Genetic analysis of *uidA* expression in enterohaemorrhagic *Escherichia coli* serotype O157:H7

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Isolates of enterohaemorrhagic *Escherichia coli* serotype O157:H7 do not exhibit β -glucuronidase (GUD) activity; however, they carry nucleotide sequences for the *uidA* gene that encodes the GUD enzyme. Polymerase chain reaction analysis using *uidA*-specific primers confirmed that a genetic region homologous in size to the *E. coli uidA* structural gene, including the regulatory region, was present in *E. coli* O157:H7 isolates. DNA sequencing analysis of the regulatory region and the 5' terminus of the *uidA* gene of *E. coli* O157:H7 showed a base substitution in the putative –10 promoter region and at 93 bases downstream from the initiation codon. Neither base alteration, however, appeared to affect *uidA* allele gene expression in the O157:H7 isolates. Immunoblotting of cell extracts with an anti-GUD antibody showed that *E. coli* O157:H7 isolates produced an antibody-reactive protein that was homologous in size to *E. coli* GUD, but no GUD activity was observed in cell-free extracts of these isolates. These results suggest that the antibody-reactive protein produced by *E. coli* O157:H7 may be an inactive GUD enzyme. Sequencing of the *uidA* structural gene in O157:H7 showed the presence of 18 additional nucleotide base substitutions, but only six altered the amino acid sequence. Also, there were two frame shift mutations, 18 bases apart, that altered the sequence of six consecutive amino acids. These genetic alterations in the *uidA* structural gene of O157:H7 may account for the absence of GUD activity in this serotype.

Keywords: *Escherichia coli* O157:H7, *uidA* expression, β -glucuronidase

INTRODUCTION

The MUG assay used to identify *Escherichia coli* in food and water is based on the activity of β -glucuronidase (GUD) on 4-methylumbelliferyl β -D-glucuronide (MUG) to release 4-methylumbelliferone, which is fluorogenic under longwave UV light (Feng & Hartman, 1982). About 94% of *E. coli* strains, including many pathogenic serogroups, produce GUD and are positive with the MUG assay (Feng & Hartman, 1982; Hartman, 1989). Exceptions, however, are isolates of enterohaemorrhagic *E. coli* of serotype O157:H7, which are consistently negative with the MUG assay. This characteristic has therefore been used to distinguish this pathogenic sero-

group from other *E. coli* isolates (Doyle & Schoeni, 1984). Isolates of *E. coli* O157:H7 have been implicated in major foodborne disease outbreaks and are the predominant cause of haemorrhagic colitis and haemolytic uraemic syndrome (HUS) (Griffin & Tauxe, 1991).

Absence of GUD activity in *E. coli* O157:H7 is not due to the lack of enzyme induction (Feng *et al.*, 1990a). Some *E. coli* isolates from the human intestine may also be MUG[–] as a result of catabolite repression by lactose (Chang *et al.*, 1989); shifting these isolates to a lactose-free minimal medium restores GUD activity (Feng *et al.*, 1990a). Isolates of *E. coli* serotype O157:H7, however, remain MUG[–] in lactose-free minimal medium (Feng *et al.*, 1990a). Incubation temperatures can also effect *E. coli* gene expression. For instance, the *cel* operon, which encodes enzymes for the utilization of cellobiose, is cryptic at 37 °C but is expressed at 48 and 54 °C (Droffner & Yamamoto, 1992). The elevated temperature is thought

Abbreviations: GUD, β -glucuronidase; MUG, 4-methylumbelliferyl β -D-glucuronide; CAT, chloramphenicol acetyltransferase; HUS, haemolytic uraemic syndrome.

Table 1. PCR primers used to amplify the specific fragments from the *uidA* gene of *E. coli* and *E. coli* O157:H7 isolates

Fragment	Expected size (bp)	Primer	Sequence
1	513	PF-20	5'-TGCGATCTATATCACGCTG-3'
		PF-19	5'-TGATGCTCCATCACTTCCT-3'
2	326	PF-21	5'-ACGTCCTGTAGAAACCCCA-3'
		PF-19	Same as above
3	415	PF-15	5'-TACAGCGAAGAGGCAGTC-3'
		PF-22	5'-GCAGCCCGGCTAACGTAT-3'
4	838	PF-25	5'-AGGAAGTGATGGAGCATCA-3'
		PF-26	5'-GACTGCCTCTTCGCTGTA-3'
5	2100	PF-20	Same as above
		PF-28	5'-AGCGACGTCGGTGTAGTAACTC-3'

to alter DNA superhelicity, enabling transcription of the operon (Droffner & Yamamoto, 1992). Incubation temperatures ranging from 4 to 54 °C had no effect on the expression of GUD activity in the *E. coli* O157:H7 serotype (P. Feng, unpublished data). These results suggest that the absence of GUD activity in *E. coli* O157:H7 may not be attributable to obvious physiological factors.

GUD is an inducible enzyme encoded by the *uidA* gene in *E. coli* (Blanco *et al.*, 1985; Novel & Novel, 1976). Cleavage of the *uidA* structural gene with the *Hin*I restriction enzyme produces three fragments, the largest of which is 900 base pairs (bp) in length (Jefferson *et al.*, 1986). Feng *et al.* (1991) showed that almost all strains of *E. coli*, including isolates of serotype O157:H7, hybridize with an oligonucleotide probe specific for the 900 bp *Hin*I fragment. Southern hybridization analyses confirmed the presence of the probe-reactive 900 bp *Hin*I fragment in the *E. coli* O157:H7 genome; therefore this serogroup, despite its MUG⁻ phenotype, carried nucleotide sequences for the *uidA* gene (Feng *et al.*, 1991).

In this study, the *uidA* gene sequences of *E. coli* O157:H7 isolates were examined to determine whether genetic factors may account for the absence of GUD activity in this serogroup.

METHODS

Bacterial strains. Most *E. coli* and enterohaemorrhagic *E. coli* serotype O157:H7 isolates used in this study were environmental or clinical specimens obtained from P. A. Hartman, Iowa State University, Ames, USA. The MUG⁺ O157:H7 isolates (Gunzer *et al.*, 1992) were kindly provided by H. Karch, University of Wurzburg, Germany. Other isolates were from the collection of the Division of Microbiological Studies, Food and Drug Administration, Washington, DC, USA.

Polymerase chain reaction. PCR primers were synthesized

with an ABI380B automated synthesizer (Applied Biosystems) based on published sequences of the *E. coli uidA* gene (Jefferson *et al.*, 1986). The first four sets of primers (Table 1), used to amplify fragments 1–4, were directed to various regions and spanned almost the entire *uidA* structural gene, including the putative regulatory region (Fig. 1). Chromosomal DNA from various MUG⁺ *E. coli* and MUG⁻ *E. coli* O157:H7 isolates were used as templates. PCR amplification was conducted for 35 cycles using established protocols (Hill *et al.*, 1991), except that 56 °C was used as the annealing temperature. PCR-amplified DNA fragments were examined in a 1% (w/v) agarose gel in Tris/borate buffer (Maniatis *et al.*, 1982).

The last set of primers shown in Table 1 was used to amplify fragment 5, which carries the entire *uidA* gene of the O157:H7 serotype. Primer PF-20 maps at position 120 on the *uidA* sequence map (Jefferson *et al.*, 1986) and is about 180 bases upstream from the initiation codon. Primer PF-28 maps at position 2220 on the 3' terminus to bracket the *uidA* structural gene, which is about 1.8 kb in size. Chromosomal DNA from E260, an O157:H7 isolate, was used as template. PCR amplification was done as described above, but used a 50 °C annealing temperature for 30 cycles.

DNA sequencing. PCR fragments 1 and 1', containing the regulatory region and the 5' terminus of the *uidA* gene of *E. coli* and *E. coli* O157:H7, respectively, and fragment 5, which

Fig. 1. *Hin*I restriction map of *E. coli uidA* gene and the position of the four specific PCR fragments. The *uidA* structural gene is 1.8 kb long and is outlined in bold.

carried the entire *uidA* structural gene from O157:H7, were sequenced using a 2.0 Sequenase kit (United States Biochemical) and [35 S]dATP α S (Amersham). About 2 μ g PCR-amplified DNA was mixed with 3 ng primer and 2 μ l 5 $\times$ reaction buffer and brought to a final reaction volume of 10 μ l. The mixture was boiled for 5 min, placed on ice for 1 min to allow primers to anneal to template DNA, and sequenced according to the protocol described by the manufacturer. Sequenced mixtures were fractionated on 6% (w/v) polyacrylamide/7 M urea gels at 60 W, dried, and exposed to X-ray film. DNA sequences were computer-analysed, using programs from the University of Wisconsin Genetics Computer Group (Devereux *et al.*, 1984).

Cloning into expression vectors. DNA fragments carrying either the *uidA* promoter region of a MUG⁺ *E. coli* or a MUG⁻ *E. coli* O157:H7 isolate were produced by PCR amplification. The primers used were modified to include a *Bam*HI and a *Hind*III site at the 5' and 3' end, respectively, of the PCR-amplified product. PCR products were digested with both restriction enzymes and cloned into pKK232-8 (Pharmacia), a promoter selection vector that carries ampicillin resistance and a promoterless chloramphenicol acetyltransferase (CAT) gene (Brosius, 1984). Vectors ligated with inserts were transformed into *E. coli* DH5 α (Maniatis *et al.*, 1982) and selected for ampicillin resistance on Luria-Bertani (LB) agar containing 30 μ g ampicillin ml⁻¹. Transformants were screened for CAT expression on LB agar containing 20, 50 or 100 μ g chloramphenicol ml⁻¹.

SDS-PAGE and Western blotting. Isolates grown at 37 °C overnight in tryptic soy broth (TSB) were pelleted by centrifugation and resuspended in sample buffer (0.12 M Tris, pH 6.8; 5% (w/v) SDS; 2% (v/v) 2-mercaptoethanol; 25% (v/v) glycerol; 0.25% bromophenol blue). Cells were lysed by heating for 5 min in a boiling water bath and analysed by SDS-PAGE using a 3% stacking gel and a 10% separating gel as described previously (Feng *et al.*, 1990).

Proteins fractionated on SDS-PAGE were electrophoretically transferred onto nitrocellulose paper by Western blotting and probed by a described protocol (Feng *et al.*, 1990b) with an affinity-purified rabbit antibody to GUD obtained from M. Wolcott and P. A. Hartman, Iowa State University, Ames, USA. Antigen-bound antibody on the nitrocellulose blot was detected by using goat anti-rabbit antibody conjugated with horseradish peroxidase (Kirkegaard & Perry Laboratories) and with the precipitable peroxidase substrate 4-chloro-1-naphthol (BioRad) (Feng *et al.*, 1990b).

GUD activity of cell-free extracts. Bacterial isolates were grown at 37 °C overnight in TSB with 100 μ g MUG (Hach) ml⁻¹ to induce GUD production. The presence of enzyme activity was confirmed by examining the medium for bluish fluorescence under longwave UV light. Cells were harvested by centrifugation, washed and resuspended in cold 0.075 M potassium phosphate buffer, pH 6.8, with 0.1% bovine serum albumin. Cells were lysed by sonication in an ice-water-bath and the cell debris was removed by centrifugation. Protein concentration of cell-free preparations was determined by absorbance at 280 nm and adjusted with buffer to equivalent protein concentrations.

The assay procedure described by Sigma Chemicals was used to determine the GUD activity of cell-free extracts. *E. coli* β -glucuronidase (EC 3.2.1.31) (Sigma) was used as a positive control. GUD activity, measured by the enzymic cleavage of

phenolphthalein glucuronide, was quantified spectrophotometrically at 540 nm.

RESULTS

PCR of *uidA* gene in *E. coli* O157:H7

The *uidA* gene of *E. coli* and *E. coli* O157:H7 were compared by PCR analysis, using four sets of primers directed to the regulatory and structural regions of the *E. coli uidA* gene. Agarose gel electrophoresis of PCR products showed that DNA fragments identical in size were amplified from the template DNA of a MUG⁺ *E. coli* isolate and a MUG⁻ *E. coli* O157:H7 isolate (Fig. 2). Analysis of several additional isolates confirmed these results. The fragment sizes, estimated from the 123 bp molecular size ladder, were 520, 320, 420 and 840 bp for PCR fragments 1, 2, 3 and 4, respectively. These sizes closely approximate the predicted sizes (Table 1) for these fragments based on the primer and known *uidA* gene sequences (Jefferson *et al.*, 1986).

Sequencing and analysis of the regulatory and 5' region

Several PCR fragments 1 and 1', amplified from various MUG⁺ *E. coli* and MUG⁻ *E. coli* O157:H7 isolates, respectively, were sequenced and compared. This 520 bp fragment included the regulatory region and the 5' terminus of the *uidA* structural gene (Fig. 1). Sequencing of about 310 bases, starting at 130 bases upstream and extending to 180 bases downstream of the initiation codon, showed that the nucleotide base sequences of

Fig. 2. Agarose gel electrophoresis of the four PCR fragments amplified from the DNA of: E227, a MUG⁺ *E. coli* (1, 2, 3, 4) and E260, a MUG⁻ *E. coli* O157:H7 isolate (1', 2', 3', 4'). The size of the fragments shown was estimated by using the 123 bp molecular size ladder (GIBCO-BRL).

Fig. 3. Nucleotide sequence of the regulatory region and the 5' terminus of the *uidA* structural gene from *E. coli* and *E. coli* O157:H7. The putative -10 promoter region is underlined and the putative ribosome binding site is outlined. Consensus sequences are indicated by asterisks, and differences in nucleotide or amino acid sequences are marked in bold.

Fig. 4. Immunoblot of bacterial cell extracts using an antibody to GUD. Lanes: 1 and 2, *Yersinia* spp.; 3, *Pseudomonas* sp.; 4 and 5, MUG⁺ *E. coli*; 6–11, isolates of *E. coli* O157:H7. The position and sizes of protein molecular mass standards (Sigma) are marked.

fragment 1, amplified from *E. coli*, correlated with the published sequences for the *uidA* gene of *E. coli* K12 (Jefferson *et al.*, 1986). However, the nucleotide sequences of fragment 1', amplified from *E. coli* O157:H7 isolates, contained two base alterations (Fig. 3). There was a T for an A substitution in the putative -10 promoter region identified by previous reports (Blanco *et al.*, 1985; Jefferson *et al.*, 1986), and a G for a T base change 93 bases downstream from the initiation codon, which altered the amino acid residue from aspartate to glutamate (Fig. 3).

To determine if the base substitution in the putative -10 promoter of the *uidA* allele of *E. coli* O157:H7 isolates affected gene expression, PCR fragments carrying the *uidA* promoter region of *E. coli* and *E. coli* O157:H7, respectively, were cloned into pKK232-8 and examined for the expression of CAT genes. Transformants carrying the *E. coli* promoter grew well overnight on LB medium with 20–100 µg chloramphenicol ml⁻¹, whereas trans-

Table 2. Activity of GUD in cell-free extracts of *E. coli*, *E. coli* O157:H7 and isolates of other species

Protein concentrations were adjusted on the basis of absorbance at 280 nm.

Bacterium	Isolate	MUG reaction	GUD activity (A_{540})
<i>E. coli</i>	E38	+	0.022
	E175	+	0.143
	E227	+	0.049
	E258	+	0.025
<i>E. coli</i> O157:H7	E121	—	0.003
	E260	—	0.001
	E264	—	0.001
	E265	—	0.005
	E267	—	0.001
	E270	—	0.005
<i>Pseudomonas</i>	P169	—	0.002
<i>Citrobacter</i>	C200	—	0.001
<i>Escherichia</i> <i>hermannii</i>	E207	—	0.001

formants carrying the *E. coli* O157:H7 promoter required 2 d incubation and grew only on medium containing 20 µg chloramphenicol ml⁻¹.

Presence of GUD-like proteins in *E. coli* O157:H7

Immunoblotting of cell extracts with an anti-GUD antibody showed an antibody-reactive protein of identical size in isolates of MUG⁺ *E. coli* (Fig. 4, lanes 4 and 5) and MUG⁻ *E. coli* O157:H7 (Fig. 4, lanes 6–11). The antibody-reactive protein was estimated to be 64 kDa, which approximated the 68 kDa size reported for a subunit of *E. coli* GUD (Jefferson *et al.*, 1986). No antibody-reactive protein was found in the isolates of *Yersinia* or *Pseudomonas* species used as negative controls (Fig. 4, lanes 1–3). All extracts of *E. coli* and O157:H7 isolates also showed a faint antibody-reactive band of higher molecular mass that was not observed in the negative controls. The appearance of such a band cannot be explained, but it may contain aggregates of GUD protein.

GUD activity of cell-free extracts

Isolates of *E. coli*, *E. coli* O157:H7 and other species were cultured in TSB with 100 µg of MUG reagent ml⁻¹ and examined under longwave UV light. Only *E. coli* isolates other than O157:H7 were MUG⁺, displaying the bluish fluorescence due to cleavage of MUG reagent by GUD. Cell-free extracts of *E. coli* showed GUD activities ranging from an absorbance (A_{540}) of 0.022 to 0.143 (Table 2). The six isolates of *E. coli* O157:H7 examined did not show fluorescence or cell-free enzyme activity. The A_{540}

Table 3. Nucleotide base substitutions in the *uidA* structural gene of *E. coli* serotype O157:H7 that effected changes in the amino acid sequence

Position	Bacterium	Codon	Amino acid
457	<i>E. coli</i> *	AAC	Asp
	<i>E. coli</i>	ACC	Thr
	O157:H7		
750	<i>E. coli</i>	TGC	Cys
	<i>E. coli</i>	TAC	Tyr
	O157:H7		
775	<i>E. coli</i>	CAG	Gln
	<i>E. coli</i>	CGG	Arg
	O157:H7		
834	<i>E. coli</i>	CAA	Gln
	<i>E. coli</i>	GAA	Glu
	O157:H7		
854	<i>E. coli</i>	CAC	His
	<i>E. coli</i>	CAG	Gln
	O157:H7		
918	<i>E. coli</i>	GAT	Asp
	<i>E. coli</i>	CAT	His
	O157:H7		

* Based on *uidA* sequence for *E. coli* K12 published by Jefferson *et al.* (1986).

sequences were essentially identical, except for 18 base changes. Those at the following positions from the initial ATG start codon did not alter the amino acid sequence: 500, 689, 800, 848, 881, 941, 1058, 1157, 1307, 1409, 1703 and 1715. However, the remaining six base substitutions resulted in changes in the amino acid sequence (Table 3). Furthermore, two frame shift mutations, affecting the sequence of six consecutive amino acids, resulted from the insertion of a G at 1257 which was corrected downstream by the insertion of a G and a C at 1276 (Fig. 5).

DISCUSSION

The *uidA* gene sequences of *E. coli* O157:H7 isolates were studied to investigate the absence of GUD enzyme activity. PCR analysis using four sets of primers that spanned the *uidA* structural gene of *E. coli*, including the regulatory region, showed that DNA fragments of identical sizes were amplified from the template DNA of MUG⁺ *E. coli* and MUG⁻ *E. coli* O157:H7 isolates. The specific amplification of similarly sized fragments from both isolates suggests that the *uidA* genes of the *E. coli* O157:H7 serotype and other *E. coli* isolates are homologous and that the entire *uidA* gene, including the regulatory region, is present in the *E. coli* O157:H7 serotype. The absence of GUD activity in *E. coli* O157:H7 therefore does not appear to be due to large genetic deletions or to the absence of complete *uidA* structural gene sequences.

Partial sequencing of the regulatory region and the 5' terminus of the *uidA* gene from several *E. coli* O157:H7 isolates revealed the presence of a base substitution in the putative -10 promoter region and another 93 bases downstream from the initiation codon. To determine if the base alteration in the -10 region affected promoter function, a PCR-amplified fragment carrying the base-altered promoter of the *uidA* gene from *E. coli* O157:H7 was cloned into an expression vector. Analysis of transformants showed low levels of gene expression (CAT activity), indicating that, although the base-altered *uidA* promoter of O157:H7 was not as efficient as the *E. coli* promoter, the base change in the -10 region did not inhibit promoter function. A review of promoter sequences showed that other *E. coli* genes also have -10 promoter region sequences similar to that observed for the *uidA* allele of *E. coli* O157:H7 isolates (Hawley & McClure, 1983). The second base substitution, which was in the *uidA* structural gene, was found to be highly conserved among isolates of the *E. coli* O157:H7 serotype (Feng, 1993). Although this base change also altered the amino acid sequence, it was not considered critical because it did not alter the net charge of the protein molecule. We concluded, therefore, that neither of these base substitutions in the 5' region was responsible for the absence of GUD activity in *E. coli* O157:H7.

The above conclusions were supported by our subsequent findings from the genetic analysis of *E. coli* O157:H7 isolates sent to us by H. Karch. Those clinical isolates, obtained from HUS patients, carried the Shiga-like toxin

Fig. 5. Partial sequence map of the *uidA* structural gene of serotype O157:H7 compared with that of *E. coli* K12 published by Jefferson *et al.* (1986). The insertion mutations in the O157:H7 sequence are shown in larger letters and the codons for the amino acids are underlined, with the amino acids indicated in single letter code above and below the nucleotide sequence.

values of cell-free extracts of *E. coli* O157:H7 isolates were below 0.005, or about 4- to 28-fold lower than those of MUG⁺ *E. coli* isolates (Table 2). Isolates of other microbial species were negative with the MUG assay and the A_{540} values of their cell-free extracts were 0.002 or less.

Sequencing of the *uidA* structural gene in *E. coli* O157:H7

PCR fragment 5, which carried the entire *uidA* gene of O157:H7, was sequenced and compared with the *uidA* sequence for *E. coli* K12 (Jefferson *et al.*, 1986). The two

gene and were serotyped as *E. coli* O157:H⁻ because they were nonmotile (Gunzer *et al.*, 1992). They were also unusual phenotypically: they fermented sorbitol, and because they exhibited GUD activity, were MUG⁺ (Gunzer *et al.*, 1992). PCR analysis, using *uidA* gene-specific primers (Table 1), verified that a *uidA* gene, homologous in size to the *E. coli* gene, was present in these isolates (data not shown). Furthermore, sequencing of the regulatory and 5' structural gene regions showed that the *uidA* gene of these *E. coli* O157:H⁻ isolates contained base changes at the -10 promoter region and at +93 that were identical to those in the *E. coli* O157:H7 isolates. The presence of identical base substitutions in both MUG⁺ *E. coli* O157:H⁻ and MUG⁻ *E. coli* O157:H7 isolates is consistent with our conclusions that neither base alteration has an effect on genetic expression and therefore does not account for the absence of GUD activity in *E. coli* O157:H7 isolates.

Because the *uidA* allele gene in the *E. coli* O157:H7 isolates appears to carry the entire structural gene and has a functional promoter, we would expect the gene to be expressed and the isolates to produce GUD. Immunoblotting of cell extracts using an antibody to GUD confirmed that an immunologically related protein identical in size to that of *E. coli* was present in the *E. coli* O157:H7 isolates. The genetic expression of the *uidA* allele, as evidenced by the production of a protein that is antigenically similar to *E. coli* GUD, supports our conclusion that the promoter sequences are functional and implies that the absence of GUD activity in *E. coli* O157:H7 may be attributable to other factors. Perhaps an active enzyme is not transported to the bacterial surface, or perhaps an inactive GUD is being produced. These hypotheses were tested by assaying ruptured cells for GUD activity. Analysis of cell-free extracts of *E. coli*, *E. coli* O157:H7 and isolates of other species showed GUD activity only with isolates of *E. coli* other than *E. coli* O157:H7. The absence of enzyme activity in the *E. coli* O157:H7 isolates, despite their production of a protein antigenically related to GUD, suggests that they may be producing an inactive GUD.

Kaspar *et al.* (1987) observed that cell lysates of some MUG⁻ *E. coli* will coagglutinate with an antibody to GUD; therefore they are producing GUD-related proteins that do not exhibit enzyme activity. Messer & Melchers (1970) isolated *E. coli* mutants that produced a defective β -galactosidase; however, enzyme activity was restored by antibody treatment. Presumably, antibody binding altered the structural conformation of the protein sufficiently to activate β -galactosidase activity. Treatment of cell-free extracts of *E. coli* O157:H7 isolates with anti-GUD antibody did not activate GUD (data not shown); therefore it does not appear to be analogous to the β -galactosidase mutations described by Messer & Melchers (1970). However, comparison of the sequence of the *uidA* allele of *E. coli* O157:H7 to that of *E. coli* K12 (Jefferson *et al.*, 1986) showed the presence of other genetic alterations that could have affected GUD activity. The frameshift mutations, or the base substitutions that resulted in the replacement of cysteine or that altered the

net charge of the protein molecule, may all affect GUD activity. We are conducting further analysis to determine whether some of these genetic alterations may be accountable for the absence of GUD activity in *E. coli* isolates of the O157:H7 serotype.

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