**ABSTRACT**

Toxin-antitoxin systems (TAS) are abundant, diverse, horizontally mobile gene modules that encode powerful resistance mechanisms in prokaryotes. We use the comparative-genomic approach to predict a new TAS that consists of a two-gene cassette encoding uncharacterized HicA and HicB proteins. Numerous bacterial and archaeal genomes encode from one to eight HicAB modules which appear to be highly prone to horizontal gene transfer. The HicB protein (COG1598/COG4228) has a partially degraded RNAse H fold, whereas HicA (COG1724) contains a double-stranded RNA-binding domain. The stable combination of these two domains suggests a link to RNA metabolism, possibly, via an RNA interference-type mechanism. In most HicB proteins, the RNAse H-like domain is fused to a DNA-binding domain, either of the ribbon-helix-helix or of the helix-turn-helix class; in other TAS, proteins containing these DNA-binding domains function as antitoxins. Thus, the HicAB module is predicted to be a novel TAS whose mechanism involves RNA-binding and, possibly, cleavage.

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**Supplementary information:** Supplementary data are available at Bioinformatics online.

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**1 INTRODUCTION**

Since the discovery of the first plasmid addiction module, numerous plasmid- and chromosome-encoded bacterial toxin-antitoxin systems (TAS) have been characterized (Buts et al., 2005; Gerdes et al., 2005; Hayes, 2003). The TAS are classified into two types on the basis of the nature of the antitoxin (Gerdes et al., 2005; Hayes, 2003). Type I TAS encompass an antisense RNA antitoxin that inactivates the toxin mRNA, whereas type II TAS employ a protein antitoxin to inactivate the toxin through the protein–protein interaction. Type I TAS typically mediate post-segregational killing of plasmid-free bacteria, with the respective toxins damaging the bacterial membrane. By contrast, in the majority of the type II TAS, the protein toxins are nucleases that target DNA gyrase (Buts et al., 2005; Gerdes et al., 2005).

Recent comparative-genomic analyses have revealed unexpected abundance of (predicted) TAS in most prokaryotic genomes (with the exception of small genomes of parasitic bacteria, especially, intracellular parasites) and remarkable horizontal mobility of TAS operons (Anantharaman and Aravind, 2003; Pandey and Gerdes, 2005). Furthermore, it has been proposed that TAS are stress-response systems beneficial to free-living prokaryotes rather than simple cell-killing machines (Buts et al., 2005; Gerdes et al., 2005; Hayes, 2003; Pandey and Gerdes, 2005). The emerging diversity of TAS suggests that additional, functionally similar but structurally unrelated modules await discovery.

Here we present a detailed computational analysis of a two-gene cassette encoding previously uncharacterized HicA and HicB proteins. The hicAB locus has been described first as an insertion into the major pilus gene cluster in several strains of *Haemophilus influenzae* (Mhlanga-Mutangadura et al., 1998). We report several lines of evidence suggesting that the HicAB module is a novel TAS that functions via RNA-binding and, possibly, cleavage.

**2 RESULTS**

**2.1 HicA/COG1724 protein family belongs to the double-stranded RNA-binding fold**

We used exhaustive PSI-BLAST searches (inclusion cutoff E-value of 0.05; non-redundant protein database; starting query HicA protein, GI: 3603335), initiated from all the sequences retrieved in any search until no new sequences could be detected, to achieve the maximum coverage of the HicA family (COG1724; this COG is annotated as ‘A predicted periplasmic or secreted lipoprotein’ but we are unaware of any evidence supporting this prediction). Altogether, ~230 HicA family proteins were detected in most major clades of bacteria and archaea. The greatest number of HicA sequences (12) was found in the draft genome of cyanobacterium *Crocospheara watsonii* (Supplementary material). Among the retrieved HicA family proteins was the THTA1913 protein from *Thermus thermophilus* HB8 for which the crystal structure has been solved as part of one of the structural-genomics projects (pdb: 1WHZ). The VAST program for structure comparison and alignment aligned the 70 residue THTA1913 protein with a dsRBD domain of PI-PfuI intein (pdb: 1DQ3 (Ichiyanagi et al., 2000),...
residues 336–414 as per the SCOP database] as the first hit. The alignment spans the entire length of the domain, with a \( P \)-value of 0.0039 and RMSD of 2.2\( \AA \) over 52 residues. This particular version of dsRBD in the intein has an additional \( \alpha \)-helix at the C-terminus, a feature shared with the TTHA1913 domain (Fig. 1A). A \( \sim 50 \) amino acid core of the HicA family proteins precisely corresponds to the characteristic \( \alpha-\beta(3)-\alpha \) structural arrangement of the dsRBD fold (Fig. 1B). With a few exceptions, the \textit{hic}A gene is located in a predicted operon with the \textit{hic}B gene.

### 2.2 HicB/COG1598/COG4226 adopts a degraded RNAse H fold which is often fused to a DNA-binding domain

Exhaustive PSI-BLAST search was also used for identification of the HicB family members (with the \textit{H.influenzae} HicB protein [GI: 3603336] as the starting query). The majority of these proteins are included in the UPF0150 domain family in the Pfam database (Bateman et al., 2004) and in COG1598 or COG4226 in the COG database (Tatusov et al., 2003). Surprisingly, we detected almost twice as many (~450 sequences) HicB proteins than HicA family proteins, with the largest number, again, found in \textit{C.watsonii} (33, Supplementary material). In part, this is due to the poor detection of the small \textit{hic}A genes during genome sequence annotation. In several cases, we identified a \textit{hic}A-like ORF in the untranslated region upstream of the \textit{hic}B gene. However, in many other genomes, we were unable to detect the missing \textit{hic}A gene, including several genomes in which no \textit{hic}A genes were found whereas at least one \textit{hic}B gene was present. This observation, together with the considerable overall excess of \textit{hic}B genes, suggest that HicB might have functions independent of HicA. For one HicB-family protein, TTHA1013 from \textit{T.thermophilus} HB8, the crystal structure has been solved (pdb: 1WV8) but no significant similarity to any proteins with known structures has been detected leading to the conclusion that TTHA1013 had a new fold (Hattori et al., 2005). The VAST program aligned the 70 residue TTHA1013 protein with domains of the RNAse H-like fold as the top hits. The RNAse HIII from \textit{Methanocaldococcus jannaschii} was found as the longest hit with RMSD of 2.1\( \AA \) over 49 residues, and \( P \)-values for some RNAse H domains were as low as 0.0007. The alignment completely covers the TTHA1013 protein but typical RNAse H domains are longer at the C-terminus. Analysis of representatives of this fold in the SCOP database showed that RNAse H-like domains in certain superfamilies of the RNAse H fold which is often fused to a DNA-binding domain.
their average length is and b

Fig. 1S). A specific feature of this family is that distant related to the RHH domains of family 2 (Supplementary

long proteins with a distinct, C-terminal RHH domain that is only

RNAse H fold is either missing or very short. (4) HicB domains

parison, with the catalytic residues indicated by asterisks. 

sequence of (1–5; for details see text). Secondary structure is shown for pdb: 1WV8. The protein is highlighted in red. The HicB

B 1WV8 1MGT

core of the HicB family. Designations are the same as in Figure 1B. The HicB subfamilies are indicated by numbers

alkyltransferase from

compared with the degraded RNAse H-like domain of O6-alkylguanine-DNA

bstrands of the

domain, these proteins contain an insertion between the

helix (RHH) DNA-binding domains. In addition to the RHH

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3 DISCUSSION

the characteristics of the hicAB module are similar to those of

known TAS (Anantharaman and Aravind, 2003; Buts et al.,

2005; Gerdes et al., 2005; Hayes, 2003; Pandey and Gerdes,

2005). Indeed, (1) HicAB is a two-component system of small

proteins, (2) RHH and HTH DNA-binding domains, which are

contained in most TAS, are often fused to HicB, (3) most type II

TAS target RNA, and the structures of both HicA domain (dsRBD)

and HicB domain (RNAse H fold) are compatible with their

involvement in RNA metabolism, (4) like other TAS, the

hicAB cassette appears to be highly mobile and prone to HGT; (5) the

distribution of the hicAB cassettes and stand-alone hicB genes are transferred horizontally. In accord with

this mode of transmission, several cassettes and stand-alone hicB genes are encoded in phages and plasmids that could serve as

vehicles for horizontal gene transfer (HGT) (Supplementary

material). Despite the abundance of hicAB modules in several

genes, we detected few demonstrable intragenomic duplications

(e.g. MM0023 and MM0032 in Methanosarcina mazei). Thus,

some genomes seem to have accumulated hicAB modules via

HGT from different sources. In agreement with this notion, we

observed virtually no colinearity in the localization of hicAB cassettes in the genomes of closely related species and even strains,

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partially degraded RNAse H fold, and it remains unclear whether or not these proteins have nuclease activity.

Further analysis of HicB-domain-containing proteins revealed at least five families with the following, distinct domain architectures (Fig. 2B). (1) Short (~70 amino acids) proteins—solo HicB domains, (2) HicB domains fused with CopG-like, ribbon-helix-helix (RHH) DNA-binding domains. In addition to the RHH domain, these proteins contain an insertion between the a-helix and b-strand 4 of the RNAse H-like domain (Fig. 2B) such that their average length is ~130 amino acids. (3) Approximately 120 aa long proteins with a distinct, C-terminal RHH domain that is only distantly related to the RHH domains of family 2 (Supplementary Fig. 1S). A specific feature of this family is that b-strand 4 of the RNAse H fold is either missing or very short. (4) HicB domains fused to helix-turn-helix (HTH) DNA-binding domains of the Xre family. (5) HicB domains fused to Shufflon-specific DNA recombinase (Rci). These proteins are encoded, mostly, in plasmids of several Enterobacteria. No specific function for this domain in the Rci recombinases has been reported but it has been proposed that it plays a role in the recognition of asymmetric sequences in recombination sites (Guyoda et al., 2004). The genes encoding HicB-family proteins of families 1–4 are, most often, linked to hicA genes; by contrast, family 5 hicB genes are not associated with hicA.

2.3 Occurrence of the HicAB module in prokaryotic genomes and its apparent horizontal mobility

The hicA and hicB genes are abundant in free-living archaea and bacteria (Fig. 2S in Supplementary material), with many genomes containing multiple copies of each, but are absent from the genomes of most obligate parasites and symbionts, in a pattern that is typical of TAS [(Pandey and Gerdes, 2005); Supplementary Table 1S]. Table 2S shows the numbers of identified HicA and HicB proteins encoded in selected genomes. As there is little, if any, correlation between the distribution of the five HicB families and taxonomy, and representatives of different HicB families are often found in the same genome, it appears likely that both hicAB cassettes and stand-alone hicB genes are transferred horizontally. In accord with this mode of transmission, several cassettes and stand-alone hicB genes are encoded in phages and plasmids that could serve as vehicles for horizontal gene transfer (HGT) (Supplementary

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plasmid- or phage-free cells. More specifically, given that the HicAB module consists of two protein components, one can predict that it is a Type II TAS. This is compatible with the fact that all Type I TAS protein components are small membrane proteins whereas neither HicA nor HicB is. Furthermore, search of the vicinities of hicAB genes for potential stable, small RNAs that could function as RNA antitoxins revealed no plausible candidates (data not shown).

The mechanism of HicAB functioning remains obscure. It is not even quite clear which protein is the toxin and which is the antitoxin. Some of the available evidence suggests that HicB is the toxin. First, despite the lack of conservation of most of the canonical catalytic residues of the RNase H fold nucleases, the possibility remains that at least some versions of HicB are RNases, like many other toxins. Second, hicB genes are found in some genomes without hicA, which is also the case for known toxins (Mittenhuber, 1999; Pandey and Gerdes, 2005). In contrast, solitary antitoxins so far have not been detected. Furthermore, most often, in TAS operons, the antitoxin gene is located upstream of the corresponding toxin gene, and in the majority of the hicAB modules, hicA precedes hicB. Other observations, however, seem to contradict this hypothesis. In particular, in all known TAS, DNA-binding regulatory domains are fused to antitoxins. The mechanism of action of HicAB might be distinct from those of known TAS and could involve a complex interplay between HicA, HicB, DNA-binding domains, and a specific target.

A parallel exists between the domain architecture of the HicAB module and that of the eukaryotic RNA interference (RNAi) machinery. The essential components of the latter include at least two versions of dsRBD (in R2D2-like proteins and the dicer dsRNA nuclease) and a distinct RNase H domain of the PIWI family in the Ago-like slicer nucleases (Filipowicz, 2005; Tang, 2005) and references therein). These observations suggest that functional similarities between HicAB and RNAi systems might exist as well. Although we cannot currently predict the specific mechanism of HicAB toxicity, it appears most likely that it involves RNA-binding and, possibly, cleavage.

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