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Short Communication

Molecular investigation of tRNA genes integrity and its relation to pathogenicity islands in Shiga toxin-producing *Escherichia coli* (STEC) strains

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Abstract

tRNA genes are known target sites for the integration of pathogenicity islands (PAI) and other genetic elements, such as bacteriophages, into bacterial genome. In most STEC (Shiga toxin-producing *Escherichia coli*), the PAI called LEE (locus of enterocyte effacement) is related to bacterial virulence and is mostly associated to the tRNA genes *selC* and *pheU*. In this work, we first investigated the relationship of LEE with tRNA genes *selC* and *pheU* in 43 STEC strains. We found that 28 strains (65%) had a disrupted *selC* and/or *pheU*. Three of these strains (637/1, 650/5 and 654/3) were chosen to be submitted to a RAPD-PCR technique modified by the introduction of specific primers (corresponding to the 5'end of genes *selC* and *pheU*) into the reaction, which we called "anchored RAPD-PCR". The PCR fragments obtained were transferred onto membranes, and those fragments which hybridized to *selC* and *pheU* probes were isolated. One of these fragments from strain 637/1 was partially sequenced. An 85-nucleotide sequence was found to be similar to the *cfxA2* gene that encodes a beta-lactamase and is part of transposon Tn4555, a pathogenicity island originally integrated into the *Bacteroides* genome.

Key words: pathogenicity islands, tRNA, STEC, RAPD-PCR, *E.coli*.

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Pathogenicity islands (PAI) are extensive clusters of virulence genes present in pathogenic bacteria, which are horizontally transferred among bacterial species and are acquired as plasmids, transposons and bacteriophages (Carniel *et al.*, 1996; Waldor and Mekalanos, 1996). This fact has an enormous importance in bacterial evolution, since it may transform a non-pathogenic strain into a pathogenic form in a single event. PAI are found in pathogenic strains, but rarely in non-pathogenic ones (Hacker *et al.*, 1997). These genetic elements have been described in bacteria such as *Escherichia coli* (McDaniel *et al.*, 1995), *Helicobacter pylori* (Censini *et al.*, 1996), *Salmonella spp* (Mills *et al.*, 1995), and *Vibrio cholerae (*Waldor and Mekalanos, 1996; Novais *et al*., 1999; Vicente *et al.*, 1997). PAI usually integrate into tRNA loci in *E.coli* (Inouye *et al.*, 1991), *Pseudomonas* spp (Hayashi *et al.*, 1993) and *Salmonella spp* (Mills *et al*., 1995), and the disruption of these genes is a potential marker for the occurrence of PAI (Hacker *et al*., 1997). Shiga toxin-producing *Escherichia coli* (STEC) colonizes the gastrointestinal tract of bovines and other animals and is mostly transmitted to humans by contaminated undercooked ground beef. The major PAI in STEC, the etiological agent of hemolytic-uremic syndrome (HUS) is the locus of enterocyte effacement (LEE) that encodes a type III secretion system and *E.coli*-secreted proteins, required for the induction of attaching and effacing lesions in intestinal cells (Paton and Paton, 1998).

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Two preferential LEE insertion sites were described in tRNA genes, *selC* (selenocysteine tRNA gene) and *pheU* (phenylalanine tRNA gene) (Sperandio *et al.*, 1998). During the insertion process, PAI interrupt these genes, making them non-functional. The insertion makes tRNA/PAI too large to be amplified, and some authors considered negative PCR results as indicating the presence of PAI inserted into tRNA genes (Sperandio *et al.*, 1998).

In this work, we analyzed the molecular integrity of these two tRNA genes and investigated its relation to PAI in LEE-positive and LEE-negative STEC strains using a

RAPD-PCR technique modified by the inclusion of specific primers in the reaction (which we called "anchored RAPD-PCR").

Bacterial strains

STEC strains of different serotypes were previously isolated from healthy cattle from Rio de Janeiro State, Brazil, and were classified, by the detection of the *eae* gene, into LEE-positive or LEE-negative (Gonzalez *et al*., 2001)

Specific PCR and anchored RAPD-PCR conditions

Blanc-Potard and Groisman (1997) and Sperandio *et al*. (1998) described the primer pairs used to amplify the *selC* gene and the *pheU* gene, respectively. The cycling parameters for specific PCR were: 30 cycles, each cycle consisting of a denaturing step at 94 °C for 1 min, an annealing step at 50 °C for 1 min, and an extension step at 72 °C for 1 min. The components for 50 µL PCR reaction solution were: 100 ng of DNA template, 30 pmoles of each primer, 10 mM Tris-HCL (pH8.3), 50 mM KCL, 3 mM $MgCl_2$, 0.1 mM of each dNTP and 2 units of Taq polymerase (Invitrogen, Carlsbad, CA, USA). Negative controls were included in each experiment. For the anchored RAPD-PCR, the following conditions were used: one cycle of 5 min each at 94 °C, 32 °C, and 72 °C, respectively; then, one cycle of 1 min at 94 °C, 5 min at 32 °C, and 5 min at 72 °C, and finally 43 cycles of 1 min at 94 °C, 1 min at 32 °C, and 2 min at 72 °C each. 12 pmoles of random primers (r1 or r2) and 30 pmoles of specific primers (for the 5'end *selC* or the 5'end *pheU*) were included in each reaction. The components were the same used in the specific reactions: primer r1: 5' GGGTAACGCC 3' and r2: 5'AGAG GGCACA 3'; primer *cfxA1*: 5' TAACATAACCTGAACC TGTC and primer *cfxA2*: 5' TCAGATAGCTTATACG GAAG 3'.

DNA extraction, DNA restriction, electrophoresis conditions and Southern blotting

Genomic DNA was extracted with TRIZOL reagent (Invitrogen), according to the manufacturer's instructions. DNA fragments were extracted from the gel by the use of the Gene Clean kit (Bio 101 Inc). 10 µg of DNA were digested with 10 U of *EcoRI* enzyme (Invitrogen). Digested DNA was submitted to electrophoresis (100 V, 2 h) in 0.8% (w/v) on agarose gel immersed in TBE buffer (90 mM Tris-borate, 2 mM EDTA, pH 8.0), and transferred onto nylon membranes, according to the Southern blotting method (Sambrok *et al.*, 1989).

Hybridization to radioactive probes

DNA fragments including *selC* and *pheU* genes were labeled with α-^{32}P dCTP using the random primer labeling Kit (Amersham Biosciences). Nylon membranes were hybridized to radioactive probes at 42 °C in the presence of 6x SSC, 0.7% SDS and 50% formamide, and the filters were washed twice with 0.3x SSC and 0.1% SDS at 42 °C for 30 min. After hybridization, the filters were exposed to X-OMAT (Kodak) films for 24 h and developed.

Sequencing

The reactions were carried out according to the manufacturer's procedures included in the ABI PRISM^{TM} Dye terminator cycle Sequencing ready reaction kit (Perkin Elmer) and run in 6% acrylamide/urea gel at 60 W, in an ABI 373 automated sequencer (Applied Biosystems, Inc.)

Our results show (Table 1) that 18 (82%) out of 22 LEE-positive strains had a disrupted *selC* and/or *pheU*. Ten (47%) out of 21 LEE-negative strains had one or both genes disrupted. We also found LEE-positive strains with both genes intact, suggesting that LEE was integrated somewhere else in the genome. These findings will be investigated further.

We submitted 14 LEE-positive and negative strains to hybridization to a *selC* probe, after digestion with *EcoRI* enzyme. There is a single copy of the *selC* gene in the bacteria genome (Hou, 1999), and has no *EcoRI* sites. After hybridization, all strains which were PCR-negative for *selC* (lanes 11, 13, 14, and 15) showed two bands (Figure 1). The *selC*$^+$ strains (lanes 2, 4, 6, 7, 8, 10, and 16) showed a single band, suggesting that, in these strains, the *selC* gene is intact, whereas it is disrupted in the others (Figure 1, lanes 11,13,14, and 15). Strains 231/3 and 234/1 will be investigated further, once they are LEE-negative, but have a disrupted *selC* gene.

In order to investigate the genetic elements integrated into *selC* and *pheU* genes, in strains with one or both of these genes disrupted, we conceived a modification of the RAPD-PCR technique. Specific primers which anneal to the 5' end of genes *selC* and *pheU* were included, along with the random primers, in the RAPD-PCR technique herein called "anchored RAPD-PCR". This approach allows the amplification of portions of the disrupted tRNA genes. Amplification products (Figure 2) submitted to hybridization confirmed this assertion (Figures 3 and 4). We were able to identify these fragments following hybridization to specific *pheU* (Figure 3) and *selC* (Figure 4) probes. Three strains [650/5 (*eae*$^+$,*selC*$^-$/*pheU*$^-$), 637/1(*eae*$^-$, *selC*$^+$/*pheU*$^-$), and 654/3 (*eae*$^-$,*selC*$^+$/*pheU*$^-$)] were initially chosen to be submitted to the modified RAPD-PCR technique, once 637/1 and 654/3 are LEE-negative but disrupted at the *pheU* gene, suggesting that a PAI or another genetic element might be inserted at this locus. Strain 650/5 is LEE-positive but has both genes (*selC* and *pheU*) disrupted, and it is possible that, besides LEE, another PAI might be inserted at one of these loci.

Some fragments hybridized to the *pheU* and *selC* probes (Figures 3 and 4). We chose one of these fragments (a 0.75 Kb fragment from strain 637/1, Figure 3) to be isolated and sequenced first. A short preliminary 85-nucleotide sequence (accession number AY191267) was

Table 1 - Characterization of *selC* and *pheU* genotypes and serotypes of 43 LEE- negative and positive strains.

LEE-negative strains	Strain numbers	Serotypes
selC$^+$/*pheU*$^+$		
	418/1	O10:H42
	784	O113:H21
	254/2	O113:H21
	603/1	O113:H21
	397/2	O113:H21
	254/6	O113:H21
	261/1	O113:H21
	226/1	O113:H21
	281/5	O113:H21
	702/1	O113:H21
	415/2	R:H8
selC$^+$/*pheU*$^-$	637/1	O22:H8
	269/1	O113:H21
	654/3	O121:H16
selC$^-$/*pheU*$^+$	420/2	NT:H42/46
	563/3	O74:H42
	565/1	O74:H42
	559/1	O171:H2
	560/1	O171:H2
selC$^-$/*pheU*$^-$	234/1	O141:H21
	231/3	O172:NM
LEE-positive strains		
selC$^+$/*pheU*$^+$	152/1	NT:NT
	296/1	NT:NM
	181/2	O20:H19
	231/1	R:H19
selC$^+$/*pheU*$^-$	300/2	NT:H38
	137/1	NT:NT
	183/1	O153:H25
	173/2	R:H2
	173/1	R:H21
selC$^-$/*pheU*$^+$	324/1	NT:H18
	187/3	O55:H25
	1770/1	O157:H7
	902/1	O157:H7
	691/1	O157:H7
	581/1	O157:H7
	2228/1	O157:H7
	581/1	O157:H7
	1728/1	O157:H7
	137/3	R:H21
	173/3	R:H21
	235/1	R:H26
selC$^-$/*pheU*$^-$	650/5	O165:NM

R: rough; NT: not typeable; NM: non-motile; +: intact gene; -: disrupted gene.

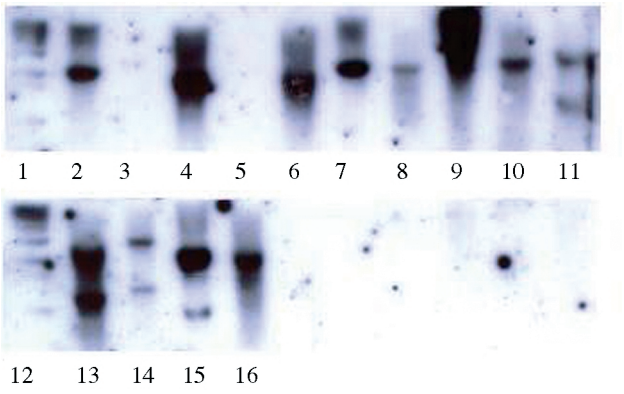
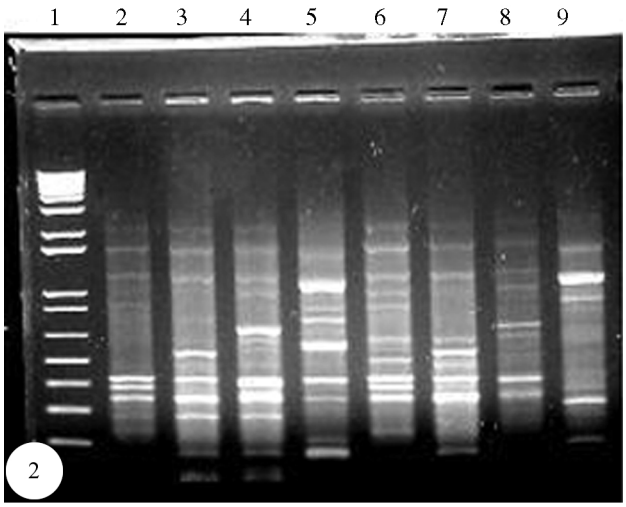


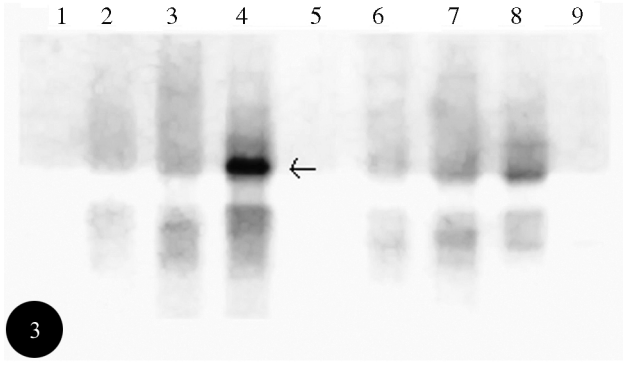
Figure 1 - Hybridization of DNA digested with *EcoRI* from LEE-positive and LEE-negative strains to probe *selC*. Lanes 1 and 12: 1Kb DNA ladder; lanes 2 to 10 and 14 to 16: LEE-positive strains: 137/1, 152/1, 173/1, 173/3, 173/2, 178/1, 183/1, 187/3, 231/1, 235/1, 269/1 and 300/2, respectively. Lanes 11 and 13 - LEE-negative strains 231/3 and 234/1, respectively.

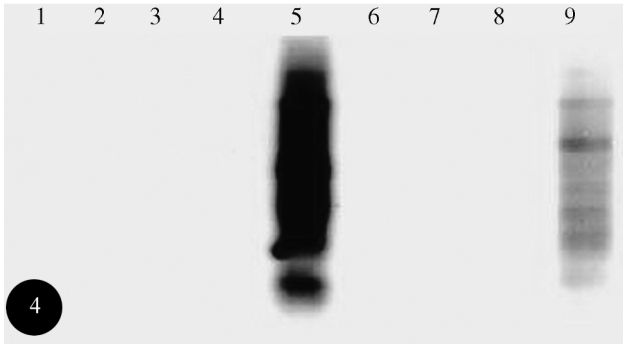
compared to homologous sequences at Genbank, and the alignment showed a similarity of 89% with gene *cfxA2* (data not shown). *cfxA2* is a beta-lactamase gene included in transposon Tn4555 (Smith and Parker, 1998), largely distributed among bacterial species and found very often in *Bacteroides* genera (Tribble *et al.,* 1999). We were also able to amplify a 250 pb fragment from strain 637/1 by using *cfxA1* and *cfxA2* primers which amplify part of the *cfxA2* gene (data not shown). Besides *Bacteroides* genera, the sequence found in strain 637/1 is similar to *cfxA2* sequences also found in six species of *Prevotella* (*P. oralis*, *P. malaninogenica*, *P. intermedia*, *P. denticola*, *P. buccae*, *P. bivia*), both genera commonly found colonizing the bovine gastrointestinal tract. Transposon Tn4555 is in fact a pathogenicity island [Morschhäuser *et al.* (2000) define similar DNA elements, which have genes responsible for antibiotic resistance, as PAI] that is not self-transmissible, but can be transferred between species via conjugation, in the presence of a helper element that supplies most of the necessary conjugation functions (Smith and Parker, 1998). We speculate that transposon Tn4555, which carries the *cfxA2* gene, might be transposed to STEC 637/1 in the bovine intestine, once strain 637/1 was originally isolated from cattle feces. Mizan *et al*. (2002) showed that cattle rumen is a favorable environment for the genetic exchange of plasmids between the indigenous micro biota and resident STEC O157:H7 in the bovine host.

In summary, our results point to *selC* and *pheU* genes as preferential target sites for the integration of LEE into the STEC genome. Eighteen out of 22 LEE-positive strains had either *selC* or *pheU* or both genes disrupted, suggesting that LEE or another genetic element is inserted into one of these loci. Results of hybridization to a *selC* probe allow us to suggest that the *selC* gene may be disrupted in *selC*-negative strains.

The use of anchored RAPD-PCR and hybridization led to the identification of fragments which include genes *selC* and *pheU* and an adjacent region. Sequencing of only 85 nucleotides was sufficient for identifying the nature of a sequence transposed to *pheU* locus in strain 637/1. The results obtained showed that transposon Tn4555, which is in fact a pathogenicity island, first described to belong to the genera *Bacteroides*, had been transposed to the STEC genome.







Figures 2, 3 and 4 - Anchored RAPD-PCR profiles of selected STEC strains (Figure 2) and hybridization against probes *pheU* (Figure 3) and *selC* (Figure 4). Primers used are described in parentheses. Samples of lanes 2, 3, 4, 6, 7 and 8 were amplified with a specific primer annealing to the 5end of *pheU* (913) and a random primer (r1 or r2). Samples of lanes 5 and 9 were amplified with a specific primer annealing to the 5end of *selC* (*selC*-F) and a random primer. **1**. 1 Kb DNA ladder **2**. 650/5 (913/r1) **3**. 654/3(913/r1) **4**. 637/1 (913/r1) **5**. 650/5 (*selC*-F/r1) **6**. 650/5 (913/r2) **7**. 654/3 (913/r2) **8**. 637/3 (913/r2) **9**. 650/5 (*selC*-F/r2). → Indicates the 0.75 Kb band in sample 4 (Figure 3), which was submitted to sequencing.

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