Escherichia coli toxin gene hipA affects biofilm formation and DNA release

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INTRODUCTION

Most bacteria live in surface-associated communities called biofilms, while free floating micro-organisms are comparatively uncommon (Costerton et al., 1995). Micro-organisms attach via appendages such as fimbriae and flagella, accumulating until microcolonies are formed, further stimulated by the production of the microbial matrix, containing polysaccharides, glycoproteins and DNA (Karanakaran et al., 2011). This multicellular behaviour is crucial for effective host colonisation, since 80% of human bacterial chronic inflammatory and infectious diseases involve biofilms (Barraud et al., 2009).

Toxin–antitoxin (TA) systems are widely present in bacterial chromosomes, including pathogens (Engelberg-Kulka et al., 2006). In Escherichia coli alone, the number of TA systems has recently increased from five to 37 (Hayes, 2003; Tan et al., 2011). However, the role of TA systems in cell physiology is controversial, with several possible roles identified (Magnuson, 2007): additive genomic debris, stabilization of genomic parasites, selfish alleles, gene regulation, growth control, persister cell formation, programmed cell arrest, programmed cell death, and anti-phage measures. Recent studies have suggested that bacterial cell death and lysis play a significant role in biofilm formation and development (Rice et al., 2007; Webb et al., 2003). However, reports on the effects of TA systems on biofilm formation are limited.

Investigation of the hip locus has revealed that it consists of two genes, hipA and hipB (Black et al., 1991; Moyed & Broderick, 1986). The hipA gene encodes a stable toxin, HipA, while hipB encodes the antitoxin HipB. The HipBA system is implicated in biofilm formation, providing multidrug tolerance (Lewis, 2007, 2008). Biochemical and structural studies have shown that the toxin HipA is a member of the phosphatidylinositol 3/4-kinase superfamily and can phosphorylate the translation factor EF-Tu (Schumacher et al., 2009). This kinase activity is required for the induction of the dormant state of persister cells (Correia et al., 2006). The hipB gene product is a small Cro-like protein with a helix–turn–helix DNA-binding domain. It functions as a repressor for the hipBA operon by binding to four operator sites on the promoter region of hipBA, and inhibits HipA activity through covalent binding to the toxin (Black et al., 1991, 1994; Schumacher et al., 2009). It has been reported that expression of HipA in the wild-type can inhibit protein, RNA and DNA synthesis (Korch & Hill, 2006). Furthermore, low-level expression of
HipA in the presence of glucose is sufficiently toxic to inhibit cell growth and affect cell viability (Korch et al., 2003). Overexpression of HipA in the ΔhipBA mutant causes almost all the cells to form filaments, with eventual lysis (Korch et al., 2003).

Currently, toxin proteins from over 30 chromosomally encoded TA modules have been identified in *E. coli*. Chromosomal TA modules can be grouped into three main superfamilies based on whether the toxin has an RNase/gyrase-like fold, RNase barnase-like structure, or Pin N terminus (PIN) domain. The corresponding antitoxins contain DNA-binding domains (Buts et al., 2005). However, the TA system hipBA shows no homology to any member of the three TA superfamilies.

While the mode of action of reported *E. coli* toxins varies, they also appear to differ in their role regarding bacterial physiology and subsequent biofilm formation (Kim et al., 2009; Kolodkin-Gal et al., 2009). Therefore, we investigated whether hipBA affected biofilm formation, even though the effects of several other TA systems on *E. coli* biofilm formation have been reported (Kim et al., 2009; Kolodkin-Gal et al., 2009).

In the study presented here, we further investigated the relationship between TA systems and biofilm formation. Our results demonstrate that the TA system HipBA significantly affects biofilm formation in *E. coli* BW25113 without antibiotic pressure. Furthermore, treatment of the BW25113 biofilm with DNase I caused significant reduction in biofilm formation, whereas similar treatment of the hipA mutant biofilm had only a minor effect. Additionally, the inactivation of HipA reduced the level of extracellular DNA (eDNA) present during biofilm formation. To our knowledge, this is the first report that HipBA can influence *E. coli* biofilm formation and that a TA system may affect biofilm formation by producing eDNA in the biofilm.

**METHODS**

**Bacterial strains, plasmid and growth conditions.** *E. coli* strains and the plasmid used in our work are listed in Table 1. We used pITGL to measure β-galactosidase activity and GFP. pITGL was constructed from pITG (Li et al., 2012) by inserting the lacZ gene at the XhoI and SacI sites. The lacZ gene was amplified with primers lacz-up and lacz-down (Table 2). HipBA genes were amplified from wild-type BW25113 with primers hipBA-F and hipBA-R (Table 2) and ligated into plasmid pBAD24, digested by EcoRI and PstI. The recombinant plasmid was named pBAD-hipBA.

All strains were incubated at 37 °C. Biofilms were grown in the liquid rich medium TSBG (Rice et al., 2007) and minimal medium ABTCAA (Reisner et al., 2006). Crystal violet and bovine pancreatic DNase I with a specific activity of 2000 Kunitz units per milligram were obtained from Sigma. SYTO 9 was obtained from Invitrogen.

**HipBA operon inactivation.** The hipBA mutant strain of BW25113 was created by using the one-step inactivation method (Datsenko & Wanner, 2000). This method includes the following steps: first, amplification of the resistance gene from pKD4 (KmR) using the primer hipBA-pKD4 and hipBA-pKDR (Table 2). The PCR products flanked by FRT (FLP recognition target) sites and homologous sequences to hipBA were purified and electroporated into *E. coli* cells carrying plasmid pKD46.

**Complementation of the hip mutation by expression of HipBA.** *E. coli* strain BW25113ΔhipBA was transformed with pBAD-hipBA or pBAD-24. The transformants were induced with 0.2 % arabinose at time zero, and biofilm formation was quantified with crystal violet stain as described below.

**Quantitative detection of crystal violet-stained biofilm.** The biofilm attached to the bottom of 96-well plates was detected by spectrophotometric measurements. In brief, cells were grown overnight in ABTCAA medium, and diluted to a final O.D.600 of 0.05 with fresh medium and incubated in 96-well polystyrene plates (200 μl culture in each well) for 8 or 24 h without shaking. After incubation, cell turbidity at 600 nm was measured. The supernatant was removed, and the wells were washed twice gently with isotonic phosphate buffer (0.15 M, pH 7.2). The adherent biofilm was stained with 0.1 % (w/v) crystal violet (200 μl) and incubated at room temperature for 20 min. The excess crystal violet was removed by

### Table 1. Strains and plasmids used in this work

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype or characteristics</th>
<th>Source (reference)</th>
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<tr>
<td><strong>Strains</strong></td>
<td></td>
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<tr>
<td>BW25113</td>
<td>rrrB3 ΔlacZ4787 hsdR514 Δ(araBAD)567 Δ(rhaBAD)568 rph-1</td>
<td>Keio collection (Baba et al., 2006)</td>
</tr>
<tr>
<td>BW25113ΔhipA</td>
<td>BW25113 ΔhipA::Kan</td>
<td>Keio collection (Baba et al., 2006)</td>
</tr>
<tr>
<td>BW25113ΔhipBA</td>
<td>BW25113 ΔhipBA::Kan</td>
<td>This study</td>
</tr>
<tr>
<td>BW25113ΔpITGL</td>
<td>BW25113 harbouring pITGL</td>
<td>This study</td>
</tr>
<tr>
<td>ΔhipAΔpITGL</td>
<td>BW25113ΔhipA harbouring pITGL</td>
<td>This study</td>
</tr>
<tr>
<td>ΔhipBAΔpAD-hipBA</td>
<td>BW25113ΔhipA harbouring pAD-hipBA</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pKD4</td>
<td>oriR6Kγ, KmR, rgnB (Ter)</td>
<td>CGSC* (Datsenko &amp; Wanner, 2000)</td>
</tr>
<tr>
<td>pKD46</td>
<td>araBp-gam-bet-exo, bla(Apr), repA10 (ts), oriR101</td>
<td>CGSC* (Datsenko &amp; Wanner, 2000)</td>
</tr>
<tr>
<td>pITGL</td>
<td>β-Galactosidase and GFP expression plasmid</td>
<td>This study</td>
</tr>
<tr>
<td>pBAD-24</td>
<td>Cloning vector with arabinose regulatory region, AmpR</td>
<td>Lab stock</td>
</tr>
<tr>
<td>pBAD-hipBA</td>
<td>pBAD-24 containing hipBA (E. coli)</td>
<td>This study</td>
</tr>
</tbody>
</table>

*E. coli* Genetic Stock Center, Department of Biology, Yale University, New Haven, CT 06510, USA.
three gentle washes with distilled water. The crystal violet was solubilized by adding 95% (v/v) ethanol, and the extent of biofilm formation was determined by the $A_{540}$ using a microplate reader (BioTek). Normalized biofilm was calculated by dividing the biofilm value ($A_{540}$) by the bacterial growth turbidity value (OD$_{600}$) for each strain.

**Effect on biofilm formation of supplementation with extra-cellular genomic DNA.** Overnight cultures were diluted to OD$_{600}$ 0.2 in the same TSBG medium containing 10 µg ml$^{-1}$ DNA extracted from E. coli BW25113. Then, diluted cultures were incubated in 96-well polystyrene plates for 8 or 24 h without shaking. Crystal violet stain was used for quantification.

**Visualization of biofilm under static conditions.** E. coli strains BW25113 and BW25113ΔhipA were transformed with p1TGL to express GFP constitutively. Overnight cultures were diluted to a final OD$_{600}$ of 0.05 and incubated in 24-well polystyrene plates (1 ml culture in each well) without shaking. After 2 days, the polystyrene plates were centrifuged at 3000 g for 10 min. Then, the supernatants were removed and fresh ABTCAA medium was added. After 4 days of culture, the polystyrene plates were washed three times with isotonic phosphate buffer (0.15 M, pH 7.2). Finally, biofilms were visualized by fluorescence microscopy (Nikon Eclipse TE2000 S) with a Plan Fluor ×10/0.30 objective.

**DNase I treatment of biofilms on polystyrene microtitre plates.** The incubation of biofilms was performed as described above. (1) For the crystal violet method, DNase I was diluted with ABTCAA medium to a final concentration of 100 U ml$^{-1}$ and 200 µl was added to each well. After 8 h, crystal violet-stained biofilm quantification was performed as described above. Normalized biofilm was calculated by dividing the biofilm value ($A_{540}$) by the bacterial growth value (OD$_{600}$) for each strain. (2) For the fluorescence microscopy method, DNase I was diluted with isotonic phosphate buffer (0.15 M, pH 7.2) to a final concentration of 100 U ml$^{-1}$. Polystyrene microtitre plates with 4 days-incubated attached cells were treated with 1 ml DNase I solution for 1 h. Biofilms were visualized by fluorescence microscopy as described above.

**Extraction and quantification of eDNA.** BW25113 and its mutants were incubated overnight, after which the cultures were diluted to an OD$_{600}$ of 0.05 in ABTCAA medium and grown for 96 h in polystyrene Petri dishes at 37 °C. eDNA in biofilms was extracted with a combination of N-glycanase and proteinase K treatment, as described before (Wu & Xi, 2009). eDNA was quantified using a spectrophotometer (NanoDrop ND-1000; Thermo Fisher Scientific) (Wu & Xi, 2009). To account for potential differences in biomass, the average OD$_{600}$ of each unwashed biofilm was determined and used to calculate the relative OD$_{600}$ of each biofilm with respect to the OD$_{600}$ of the untreated BW25113 biofilm (whereby the relative OD$_{600}$ of the BW25113 biofilm was taken as 1). The mass of eDNA per relative biomass of each biofilm was then calculated by dividing its total eDNA (ng) by its relative OD$_{600}$ (Rice et al., 2007).

**Measurement of β-Galactosidase activity in culture supernatants.** β-Galactosidase activity was measured as described elsewhere (Rice et al., 2007; Steinmoen et al., 2002). BW25113 and BW25113ΔhipA, both harbouring p1TGL, were incubated overnight, the cultures were diluted to an OD$_{600}$ of 0.05 in ABTCAA medium and grown for 48 h. Samples were taken every 12 h and cell turbidity was measured at 600 nm. Cells were then removed by centrifugation, and β-galactosidase activity in the supernatants was determined with ONPG as the substrate.

**Statistical analysis.** Statistical analyses were performed by using SPSS for Windows (version 13.0). A P value of <0.05 was considered statistically significant.

## RESULTS

### The E. coli TA system HipBA affects biofilm formation

The hipBA system is implicated in biofilm formation in response to several drugs (Lewis, 2007, 2008). However, whether HipBA affects biofilm formation without antibiotic supplementation has not been reported. Using the E. coli deletion mutants described in Methods, we studied the effect of HipBA on biofilm formation. Biofilm formation in polystyrene wells under static conditions was tested. The normalized biofilm formation by the hipA mutant and the hipBA mutant (0.50 ± 0.09 and 0.27 ± 0.01) was significantly less than the wild-type strain (1.02 ± 0.16 and 0.51 ± 0.02) after 8 and 24 h incubation (Fig. 1). To quantify the specific biofilm-forming ability, normalized biofilm was calculated by dividing the total biofilm by the bacterial growth for each strain. Furthermore, the mutant strain BW25113ΔhipBA was complemented by expression of HipBA and biofilm formation was measured in the 96-well plate crystal violet assay: the normalized biofilm of the hipBA mutant expressing HipBA was 0.98 ± 0.13 and 0.49 ± 0.01 after 8 and 24 h incubation. Hence, the hipBA mutant carrying plasmid pAD-hipA showed suppression of the mutator phenotype.

### The TA system may affect biofilm formation by controlling eDNA release

Recent studies have shown that eDNA can be a structural component of the biofilm matrix (Heijstra et al., 2009; Rice et al., 2007; Spoering & Gilmore, 2006). However, an

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**Table 2. Primers used in this work**

<table>
<thead>
<tr>
<th>Primer</th>
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<tbody>
<tr>
<td>hipBA-pKDF</td>
<td>5′-TAAAGCCGATAAACTTGTGGACGTATGACATGTGATGCGGTGACTGCTC-3′</td>
</tr>
<tr>
<td>hipBA-pKDRI</td>
<td>5′-CCCAACCCCATATCTCTTCAATACGTATTCTGATGGGAATTGACCCATTGCC-3′</td>
</tr>
<tr>
<td>hipBA-F</td>
<td>5′-CGGAATTCGAAAGGAGATATCATATGAGGGTTTCAGGAAGATCTATAG-3′</td>
</tr>
<tr>
<td>hipBA-R</td>
<td>5′-AAGCTGGACTCATTCACTACGTTATTCT-3′</td>
</tr>
<tr>
<td>lacZ-up</td>
<td>5′-GCCCTCGAGTTGCGAATATCACTCGGCTGTAATGTGTGGAAATTGTGAAAGGAGAAATACTAG-3′</td>
</tr>
<tr>
<td>lacZ-down</td>
<td>5′-GCCGGCGGGCTCGCCCGTTATTATTA-3′</td>
</tr>
</tbody>
</table>
earlier study indicated that the addition of DNase did not reduce biofilm formation of *E. coli* MC4100 WT and its Δ5 TA system mutants (Kolodkin-Gal *et al.*, 2009). We investigated whether eDNA was present in biofilm structures of *E. coli* BW25113 and studied the link between biofilm formation and DNA release mediated by the hipBA system. First, BW25113 and the hipA mutant were able to produce significantly more biofilm mass (all *P* < 0.05) by adding 10 μg ml⁻¹ genomic DNA (Fig. 2). This result suggests that extracellular genomic DNA directly enhances biofilm formation and that eDNA is a structural component of the biofilm matrix in *E. coli* BW25113. The effect of DNase I on biofilm formation was further investigated by utilizing polystyrene microtitre plates. When biofilm was grown on polystyrene microtitre plates, we observed a significant decrease in BW25113 biofilm formation after DNase I treatment for 8 h (Fig. 3). However, DNase I had only a minor effect on the hipA mutant biofilm (Fig. 3). Further, DNase I treatment for 1 h led to the formation of similar biofilm densities of BW25113 and its hipA mutant under a fluorescence microscope (Fig. 4 c, d). The addition of DNase I to growing cultures had no obvious effect on cell growth for any of the BW25113 strains (data not shown). Last, measurement of the eDNA level during biofilm formation is a possible way to investigate the link between the release of eDNA and the hipA mutant, so the eDNA from 96 h-old biofilms was obtained and quantified. This analysis indicated that the amount of eDNA present in a BW25113 biofilm (9.82 ± 0.544 ng) was almost threefold greater than that present in the both the hipBA and hipA mutant biofilms (3.50 ± 0.239 and 3.335 ± 0.243 ng, respectively) when grown under similar conditions (Fig. 5). These results imply that eDNA is an important structural component in biofilms of *E. coli* BW25113 and that the inactivation of HipA reduces the level of eDNA present during biofilm formation.

It has been suggested that hipA is a lethal toxin, because overproduction of HipA in the absence of HipB leads to decreased viability of *E. coli* BL21, K-12 and MG1655 (Black *et al.*, 1994; Falla & Chopra, 1998; Korch *et al.*, 2003). However, Korch & Hill (2006) have disputed this claim using *E. coli* MG1655. To determine whether HipA can mediate cell lysis in *E. coli* BW25113, we transferred p1TGL into BW25113 and the hipA mutant (p1TGL expresses the lacZ reporter constitutively). Cell lysis was measured by β-galactosidase activity in culture supernatants at 12 h intervals. Low levels of β-galactosidase activity were found in the hip mutant containing p1TGL, whereas higher levels of β-galactosidase activity in BW25113 containing p1TGL were observed at most time points (Fig. 6). No β-galactosidase activity was detected in...
BW25113 and the hipA mutant without p1TGL. These results indicate that the presence of HipA affects the integrity of E. coli BW25113 cells. Apparently, the lysis of a subpopulation of cells had an influence on eDNA concentrations. Therefore, HipA could trigger cell death and influence the amount of eDNA in the biofilm.

**DISCUSSION**

The first TA system reportedly linked to biofilm formation was the MqsR/MqsA pair of E. coli, where, upon deletion of mqsRA, biofilm formation decreased (Kasari et al., 2010; Ren et al., 2004). Further evidence of the role of TA systems in biofilm formation was obtained with E. coli Δ5 which had five of the most-studied TA systems deleted (Δ5, MazF/MazE, RelE/RelB, ChpB, YoeB/YeiM and YafQ/DinJ) from E. coli MG1655 (Kim et al., 2009; Kolodkin-Gal et al., 2009). Kim and co-workers have reported that strain Δ5, compared with the wild-type, shows decreased biofilm formation at 8 h and increased biofilm formation at 24 h of incubation. This result is caused by over-expression of YigK, a protein that affects the production of fimbriae involved in both biofilm attachment and dispersal (Kim et al., 2009). The five TA systems affect cell death differently during biofilm formation in E. coli strain MC4100 (Kolodkin-Gal et al., 2009). First, the mazEF system is the regulatory module, mediating cell death both in liquid media and during biofilm formation; second, the relBE system is a principal mediator of cell death only in liquid media, but not in biofilm formation; third, the chpBIK system is a back-up death system for the mazEF system in a ppGpp-independent manner; fourth, the yefM-yoeB system mediates cell death only in liquid media under certain conditions, but not in biofilm formation; fifth, the dinJ-yafQ system is a principal mediator of cell death only in biofilm formation. These results, when compared with earlier studies with MG1655 (Kim et al., 2009) and our studies with BW25113, provide different mechanisms for the effects of TA systems on biofilm formation.

Although HipA is implicated in biofilm formation in the presence of several drugs (Lewis, 2007, 2008), its enhancement of biofilm formation in the absence of antibiotics and the mechanism have not been reported. Our results show that the deletion of HipBA of E. coli BW25113 significantly reduced the biofilm biomass.
eDNA is an important component of the biofilm matrix (Heijstra et al., 2009; Rice et al., 2007; Spoering & Gilmore, 2006). Recent studies have shown that bacterial programmed cell death has multiple functions, including releasing eDNA that could have a function in structuring biofilms (Rice et al., 2007; Webb et al., 2003; West et al., 2007). In E. coli, some TA systems can mediate programmed cell death to influence cell lysis (Engelberg-Kulka et al., 2005). However, no evidence indicates that the TA system can affect biofilm formation through DNA release. Although Kolodkin-Gal and co-workers attempted to study the link between biofilm and DNA release mediated by TA-mediated cell lysis, they found that the addition of DNase did not reduce biofilm formation of MC4100 wild-type and Δ5. They suggested that the role of cell death in E. coli is not through the release of DNA; thus, MazEF and DinJ-YafQ-mediated cell death has a possible function in biofilm formation through novel, yet unknown, mechanism(s) (Kolodkin-Gal et al., 2009). However, the contribution of cell lysis to biofilm formation appears to vary from species to species and strain to strain (Rice et al., 2009; Wang et al., 2009; Zegans et al., 2009). We suspected hipA-mediated cell lysis and eDNA release to play an important role in biofilm formation in BW25113. To investigate this hypothesis, treatment with DNase I of biofilms of BW25113 and its hipA mutant was investigated. The results showed that DNase I treatment significantly decreased biofilm formation for E. coli strain BW25113, but that the treatment had only a minor effect on the hipA mutant. This hypothesis was also supported by the unequal level of cell lysis in the cultures with or without the presence of hipA in the genome. Additionally, by measuring eDNA levels in BW25113 and mutant biofilms, we directly demonstrated that hipA influences the production of eDNA and that a supplement of extracellular genomic DNA enhances biofilm development in both strain BW25113 and its hipA mutant. These results demonstrate that HipA plays a significant role during biofilm development and that released genomic DNA is an important structural component of the E. coli biofilm.

Based on this study and earlier reported studies, it is clear that the E. coli TA systems differ in their role in bacterial physiology and biofilm formation (Kim et al., 2009; Kolodkin-Gal et al., 2009). In addition, the roles of TA systems in biofilm formation, cell lysis and DNA release also appear to vary within species and strains (Kim et al., 2009; Kolodkin-Gal et al., 2009; Rice et al., 2009; Wang et al., 2009; Zegans et al., 2009). Therefore, we need careful clarification on a case-by-case basis in future studies of the link between TA systems and their function in biofilm formation.

**ACKNOWLEDGEMENTS**

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