Modulation of transcription and characterization of the promoter organization of the autotransporter adhesin heptosyltransferase and the autotransporter adhesin AIDA-I

Inga Benz, Tessa van Alen, Julia Bolte, Mirka E. Wörmann and M. Alexander Schmidt

Correspondence

M. Alexander Schmidt
infekt@uni-muenster.de

Institut für Infektiologie, Zentrum für Molekularbiologie der Entzündung (ZMBE), Westfälische Wilhelms-Universität Münster, D-48149 Münster, Germany

In Gram-negative bacteria, autotransporter proteins constitute the largest family of secreted proteins, and exhibit many different functions. In recent years, research has largely focused on mechanisms of autotransporter protein translocation, where several alternative models are still being discussed. In contrast, the biogenesis of only a few autotransporters has been studied and, likewise, regulation of expression has received only very limited attention. The glycosylated autotransporter adhesin involved in diffuse adherence (AIDA)-I system consists of the *aah* gene, encoding a specific autotransporter adhesin heptosyltransferase (AAH), and the *aidA* gene, encoding the autotransporter protein (AIDA-I). In this study, we investigated the promoter organization and transcription of these two genes using reporter plasmids carrying *lacZ* transcriptional fusions. The two genes, *aah* and *aidA*, are transcribed as a bicistronic message. However, *aidA* is additionally transcribed from its own promoter. There are two distinct start sites for each of the two genes. Interestingly, transcription of both genes is enhanced in *hns* and *rfaH* mutant backgrounds. Furthermore, we addressed the influence of environmental factors and different genetic backgrounds of *Escherichia coli* K-12 strains on transcription activity. We found that transcription varied considerably in different *E. coli* K-12 laboratory strains and under different growth conditions.

Received 2 July 2010
Revised 21 December 2009
Accepted 26 December 2009

INTRODUCTION

With now more than 800 members, autotransporter proteins constitute the largest family of secreted proteins in Gram-negative bacteria (Dautin & Bernstein, 2007; Wells *et al.*, 2007). Autotransporters mediate a wide variety of functions, such as adhesion, host cell actin polymerization, and serum resistance. In addition, they may also express or shed enzymes with, e.g. protease, esterase and lipase activities (Dautin & Bernstein, 2007; Henderson *et al.*, 2004). Plasmid-encoded adhesin involved in diffuse adherence (AIDA)-I was isolated from the clinical *Escherichia coli* strain 2787 (O126:H27) and mediates the diffuse adherence phenotype of the wild-type bacterium to HeLa cells (Benz & Schmidt, 1989).

The distribution of AIDA-I is not restricted to human isolates; on the contrary, AIDA-I is more often identified in porcine *E. coli* isolates, indicating a putative food-borne infection chain. In these animals, the presence of AIDA-I-

positive strains appears to be associated with F18 fimbriae and oedema disease (Niewerth *et al.*, 2001; Mainil *et al.*, 2002). That AIDA-I augments virulence in intestinal disorders is supported by findings *in vivo* that show that expression of AIDA-I in porcine strains increases the onset of diarrhoea in weaned piglets (Pritchard *et al.*, 2004).

AIDA-I is encoded by the *aidA* gene and is synthesized as a 132 kDa pre-pro-protein featuring an unusually long 49 aa signal peptide (Benz & Schmidt, 1992a), as has also been found in other autotransporters. For IcsA of *Shigella flexneri*, it has been shown that the protein is cleaved during Sec-dependent transport through the inner membrane (Brandon *et al.*, 2003). In the periplasm, the C-terminal part ('translocator') of the protein is integrated as a β -barrel into the outer membrane (Suhr *et al.*, 1996). The mechanism of surface translocation of the adhesin ('passenger' domain) involving the translocator domain still remains elusive and various alternative mechanistic models have been proposed (Jain & Goldberg, 2007). During the translocation process the AIDA pro-protein is cleaved autocatalytically and the mature AIDA-I adhesin remains associated with the outer

Abbreviations: AAH, autotransporter adhesin heptosyltransferase; AIDA, adhesin involved in diffuse adherence; 5'-RACE, rapid amplification of 5' complementary DNA ends.

membrane on the bacterial surface. A 119 kDa glycoprotein on the surface of HeLa cells has been identified as a docking protein for the AIDA adhesin (Laarmann & Schmidt, 2003).

For full adhesive function and/or stability, AIDA-I has to be modified by a second protein encoded directly upstream of the *aidA* gene (Benz & Schmidt, 2001). This protein has been shown to function as a protein heptosyltransferase, and is accordingly termed the 'autotransporter adhesin heptosyltransferase' (AAH). AAH catalyses the addition of, on average, 19 heptose residues to the AIDA protein (Benz & Schmidt, 2001). For this, AAH uses ADP-glycero-manno-heptopyranose as a donor from the lipopolysaccharide synthesis pathway. AAH appears to be the only protein-heptosylating enzyme known thus far. Not surprisingly, the amount of AIDA-I substrate present in the bacterial cell (Benz & Schmidt, 1992b) by far exceeds the amount of the heptosylating enzyme AAH. How the balance between the two proteins is maintained to ensure the optimal function of the glycosylated AIDA-I adhesin is not known.

Thus far, expression of bacterial autotransporters has been mostly addressed with respect to modulation by environmental conditions (e.g. Henderson *et al.*, 2004). However, the expression of bacterial glycosylated autotransporters

such as TibA or AIDA-I has not been addressed in any detail. The need for a better understanding of autotransporter expression is further underlined by the increasing interest in the AIDA-I translocator (AIDA^C) for the expression of heterologous proteins on the surface of Gram-negative bacteria (Konieczny *et al.*, 2000; Casali *et al.*, 2002; Jose & Meyer, 2007).

In this study, we present evidence that the two genes constituting the AIDA system are transcribed as a bicistronic mRNA from a regulated promoter upstream of *aah*. In addition, mRNA is also synthesized from a promoter directly upstream of the *aidA* gene.

METHODS

Bacterial strains, plasmids and media. *E. coli* strains used in this study are listed in Table 1. Plasmid pIB264 (Benz & Schmidt, 1989) is a pBR322 derivative (Amp^r) and harbours the genes for the expression of the authentic AIDA-I system (*aah* and *aidA*). pCB192 (Amp^r) derived from pBR322 is a promoter test vector and harbours the promoterless genes *lacZ* and *galK* (Schneider & Beck, 1986). Bacteria were generally grown at 37 °C in Standard I medium (Merck). For the maintenance of plasmids, the medium was supplemented with 100 µg ampicillin ml⁻¹. Salt medium was prepared as described by Bäcklund *et al.* (2008). The minimal medium was supplemented with 0.02 % thiamine and 0.03 % Bacto casamino acids.

Table 1. *E. coli* strains employed in this study

Strain	Genotype	Source
BW25113	$\Delta(\text{araD-araB})567 \Delta\text{lacZ4787} (:: \text{rrnB-4}) \text{rph-1} \Delta(\text{rhaD-rhaB})568 \text{rrnB-4 hsdR514}$	Datsenko & Wanner (2000)
BW25141	$\Delta(\text{araD-araB})567 \Delta\text{lacZ4787} (:: \text{rrnB-4}) \text{rph-1} \Delta(\text{rhaD-rhaB})568 \text{rrnB-4 hsdR514}$	Datsenko & Wanner (2000)
C600	$\Delta(\text{phoB-phoR})580 \text{galU95} \Delta\text{uidA3} :: \text{pir+ recA1 endA9}(\text{del-ins}) :: \text{FRT}$	
DH5 α	F ⁻ <i>thi-1 thr-1 leuB6 lacY1 cyn101 glpR200 e14- supE44 rfbC1 fhuA21</i>	Appleyard (1954)
	F ⁻ <i>supE44</i> $\Delta(\text{lacZYA-argF})\text{U169 deoR nupG } \Phi 80\text{lacZ}\Delta\text{M15 hsdR17}(\text{r}_{\text{K}}^{\text{m}}\text{m}_{\text{K}}^{\text{+}}) \text{recA1 endA1 gyrA96 thi-1 relA1}$	Hanahan (1983)
GM2163	F ⁻ <i>araC14 leuB6 fhuA13 lacY1 tsx-78 glnV44 galK2 galT22 mcrA0 dcm-6 hisG4 rfbC1 rpsL136 dam-13 :: Tn9 xylA5 mtl-1 thi-1 mcrB9999 hsdR2</i>	Marinus <i>et al.</i> (1983)
JM105	<i>supE44 endA1 thi-1 rpsL sbcB15 hsdR4</i> $\Delta(\text{lac-pro})$ [F' <i>traD36 pro AB</i> ⁺ <i>lacIqZ</i> Δ M15]	Yanisch-Perron <i>et al.</i> (1985)
JM109	<i>recA1 endA1 gyrA96 thi-1 hsdR17</i> (r _K ⁺ m _K ⁺) <i>supE44 relA1 e14- </i> $\Delta(\text{lac-proAB})$ [F' <i>traD36 proAB</i> ⁺ <i>lacIqZ</i> Δ M15]	Promega
MC4100	F ⁻ $\Delta(\text{lacZYA-argF})\text{U169 araD139 rpsL150 flhD5301 deoC1 fruA25 relA1 e14- rph-1}$	Powers & Randall (1995)
MG1655	MG1655 $\Delta\text{rfaH} :: \text{cat}$, cat resistance removed by FLP flippase carried by pCP20	Jensen (1993)
MG1655 ΔrfaH		Beloin <i>et al.</i> (2006)
ORN103	F ⁻ <i>thr-1 leuB6 thi-1 lacY1 xyl-7 ara-13 mtlA2 gal-6 rpsL tonA2 minA minB-2</i> $\Delta\text{pil } \Delta(\text{lacZYA-argF})\text{U169 recA13}$	Orndorff <i>et al.</i> (1985)
PD145	MC4100 <i>hms205 :: Tn10</i>	Dersch <i>et al.</i> (1994)
RH90	MC4100 <i>rpoS359 :: Tn10</i>	Lange & Hengge-Aronis (1991)
Sure	$\Delta(\text{mrr-hsdRMS-mcrBC}) \text{endA1 supE44 thi-1 gyrA96 relA1 lac recB recJ sbcC umuC} :: \text{Tn5 e14- uvrC}$ [F' <i>proAB</i> ⁺ <i>lacIqZ</i> Δ M15 Tn10 (tet ^r)]	Stratagene
TB1	<i>ara</i> $\Delta(\text{lac-araAB}) \text{rpsL } \Phi 80\text{lacZ}\Delta\text{M15 hsdR}$	Biolabs
TG1	<i>supE hsdD5 thi</i> $\Delta(\text{lac-proAB})$ [F' <i>traD36 proAB</i> ⁺ <i>lacIqZ</i> Δ M15]	Gibson (1984)
top10	F ⁻ <i>mcrA</i> $\Delta(\text{mrr-hsdRMS-mcrBC}) \Phi 80\text{lacZ}\Delta\text{M15 } \Delta\text{lacX74 recA1 araD139 } \Delta(\text{ara-leu})7697 \text{galU galK rpsL endA1 nupG}$	Invitrogen
XL1 blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17</i> (r _K ⁺ m _K ⁺) <i>supE44 relA1 lac</i> [F' <i>proAB</i> ⁺ <i>lacIqZ</i> Δ M15 Tn10 (tet ^r)]	Stratagene

Generation of plasmids, mutagenesis, and oligonucleotide primers for PCR. Plasmids were constructed using established methods (Sambrook *et al.*, 1989). Plasmid transfer to bacteria was performed according to the protocol of Mandel & Higa (1970). For *in vitro* mutagenesis, the QuikChange Site-Directed Mutagenesis kit (Stratagene) was used according to the manufacturer's instructions. DNA fragments were amplified employing *Taq* polymerase following the instructions of the manufacturer (Segetec). Oligonucleotide primers used for PCR are summarized in Table 2.

β -Galactosidase assay. Liquid medium was inoculated 1:10 with an overnight culture of the respective strain. To determine the influence of growth conditions on the transcriptional activity, overnight cultures were grown under the same conditions as the culture to be investigated. The culture was grown at 37 °C with agitation to OD₆₀₀ 0.3–0.7. To investigate the possible influence of oxygen pressure, static cultures were compared with cultures grown with intense shaking (10 ml culture in a 100 ml Erlenmeyer flask at 180 r.p.m.). The β -galactosidase assays were performed as described

Table 2. Oligonucleotides used as primer pairs

Sequences shown in upper-case type are homologous to *aah* or *aidA* sequences. Recognition sequences for restriction enzymes are underlined.

Oligonucleotide	Sequence (5'–3')	Restriction enzyme
Amplification of promoter region fragments		
A-515	<u>gctctaga</u> GCTTTTATCCACGATGGC	<i>XbaI</i>
A-443	<u>gctctaga</u> AGCAACATGTTTCATGGCC	<i>XbaI</i>
A-319	<u>gctctaga</u> CTTGCATGGCATCTTGTG	<i>XbaI</i>
A-230	<u>gctctaga</u> GATAACGTCAGATCTCATC	<i>XbaI</i>
A-194	<u>gctctaga</u> CTTTATTTCGCCACGACAC	<i>XbaI</i>
A-133	<u>gctctaga</u> TATTGGCACATGGTATTATCC	<i>XbaI</i>
A + 114	<u>tcggatcc</u> GTTAACGTGCCATTCTCC	<i>Bam</i> HI, <i>Hpa</i>
B-390	<u>gctctaga</u> ATCATATTCATGGGGAGC	<i>XbaI</i>
B-334	<u>gctctaga</u> GTAGATTATTACGACATGCC	<i>XbaI</i>
B-271	<u>gctctaga</u> ATGGGCAAGTAGAATACCC	<i>XbaI</i>
B-185	<u>gctctaga</u> CATGGCTGTAATGGATGC	<i>XbaI</i>
B-137	<u>gctctaga</u> ACTGACTTTCTCTGGTGC	<i>XbaI</i>
B + 85	<u>tcggatc</u> CAAAACCATGTCCTCTGG	<i>Bam</i> HI
Inverse PCR		
IB-galK	<u>aggta</u> CCTCGCGCGTTTCGG	<i>KpnI</i>
B-317	<u>aggatcc</u> GCCAGTTTTTTTGTGG	<i>KpnI</i>
B-290	<u>aggatcc</u> GGATTGTCATGGCTG	<i>KpnI</i>
B-118	<u>aggta</u> CCCTGTACACAAAGG	<i>KpnI</i>
B-97	<u>aggatcc</u> GACAGTTTGAATGTACC	<i>KpnI</i>
B-80	<u>aggta</u> CCGGCTTATTACAGGG	<i>KpnI</i>
B-58	<u>aggatcc</u> TGTGGTGTGATTAGAAC	<i>KpnI</i>
Site-directed mutagenesis		
mut1for	CGTCAGATCTCATCTc <u>Tg</u> CAGAAAAAGATATC	<i>PstI</i>
mut1rev	GATATCTTTTTCTGc <u>Ag</u> AGATGAGATCTGACG	<i>PstI</i>
mut2for	GATCTCATCTTTTTCAGc <u>Agct</u> GATATCTTTTATTCGCCAC	<i>PvuII</i>
mut2rev	GTGGCGAATAAAAAGATATC <u>cagc</u> TgCTGAAAAGATGAGATC	<i>PvuII</i>
mut3for	CTTTTCAGAAAAAGATAT <u>Cga</u> TcATTTCGCCACGACACAAG	<i>Clal</i>
mut3rev	CTTGTGTCGTGGCGAATg <u>Atc</u> GATATCTTTTCTGAAAAG	<i>Clal</i>
mut13for	CTc <u>Tg</u> CAGAAAAAGATATC <u>cga</u> TcATTTCGCCACGACACAAG	<i>Clal</i> / <i>PstI</i>
mut13rev	CTTGTGTCGTGGCGAATg <u>Atc</u> GATATCTTTTCTGc <u>Ag</u> AG	<i>Clal</i> / <i>PstI</i>
Multiplex PCR		
aah/aidAfor	<u>gctctaga</u> ATGGGCAAGTAGAATACCC	
aah/aidArev	GTTGTAGCAGTGGTTTTCCC	
aahfor	CTGGGTGACATTATTGCTTGG	
aahrev	TTTGCTTGTGCGGTA	
aidAfor	GGTGGCATTGCATTCCGG	
aidArev	CCGGAAACATTGACCATACC	
5'-RACE		
lacZ	CAAGGCGATTAAGTTGG	
B + 571	TCGAGGCAACGCCACCACTACTGACAC	
polyC	CCCCCCCCCCCCCCCCCCCC	

by Miller (1972). Enzyme activities were expressed as arbitrary Miller units and were derived from at least three independent experiments, each performed in triplicate.

RNA isolation and cDNA synthesis. RNA was isolated from *E. coli* strains with the RNeasy kit (Qiagen) following the addition of RNaProtect (Qiagen). To eliminate remaining plasmid and chromosomal DNA the Turbo DNA-free kit (Ambion) was used. Transcription from RNA to cDNA was performed with the RevertAid H Minus First Strand cDNA Synthesis kit (Fermentas). Random hexamers were used as primers.

Northern blotting. RNA molecules were separated by electrophoresis in agarose gels containing 2% formaldehyde, followed by incubation in 50 mM NaOH for 20 min to denature the RNA and a further 20 min in $10\times$ SSC (1.5 M NaCl, 0.15 M sodium citrate, pH 7.0) for neutralization. The RNA was then transferred to a nylon membrane. After blotting, the membrane was incubated with $2\times$ SSC for 5 min and dried, and the RNA was cross-linked to the membrane by UV radiation (Stratalinker, Stratagene). Hybridization was performed with the ECL Direct Nucleic Acid Labeling and Detection System (Amersham Bioscience). As a molecular mass standard, the RiboRuler High Range RNA ladder (Fermentas) was used. The RNA was visualized after ethidium bromide staining in a Lumi-Imager (Roche).

Rapid amplification of 5' complementary DNA ends (5'-RACE). 5'-RACE was carried out according to Frohman (1993). RNA was isolated as described above. cDNA synthesis was performed with Avian Myeloblastosis Virus (AMV) reverse transcriptase (Promega). Total RNA (5 µg) was incubated with 20 pmol of specific oligonucleotide primers 1 in a total volume of 13.5 µl at 65 °C for 5 min. After 5 min on ice, 4 µl AMV RT-buffer, 2 µl 10 mM dNTP mix and 5 U AMV reverse transcriptase were added. After incubation at 50 °C for 1 h, the enzyme was inactivated at 85 °C for 5 min. The tailing reaction was performed with terminal transferase (BioLabs) and dGTP according to the manufacturer's instructions. The tailed cDNA was amplified by PCR using a polyC oligomer and a gene-specific primer 2. Primers and templates used are summarized in Table 3. PCR products were verified by sequencing (SeqLab, Göttingen).

Table 3. Primers used to determine the transcription start site

cDNA was synthesized from total RNA with specific primers (primer 1). After tailing of the cDNA, the DNA was further amplified with a gene-specific nested primer (primer 2) and a polyC oligonucleotide. The primers were chosen in accordance with the gene to be investigated and the plasmid of the recombinant strain used for RNA isolation.

Gene	Plasmid	Primer
<i>aah</i>	pIB264	Primer 1 <i>aah</i> rev
<i>aah</i>	pIB264	Primer 2 A + 114
<i>aah</i>	pTvA515	Primer 1 <i>lacZ</i>
<i>aah</i>	pTvA515	Primer 2 A + 114
<i>aidA</i>	pIB264	Primer 1 B + 571
<i>aidA</i>	pIB264	Primer 2 B + 85
<i>aidA</i>	pMW334	Primer 1 <i>lacZ</i>
<i>aidA</i>	pMW334	Primer 2 B + 85

RESULTS

Identification of *aah* and *aidA* promoter regions

For stability and/or activity of AIDA-I as an adhesin, both the *aah* and the *aidA* genes need to be functional (Benz & Schmidt, 2001). Recombinant *E. coli* strains harbouring *aah* and *aidA* genes either on the same (pIB264) or on different plasmids exhibit the same phenotype as the wild-type *E. coli* 2787 (Benz & Schmidt, 1989, 2001). This indicates that both genes are transcribed from their own promoters. To identify the promoter regions of the two genes, transcriptional fusions of DNA fragments of varying lengths upstream of the *aah* or *aidA* start codon with the *lacZ* coding region in the promoter test vector pCB192 were tested for activity. The various fragments included the upstream region (133–515 nt) and 114 nt of the coding region of *aah*, and 58–390 nt of the upstream region and 85 nt of the coding region of *aidA*. Immediately upstream of the *lacZ* coding regions are stop codons in all three reading frames, so that we can exclude the synthesis of a fusion protein. Some of the plasmids harbouring small upstream regions were amplified by inverse PCR. In addition, we also obtained a construct containing 218 nt of the upstream region of *aah*.

To monitor transcription, β -galactosidase activities of transcriptional fusion constructs were determined in the *E. coli* laboratory strain DH5 α . For both *aah* and *aidA* genes, a correlation of the length of the upstream region to the β -galactosidase activity was observed. For *aah*, 218 nt upstream of the start codon were found to be necessary for optimal transcription. Upstream of the start codon of *aidA*, 290 nt were needed for efficient mRNA synthesis (Fig. 1). Within these regions the ribosome-binding site, the transcription start site, the promoter region, and possibly also binding sites for putative regulatory proteins, were located. The transcription activities of the two promoter regions in the β -galactosidase assays differed by a factor of approximately three.

Determination of transcription start sites

To identify the transcription start sites, 5'-RACE experiments were performed. Total RNA from different recombinant DH5 α strains was isolated and reverse-transcribed to cDNA. Amplification of the 5' fragment of the mRNA was done with polyG-tailed cDNA as template, polyC oligonucleotides, and a gene-specific primer 2 (Table 3). The polyG fragments were sequenced and the transcription start sites determined. For the *aah* gene, two transcription start sites, namely 128 and 149 nt upstream of the start codon, were identified. This was independent of whether the RNA was isolated from DH5 α harbouring pIB264 or the promoter test vector *paah*515. Sequences representing the –10 and –35 regions were located upstream of both sites (Fig. 2). For the start site at 128 nt, part of a UP element could also be detected. For the *aidA* gene also, two start sites were identified at 66 and 252 nt upstream of the

Fig. 1. Localization of the promoter region. (a) Schematic view of inserts containing a constant part of the coding region of either *aah* or *aidA* and upstream regions of various lengths. (b) β -Galactosidase activity of recombinant *E. coli* DH5 α with a promoter test vector harbouring inserts containing part of the coding region of either *aah* (left) or *aidA* (right) and upstream regions of various lengths. On the x axis, the number of nucleotides upstream of the start codon is indicated. As a negative control, the vector pCB192 was used (C). Mean enzymatic activities are shown from at least three independent experiments performed in triplicate; error bars, SD.

start codon in DH5 α /pIB264. In the case of DH5 α /paidA334, only the start site at position 252 could be identified. Upstream of both sites, sequences representing the -10 and -35 motifs were identified (Fig. 2).

Evidence for a bicistronic mRNA encompassing *aah* and *aidA* transcripts

To obtain a functional AIDA-I adhesin, both gene products have to be present and functional in the appropriate ratio, as a low amount of the AAH enzyme might be sufficient to modify the AIDA protein. As both genes carry promoter sequences upstream of their start codons we were interested to see whether a basal level of both proteins could be synthesized via a bicistronic mRNA. To provide evidence for the additional presence of a bicistronic message, PCR and Northern blot analysis were performed.

RNA was isolated from *E. coli* DH5 α /pIB264 and transcribed into cDNA, and a multiplex PCR was performed with total cDNA as template. Plasmid pIB264 harbours the complete coding sequences for both genes, *aah* and *aidA*. The primers used lead to the amplification of an internal fragment for both *aah* (370 bp) and *aidA* (452 bp) (Niewerth *et al.*, 2001) and, in addition, in the case of the presence of a bicistronic mRNA, to a fragment comprising the 3' end of *aah* and the 5' end of *aidA* (576 bp). As shown in Fig. 3(a), all three

expected fragments were amplified as expected, thus providing evidence for a bicistronic mRNA.

To confirm these results, total RNA isolated from *E. coli* C600/pIB264 was separated by electrophoresis in a formaldehyde gel and subsequently blotted to a nylon membrane. Hybridization was performed with an *aah*-specific probe. RiboRuler High Range RNA was used as a size standard. As shown in Fig. 3(b), *aah*-specific mRNA with a size of about 5300 nt could be detected. Transcription from the *aah* promoter apparently leads to a bicistronic mRNA comprising *aah* (1173 nt) and *aidA* (3858 nt).

Influence of growth conditions on transcription activity

Experiments to identify the minimal regions necessary for transcription of the two genes were performed with bacteria grown in rich medium. To investigate the expression of *aah* and *aidA* under different conditions, bacteria were also grown in minimal medium, at different temperatures and oxygen pressures, and were further analysed in different growth phases. To ensure that no regulatory region(s) might be deleted, the experiments were performed with DH5 α /paah515 and DH5 α /paidA334. Expression of the *aah* and *aidA* genes was found to be highly susceptible to variations in growth conditions (Fig. 4). By monitoring expression

Fig. 4. Influence of growth conditions on transcription activity. β -Galactosidase activity of recombinant *E. coli* DH5 α with a promoter test vector harbouring 515 nt upstream of the start codon of *aah* or 334 nt upstream of the start codon of *aidA*. The bacteria were grown at different growth phases (a), at different temperatures (b), in different oxygen conditions (c) and in different media (d). Mean enzymatic activities are shown from at least three independent experiments performed in triplicate; error bars, SD.

growth in salt medium, which indicated that the sigma factor RpoS might be involved in transcription. To investigate this further, we transformed *E. coli* strain RH90, an *E. coli* *rpoS* mutant, with the promoter test vector pCB192 containing 515 nt upstream of *aah*, and subsequently assessed the expression of the *lacZ* gene. We found a significant reduction in transcription activity in the *rpoS* mutant compared to the wild-type isogenic strain MC4100 (Fig. 6a). Interestingly, transcription from the *aidA* promoter appeared not to be influenced by RpoS.

Another global regulator is the histone-like protein H-NS (e.g. Dorman, 2004). The influence of this protein on the expression from *aah* and *aidA* promoters was determined in an *hns* mutant (Dersch *et al.*, 1994). In the absence of H-NS, both promoters showed reduced activity (Fig. 6b). Although H-NS has been reported in most cases to exert a repressor function (Fang & Rimsky, 2008), the AIDA system seems to be one of the exceptions where H-NS

appears to act as an activator. At present, however, it cannot be determined whether H-NS functions directly or, alternatively, indirectly influences expression of the *aah* and *aidA* genes.

In addition, based on reports that *rfaH* influences biofilm formation by modulating expression of the abundant *E. coli* autotransporter Ag43 (Beloin *et al.*, 2006; Van der Woude & Henderson, 2008), we were interested to investigate the effect of *rfaH* on the expression of *aah* and *aidA* (Fig. 6c). Interestingly, in *rfaH* mutants, expression of *aah* and of *aidA* was enhanced as compared with wild-type bacteria.

Influence of upstream repeat motifs on transcriptional activity

An inverted repeat region has been identified at the extreme 5' end of the fragment upstream of the *aah* start

Fig. 5. Influence of *E. coli* strain on transcription activity. β -Galactosidase activities of recombinant *E. coli* strains with either a promoter test vector harbouring 515 nt upstream of the start codon of *aah* or 334 nt upstream of the start codon of *aidA*. Mean enzymatic activities are shown from at least three independent experiments performed in triplicate; error bars, SD.

codon mediating maximum transcription rates. This region exhibited two identical motifs and one motif harbouring an inverted nucleotide sequence in the middle (Fig. 7), constituting two different inverted repeats. Deletion of this region resulted in a remarkable decrease in activity (Fig. 1). To examine whether the inverted repeats might be important for transcription, site-directed mutagenesis was performed. Here, either a single motif was altered or the two identical motifs were mutated in such a way that at least one of the inverted repeats was destroyed. Every mutation introduced showed reduced transcription rates (Fig. 7). Moreover, a mutation in the second motif leading to the loss of the inverted repeat sequence also resulted in a nearly complete loss of promoter activity.

DISCUSSION

The products of the *aah* and *aidA* genes, AAH and AIDA-I, constitute the AIDA system, which mediates the diffuse

Fig. 6. Influence of RpoS, H-NS and RfaH on transcription activity. β -Galactosidase activities of recombinant *E. coli* strains with either a promoter test vector harbouring 515 nt upstream of the start codon of *aah* or 334 nt upstream of the start codon of *aidA* were determined. The *E. coli* strains were *rpoS* (a), *hns* (b) (both in a MC4100 background) or *rfaH* (c) mutants in a MG1655 background. The MC4100 wild-type strains were derived from different laboratories and were compared with the appropriate mutant strain. Mean enzymatic activities are shown from at least three independent experiments performed in triplicate; error bars, SD.

adherence phenotype of *E. coli* 2787 to HeLa cells. For full activity it is imperative that both proteins are present and functional (Benz & Schmidt, 2001). In this study, we investigated the promoter organization and transcription of the two genes to gain further insight into their regulation.

Fig. 7. Influence of the inverted repeat region on transcription activity. (a) β -Galactosidase activity of recombinant *E. coli* DH5 α with a promoter test vector harbouring 230 nt upstream of the start codon of *aah* was determined and compared with that of strains harbouring plasmids with point mutations within this region. Mean enzymatic activities are shown from at least three independent experiments performed in triplicate; error bars, SD. (b) Sequence of the repeat region; locations of the mutations are indicated. The GATC motif for methylation is underlined.

Previous studies from our laboratory have indicated that 220 nt upstream of the start codon of *aah* (Benz & Schmidt, 2001) and 384 nt upstream of *aidA* (unpublished results) are sufficient for synthesis of the functional AAH and AIDA-I proteins. To further characterize the promoter region(s) and to identify possible regulator sites, deletion mutants of the upstream regions of *aah* and *aidA* were created and 5'-RACE experiments were performed. For both genes, the transcription start sites were identified, the promoter regions were localized, and additional insight concerning regulatory structures was obtained.

The transcription of the *aah* gene starts at two different sites. For both start sites, -10 and -35 regions were identified. Comparison of the sequences upstream of the start sites with the consensus sequences of σ^{70} and σ^S promoters (Table 4) showed a high homology to both of the promoters at position 149 and only homology to the

-10 region for the start site at position 128. It is known that both factors can bind to the same promoter, albeit with different efficiencies. Transcription activity in a *rpoS*-negative *E. coli* strain was markedly reduced, indicating that σ^S apparently takes part in transcription initiation. It has been demonstrated that this alternative sigma factor can also bind to a degenerate -35 region (Typas *et al.*, 2007).

Besides the binding sites for the RNA polymerase there are two regions located upstream of *aah* that might be involved in regulation. One region contains three sequence motifs that would be sufficient for the formation of two inverted repeats. As sequences with dyad symmetry in the neighbourhood of a promoter are known to be binding sites for regulatory proteins this might also be the case for the *aah* promoter. Mutations in this region led to a dramatic decrease in transcription activity that clearly

Table 4. Comparison of the sequence of the upstream region of *aah* with the consensus sequences of σ^{70} and σ^S promoter regions according to Typas *et al.* (2007)

Promoter region	Sequence
Promoter region upstream of position -149	
<i>aah</i> sequence	AAAAGATATCTTTTATTCGCCACGACACAAGAAAAAACTATTATTAAGAATG
σ^{70} consensus	AAWWWTWTTTNNAAAA—TTGACA—TRTGNTATAAT—R
σ^S consensus	AAAWWWWT—tTGaCA—TRTKCTAYaCTTaa—R
Promoter region upstream of position -128	
<i>aah</i> sequence	CACGACACAAGAAAAAACTATTATTAAGAATGTATTTGATTATTACTATTGG
σ^{70} consensus	AAWWWTWTTTNNAAAA—TTGACA—TRTGNTATAAT—R
σ^S consensus	AAAWWWWT—tTGaCA—TRTKCTAYaCTTaa—R

indicated an important role for this motif in transcription regulation. Interestingly, in a database search we could not identify sequence homologies to known regulator-binding sites.

Further, we wondered whether the AT-rich region might harbour structural elements that would allow the binding of additional regulatory factors. A protein that is known to bind to bent DNA is the histone-like protein H-NS. Normally, H-NS serves as a negative regulator (Fang & Rimsky, 2008), and in *hns*-negative strains transcription activity is usually increased as compared with the wild-type strain. In some cases, such as for the *csiD* promoter of *E. coli*, H-NS has also been reported to actually activate transcription (Germer *et al.*, 2001). However, for *csiD*, as for *aah* and *aidA*, conclusions about whether H-NS exerts its positive effect directly or indirectly cannot be drawn.

The influence of RfaH on transcription of *aah* and *aidA* was found to be opposite to the usually observed effect of this regulator. RfaH is described as a transcriptional anti-terminator interacting with the elongation complex of the RNA polymerase for efficient transcription of polycistronic mRNAs (Xue *et al.*, 2009). Normally, interaction of RfaH with the RNA polymerase depends on the *ops* element located in the 5' untranslated region (UTR) of the mRNA (Artsimovitch & Landick, 2002). This *ops* element cannot be detected in the 5' UTR of *aah* and *aidA* mRNA. Nevertheless, there was an increase in transcription activity for both genes in an *rfaH* mutant. Similar results have been reported for the related *E. coli* autotransporter Ag43 encoded by the *flu* gene (Beloin *et al.*, 2006). The 5' UTR of the *flu* mRNA also shows no homology to the *ops* element; however, increased transcription of the gene in an *rfaH* mutant has been found. As in the case of H-NS, the effect of RfaH on *aah* and *aidA* transcription might be indirect.

Initiation of transcription from the *aah* promoter(s) leads to the synthesis of a bicistronic mRNA for *aah* and *aidA*. Both genes have to be expressed to mediate and/or stabilize the adherence function of AIDA-I. As AAH is an enzyme it does not need to be expressed in equimolar amounts. Therefore, a possibility to increase the amount of the AIDA-I adhesin might be via the synthesis of additional *aidA* mRNA, utilizing the specific *aidA* promoter.

Investigating the upstream region of *aidA* by 5'-RACE we identified two distinct transcription start sites. Both sites have -10 and -35 regions with high similarity to the consensus sequence of a σ^{70} promoter, and this is further supported by the distance between the two regions (Typas *et al.*, 2007). Interestingly, the start site at 66 nt upstream of the start codon could only be identified in the strain harbouring a plasmid with the complete coding sequence for *aah* and *aidA*, and not in the promoter test vector harbouring only 85 nt encoding the N-terminal sequence. This result points to an additional influence of the downstream sequence and/or the gene products on expression. The transcription start site at 252 nt could be identified in both recombinant strains.

Gene regulation in micro-organisms is influenced by various environmental factors, and pathogenic micro-organisms are no exception (Finlay & Falkow, 1997). Even if specific regulators involved in transcription of *aah* and *aidA* have not been identified up to now, differences in expression were detected depending on growth phase, nutrition, oxygen and, particularly, temperature. The involvement of σ^S in the transcription of *aah* could be verified by experiments performed in an *rpoS*-negative strain. σ^S is a stationary phase-specific sigma factor and functions as a global regulator of gene expression under various stress conditions (Klauck *et al.*, 2007). Transcription of *aah* by a σ^S RNA polymerase fits quite well with the environment normally colonized by enteropathogenic *E. coli*, and such transcription is often modulated further by additional regulators and histone-like proteins (Germer *et al.*, 2001). The changes found in the transcription activity of *aidA* could not be referred to one single factor.

Interestingly, we observed a difference in transcription activity in *E. coli* MC4100 strains derived from different laboratory stocks. Such differences in transcription activity have also been reported by other investigators (Jishage & Ishihama, 1997; Soupene *et al.*, 2003), as bacteria from a single origin are subject to genome plasticity and, therefore, exhibit frequent changes, resulting in laboratory-specific alterations and characteristics. Much greater differences in activity have been detected by comparing different *E. coli* K-12 strains. This might be of practical interest, as the AIDA system is employed by numerous groups for surface expression studies with heterologous antigens. Not only did we measure different transcription activities for *aah* (by a factor of five) and *aidA* (by a factor of 20), but also the ratio between the two genes differed by a factor of approximately 60. This further supports the finding that the transcription of *aah* and *aidA* is modulated in different ways. In view of the known genotypes of the different *E. coli* strains examined in this study, a common reason for these differences cannot be given. The observed differences could be due to a polymorphism(s) in a varying number of genes. Spira *et al.* (2008) have shown strain variations in ppGpp concentration and σ^S level in laboratory strains of *E. coli* K-12 due to mutations in *spoT* and *rpoS*. This kind of microevolution might also take place in other genes and influence global bacterial regulatory networks. To study the regulation of the AIDA system not only with respect to the two genes of the AIDA autotransporter system but also in the context of pathogenicity it will be interesting to investigate expression in the wild-type strain *E. coli* 2787.

Up to now, a correlation between transcription of the *aah* and *aidA* genes and protein synthesis has not been experimentally proven. However, there is a first hint that the amount of mRNA correlates with the amount of protein detectable. Berthiaume *et al.* (2007) attempted to isolate AIDA-I from the recombinant *E. coli* strain ED9 and failed. Strain ED9 is an MC4100 derivative, and in transcription assays it has been shown that in MC4100 only a very low activity can be detected for *aidA*, whereas in

strain C600, which is normally used to isolate usable amounts of AIDA-I, the activity of the *aidA* promoter is eightfold higher.

In conclusion, this study shows that both genes of the AIDA system are transcribed as a bicistronic mRNA. There is additional transcription from the *aidA* promoter, increasing the amount of mRNA. However, this does not reflect the differences at the protein level seen in the bacterium. Therefore, to explain the observed differences at the protein level there have to be other, additional factors involved, such as mRNA stability, translation efficiency and/or half-life of the specific gene products. Not surprisingly, transcription varied considerably in different *E. coli* K-12 laboratory strains and under different environmental conditions. Interestingly, transcription of both genes was enhanced in *hns* and *rfaH* mutant strains, a finding that needs to be investigated in future studies.

ACKNOWLEDGEMENTS

We are indebted to P. Dersch, U. Dobrindt, R. Hengge and G. Heusipp for providing mutant *E. coli* strains. This study was supported in part by grants from the Deutsche Forschungsgemeinschaft (DFG SFB 293 TP B5) and by a grant from the EU Network ERA-NET PathoGenoMics (project no. 03113937C).

REFERENCES

- Appleyard, R. K. (1954). Segregation of new lysogenic types during growth of a doubly lysogenic strain derived from *Escherichia coli* K12. *Genetics* **39**, 440–452.
- Artsimovitch, I. & Landick, R. (2002). The transcriptional regulator RfaH stimulates RNA chain synthesis after recruitment to elongation complexes by the exposed nontemplate DNA strand. *Cell* **109**, 193–203.
- Bäcklund, E., Markland, K. & Larsson, G. (2008). Cell engineering of *Escherichia coli* allows high cell density accumulation without fed-batch process control. *Bioprocess Biosyst Eng* **31**, 11–20.
- Beloin, C., Michaelis, K., Lindner, K., Landini, P., Hacker, J., Ghigo, J.-M. & Dobrindt, U. (2006). The transcriptional anti-terminator RfaH represses biofilm formation in *Escherichia coli*. *J Bacteriol* **188**, 1316–1331.
- Benz, I. & Schmidt, M. A. (1989). Cloning and expression of an adhesin (AIDA-I) involved in diffuse adherence of enteropathogenic *Escherichia coli*. *Infect Immun* **57**, 1506–1511.
- Benz, I. & Schmidt, M. A. (1992a). AIDA-I, the adhesin involved in diffuse adherence of the diarrhoeagenic *Escherichia coli* strain 2787 (O126:H27), is synthesized via a precursor molecule. *Mol Microbiol* **6**, 1539–1546.
- Benz, I. & Schmidt, M. A. (1992b). Isolation and serologic characterization of AIDA-I, the adhesin mediating the diffuse adherence phenotype of the diarrhea-associated *Escherichia coli* strain 2787 (O126:H27). *Infect Immun* **60**, 13–18.
- Benz, I. & Schmidt, M. A. (2001). Glycosylation with heptose residues mediated by the *aah* gene product is essential for adherence of the AIDA-I adhesin. *Mol Microbiol* **40**, 1403–1413.
- Berthiaume, F., Rutherford, N. & Mourez, M. (2007). Mutations affecting the biogenesis of the AIDA-I autotransporter. *Res Microbiol* **158**, 348–354.
- Brandon, L. D., Goehring, N., Janakiraman, A., Yan, A. W., Wu, T., Beckwith, J. & Goldberg, M. B. (2003). IcsA, a polarly localized autotransporter with an atypical signal peptide, uses the Sec apparatus for secretion, although the Sec apparatus is circumferentially distributed. *Mol Microbiol* **50**, 45–60.
- Casali, N., Konieczny, M., Schmidt, M. A. & Riley, L. W. (2002). Invasion activity of a *Mycobacterium tuberculosis* peptide presented by the *Escherichia coli* AIDA autotransporter. *Infect Immun* **70**, 6846–6852.
- Datsenko, K. A. & Wanner, B. L. (2000). One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A* **97**, 6640–6645.
- Dautin, N. & Bernstein, H. D. (2007). Protein secretion in Gram-negative bacteria via the autotransporter pathway. *Annu Rev Microbiol* **61**, 89–112.
- Dersch, P., Kneip, S. & Bremer, E. (1994). The nucleoid-associated DNA-binding protein H-NS is required for the efficient adaptation of *Escherichia coli* K-12 to a cold environment. *Mol Gen Genet* **245**, 255–259.
- Dorman, C. J. (2004). H-NS: a universal regulator for a dynamic genome. *Nat Rev Microbiol* **2**, 391–400.
- Fang, F. C. & Rimsky, S. (2008). New insights into transcriptional regulation by H-NS. *Curr Opin Microbiol* **11**, 113–120.
- Finlay, B. B. & Falkow, S. (1997). Common themes in microbial pathogenicity revisited. *Microbiol Mol Biol Rev* **61**, 136–169.
- Frohman, M. A. (1993). Rapid amplification of complementary DNA ends for generation of full-length complementary DNAs: thermal RACE. *Methods Enzymol* **218**, 340–356.
- Germer, J., Becker, G., Metzner, M. & Hengge-Aronis, R. (2001). Role of activator site position and a distal UP-element half-site for sigma factor selectivity at a CRP/H-NS-activated σ^S -dependent promoter in *Escherichia coli*. *Mol Microbiol* **41**, 705–716.
- Gibson, T. J. (1984). *Studies on the Epstein-Barr virus genome*. PhD thesis, University of Cambridge.
- Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166**, 557–580.
- Henderson, I. R., Navarro-Garcia, F., Desvaux, M., Fernandez, R. C. & Ala'aldien, D. (2004). Type V protein secretion pathway: the autotransporter story. *Microbiol Mol Biol Rev* **68**, 692–744.
- Jain, S. & Goldberg, M. B. (2007). Requirement for YaeT in the outer membrane assembly of autotransporter proteins. *J Bacteriol* **189**, 5393–5398.
- Jensen, K. F. (1993). The *Escherichia coli* K-12 “wild types” W3110 and MG1655 have an *rph* frameshift mutation that leads to pyrimidine starvation due to low *pyrE* expression levels. *J Bacteriol* **175**, 3401–3407.
- Jishage, M. & Ishihama, A. (1997). Variation in RNA polymerase sigma subunit composition within different stocks of *Escherichia coli* W3110. *J Bacteriol* **179**, 959–963.
- Jose, J. & Meyer, T. F. (2007). The autodisplay story: from discovery to biotechnical and biomedical applications. *Microbiol Mol Biol Rev* **71**, 600–619.
- Klauck, E., Typas, A. & Hengge, R. (2007). The sigmaS subunit of RNA polymerase as a signal integrator and network master regulator in the general stress response in *Escherichia coli*. *Sci Prog* **90**, 103–127.
- Konieczny, M. P. J., Suhr, M., Noll, A., Autenrieth, I. B. & Schmidt, M. A. (2000). Cell surface presentation of recombinant (poly-)peptides including functional T cell epitopes by the AIDA autotransporter system. *FEMS Immunol Med Microbiol* **27**, 321–332.

- Laarmann, S. & Schmidt, M. A. (2003).** The *Escherichia coli* AIDA autotransporter adhesin recognizes an integral membrane glycoprotein as receptor. *Microbiology* **149**, 1871–1882.
- Lange, R. & Hengge-Aronis, R. (1991).** Identification of a central regulator of stationary-phase gene expression in *Escherichia coli*. *Mol Microbiol* **5**, 49–59.
- Mainil, J. G., Jacquemin, E., Pohl, P., Kaeckenbeeck, A. & Benz, I. (2002).** DNA sequences coding for the F18 fimbriae and AIDA adhesin are localised on the same plasmid in *Escherichia coli* isolates from piglets. *Vet Microbiol* **86**, 303–311.
- Mandel, M. & Higa, A. (1970).** Calcium dependent bacteriophage DNA infection. *J Mol Biol* **53**, 159–162.
- Marinus, M. G., Carraway, M., Frey, A. Z., Brown, L. & Arraj, J. A. (1983).** Insertion mutations in the *dam* gene of *Escherichia coli* K-12. *Mol Gen Genet* **192**, 288–289.
- Miller, J. H. (1972).** *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Niewerth, U., Frey, A., Voss, T., Le Bouguénec, C., Baljer, G., Franke, S. & Schmidt, M. A. (2001).** The AIDA autotransporter system is associated with F18 and Stx2e in *Escherichia coli* isolates from pigs diagnosed with edema disease and postweaning diarrhea. *Clin Diagn Lab Immunol* **8**, 143–149.
- Orndorff, P. E., Spears, P. A., Schauer, D. & Falkow, S. (1985).** Two modes of control of *pilA*, the gene encoding type 1 pilin in *Escherichia coli*. *J Bacteriol* **164**, 321–330.
- Powers, E. L. & Randall, L. L. (1995).** Export of periplasmic galactose-binding protein in *Escherichia coli* depends on the chaperone SecB. *J Bacteriol* **177**, 1906–1907.
- Pritchard, J., Ngeleka, M. & Middleton, D. M. (2004).** In vivo and in vitro colonization patterns of AIDA-I-positive *Escherichia coli* isolates from piglets with diarrhea. *J Vet Diagn Invest* **16**, 108–115.
- Sambrook, J., Frisch, E. F. & Maniatis, T. (1989).** *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Schneider, K. & Beck, C. F. (1986).** Promoter-probe vectors for the analysis of divergently arranged promoters. *Gene* **42**, 37–48.
- Soupe, E., van Heeswijk, W. C., Plumbridge, J., Stewart, V., Bertenthal, D., Lee, H., Prasad, G., Paliy, O., Charernnoppakul, P. & Kustu, S. (2003).** Physiological studies of *Escherichia coli* strain MG1655: growth defects and apparent cross-regulation of gene expression. *J Bacteriol* **185**, 5611–5626.
- Spira, B., Hu, X. & Ferenci, T. (2008).** Strain variation in ppGpp concentration and RpoS levels in laboratory strains of *Escherichia coli* K-12. *Microbiology* **154**, 2887–2895.
- Suhr, M., Benz, I. & Schmidt, M. A. (1996).** Processing of the AIDA-I precursor: removal of AIDA^c and evidence for the outer membrane anchoring as a β -barrel structure. *Mol Microbiol* **22**, 31–42.
- Typas, A., Becker, G. & Hengge, R. (2007).** The molecular basis of selective promoter activation by the σ^S subunit of RNA polymerase. *Mol Microbiol* **63**, 1296–1306.
- Van der Woude, M. W. & Henderson, I. R. (2008).** Regulation and function of Ag43 (Flu). *Annu Rev Microbiol* **62**, 153–169.
- Wells, T. J., Tree, J. J., Ulett, G. C. & Schembri, M. A. (2007).** Autotransporter proteins: novel targets at the bacterial surface. *FEMS Microbiol Lett* **274**, 163–172.
- Xue, P., Corbett, D., Goldrick, M., Naylor, C. & Roberts, I. S. (2009).** Regulation of expression of the region 3 promoter of the *Escherichia coli* K5 capsule gene cluster involves H-NS, SlyA, and a large 5' untranslated region. *J Bacteriol* **191**, 1838–1846.
- Yanisch-Perron, C., Vieira, J. & Messing, J. (1985).** Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mpl8 and pUC19 vectors. *Gene* **33**, 103–111.

Edited by: R. G. Sawers