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Original Article

Global effect of CsrA on gene expression in enterohemorrhagic *Escherichia coli* O157:H7

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Abstract

The post-transcriptional regulator CsrA regulates multiple unrelated processes such as central carbon metabolism, motility, biofilm formation and bacterial virulence in different bacteria. However, regulation by CsrA in enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 is still largely unknown. In this study, we performed a detailed analysis of gene expression differences between the EHEC O157:H7 wild-type strain and a corresponding *csrA::kan* mutant using RNA-seq technology. Genes whose expression was affected by CsrA were identified and grouped into different clusters of orthologous group categories. Genes located in the locus of enterocyte effacement (LEE) pathogenicity island were significantly upregulated, whereas expression of flagella-related genes was significantly reduced in the *csrA::kan* mutant. Subsequent bacterial adherence and motility assays showed that inactivation of CsrA in EHEC O157:H7 resulted in a significant increase in bacterial adherence to host epithelial cells, with a concomitant loss of swimming motility on semi-solid agar plates. Furthermore, we also found that CsrA regulates genes not previously identified in other bacterial species, including genes encoding cytochrome oxidases and those required for nitrogen metabolism. Our results provide essential insight into the regulatory function of CsrA.

Keywords: Comparative transcriptome; Bacterial virulence; Motility; Nitrogen metabolism; Cytochrome oxidases

1. Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 is a clinically important food and waterborne pathogen [1]. Infection with EHEC O157:H7 can result in a wide spectrum of clinical manifestations, including asymptomatic infection, mild diarrhea or severe diseases such as hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) [2]. The hallmark of EHEC O157:H7 pathogenesis is attaching and effacing (A/E) lesions, which are characterized by localized destruction of brush border microvilli and intimate attachment of the bacteria to epithelial cells [3]. Upon adhering to host cells, EHEC O157:H7 utilizes a type III secretion (TTSS) to subvert eukaryotic signaling pathways by injecting bacterial effector proteins into the host cell cytoplasm. The genes involved in the formation of A/E lesions and the TTSS are located in a 35-kb pathogenicity island termed the locus of enterocyte effacement (LEE) [4]. In EHEC O157:H7, the LEE region consists of 41 open reading frames (ORFs) organized into at least five operons (LEE1, LEE2, LEE3, LEE5 and LEE4) [5]. The operons LEE1, LEE2 and LEE3 encode a positive regulator, Ler, and the main structural components of the bacterial TTSS [3]. The LEE4 operon mainly encodes additional TTSS structural proteins, translocators and secreted Esp proteins (EspA, EspB, EspD and EspF) [3]. The LEE5 operon contains the *eae* gene, encoding intimin, and the *tir* gene, encoding the translocated intimin receptor (Tir) [3]. Another principal virulence characteristic of EHEC O157:H7 is the production of Shiga toxins (Stxs) [2]. Different isolates of EHEC O157:H7 can express only one or both Stx1 and

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Stx2 [6]. Stx2 is more often associated with HC or HUS in human infections; thus, it is thought to be more toxic than Stx1 [7]. The genes required for the production of Shiga toxins are located on intact or partial genomes of lambda prophages that have been inserted into the bacterial chromosome [5,8].

Bacteria have evolved several efficient regulatory strategies to ensure their survival in response to changes in their growth environment [9]. This is achieved by global transcriptional and post-transcriptional regulators that allow bacteria to coordinately control the expression of genes or operons encoding unrelated functions, and which are scattered throughout the genome [10]. CsrA is a well-documented RNA binding protein that post-transcriptionally controls numerous genes and processes [11,12]. CsrA and its orthologues are involved in regulating carbon metabolism, biofilm formation, motility, quorum sensing, epithelial cell invasion and virulence factor production [13]. CsrA is a homodimeric protein that regulates gene expression post-transcriptionally by binding to sites containing the AGGA/ANGGA motif in the leader segment of transcripts to alter their stability and/or translation [13,14]. For transcripts that are negatively regulated, CsrA binds to a site near the Shine–Dalgarno sequence blocking ribosome binding and facilitating mRNA decay [13,15]. For transcripts that are positively regulated, CsrA binds to a site distant from the Shine–Dalgarno sequence and subsequently increases the translation of certain target mRNAs [13,16]. CsrA activity is modulated by the action of two non-coding small RNAs, CsrB and CsrC, which contain multiple copies of an imperfectly repetitive sequence element that serves as a CsrA binding site [17,18]. In this manner, CsrB and CsrC bind and sequester up to 18 molecules of CsrA, thereby preventing its binding to target mRNAs [17,18]. The expression of CsrB and CsrC is activated by CsrA through the BarA-UvrY two-component regulatory system in *E. coli* [19,20].

Although the mechanism through which CsrA regulates target gene expression is well documented, the full regulatory impact of CsrA on cellular activities in EHEC O157:H7 is not fully understood. In this study, we investigated the global effects of CsrA in EHEC O157:H7 using RNA-seq technology. Genes whose expression was affected by CsrA were identified and grouped into different clusters of orthologous group (COG) categories. Specifically, the expression of LEE genes was significantly upregulated, whereas the transcript levels of flagella-related genes were reduced in the *csrA::kan* mutant compared to those in the EHEC O157:H7 wild-type strain. Subsequent experiments confirmed that the *csrA::kan* mutant was hyper-adherent to host epithelial cells, but was non-motile on semi-solid agar plates. Furthermore, we also found that CsrA is involved in the regulation of genes not previously identified in other bacterial species, including genes required for nitrogen metabolism and those encoding cytochrome oxidases. Our data provide additional information to further understand the regulatory function of CsrA in EHEC O157:H7.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

Bacterial strains, plasmids and primers used in this study are summarized in Supplementary Tables 1 and 2 . Because the deletion of *csrA* is lethal, we inserted *kan* at codon 51 in the *csrA* gene using the λ Red recombinase system [21,22], and the mutant strains were verified by PCR amplification and sequencing. The truncated form of CsrA has reduced RNA affinity and retains partial activity [23]. A complementary strain was constructed by cloning *csrA* into the low-copy plasmid pwsk129, and the resulting constructs were electroporated into EHEC O157:H7 parental strains. A *csrA*-overexpression strain was constructed by cloning *csrA* into the pTRC99a expression vector, and the resulting constructs were then electroporated into EHEC O157:H7 parental strains. All strains were maintained at −80 °C in LB broth with 20% glycerol and were grown overnight at 37 °C in LB broth when required. As required, antibiotics were added at the following final concentrations: ampicillin, 100 μg ml^{-1}; chloramphenicol, 15 μg ml^{-1}; kanamycin, 50 μg ml^{-1}.

2.2. RNA preparation

Cultures of wild-type and *csrA::kan* mutant EHEC O157:H7 were grown in LB broth to an OD_{600} of 0.8, and 3 ml of each mid-log culture was treated with 6 ml of RNAprotect bacterial reagent (Qiagen; #76506). Bacterial cells were lysed with TRIzol® LS Reagent (Invitrogen; #10296-028) and total RNA was purified according to the manufacturer's instructions. The extracted RNA was dissolved in RNase-free water and quantified using a NanoDrop spectrophotometer (Thermo). The concentrations of total RNA from different samples were adjusted to 500 ng/μl, and 20 μg was used for further purification and rRNA elimination. RNA samples were purified using an RNeasy Mini Kit (Qiagen; #74104) and were treated with RNase-Free DNase I (Qiagen; #79254) to eliminate genomic DNA contamination, according to the manufacturer's instructions. Bacterial 23S and 16S rRNA were removed using the MICROB*Express*™ Bacterial mRNA Enrichment Kit (Ambion; #AM1905). The assays were performed using three independent experiments.

2.3. cDNA library construction and sequencing

Double-stranded cDNA was synthetized using hexameric random primers and the SuperScript® Double-Stranded cDNA Synthesis Kit (Invitrogen; #11917-020) according to the manufacturer's instructions. The enriched cDNA was purified and sheared by nebulization at 35 psi for 6 min. The ends of these cDNA fragments were then end-repaired and A-tailed. The A-tailed fragments were ligated to Illumina adapters and then loaded onto a 2% low melting point agarose gel. cDNA fragments between 200 and 250 bp were selected and purified using the Qiaquick gel extraction kit (Qiagen; #28704). After purifi-