Overexpression of an antisense RNA, ArrS, increases the acid resistance of Escherichia coli

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The antisense RNA ArrS is complementary to a sequence in the 5’ untranslated region of the gadE T3 mRNA, the largest transcript of gadE, which encodes a transcriptional activator of the glutamate-dependent acid resistance system in Escherichia coli. Expression of arrS is strongly induced during the stationary growth phase, particularly under acidic conditions, and transcription is dependent on σS and GadE. The aim of the present study was to clarify the role of ArrS in controlling gadE expression by overexpressing arrS in E. coli. The results showed a marked increase in the survival of arrS-overexpressing cells at 2 h after a shift to pH 2.5. This was accompanied by increased expression of gadA, gadBC and gadE. The level of gadE T3 mRNA decreased markedly in response to arrS overexpression, and was accompanied by a marked increase in gadE mRNA T2. T2 mRNA had a monophosphorylated 5’ terminus, which is usually found in cleaved mRNAs, and no T2 mRNA was observed in an RNase III-deficient cell strain. In addition, T2 mRNA was not generated by a P3-deleted gadE–luc translational fusion. These results suggest strongly that T2 mRNA is generated via the processing of T3 mRNA. Moreover, the T2 mRNA, which was abundant in arrS-overexpressing cells, was more stable than T3 mRNA in non-overexpressing cells. These results suggest that overexpression of ArrS positively regulates gadE expression in a post-transcriptional manner.

INTRODUCTION

In contrast to most Enterobacteriaceae, Escherichia coli is resistant to extremely low pH, as demonstrated by its ability to survive at pH 2.5 for at least 2 h (Gorden & Small, 1993). E. coli employs four acid resistance mechanisms to survive in acidic environments, with the glutamate-dependent acid resistance (GDAR) system playing a central role (Castanie-Cornet et al., 1999; Foster, 2004). The critical components of GDAR are the glutamate decarboxylase isozymes GadA and GadB, which replace the ω-carboxyl group of glutamate with a proton, and the putative antiporter GadC, which exchanges γ-amino butyric acid for extracellular glutamate. GadE is an essential transcription factor within the GDAR system, and its expression is increased by acidic stimuli (Tucker et al., 2002). The transcription of gadE itself is controlled by a 798 bp upstream intergenic region and at least nine regulators (Bordi et al., 2003; Gong et al., 2004; Ma et al., 2004; Masuda & Church, 2003; Sayed et al., 2007; Tucker et al., 2003; Zwir et al., 2005). In an acidic environment, three gadE transcripts, T1, T2 and T3, are generated as the products of three possible promoters, P1, P2 and P3, respectively (Hommais et al., 2004; Sayed & Foster, 2009). ArrS is a cis-encoded antisense RNA that binds a target sequence within the unusually long 5’ untranslated region of gadE T3 mRNA (Aiso et al., 2011). Transcription of arrS typically occurs during the stationary growth phase and is dependent upon the factors σS and GadE. Transcription of arrS is increased by acid stimuli. Under acidic conditions, ArrS expression is induced independently of GadE during the exponential growth phase. Although most of the initially identified natural antisense RNAs were plasmid- or phage-encoded, numerous genomic-encoded antisense RNAs have since been identified in eukaryotes, archaea and bacteria using high-throughput RNAomic approaches such as deep RNA sequencing and high-resolution tiling arrays (Faghihi & Wahlestedt, 2009; Georg & Hess, 2011; Thomason & Storz, 2010). Bacterial transcriptomes were once thought to be much simpler than those of eukaryotes due to their smaller non-coding regions; however, the frequent discovery of antisense RNAs has changed this opinion. For example, antisense transcripts were identified in 46 % of annotated ORFs within the 1.67 Mbp genome of Helicobacter pylori strain 26695, which contains 1576 ORFs and was thought to
lack non-coding RNAs (Sharma et al., 2010). Compared with the continuing identification of novel antisense RNAs, evidence for the function of individual antisense RNAs is accumulating slowly. Studies investigating individual antisense RNAs show that they appear to function in a manner similar to trans-acting non-coding RNAs (Sesto et al., 2013). Although arrS is induced by acidic environments, the effect of ArrS on gadE expression and acid resistance in E. coli remains unclear.

Here, we examined both acid resistance and the expression of GDAR genes in arrS-overexpressing E. coli cells, and suggest a possible mechanism that accounts for the increased acid resistance induced by arrS overexpression.

METHODS

Bacterial strains, plasmids and culture conditions. E. coli strains JM109, MG1693 (thyA715, rplK1), SK7622 (MG1693 Arrc38 Kan+) (Arraiano et al., 1988; Babitzke et al., 1993), KT7600 [F' lacI2 lacZ2ΔM15 galK2 galT22 Δ (rnuD–rnuE)1] and JD25278 (KT7600, gad:: mini Tn10) (National Bio Resource Project, National Institute of Genetics, Japan) were used in the study. The recombinant plasmid pBR322-arrS was used to overexpress arrS (Asio et al., 2011). Cells were grown overnight at 37 °C in Luria–Bertani (LB) medium, inoculated into fresh LB medium (OD600 of 0.07) and then cultivated for a further 2 h (exponential growth phase) or 6 h (stationary phase). The pH of cultures grown in LB medium for 6 h was approximately 8.0. Appropriate amounts of HCl were added to the cultures to adjust the pH to 2.5. Alternatively, cells were inoculated into LB medium buffered with 100 mM MES (MES-LB, pH 5.5) and then cultivated for 7 h. Thymine, ampicillin, kanamycin and rifampicin were added to the culture medium as needed at 50, 50, 12.5 and 500 μg ml⁻¹, respectively.

Primers. The names and sequences of primers used in this study are listed in Table S1 (available in the online Supplementary Material).

Acid resistance assay. Cells grown overnight were inoculated into fresh LB medium at an OD600 of 0.07 and cultivated for 2 h (exponential growth phase) or 6 h (stationary phase) at 37 °C. Each culture was mixed with nine volumes of fresh LB medium, adjusted to pH 2.5 with HCl, and then cultivated for a further 2 h. A portion of the culture was serially diluted and plated onto LB plates containing ampicillin. The number of colonies was counted after 18 h of incubation at 37 °C and the percentage of surviving colonies was calculated using the equation \( \frac{[C.I.u. \text{ ml}^{-1}] \times 100}{[C.I.u. \text{ ml}^{-1} \text{ at time 0}]} \). Data were expressed as the mean value from triplicate experiments.

Real-time RT-PCR. Total RNA was treated with RQ1 RNase-free DNase (Promega) in the presence of RNasin (Promega) to obtain DNA-free total RNA. cDNAs were synthesized using 200 units of SuperScript II (Invitrogen) and 2 pmol of RT primer at 43 °C. PCR primers were designed using Primer Express (Life Technologies) and real-time PCR was performed using ABI7500Fast (Life Technologies). Power SYBR Green PCR Master Mix (Life Technologies) and primer pairs gadA386/gadA331, gadB451/gadB398, gadE(−4)/gadE(−70) and 16S711/16S328 were used for amplification. Alternatively, SYBR Premix Dimer Eraser (Takara Bio) and primer pairs gadE(−209)/luc115 and 16S711/16S328 were used. cDNA was diluted 1:50, 1:250 and 1:20000–50000 to amplify gadA, gadBC, gadE and 16S rRNA, respectively. The Δ∆Ct method was used to quantify the RNA.

Construction of gadE–luc translational fusions. A 785 bp PCR fragment containing a region between positions −749 and +16 relative to the translation start point of gadE was amplified using PrimeSTAR HS DNA polymerase (Takara Bio) and the PCR primers Ndel–gadEP3–2 and gadE16–HindIII. The PCR fragment was then blunt-ended, phosphorylated at the 5′-end and ligated into pUC118 HindII/BAP (Takara Bio) to obtain pUC118-gadEP3–T3. An Hpal–HindIII fragment derived from pSP-luc+NF plasmid (Promega), which contains the luc coding region, was cloned into the Small–HindIII sites within the low-copy number plasmid pMW218 (Nippon Gene) to obtain pMW218-luc. An HindIII fragment derived from pUC118–gadEP3–T3 was cloned into the HindIII site in pMW218-luc to obtain pMW218-gadE-luc. pMW218-gadE-luc contains a fragment of gadE from positions −749 to +16 that is connected in frame to the coding region of luc. To construct pMW218-APigadE-luc, a region between positions −590 and +16 was amplified and used as the gadE fragment.

Luciferase assay. The luciferase assay was performed according to the manufacturer's protocol (Promega). Briefly, 50 μl of each transformed cell culture was mixed with 40 μl of host cell culture. Next, 10 μl of 1 M K2HPO4 (pH 7.8) and 20 mM EDTA was added to the mixed culture, which was then stored for 10 min at −80 °C. A 300 μl aliquot of freshly prepared lysis mix was added. After incubating for 10 min at room temperature, 10 μl of the cell lysate was mixed with 50 μl of luciferase assay reagent and the light produced was measured in a Lumat LB9507 (EG & G Berthold).

Northern blot analysis. Total RNA was extracted using hot phenol as reported by Aiba et al. (1981). To analyse gadE mRNA, total RNA (10 μg) was fractionated by electrophoresis in a 1.5% agarose gel containing 6% formaldehyde. The separated RNAs were then transferred to a nylon membrane (Hybond-N’; GE Healthcare) in 20 × saline-sodium citrate (SSC). To analyse ArrS, total RNA (10 μg) was fractionated in a 6% polyacrylamide gel containing 7 M urea and then electroblotted onto a nylon membrane. DIG-labelled ribo-probe ribo-6H57 and gadE-probes 1, 2 and 3 were synthesized using the DIG RNA Labelling kit (SP6/T7) (Roche). The probe binding sites are shown in the genetic map presented in Fig. S1. The DIG Luminescent Detection kit (Roche) was used for signal detection.

RT-PCR. RT-PCR was performed to identify whether the 5′-end of each gadE mRNA carried a monophosphate or a triphosphate group as follows: the 5′-adaptor in the small RNA cloning kit (Takara Bio) was ligated to the 5′-ends of DNA-free total RNA (2 μg) at 15 °C overnight in the presence of 40 units of T4 RNA ligase (Takara Bio), 40 units of RNase Inhibitor (Takara Bio) and 0.006% BSA. Alternatively, the 5′-ends of the DNA-free total RNA were first treated with 0.8 units of T4 polynucleotide kinase (Takara Bio) to yield monophosphorylated 5′-ends. The adaptors were then ligated to the 5′-ends of the RNA. cDNAs were synthesized using 200 units of SuperScript II (Invitrogen) and 2 pmol of RT primer RT4 at 43 °C. PCR was performed using PrimeStar (Takara Bio) and primer pairs RT4/Ad1, RT4/Ad2 or RT4/Ad3 for 16 cycles. The primer binding sites are shown in Fig. S2.

RT-PCR to detect T1-luc, T2-luc and T3-luc in the gadE–luc translational fusion was performed using primer pairs gadE(−558)/luc158, gadE(−177)/luc158 and gadE(−184)/luc158. The primer binding sites are shown in Fig. S3. PCR was performed for 16 cycles.

RESULTS

Cells overexpressing arrS show increased acid resistance

First, we examined the effects of arrS overexpression on acid resistance (Fig. 1a). A recombinant plasmid, pBR322-arrS,
expressing arrS under the control of the lac promoter, was used to overexpress arrS in E. coli (Aiso et al., 2011). The percentage of colonies surviving after culture for 2 h in LB medium (pH 2.5) was markedly increased by arrS overexpression during the exponential and stationary growth phases. We next quantified the amounts of gadA, gadBC and gadE mRNAs in arrS-overexpressing cells using real-time RT-PCR (Fig. 1b). Levels of gadA and gadBC mRNA increased 4.6- and 4.2-fold, respectively, upon arrS overexpression during the stationary phase. The total amount of gadE mRNA was 3.5-fold higher in overexpressing cells. We then constructed a gadE-luc translational fusion to examine the effects of arrS overexpression on GadE expression (Fig. 1c). Luciferase activity in JM109/pMW218-gadE-luc cells increased by ~2-fold upon arrS overexpression. In the case of a gadE-deleted strain, JD25278, arrS overexpression did not increase the survival of stationary phase cells when cultured in LB medium (pH 2.5), or their mRNA levels of gadA and gadBC (Fig. 1d, e). These results suggest that overexpression of arrS indirectly increases expression of gadA and gadBC via increasing expression of gadE. In exponential growth phase, cells of JD25278 and its parental strain KT7600 showed extremely poor survival in LB medium (pH 2.5) with or without overexpression of arrS (KT7600, ≤0.01%; JD25278, ≤0.0001%; data not shown).

Levels of each gadE mRNA are affected by acid stimulation and by overexpression of arrS

The amounts of gadE mRNAs T1, T2 and T3 were examined by Northern blot analysis of total RNA derived from cells cultivated in LB medium (pH 5.5) (Sayed & Foster, 2009). First, we looked at whether the level of each mRNA was affected by pH (Fig. 2). T1 and T3 mRNA were detected in stationary phase cells cultivated in LB medium (Fig. 2a, lane 1). When the cells were cultured in MES-LB (pH 5.5), the level of T1 mRNA increased, whereas that of T3 mRNA decreased markedly (Fig. 2a, lane 1). However, we also observed a band for T2 mRNA and an additional fragment (200 nt; denoted by the asterisk).

Next, we examined the effects of arrS overexpression on levels of the gadE mRNAs (Fig. 2b). Using probe 1, we found that expression of T1 mRNA, T2 mRNA and the ~200 nt fragment increased, whereas that of T3 mRNA decreased, upon arrS overexpression during the stationary phase (Fig. 2b, lanes 3 and 4). These results are similar to those obtained for acid-stimulated cells. We presumed that the ~200 nt fragment contained a portion of the 5′-end-region of T3 mRNA, because the fragment was also detected by probe 2 (lanes 5 and 6) but not by probe 3.
Overexpression of ArrS antisense RNA

**Fig. 2.** Northern blot analyses of gadE mRNA and ArrS RNA in acid-stimulated cells or in arrS-overexpressing cells. (a) Total RNAs derived from stationary phase cells of JM109/pBR322 cultivated in LB medium (pH 8.0) (lane 1) or in MES-LB (pH 5.5) (lane 2) were analysed using gadE probe 1. SP, stationary phase. (b) Total RNAs isolated from JM109/pBR322 (–) or JM109/pBR322-arrS (+) cells were analysed using gadE probes 1, 2 and 3. EP, exponential growth phase; SP, stationary phase. (c) JM109/pBR322 (–) or JM109/pBR322-arrS (+) cells were cultivated in LB medium (pH 8.0) or in MES-LB (pH 5.8). Total RNA was isolated from the cells and used to examine ArrS RNA. The size of the fragments is shown to the left of gels. SP, stationary phase.

(lanes 7 and 8). ArrS expression in overexpressing cells was much higher than that in acid-stimulated cells (Fig. 2c).

**Generation of T2 mRNA is dependent on the gadE P3 promoter and RNA processing**

The results above suggest that T2 mRNA is not a primary transcript but, rather, is generated via RNA processing, which removes a short RNA fragment from the 5′-end of T3 mRNA. To verify this hypothesis, we performed three additional experiments (Figs 3 and 4). First, we investigated whether RNase III, one of the major RNA processing enzymes, participates in the generation of T2 mRNA. We did not detect any T2 mRNA or the 200 nt fragment in RNase III-deficient cells, but did detect a fragment that was slightly larger than 200 nt (Fig. 3a, lanes 2 and 4). This suggests that the generation of T2 mRNA is dependent upon RNase III.

Next, we analysed the 5′-end of T2 mRNA to clarify whether T2 mRNA is generated via cleavage at the 5′-end (Fig. 3b). Bacterial primary transcripts carry a triphosphate 5′-end, which can be ligated into the 5′-adaptor (Couttet et al., 1997; Yonesaki, 2002). DNA-free total RNA derived from arrS-overexpressing cells was mixed with a 5′-adaptor followed by treatment with T4 RNA ligase. cDNA was synthesized using primer RT4 and the region containing the junction was amplified. DNA fragments of 461 and 666 bp were amplified by primer pairs RT4/Ad1 and RT4/Ad2, respectively (Fig. 3b, lanes 1 and 2); however, no band was amplified by primer pair RT4/Ad3 (lane 3). A 903 bp fragment was amplified by primer pair RT4/Ad3 when the triphosphorylated 5′-ends were converted to monophosphorylated 5′-ends (lane 6). These results show that T1 and T2 mRNA, the 5′-ends of which are located at positions −124 and −324, respectively, are generated via RNA processing because both have a monophosphate at the 5′-end, and that T3 mRNA is a primary transcript of gadE.

If T2 mRNA is derived from T3 mRNA, neither T3 nor T2 mRNA would be generated by a P3-deleted gadE–luc fusion. Therefore, we constructed another recombinant plasmid, pMW218–P3gadE–luc, in which a consensus sequence at position −35 of the P3 promoter was deleted. We then performed RT-PCR to detect transcripts of T3-luc mRNA and/or T2-luc mRNA in the gadE–luc and ΔP3gadE–luc fusions (Fig. 4). Primer pair gadE(−558)/luc158 detects only cDNA derived from T3-luc mRNA. A PCR fragment of 699 bp was amplified from the gadE–luc fusion (Fig. 4, upper panel); by contrast, nothing was amplified from the ΔP3gadE–luc fusion. Primer pair gadE(−177)/luc158 detects both T3-luc mRNA and T2-luc mRNA. Similarly, a 318 bp fragment was amplified from the gadE–luc fusion but not from the ΔP3gadE–luc fusion by this primer pair (Fig. 4, middle panel). On the other hand, a 159 bp fragment was amplified from both fusion genes using primer pair gadE(−18q)/luc158, which amplifies all cDNAs derived from T3-luc, T2-luc and T1-luc mRNA (Fig. 4, lower panel). These results show that transcription of T3-luc and T2-luc mRNAs is dependent on P3. The results presented in Figs 3 and 4 suggest strongly that T2 mRNA is generated via processing of T3 mRNA. A promoter other than P3 (i.e. P1) that is located between positions −177 and −18 (Fig. S3) seems to be responsible
for transcription of T1-luc mRNA. As mentioned above, T1 had a processed 5’ end. Therefore, P1 must reside between positions −116 and −320, as previously reported (Sayed & Foster, 2009); however, the consensus sequence of P1 may lie upstream of the predicted position. The primary P1 transcript is probably processed at −124 to be T1 mRNA.

**Effects of arrS overexpression on expression of gadE-luc and ΔP3gadE-luc fusions**

Fig. 1(b) shows that the level of gadE mRNA increased, and Fig. 2(b) shows that the levels of the gadE mRNAs T1 and T2 increased, whereas that of T3 mRNA decreased in arrS-overexpressing cells. We next measured the total amount of T2-luc and T3-luc mRNA synthesized via the P3 promoter (Fig. 5). The total amount of T2-luc mRNA and T3-luc mRNA increased 2-fold upon arrS overexpression (Fig. 5a), although the total amount was extremely low in the ΔP3gadE-luc fusions. Next, we examined the fusions in a luciferase assay (Fig. 5b). Luciferase activity of the gadE-luc fusion increased 3.4-fold upon arrS overexpression (Fig. 5b). In addition, luciferase activity in the ΔP3gadE-luc fusion, expressed via the P1 promoter, increased 3.2-fold upon overexpression. These results show that arrS overexpression increases the amount of gadE mRNAs synthesized via the P3 promoter and that of gadE mRNAs expressed via the P1 promoter.

**Effects of arrS overexpression on the stability of native gadE mRNAs**

The results shown in Fig. 5 suggest that the total amount of T2 and T3 mRNA is increased in arrS-overexpressing cells. To investigate whether the processing of T3 plays a role in this increase, we examined the half-lives of T2 and T3 mRNAs (Fig. 6). The intensity of the chemical luminescence generated by the bands shown in Fig. 6(a) was plotted against time after the addition of rifampicin (Fig. 6b). The half-life of native T3 mRNA was 1.6 min in cells that did not harbour a recombinant plasmid for arrS overexpression, whereas that of T2 mRNA generated via...
the processing of T3 mRNA in arrS-overexpressing cells was 2.5 min. The half-lives of T1 mRNA in cells with and without the overexpression plasmid were 3.4 and 2.5 min, respectively.

DISCUSSION

The results of this study showed that the overexpression of ArrS: (1) increases the acid resistance of cells; (2) increases the expression of gadE, which encodes a major transcriptional activator in GDAR; (3) increases the cellular levels of gadE mRNA T1 and T2; and (4) promotes the processing of T3 mRNA to T2 mRNA by RNase III. Moreover, the results suggest that the increase in gadE expression results from the increased stability of gadE transcripts.

To investigate the effects of arrS overexpression on gadE expression, we first performed quantitative real-time RT-PCR and luciferase assays using the gadE–luc translational fusions. It was implied that both known and unknown cis-factors for gadE expression reside within a 798 bp region upstream of gadE (Ma et al., 2004; Sayed & Foster, 2009). Therefore, we used long gadE fragments containing the region between positions −749 and +16 or positions −590 and +16 to construct translational fusions of gadE–luc and P3-deleted gadE–luc, respectively.

Fig. 5. Effects of arrS overexpression on expression of the gadE–luc and ΔP3gadE–luc fusions. (a) Total RNA derived from cells containing pMW218-gadE-luc (wt) or pMW218-ΔgadE-luc (Δ) was used for cDNA synthesis. The total amount of T3-gadE-luc was quantified by real-time RT-PCR using primer pair gadE(-209)/luc115. RQ, relative quantity. (b) Lysates of cells containing pMW218-gadE-luc (wt) or pMW218-ΔgadE-luc (Δ) were tested in luciferase assays. Data are expressed as relative light units (RLU) per cell. Asterisks indicate a significant difference between non-overexpressing cells and arrS-overexpressing cells (*P<0.05, **P<0.01).

Promoters involved in the transcription of gadE mRNAs T1, T2 and T3

T1, T2 and T3 mRNAs were detected by Northern blotting of total RNA derived from acid-stimulated cells, and the 5'-ends were located at positions −124, −324 and −566, respectively (Sayed & Foster, 2009). These authors then predicted the promoters responsible for expressing T1, T2 and T3, and detected the activity of these promoters in regions between positions −116 and −320, −312 and −561, and −556 and −750, respectively. Here, we demonstrated that T2 mRNA is generated via the processing of T3 mRNA. This disagrees with the finding of this previous study that transcription of T2 mRNA is initiated at the P2 promoter. We found that: (1) a ~200 nt fragment, the appearance of which coincided with the appearance of T2 mRNA, resides within the 5'-region of T3 in arrS-overexpressing cells (Fig. 2b); (2) no T2 mRNA was generated in RNase III-deficient cells (Fig. 3a); (3) the 5'-end at position −324 was monophosphorylated (Fig. 3b); and (4) no T2-luc mRNA was...
generated in cells harbouring the ΔP3gadE–luc fusion (Figs 4 and 5a).

**Mechanisms underlying gadE activation via arrS overexpression**

The overexpression of arrS was achieved by using an arrS-containing plasmid. Therefore, the effects observed in overexpressing cells were caused by excess cellular ArrS. The arrS located in the strand opposite that containing gadE was not directly affected. The results of the present study showed that the levels of T2 and T1 mRNA increased (Figs 2b and 5). First, we discuss the increase in T2 mRNA and describe the mechanisms involved in increasing gadE expression in arrS-overexpressing cells. As described above, T2 mRNA is generated from T3 mRNA via RNA processing. Accordingly, the increase in T2 mRNA level could be caused by an increase in P3 promoter activity, by an increase in the processing of T3 mRNA and/or by an increase in the stability of T2 mRNA. The results shown in Fig. 6 suggest that T2 mRNA in overexpressing cells was more stable than T3 mRNA in non-overexpressing cells. Therefore, the increased processing of T3 mRNA increases the levels of gadE mRNA. ArrS may function by base-pairing with a sequence within the 5′ untranslated region of gadE T3 mRNA, like any other antisense RNAs (Sesto et al., 2013). It is not clear whether activation of the P3 promoter plays a role in increased gadE expression. Next, we discuss the increase in T1 mRNA. The results shown in Fig. 6 suggest that T1 mRNA was more stable in arrS-overexpressing cells. It is possible that the stabilization of T1 mRNA results in an increase in T1 mRNA levels; however, it is more probable that P1 is positively regulated by GadE (Sayed & Foster, 2009). A GAD box resides upstream of the P1 promoter (Ma et al., 2003); therefore, increased GadE levels, which are attributable to the increase in T2 mRNA levels, may increase P1 promoter activity.

**Role of ArrS in acid resistance**

The transcription of ArrS was induced by an acidic environment (pH 5.5), and the cellular levels of ArrS were comparable to those observed upon arrS overexpression (Fig. 2c). We also observed increases in both T1 and T2 mRNA in non-overexpressing cells under acidic conditions (Fig. 2a, b). It is possible that ArrS acts as a regulator of acid resistance. It is reported that at least nine regulators reside upstream of the P1 promoter (Ma et al., 2003); therefore, increased GadE levels, which are attributable to the increase in T2 mRNA levels, may increase P1 promoter activity.

Antisense RNAs are involved in several regulatory mechanisms, including transcription interference (Callen et al., 2004), transcription attenuation (Stork et al., 2007), degradation of sense transcripts (Dühring et al., 2006), stabilization of sense transcripts (Opdyke et al., 2004, 2011; Tramonti et al., 2008), and protection of sense transcripts from RNase E cleavage (Stazic et al., 2011), translation activation (Majdalani et al., 1998, 2001) and translation repression (Kawano et al., 2007); however, the mechanisms by which they increase target gene expression are limited. The antisense RNA GadY is a small RNA that is highly expressed during the stationary growth phase and is upregulated by acidic stimuli (Chen et al., 2002; Opdyke et al., 2004). GadY is involved in processing gadXW mRNA, and the message of gadX and gadW fragments generated via this processing is more stable than gadXW (Opdyke et al., 2011). RNAIII is responsible for this processing (Opdyke et al., 2011). Both gadE and arrS are found only in *E. coli* and *Shigella* strains. Recent studies report that the expression of LEE (locus of enterocyte effacement) is negatively controlled by GadE in enterohaemorrhagic *E. coli* O157:H7 (Kailasan Vanaja et al., 2009; Tree et al., 2011). This raises the possibility that ArrS is involved in pathogenicity of *E. coli* O157:H7 via modulating on of gadE.

**ACKNOWLEDGEMENTS**

We thank Hironori Niki (National Institute of Genetics) for providing *E. coli* strains KT7600 and JD25278. This work was supported by a Grant-in-Aid from the Research Promotion Award of the Faculty of Health Sciences, Kyorin University, Japan.

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Edited by: D. Grainger