The *Haemophilus influenzae* HipBA toxin–antitoxin system adopts an unusual three-component regulatory mechanism

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Type II toxin–antitoxin (TA) systems encode two proteins: a toxin that inhibits cell growth and an antitoxin that neutralizes the toxin by direct intermolecular protein–protein interactions. The bacterial HipBA TA system is implicated in persister formation. The *Haemophilus influenzae* HipBA TA system consists of a HipB antitoxin and a HipA toxin, the latter of which is split into two fragments, and here we investigate this novel three-component regulatory HipBA system. Structural and functional analysis revealed that HipAN corresponds to the N-terminal part of HipA from other bacteria and toxic HipA C is inactivated by HipAN, not HipB. This study will be helpful in understanding the detailed regulatory mechanism of the HipBA N+C system, as well as why it is constructed as a three-component system.

1. Introduction

Prokaryotic toxin–antitoxin (TA) systems are encoded by small operons that are composed of two elements, a toxic subunit that causes cell-growth arrest and an antitoxic subunit that neutralizes the harmful effect of the toxin (Cheverton et al., 2016). Depending on the molecular mechanism by which the antitoxins neutralize their cognate toxins, TA systems have been classified into diverse types (Hayes, 2003). In the most abundant type II TA systems, the antitoxin is a protein that directly binds and neutralizes its cognate toxin through strong molecular interactions (Hayes & Van Melderen, 2011). When freed from inhibition by antitoxins, the toxin acts on various targets to suppress cell growth or even induce cell death (Hall et al., 2017). The toxin is relatively long-lived, whereas the antitoxin is degraded by cellular proteases due to its labile nature. Accordingly, the antitoxin should be constantly produced to regulate the toxin (Hörak & Tamman, 2017). Type II antitoxins commonly contain a DNA-binding domain to bind at operators in the promoter region and repress the TA operon (Chan et al., 2016). Therefore, as the amount of antitoxin is reduced, repression stops and the operon is transcribed to supply the antitoxin (Loris & Garcia-Pino, 2014). The cellular activity of a toxin is thus driven by the quantity of the cognate antitoxin (Hörak & Tamman, 2017).

Type II TA systems are greatly abundant in nearly all free-living bacteria, raising debates about their biological functions (Pandey & Gerdes, 2005). Many studies point to additional functions of TA systems, including phage resistance, stress responses, biofilm formation and antibiotic persistence.
(Keździerska & Hayes, 2016; Lobato-Márquez et al., 2016; Díaz-Orejas et al., 2017). Stress-induced activation of TA systems protects bacteria from adverse environmental conditions, such as antibiotic treatment, by causing persister formation (Radzikowski et al., 2016). Antibiotic persistence results in a transient slow-growing state of bacteria, which allows antibiotics to be tolerated (Harms et al., 2016). This persister formation is a bet-hedging strategy in which the bacteria temporarily enters a nonreplicating state to gain stress tolerance against unfavourable environmental threats (Balaban et al., 2004; Veening et al., 2008; Lewis, 2010). Moreover, antibiotic persistence plays a crucial role in chronic infections and stimulates the evolution to antibiotic resistance (Levin-Reisman et al., 2017).

The HipBA TA system was first identified in *Escherichia coli*, in which an increase in persister formation is associated with the *hipBA* operon (Moyed & Bertrand, 1983). As an abbreviation of ‘high incidence of persistence’, *hipB* (antitoxin gene) and *hipA* (toxin gene) constitute a type II TA module that encodes two proteins: HipB (antitoxin) and HipA (toxin) (Korch & Hill, 2006). HipA acts as a serine–threonine kinase, and the expression of HipA triggers considerable cell-growth arrest (Hanks et al., 1988; Black et al., 1991, 1994; Stancik et al., 2018). On the other hand, HipB counteracts HipA by directly binding it and forming a stable protein complex (Korch & Hill, 2006). This HipBA protein complex binds to operators in the promoter region via the DNA-binding domain of HipB antitoxin and represses the transcription of *hipBA* (Schumacher et al., 2015). Upon HipB degradation, free HipA induces multidrug tolerance, leading to the dormant state of cells (Veening et al., 2008; Wood et al., 2013).

This study elucidates the crystal structure of HI0666, which exhibits sequence similarity with the N-terminal part of HipA (HipAN). To date, the HipBA system has been structurally studied in only *E. coli* (Schumacher et al., 2015) and *Shewanella oneidensis* (Wen et al., 2014). Structural information obtained in this study shows that HI0666 is part of an unusual three-component TA system resembling the HipBA system. Compared with sequences of HipAs, whose structures were known previously, the HI0665 sequence exhibits similarity with the C-terminal sequence of HipA (HipAC) from other species. In the genome map, *hipA* (encoding the C-terminus of HipA) is preceded by *hipAN* (encoding the N-terminus of HipA), and *hipAN* is preceded by *hipB* (encoding HipB). The results confirmed that HipAN and HipAC form a binary complex, and that HipB, HipAN and HipAC form a tertiary complex. HipAC-mediated growth inhibition was rescued by the expression of HipAN but not by HipB. HipAN functions as an untraditional antitoxin to HipAC, and HipB increases the ability of HipAN to counteract HipA. Taken together, these results outline a nontraditional three-component TA system, and a putative regulatory mechanism of the *H. influenzae* HipBA system is proposed.

### 2. Materials and methods

#### 2.1. Gene cloning and transformation

*hi0666* (*hipAN*), a gene encoding HipAN from *H. influenzae*, was amplified by polymerase chain reaction (PCR) using *H. influenzae* (KW20 strain, ATCC 51907) genomic DNA as a template. The oligonucleotide primers used for PCR are given in Table 1. After PCR amplification, *hipAN* was cloned into the pET28a vector (Novagen) using the restriction enzymes NdeI and Xhol. *hi0666* (*hipB*), a gene encoding HipB, was amplified in the same way and cloned into the pET21a vector (Novagen) using the same enzymes. To express HipAN and HipAC together, *hipAN* was first cloned into the pETDuet vector (Addgene), and *hi0665* (*hipAC*), a gene encoding HipAC, was subsequently introduced to follow *hipAN* in the pETDuet vector. For the cloning of *hipAC* into the pETDuet vector, the restriction enzymes BamHI and HindIII were used to digest PCR products and vector. A recombinant pET28a vector containing *hipAC* was also produced using the same approach. All recombinant plasmids were transformed into *E. coli* BL21(DE3) competent cells (Novagen).

#### 2.2. Protein expression and purification

Transformed cells harbouring *hipAN* were grown to an optical density at 600 nm (OD_{600}) of approximately 0.5 in LB medium containing 50 mg l \(^{-1}\) kanamycin at 37°C. At this point, expression was induced by adding 0.5 mM isopropyl \(\beta\)-d-1-thiogalactopyranoside (IPTG) and cells were grown for an additional 4 h at the same temperature. Growth cells were harvested by centrifugation at 5600g. The harvested cells were resuspended in buffer A [50 mM Tris–HCl, pH 7.9, and...
Table 2
Statistics for data collection and model refinement.

| HipAN (PDB code: 7czo) | Data collection | Protein atoms | Water oxygen atoms | R.m.s. deviation from ideal geometry |
|-------------------------|-----------------|---------------|------------------|-----------------------------------|
| X-ray wavelength (Å) | 0.9795 | 882/41.43 | 30/45.88 | 0.011/1.473 |
| Space group | P41212 | | | |
| Unit-cell length (a, b, c, Å) | 66.25, 66.25, 103.62 | | | 0.000 |
| Unit-cell angle (α, β, γ, °) | 90.00, 90.00, 90.00 | | | 0.000 |
| Resolution range (Å) | 50.00–2.70 (2.75–2.70) | | | |
| Total/unique reflections | 59,246/6,880 (345) | | | |
| Completeness (%) | 99.9 (100.0) | | | |
| Merge CC1/2† | 0.998 (0.973) | | | |
| I/σ(I) | 27.9 (3.2) | | | |
| Rmerge‡ | 0.096 (0.766) | | | |
| Table 2. | | | | |

500 mM NaCl containing 10%(v/v) glycerol] and lysed by sonication. The cell lysate was centrifuged at 18 000g for 1 h, and the supernatant was applied to an affinity chromatography column of nickel–nitritolacteric acid (Roche) equilibrated with buffer A. The trapped protein in the column was washed with buffer A containing 20 mM imidazole and eluted with buffer A containing 100–500 mM imidazole. Final purification of HipAN was achieved by size-exclusion chromatography using a Superdex 200 (16/600PG) column (GE Healthcare) equilibrated with 20 mM HEPES, pH 7.5, and 500 mM NaCl. The purity of HipAN was verified by SDS–PAGE.

2.3. Crystallization and diffraction data collection

The purified HipAN was concentrated to 10 mg ml⁻¹ using an Amicon Ultra Centrifugal Filter Unit (Millipore) for crystallization. Initial crystallization was performed with publicly available screening kits using a 384-well plate by the sitting-drop vapour-diffusion method. Each well of plates contained 0.5 μl of protein solution and 0.5 μl of reservoir solution. The plates were cultured at 4 °C. The crystals used for data collection were grown in reservoirs with 2 M ammonium sulfate and 0.1 M Tris, pH 8.5. The X-ray diffraction data used for structure calculation were collected at 100 K using an ADSC Quantum 315r CCD detector at the 5C beamline of the Pohang Light Source, Republic of Korea. Collected raw data were processed and scaled with the HKL2000 program suite (Otwinowski & Minor, 1997). Statistics for the data collection are described in Table 2.

2.4. Structure determination and refinement

To determine the structure of HipAN, PhaserMR in Phenix (Adams et al., 2010) was employed using the Protein Data Bank (PDB) entry 2wiu (Evdokimov et al., 2009) structure as a model. Iterative cycles of initial model building were performed by Coot (Emsley et al., 2010), and further refinement was conducted using REFMACS (Murshudov et al., 1997). The HipAN crystal structure belongs to the P41212 space group at 2.70 Å. The coordinates and structure factors are deposited in the PDB under the accession code 7czo for HipAN, the N-terminal component of HipA toxin from H. influenzae. Detailed refinement statistics are available in Table 2.

2.5. Bioinformatics

Structure figures were generated using PyMol (The PyMOL Molecular Graphics System, Version 1.8, Schrödinger, LLC). Superimpositions of structures were performed with the SSM (Krivsine & Henrick, 2004) option within Coot. The solvent-accessible areas of the protein surface were calculated by PISA (Krivsine & Henrick, 2007). Genome maps were utilized from the KEGG database (Kanehisa et al., 2017). Sequence and structural similarity were searched using DALI (Holm & Rosenstron, 2010). Structure-assisted alignment was carried out using ESPript (Robert & Gouet, 2014), with the help of ClustalW (Chenna et al., 2003) for sequence alignments. The quality of the final structure was assessed at the wwPDB X-ray structure validation server (Berman et al., 2003).

2.6. Copurification of HipAN, HipAC and HipB

To determine whether HipB, HipAN and HipAC might form a protein complex, the pETDuet vector containing both hipAN and hipAC, and the pET21a vector containing hipB were used. For this study, a hexahistidine tag was attached to the N-terminus of HipAC. These plasmids were cotransformed above, with 50 mg l⁻¹ streptomycin. Protein expression and affinity chromatography were also carried out in a similar manner to the above methods, but the three proteins were eluted carefully from being bound to the column with a gradient of 50–700 mM imidazole.
2.8. Cell-growth assay

To validate the three-component regulatory mechanism of the *H. influenzae* HipBA system, a cell-growth assay was performed using the following plasmids resistant to different antibiotics: pET21a containing *hipB*, pETDuet containing only *hipAN* and pET28a containing *hipAC*. For this assay, all plasmids were transformed into *E. coli* strain BL21(DE3). Single colonies from transformed cells grown in M9 medium plates containing 0.1% glucose were further grown overnight. These overnight cultures were then diluted to an OD600 of 0.1. The diluted cells were freshly grown until the OD600 reached 0.5; 0.5 mM IPTG was then added to the culture medium to induce protein expression. The cells were incubated at 37°C and monitored at 1 h intervals.

3. Results and discussion

3.1. Overall structure of *H. influenzae* HipAN and the hipBA genome map

There are four α-helices and five β-strands arranged as a β-barrel in the structure of *H. influenzae* HipAN [Fig. 1(a)]. Among those β-strands, five β-strands (β1–β5) form a β-sheet antiparallel to each other and these antiparallel β-sheets are flanked by four α-helices. The total solvent-accessible surface area of the monomeric structure is 7119 Å². In the reported structures of HipBA systems, we discovered an additional locus in the genome map of *H. influenzae* immediately upstream from *hipAC*. This locus is called *hipAN* in this article and encodes a 106 aa protein corresponding to the N-terminal part of HipA from *E. coli* and *S. oneidensis* [Fig. 1(b)]. In all these cases, *hipB* was located upstream of *hipA* and these putative HipB homologs might thus auto-regulate the *hipBA* operon from *H. influenzae* contains three genes, *hipB*, *hipAN* and *hipAC*.

3.2. Comparative structural analysis of the HipBA system

To date, structures of HipA have been reported from two species: *E. coli* (PDB code 5k98; Z score of 9.2, r.m.s. deviation of 2.4 Å, sequence identity of 24%) (Schumacher *et al.*, 2015) and *S. oneidensis* (PDB code 4pu3; Z score of 9.9, r.m.s. deviation of 2.7 Å, sequence identity of 23%) (Wen *et al.*, 2014) [Fig. 2(a)]. To compare the structural characteristics of *H. influenzae* HipAN and its homologs, a comparative analysis was performed with these two reported structures. *H. influenzae* HipAN superimposed well with the N-terminal parts of the HipA proteins from *E. coli* and *S. oneidensis* [Fig. 2(b)]. In addition, *H. influenzae* HipB showed a 48% sequence similarity to HipB of the *E. coli* *O127:H6* tripartite HipBA system (PDB code: 7ab3), which in turn showed high structural homology with *E. coli* HipB (PDB code: 5k98, Z score of 12.0, r.m.s. deviation of 1.2 Å) (Schumacher *et al.*, 2015) and *S. oneidensis* HipB (PDB code: 4pu3, Z score of 9.7, r.m.s. deviation of 1.8 Å) (Wen *et al.*, 2014) [Fig. 2(c)]. Regardless of the binary or tertiary nature of the HipBA systems, dimeric HipB antitoxins were highly conserved in their DNA-binding HTH motif [Fig. 2(c)], which also indicated their conserved role as transcriptional regulators. Furthermore, although the HipA toxins had similar folds, the N-terminal of HipA bound HipB antitoxins differently with respect to *E. coli* (loop between β3 and β4, containing α1) and *S. oneidensis* (α3–α4) proteins. This may suggest that the toxin neutralization mechanism of the HipBA system might be different among its homologues.

3.3. Complex formation of HipB, HipAN and HipAC

To show that HipB, HipAN and HipAC from *H. influenzae* form a complex similar to those of other type II TA systems, analytical gel filtration was conducted [Fig. 3(a)], and the eluted fractions were analyzed by SDS–PAGE [Fig. 3(b)]. His-tagged HipAC was pulled down with both HipAN and HipB. Size-exclusion chromatography further confirmed that two forms of complex [(HipB + HipAN + HipAC) and (HipAN + HipAC)] are monodispersed in solution. The second peak eluted between the 75 and 43 kDa molecular weight standards, which corresponded to the heterodimeric HipAN+C complex (~52.3 kDa). This was consistent with the relative masses of HipA toxin structures in the binary *E. coli* HipBA systems (Schumacher *et al.*, 2015). In addition, chemical crosslinking of *H. influenzae* HipB showed the predominant multimeric form of HipB as the dimer [Fig. 3(c)], as the other HipB antitoxins and thus the results of the first peak which eluted between the 158 and 75 kDa molecular weight standards were presumed to be the hexameric complex consisting of one HipB dimer bound to two HipAN+C heterodimers (Baerentsen *et al.*, 2022). Indeed, the expected
Figure 2
Comparative structural analysis of HipBA systems. (a) Structure-guided sequence alignment of \textit{H. influenzae} HipAN with the N-terminal parts of other HipA\textsubscript{s}. The secondary structural organization of \textit{H. influenzae} HipAN\textsuperscript{N} is displayed on the alignment. Conserved residues are highlighted in red and yellow. (b) Structural comparison of \textit{H. influenzae} HipAN\textsuperscript{N} and HipBA systems from other organisms. Residues showing high conservation are marked in the overlay. HipBA binding interfaces of \textit{E. coli} and \textit{S. oneidensis} are identified in circles and enlarged in squares. (c) Structural comparison of HipB antitoxins from \textit{E. coli O127:H6} (PDB code: 7ab3) and \textit{S. oneidensis} (PDB code: 4pa3) using \textit{E. coli} HipB antitoxin bound to the DNA (PDB code: 5k98) structure as model.
molecular weights of the copurified three proteins suggested that *H. influenzae* (HipB + HipAN + HipAC) forms a heterohexamer and (HipAN + HipAC) forms a heterodimer in solution.

3.4. Three-component regulatory mechanism of the *H. influenzae* HipBA system

We validated the components constituting the *H. influenzae* HipBA system and their regulatory mechanism through a cell-growth assay [Fig. 4(a)]. Expression of HipAC resulted in strong inhibition of cell growth, indicating that HipAC functions as a toxin. Surprisingly, the growth was not rescued by the traditional HipB antitoxin but was rescued upon the expression of HipAN, suggesting that HipAN functions as the antitoxin. In addition, co-induction of HipB and HipAN provided a slightly advantageous growth rescue compared to HipAN alone, giving rise to the possibility that HipB may augment the antitoxin activity of HipAN to quickly restore cell growth [Fig. 4(b)]. The co-induction of HipB and HipAN in the *E. coli* O127:H6 HipBA tripartite TA system also showed improved growth over induction of HipAN alone (Vang Nielsen *et al.*, 2019), further supporting the augmentative role of HipB along with the HipAN component to further inhibit the toxic activity of HipAC toxins.

4. Conclusions

Of the three proteins constituting the *H. influenzae* HipBA system, the function of HipAN as the third component is quite striking. We found that HipAN counteracts the toxin HipAC, while the conserved HipB does not have an antitoxic effect. However, HipB is proposed to strengthen the activity of the HipAN-mediated neutralization of HipAC, supporting its
augmentative role. For example, HipB might intensify the efficacy of HipA\textsuperscript{N} by stabilizing its interaction with HipA\textsuperscript{C}. This hypothesis is consistent with the complex formation of HipB, HipA\textsuperscript{N} and HipA\textsuperscript{C}. In addition, hipA might have been split into two fragments during evolution, but the reason for this is unknown.

In conclusion, our work here reveals a novel type of three-component TA module with unknown regulator properties that is important and exciting to study.

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References

Adams, P. D., Afonine, P. V., Bunkóczi, G., Chen, V. B., Davis, I. W., Echols, N., Headd, J. J., Hung, L.-W., Kapral, G. J., Grosse-Kunstleve, R. W., McCoy, A. J., Moriarty, N. W., Oeffner, R., Read, R. J., Richardson, D. C., Richardson, J. S., Terwilliger, T. C. & Zwart, P. H. (2010). Acta Cryst. D66, 213–221.

Baerentsen, R. L., Nielsen, S. V., Lyngsø, J., Bisiak, F., Pedersen, J. S., Gerdes, K., Sørensen, M. A. & Brodersen, D. E. (2022). bioRxiv, https://doi.org/10.1101/2022.01.28.478185.

Balaban, N. Q., Merrin, J., Chait, R., Kowalik, L. & Leibler, S. (2004). Science, 305, 1622–1625.

Berman, H., Henrick, K. & Nakamura, H. (2003). Nat. Struct. Mol. Biol. 10, 980.

Black, D. S., Irwin, B. & Moyed, H. S. (1994). J. Bacteriol. 176, 4081–4091.

Black, D. S., Kelly, A. J., Mardis, M. J. & Moyed, H. S. (1991). J. Bacteriol. 173, 5732–5739.

Chan, W. T., Espinosa, M. & Yeo, C. C. (2016). Front. Mol. Biosci. 3, 9.

Cheverton, A. M., Gollan, B., Przydzial, M., Wong, C. T., Mylonas, A., Hare, S. A. & Helaine, S. (2016). Mol. Cell. 63, 86–96.

Díaz-Orejas, R., Espinosa, M. & Yeo, C. C. (2017). Front. Microbiol. 8, 1479.

Emsley, P., Lohkamp, B., Scott, W. G. & Cowtan, K. (2010). Acta Cryst. D66, 486–501.

Evdokimov, A., Voznesensky, I., Fennell, K., Anderson, M., Smith, J. F. & Fisher, D. A. (2009). Acta Cryst. D65, 875–879.

Hall, A. M., Gollan, B. & Helaine, S. (2017). Curr. Opin. Microbiol. 36, 102–110.

Hanks, S. K., Quinn, A. M. & Hunter, T. (1988). Science, 241, 42–52.

Harms, A., Maisonneuve, E. & Gerdes, K. (2016). Science, 354, aaf4268.

Hayes, F. (2003). Science, 301, 1496–1499.

Hayes, F. & Van Melderen, L. (2011). Crit. Rev. Biochem. Mol. Biol. 46, 386–408.

Holm, L. & Rosenstrøm, P. (2010). Nucleic Acids Res. 38, W545–W549.

Hörak, R. & Tamman, H. (2017). Curr. Genet. 63, 69–74.

Kanehisa, M., Furumichi, M., Tanabe, M., Sato, Y. & Morishima, K. (2017). Nucleic Acids Res. 45, D353–D361.

Karpus, P. A. & Diederichs, K. (2012). Science, 336, 1030–1033.

Kędzierska, B. & Hayes, F. (2016). Molecules, 21, 790.

Korch, S. B. & Hill, T. M. (2006). J. Bacteriol. 188, 3826–3836.

Krisinel, E. & Henrick, K. (2004). Acta Cryst. D60, 2256–2268.

Krisinel, E. & Henrick, K. (2007). J. Mol. Biol. 372, 774–797.

Levin-Reisman, I., Ronin, I., Gelen, O., Braniss, I., Shoresh, N. & Balaban, N. Q. (2017). Science, 355, 826–830.

Lewis, K. (2010). Annu. Rev. Microbiol. 64, 357–372.

Lobato-Márquez, D., Díaz-Orejas, R. & García-del Portillo, F. (2016). FEMS Microbiol. Rev. 40, 592–609.

Loris, R. & Garcia-Pino, A. (2014). Chem. Rev. 114, 6933–6947.

Moyed, H. S. & Bertrand, K. P. (1983). J. Bacteriol. 155, 768–775.

Murshudov, G. N., Vagin, A. A. & Dodson, E. J. (1997). Acta Cryst. D53, 240–255.

Otwinowski, Z. & Minor, W. (1997). Methods Enzymol. 276, 307–326.

Pandey, D. P. & Gerdes, K. (2005). Nucleic Acids Res. 33, 966–976.

Radzickowski, J. L., Vedelaar, S., Siegel, D., Ortega, A. D., Schmidt, A. & Heinemann, M. (2016). Mol. Syst. Biol. 12, 882.

Robert, X. & Gouet, P. (2014). Nucleic Acids Res. 42, W320–W324.

Schumacher, M. A., Balani, P., Min, J., Chinnam, N. B., Hansen, S., Vučić, M., Lewis, K. & Brennan, R. G. (2015). Nature, 524, 59–64.

Stancik, I. A., Šestak, M. S., Ji, B., Axelson-Fisk, M., Franjevic, D., Jers, C., Domazet-Lošo, T. & Mijakovic, I. (2018). J. Mol. Biol. 430, 27–32.

Vang Nielsen, S., Turnbull, K. J., Roghmann, M., Baerentsen, R., Sensmäki, M., Brodersen, D. E., Macek, B. & Gerdes, K. (2019). mBio, 10, e01138-19.

Veening, J. W., Smits, W. K. & Kuipers, O. P. (2008). Annu. Rev. Microbiol. 62, 193–210.

Wen, Y., Behiels, E., Felix, J., Elgeheert, J., Vergauwen, B., Devreese, B. & Savvides, S. N. (2014). Nucleic Acids Res. 42, 10134–10147.

Wood, T. K., Knabel, S. J. & Kwan, B. W. (2013). Appl. Environ. Microbiol. 79, 7116–7121.