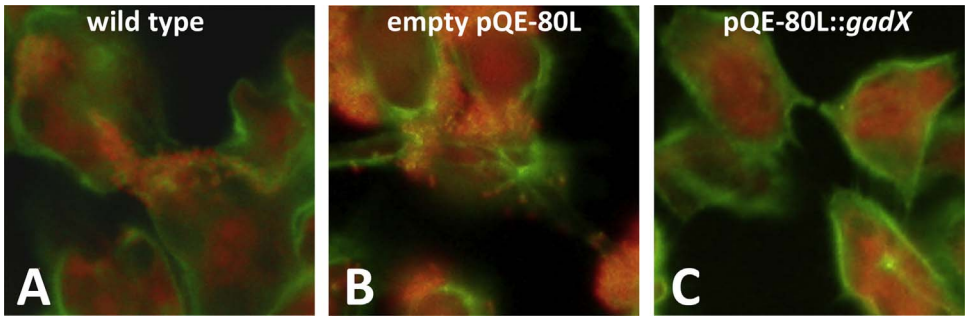

Fig. 5. IPEC-J2 cells in the fluorescent-actin staining (FAS) test of EPEC strain 4181/05-2 and its transformants. In an IPTG-containing medium, IPEC-J2 cells (red nuclei, green actin/cell membranes) had been incubated with the (A) wild type strain, (B) pQE-80L transformant, or (C) pQE-80L:*gadX* transformant. Localized adherence of bacteria (conglomerates of tiny red spots) and accumulated actin (green accumulations right underneath bacteria) were only visible in A and B. Original magnifications × 1000.

to our study. This could explain why interaction of transformed EPEC with cultured epithelial cells (HeLa) was not completely abolished in their study.

Dose effects of GadX may also be expected from the results of an analysis of the LEE-encoding EHEC strain O157:H7 (Branchu et al., 2014). This study showed that GadX is not only a negative regulator of LEE1 (thereby suppressing the expression of all other LEE genes) but also a positive regulator of LEE4 and LEE5. For this reason, we focused especially on LEE5 genes (*eae*, *tir*, *cesT*) to evaluate the net effect of *gadX* on these genes. The net effect was consistently negative, although not always statistically significant.

To date, it is well known that GABA - as an exemplary neurotransmitter - has many functions outside the mammalian brain (Erdo & Wolff, 1990). Even for the gut, various effects of GABA have already been described, as for example the alteration of smooth muscle activity (Bayer, Crenner, Aunis, & Angel, 2002) or the activation of antibody secretion by the intestinal epithelium (Jin, Guo, & Houston, 1989). A recent study demonstrated the modulation of abdominal pain by GABA derived from enteric Bifidobateria (Pokusaeva et al., 2016). In accordance with our recent hypothesis that GABA levels influence mucin-1 expression, McGuckin, Linden, Sutton and Florin (2011) described that the mucus layer is thickest in the stomach and colon. Apparently this does not only correlate with the number and distribution of mucus-producing cells throughout the gastrointestinal tract but also with our finding that GABA levels are highest in the stomach and colon (Braun et al., 2015). These GABA levels were derived from the same animals and correlate well with measured pH levels. At least for the hindgut, this supports our hypothesis that there is a link between pH and resulting GABA production by enteric bacteria.

Considering these facts, we assume that the GABA content in the intestinal fluid, which might – at least in parts - be derived from bacterial production, can remarkably influence the gastrointestinal physiology via specific GABA receptors. An increase of intestinal GABA production could lead to increased GABA resorption and resulting higher blood levels, however, effects would probably be restricted to the gastrointestinal tract since GABA as a zwitterion cannot pass the blood brain barrier. Thus, effects on the central nervous systems, as they are known from commonly available drugs like GABA receptor agonists or modulators of the GABA synthesis in the brain, are rather unlikely.

However, the interplay between bacterial GABA and the intestine is poorly understood so far. The lack of knowledge in this field offers another interesting subject for further research.

Finally, we have to answer the question of the applicability of our results. The signal that the environment is unfavorably acidic is translated via GadX into reduced efforts of the bacterium to adhere and stay at this unfavorable site as well as into increased efforts to defend (real or fake) intracellular bacterial pH via GABA production. Mimicking such an unfavorable environment by intentional activation of the GadX regulon may reprogram bacteria so that virulence is suppressed. Drugs activating the bacterial GadX regulon are not available so far but there might be other options to modulate this pathway. For instance, Branchu et al. (2014) postulate that the nitric oxide (NO) sensor nitrite-sensitive repressor (NsrR) is a direct positive regulator of the transcription of LEE1, LEE4 and LEE5 genes and an indirect repressor of *gadE* and *gadX* genes. However, supporting antibiotics in their mode of action or even replacing them by GadX modulators is still a long way off and requires further research.

5. Conclusions

We conclude that intensified *gadX* activation in EPEC can abolish EPEC´s ability to adhere to the intestinal epithelium by reducing expression of virulence genes like *intimin*. Since production of GABA, a neurotransmitter with potentially positive effects on the mucosal barrier, was increased at the same time, the GadX regulon could be an interesting target for the prevention of EPEC-associated postweaning diarrhea in piglets.

Funding

The study was supported by Pfizer Animal Health (Zoetis).

Declarations

The authors declare no conflict of interest related to this work.

The study was financially supported by Pfizer Animal Health (Zoetis).

Authors' contributions

JRA and CD conceived and designed the experiments. HSB, GS, KK and CD performed the laboratory work. KK and RB acquired and provided EPEC isolates. GS performed the transformation of the EPEC isolates. HSB, JRA, KK, RB, and CD analyzed the data. HSB, JRA and CD prepared the manuscript. All authors participated in interpretation and discussion of results and have read and approved the final manuscript.

Acknowledgements

The authors would like to thank Anja Schwanitz (Institute of Hygiene and Infectious Diseases of Animals, Faculty of Veterinary Medicine, Justus Liebig University Gießen) for excellent technical assistance.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.vas.2017.04.001.

References

An, H., Fairbrother, J. M., Desautels, C., Mabrouk, T., Dugourd, D., Dezfulian, H., & Harel, J. (2000). Presence of the LEE (locus of enterocyte effacement) in pig attaching and effacing *Escherichia coli* and characterization of eae, espA, espB and espD genes of PEPEC (pig EPEC) strain 1390. *Microbial Pathogenesis*, *28*(5), 291–300.

Bayer, S., Crenner, F., Aunis, D., & Angel, F. (2002). Effects of GABA on circular smooth

muscle spontaneous activities of rat distal colon. *Life Sci. 71*(8), 911–925.
Beaudry, M., Zhu, C., Fairbrother, J. M., & Harel, J. (1996). Genotypic and phenotypic characterization of *Escherichia coli* isolates from dogs manifesting attaching and effacing lesions. *Journal of Clinical Microbiology*, *34*(1), 144–148.
Branchu, P., Matrat, S., Vareille, M., Garrivier, A., Durand, A., Crepin, S., & Gobert, A. P. (2014). NsrR, GadE, and GadX interplay in repressing expression of the *Escherichia coli* O157:H7 LEE pathogenicity island in response to nitric oxide. *PLOS Pathogens*, *10*(1), e1003874. http://dx.doi.org/10.1371/journal.ppat.1003874.
Braun, H. S., Sponder, G., Pieper, R., Aschenbach, J. R., & Deiner, C. (2015). GABA selectively increases mucin-1 expression in isolated pig jejunum. *Genes & Nutrition*, *10*(6), 47. http://dx.doi.org/10.1007/s12263-015-0497-8.
Bruant, G., Zhang, Y., Garneau, P., Wong, J., Laing, C., Fairbrother, J. M., & Harel, J. (2009). Two distinct groups of porcine enteropathogenic *Escherichia coli* strains of serogroup O45 are revealed by comparative genomic hybridization and virulence gene microarray. *BMC Genomics*, *10*, 402. http://dx.doi.org/10.1186/1471-2164-10-402.
Carey, C. M., Kostrzynska, M., & Thompson, S. (2009). *Escherichia coli* O157:H7 stress and virulence gene expression on Romaine lettuce using comparative real-time PCR. *Journal of Microbiological Methods*, *77*(2), 235–242. http://dx.doi.org/10.1016/j.mimet.2009.02.010.
Castanie-Cornet, M. P., Penfound, T. A., Smith, D., Elliott, J. F., & Foster, J. W. (1999). Control of acid resistance in Escherichia coli. *Journal of Bacteriology*, *181*(11), 3525–3535.
Chandran, A., & Mazumder, A. (2013). Prevalence of diarrhea-associated virulence genes and genetic diversity in *Escherichia coli* isolates from fecal material of various animal hosts. *Applied and Environmental Microbiology*, *79*(23), 7371–7380. http://dx.doi.org/10.1128/AEM.02653-13.
Cohen, S. A., & Michaud, D. P. (1993). Synthesis of a fluorescent derivatizing reagent, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate, and its application for the analysis of hydrolysate amino acids via high-performance liquid chromatography. *Analytical Biochemistry*, *211*(2), 279–287. http://dx.doi.org/10.1006/abio.1993.1270.
De Biase, D., Tramonti, A., Bossa, F., & Visca, P. (1999). The response to stationary-phase stress conditions in *Escherichia coli*: Role and regulation of the glutamic acid decarboxylase system. *Molecular Microbiology*, *32*(6), 1198–1211 (doi: mmi1430)(pii).
De Biase, D., Tramonti, A., John, R. A., & Bossa, F. (1996). Isolation, overexpression, and biochemical characterization of the two isoforms of glutamic acid decarboxylase from *Escherichia coli*. *Protein Expression and Purification*, *8*(4), 430–438 (doi: S1046592896901217)(pii).
DebRoy, C., & Maddox, C. W. (2001). Identification of virulence attributes of gastrointestinal *Escherichia coli* isolates of veterinary significance. *Animal Health Research Reviews*, *2*(2), 129–140.
Elliott, S. J., Wainwright, L. A., McDaniel, T. K., Jarvis, K. G., Deng, Y. K., Lai, L. C., & Kaper, J. B. (1998). The complete sequence of the locus of enterocyte effacement (LEE) from enteropathogenic *Escherichia coli* E2348/69. *Molecular Microbiology*, *28*(1), 1–4.
Erdo, S. L., & Wolff, J. R. (1990). gamma-Aminobutyric acid outside the mammalian brain. *Journal of Neurochemistry*, *54*(2), 363–372.
Fairbrother, J. M., Nadeau, E., & Gyles, C. L. (2005). *Escherichia coli* in postweaning diarrhea in pigs: An update on bacterial types, pathogenesis, and prevention strategies. *Animal Health Research Reviews*, *6*(1), 17–39.
Foster, J. W. (2004). *Escherichia coli* acid resistance: Tales of an amateur acidophile. *Nature Reviews Microbiology*, *2*(*11*), 898–907. http://dx.doi.org/10.1038/nrmicro1021.
Frohlicher, E., Krause, G., Zweifel, C., Beutin, L., & Stephan, R. (2008). Characterization of attaching and effacing *Escherichia coli* (AEEC) isolated from pigs and sheep. *BMC Microbiology*, *8*, 144.
Girard, F., Batisson, I., Frankel, G. M., Harel, J., & Fairbrother, J. M. (2005). Interaction of enteropathogenic and Shiga toxin-producing *Escherichia coli* and porcine intestinal mucosa: Role of intimin and Tir in adherence. *Infection and Immunity*, *73*(9), 6005–6016. http://dx.doi.org/10.1128/IAI.73.9.6005-6016.2005.
Gomes, T. A., Rassi, V., MacDonald, K. L., Ramos, S. R., Trabulsi, L. R., Vieira, M. A., et al. (1991). Enteropathogens associated with acute diarrheal disease in urban infants in Sao Paulo, Brazil. *Journal of Infectious Diseases*, *164*(2), 331–337.
Gomez-Duarte, O. G., & Kaper, J. B. (1995). A plasmid-encoded regulatory region activates chromosomal eaeA expression in enteropathogenic *Escherichia coli*. *Infection and Immunity*, *63*(5), 1767–1776.
Hampson, D. J., Woodward, J. M., & Connaughton, I. D. (1993). Genetic analysis of *Escherichia coli* from porcine postweaning diarrhoea. *Epidemiology and Infection*, *110*(3), 575–581.
Hersh, B. M., Farooq, F. T., Barstad, D. N., Blankenhorn, D. L., & Slonczewski, J. L. (1996). A glutamate-dependent acid resistance gene in Escherichia coli. *Epidemiology and Infection*, *178*(13), 3978–3981.
Jarvis, K. G., Giron, J. A., Jerse, A. E., McDaniel, T. K., Donnenberg, M. S., & Kaper, J. B. (1995). Enteropathogenic *Escherichia coli* contains a putative type III secretion system necessary for the export of proteins involved in attaching and effacing lesion formation. *Proceedings of the National Academy of Sciences USA*, *92*(17), 7996–8000.
Jerse, A. E., Yu, J., Tall, B. D., & Kaper, J. B. (1990). A genetic locus of enteropathogenic *Escherichia coli* necessary for the production of attaching and effacing lesions on tissue culture cells. *Proceedings of the National Academy of Sciences USA*, *87*(20), 7839–7843.
Jin, G. F., Guo, Y. S., & Houston, C. W. (1989). Stimulation of specific Aeromonas antibody secretion in rat intestine by gamma-aminobutyric acid. *Digestive Diseases and Sciences*, *34*(4), 617–622.
Kalman, D., Weiner, O. D., Goosney, D. L., Sedat, J. W., Finlay, B. B., Abo, A., & Bishop, J. M. (1999). Enteropathogenic *E. coli* acts through WASP and Arp2/3 complex to form actin pedestals. *Nat. Cell Biol. 1*(6), 389–391. http://dx.doi.org/10.1038/14087.
Kaper, J. B., McDaniel, T. K., Jarvis, K. G., & Gomez-Duarte, O. (1997). Genetics of virulence of enteropathogenic *E. coli*. *Advances in Experimental Medicine and Biology*, *412*, 279–287.
Kaper, J. B., Nataro, J. P., & Mobley, H. L. (2004). Pathogenic *Escherichia coli*. *Nature Reviews Microbiology*, *2*(2), 123–140. http://dx.doi.org/10.1038/nrmicro818.
Katouli, M., Jaafari, A., Farhoudi-Moghaddam, A. A., & Ketabi, G. R. (1990). Aetiological studies of diarrhoeal diseases in infants and young children in Iran. *Journal of Tropical Medicine and Hygiene*, *93*(1), 22–27.
Kaufmann, M., Zweifel, C., Blanco, M., Blanco, J. E., Blanco, J., Beutin, L., & Stephan, R. (2006). *Escherichia coli* O157 and non-O157 Shiga toxin-producing *Escherichia coli* in fecal samples of finished pigs at slaughter in Switzerland. *Journal of Food Protection*, *69*(2), 260–266.
Kenny, B. (2002). Mechanism of action of EPEC type III effector molecules. *International Journal of Medical Microbiology*, *291*(6–7), 469–477.
Kenny, B., DeVinney, R., Stein, M., Reinscheid, D. J., Frey, E. A., & Finlay, B. B. (1997). Enteropathogenic *E. coli* (EPEC) transfers its receptor for intimate adherence into mammalian cells. *Cell*, *91*(4), 511–520.
Khan, M. M., Iqbal, J., Ghafoor, A., & Burney, M. I. (1988). Aetiologic agents of diarrhoeal diseases in hospitalised children in Rawalpindi, Pakistan. *Journal of Diarrhoeal Diseases Research*, *6*(3–4), 228–231.
Kim, Y. J., Kim, J. H., Hur, J., & Lee, J. H. (2010). Isolation of *Escherichia coli* from piglets in South Korea with diarrhea and characteristics of the virulence genes. *Canadian Journal of Veterinary Research*, *74*(*1*), 59–64.
Knutton, S., Phillips, A. D., Smith, H. R., Gross, R. J., Shaw, R., Watson, P., & Price, E. (1991). Screening for enteropathogenic *Escherichia coli* in infants with diarrhea by the fluorescent-actin staining test. *Infection and Immunity*, *59*(1), 365–371.
Levine, M. M., & Edelman, R. (1984). Enteropathogenic *Escherichia coli* of classic serotypes associated with infant diarrhea: Epidemiology and pathogenesis. *Epidemiologic Reviews*, *6*(1), 31–51.
McDaniel, T. K., Jarvis, K. G., Donnenberg, M. S., & Kaper, J. B. (1995). A genetic locus of enterocyte effacement conserved among diverse enterobacterial pathogens. *Proceedings of the National Academy of Sciences USA*, *92*(5), 1664–1668.
McDaniel, T. K., & Kaper, J. B. (1997). A cloned pathogenicity island from enteropathogenic *Escherichia coli* confers the attaching and effacing phenotype on E. coli K-12. *Molecular Microbiology*, *23*(2), 399–407.
McGuckin, M. A., Linden, S. K., Sutton, P., & Florin, T. H. (2011). Mucin dynamics and enteric pathogens. *Nature Reviews Microbiology*, *9*(4), 265–278. http://dx.doi.org/10.1038/nrmicro2538.
Mellies, J. L., Elliott, S. J., Sperandio, V., Donnenberg, M. S., & Kaper, J. B. (1999). The Per regulon of enteropathogenic *Escherichia coli*: Identification of a regulatory cascade and a novel transcriptional activator, the locus of enterocyte effacement (LEE)-encoded regulator (Ler). *Molecular Microbiology*, *33*(2), 296–306.
Mubashir, M., Khan, A., Baqai, R., Iqbal, J., Ghafoor, A., Zuberi, S., & Burney, M. I. (1990). Causative agents of acute diarrhoea in the first 3 years of life: hospital-based study. *Journal of Gastroenterology and Hepatology*, *5*(3), 264–270.
Nataro, J. P., & Kaper, J. B. (1998). Diarrheagenic *Escherichia coli*. *Clinical Microbiology Reviews*, *11*(1), 142–201.
Pokusaeva, K., Johnson, C., Luk, B., Uribe, G., Fu, Y., Oezguen, N., & Versalovic, J. (2016). GABA-producing Bifidobacterium dentium modulates visceral sensitivity in the intestine. *Neurogastroenterology & Motility*. http://dx.doi.org/10.1111/nmo.12904.
Richard, H. T., & Foster, J. W. (2003). Acid resistance in *Escherichia coli*. *Advances in Applied Microbiology*, *52*, 167–186.
Rowe, B. (1979). The role of *Escherichia coli* in gastroenteritis. *Clinical Gastroenterology*, *8*(3), 625–644.
Sambrook, J., & Russell, D. W. (2006). The Hanahan method for Preparation and transformation of competent *E. coli*: High-efficiencyTransformation. *CSH Protocols*, *2006*(1), http://dx.doi.org/10.1101/pdb.prot3942.
Shaw, R. K., Cleary, J., Murphy, M. S., Frankel, G., & Knutton, S. (2005). Interaction of enteropathogenic *Escherichia coli* with human intestinal mucosa: Role of effector proteins in brush border remodeling and formation of attaching and effacing lesions. *Infection and Immunity*, *73*(2), 1243–1251. http://dx.doi.org/10.1128/IAI.73.2.1243-1251.2005.
Shin, S., Castanie-Cornet, M. P., Foster, J. W., Crawford, J. A., Brinkley, C., & Kaper, J. B. (2001). An activator of glutamate decarboxylase genes regulates the expression of enteropathogenic *Escherichia coli* virulence genes through control of the plasmid-encoded regulator, Per. *Molecular Microbiology*, *41*(5), 1133–1150.
Shukuya, R., & Schwert, G. W. (1960). Glutamic acid decarboxylase. I. Isolation procedures and properties of the enzyme. *Journal of Biological Chemistry*, *235*, 1649–1652.
Smith, D. K., Kassam, T., Singh, B., & Elliott, J. F. (1992). *Escherichia coli* has two homologous glutamate decarboxylase genes that map to distinct loci. *Journal of Bacteriology*, *174*(18), 5820–5826.
Trabulsi, L. R., Keller, R., & Tardelli Gomes, T. A. (2002). Typical and atypical enteropathogenic *Escherichia coli*. *Emerging Infectious Diseases*, *8*(5), 508–513. http://dx.doi.org/10.3201/eid0805.010385.
Tramonti, A., Visca, P., De Canio, M., Falconi, M., & De Biase, D. (2002). Functional characterization and regulation of *gadX*, a gene encoding an AraC/XylS-like transcriptional activator of the *Escherichia coli* glutamic acid decarboxylase system. *Journal of Bacteriology*, *184*(10), 2603–2613.
Tsiloyiannis, V. K., Kyriakis, S. C., Vlemmas, J., & Sarris, K. (2001). The effect of organic acids on the control of porcine post-weaning diarrhoea. *Research in Veterinary Science*, *70*(3), 287–293. http://dx.doi.org/10.1053/rvsc.2001.0476.
Yokoigawa, K., Takikawa, A., Okubo, Y., & Umesako, S. (2003). Acid tolerance and gad mRNA levels of *Escherichia coli* O157:H7 grown in foods. *International Journal of Food Microbiology*, *82*(3), 203–211.
Zhou, K., Zhou, L., Lim, Q., Zou, R., Stephanopoulos, G., & Too, H. P. (2011). Novel reference genes for quantifying transcriptional responses of *Escherichia coli* to protein overexpression by quantitative PCR. *BMC Molecular Biology*, *12*, 18. http://dx.doi.org/10.1186/1471-2199-12-18.