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Identifying Hfq-binding small RNA targets in *Escherichia coli*

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Abstract

The Hfq-binding small RNAs (sRNAs) have recently drawn much attention as regulators of translation in *Escherichia coli*. We attempt to identify the targets of this class of sRNAs in genome scale and gain further insight into the complexity of translational regulation induced by Hfq-binding sRNAs. Using a new alignment algorithm, most known negatively regulated targets of Hfq-binding sRNAs were identified. The results also show several interesting aspects of the regulatory function of Hfq-binding sRNAs.

Keywords: Hfq-binding sRNA; Target identification; Translational regulation; Loop-centered extension alignment

Hfq-binding small RNAs (sRNAs), which require the Sm-like protein Hfq for activity [1], direct posttranscriptional regulation of gene expression by discontinuous base pairing with target mRNAs [2,3]. Hfq is conserved in a wide range of bacteria [4,5], suggesting that Hfq-binding sRNAs are also widely distributed. Up to now, more than 30% of the known sRNAs in *Escherichia coli* K-12, including some of the best understood sRNAs, are found to be Hfq-binding [6]. The best understood Hfq-binding sRNAs are all induced under stress conditions [3,7], indicating that sRNAs act as central regulators in response to adverse environmental conditions. Less than half of the known Hfq-binding sRNAs in *E. coli* have been characterized in detail, and for each of these, one or more targets have been validated [2,3,8–10]. Therefore, with the availability of the complete genome sequence of *E. coli* [11], developing a method which can identify the target genes that are regulated by Hfq-binding sRNAs in genomic scale will greatly facilitate the study of how Hfq-binding sRNAs function in bacteria.

Although the mechanism by which Hfq facilitates base pairing between sRNAs and their target mRNAs is still under debate, it is clear that Hfq not only binds to the sRNAs via AU-rich sequences, but also binds to some mRNA targets as well [3]. The base pairing details, which are partially validated in several cases, suggest that the loops of Hfq-binding sRNAs are likely to initiate the base pairing, and that short, interrupted base pairings are sufficient to allow specific regulation [2]. In most cases, Hfq-binding sRNAs negatively regulate the translation activity of target mRNAs by base pairing near or across the Shine–Dalgarno (SD) sequence, thereby blocking ribosome binding [3] or triggering degradation of both sRNA and its mRNA target [10,12]. However, positive regulation also occurs when an Hfq-binding sRNA base pairs with one side of an inhibitory mRNA, thus promoting ribosome binding [3]. However, only two instances of positive regulation have been detailed studied up to now [13,14], and we have therefore focused on identifying the targets that are negatively regulated by Hfq-binding sRNAs.

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The discontinuous duplexes of Hfq-binding sRNAs and their known targets are variable in length and position, which makes it difficult to identify targets on a genome-wide scale. To avoid the many false positives that will derive from standard alignment methods, we developed a new algorithm for identifying negatively regulated targets. This algorithm incorporates five different features, (i) the structure of sRNAs, (ii) Hfq-binding sites, (iii) the 5′ end of the mRNAs, (iv) loop-centered extension alignments, and (v) conservation profiles. Applying this algorithm to the genome sequence of *E. coli*, we identified most of the previously known negatively regulated Hfq-binding sRNA targets, and gave insight into the complexity of translational regulation induced by Hfq-binding sRNAs.

Methods

Hfq-binding sRNA sequences. Up to now, a total of 23 *E. coli* sRNAs have been experimentally validated to interact with Hfq [6,9,15] (S-Table 1 in supplementary materials). For 20 of these, their precise genomic locations are available in RFAM Genome Annotation [16], from which the sequences of these 20 sRNAs were obtained. The sequence of an additional Hfq-binding sRNA, SgrS, was obtained from the literature [10], while the remaining two Hfq-binding sRNAs, RybD and RyeF, were not considered in this work for lack of precise information on their genomic locations.

Orthologous partners of Hfq-binding sRNAs in other organisms were also identified. Eight close relatives of *E. coli* K-12 were selected, shown in the supplementary materials, as most of Hfq-binding sRNAs in *E. coli* have orthologous partners in all these eight organisms. The genomes of these eight, along with their annotations, were obtained from NCBI (ftp://ftp.ncbi.nlm.nih.gov). The sequences of the 21 Hfq-binding sRNAs of *E. coli* were used as queries in Blast [17] to search in the selected organism. For each sRNA, its orthologous partner in each selected organism, if extant, was identified by a combination the BLAST results, the RFAM Genome Annotation and literature mining.

mRNA 5′ end sequences. Hfq-binding sRNAs are likely to negatively regulate their target mRNAs by base pairing near or across the SD sequence thus blocking the ribosome binding [3]. The sequence from −35 to +15 nt of the translation initiation site, including the SD sequence in most cases, was regarded as the mRNA 5′ end for each protein coding genes. To avoid false positive caused by alignments between Hfq-binding sRNAs and full-length mRNAs, only mRNA 5′ end sequences were considered. All mRNA 5′ end sequences from *E. coli* were created based on the gene annotations in the EcoGene18 release of the EcoGene database [18].

For each mRNA 5′ end sequence in *E. coli*, orthologous partner in the eight selected organisms was also identified. The orthologous partner of an mRNA 5′ end sequence in each selected organisms, if present, was defined as follows: (a) the 5′ end partner must be identified by BLAST (identity >80%, length at least 25 nt) with the *E. coli* mRNA 5′ end sequence used as query; (b) the partner must be located within the interval from −185 to +115 nt of the translation initiation site of the orthologous protein coding gene in the selected organism. The larger interval was used to decrease the potential influence of genome annotation mistakes. Orthologous proteins in *E. coli* and each selected organism were identified by reciprocal best match of predicted protein sequences, while the best match in each direction was identified using BLASTP.

Conservation profiles. Due to the widespread occurrence of Hfq [4,5] and because most Hfq-binding sRNAs in *E. coli* have orthologous partners in the eight selected organisms, the regulatory relationship between Hfq-binding sRNAs and their targets was assumed to be conserved in these fairly close relatives. To permit global searches for targets of Hfq-binding sRNAs in *E. coli*, conservation profiles of Hfq-binding sRNAs and mRNA 5′ ends were introduced. The conservation profile for each sRNA or mRNA 5′ end was defined as an 8-bit-length binary string, corresponding to the eight selected organisms. If an orthologous partner was identified in organism i, 1 was entered into the ith position, otherwise 0. Hamming distance was used to compare the conservation profiles. If the distance between the conservation profile of an Hfq-binding sRNA and that of an mRNA 5′ end was larger than two, the mRNA was not considered as the candidate target of the sRNA.

Hfq-binding sRNA structures. Experimentally determined secondary structures, including those of Hfq-binding sRNAs DsrA [19], OxyS [20,21], RyhB [22], Spot 42 [1,23], and MicF [24], were obtained from the literature. For all other Hfq-binding sRNAs in *E. coli*, CLUSTALW [25] was used to perform multiple sequence alignment of the sRNA and its orthologous partners, and the consensus secondary structure was obtained from these aligned sequences by using program RNAalifold in Vienna RNA Package [26]. Correction of some obviously contradictory details gave the final secondary structures of these Hfq-binding sRNAs.

Hfq-binding sites. The Hfq-binding sites of some sRNAs, including DsrA [27], OxyS [28], RyhB [22], and Spot 42 [1], have been determined, and gave clues to the prediction of the Hfq-binding sites of other sRNAs. For each sRNA, the binding sites for Hfq were identified as follows: (a) the candidate Hfq binding sites must be an A/U rich sequence in a single-stranded region, which was identified by scanning all single-stranded sequences of sRNA with a window length of 5 nt, a step length of 1 nt, and a A/U rich threshold of 4 nt; (b) if there were more than one candidate binding site, the candidate with more characters was preferred as the Hfq-binding sites. (These characters including conservation in the close relatives of *E. coli*, location between two hairpin structures, and being flanked by a hairpin structure); (c) if there still remained more than one candidate which could not be distinguished by those characters, all were regarded as binding sites for Hfq. To avoid false positive in the alignments, the Hfq binding sites were masked by "X". Also some sRNAs have poly U tracts in their single-stranded 3'ends, and these were also masked by "X" to avoid the false positive.

Loop-centered extension alignment. To examine the base pairing between an sRNA and the 5′ end sequence of an mRNA, all matches that meet the requirements of minimum matching in loop or bulge regions of the sRNA are treated as candidates for extension. An sRNA sequence is $a_1a_2\cdots a_{k+1}a_{k+2}\cdots a_{k+l}\cdots a_m$ and the reverse of the 5′ end sequence of an mRNA is $b_nb_{n-1}\cdots b_1$. If $a_{k+1}\cdots a_{k+l}$ (l is larger than the minimum requirement) is a subsequence of the loop or bulge regions and it precisely base pairs with $b_{j+l}b_{j+l-1}\cdots b_{j+1}$, the match is a candidate for extension. For each candidate, the following five steps were performed, respectively: (a) The Smith–Waterman algorithm was used to calculate a complementarily alignment matrix between $a_1\cdots a_{k+1}a_{k+2}\cdots a_{k+l-1}$ and $b_n\cdots b_{j+l}b_{j+l-1}\cdots b_{j+2}$. (b) The path from the matrix was traced back from the element (a_{k+l-1}, b_{j+2}) until the element was equal to zero, thus leftward half of the alignment was obtained, and the score of the leftward alignment is the element (a_{k+l-1}, b_{j+2}). (c) The Smith-Waterman algorithm was used to calculate $a_ma_{m-1}\cdots a_{k+l}$ and $b_1b_2\cdots b_{j+1}$. (d) The path from the matrix was traced back from the matrix from the element (a_{k+l}, b_{j+1}) until the element was equal to zero, and after reversing the alignment, the rightward half of the alignment was obtained and the score of the rightward alignment is the value of the element (a_{k+l}, b_{j+1}). (e) The loop-centered alignment of the candidate was obtained by combining the alignment of steps b and d, and the alignment score is the sum of scores in steps b and d. After these steps, for each candidate, we obtained an alignment and a corresponding score. For a pair of sRNA and mRNA 5′ end sequences, we select the highest score from the candidates as the score of this pair.

Results and discussion

Most known targets of Hfq-binding sRNAs were identified

Several partially validated cases of base pairing details suggest that the loops of Hfq-binding sRNAs are likely to initiate the base pairing [2]. We hypothesize (a) the loops

or bulges (>2 nt) of the stem-loop structures of Hfq-binding sRNAs are the potential sites to initiate base pairing with the 5′ ends of the target mRNAs; (b) five consecutive complementary bases (or $n-1$ bases if the length of a loop or bulge, that is n, is less than 6) are required for initiation of base pairing; (c) after initiation of a base pairing, the loop or bulge is opened, and the base pairing region is extended in both directions; and (d) the distance between the conservation profile of an Hfq-binding sRNA and that of its target mRNA 5′ end must be equal to or less than 2. To test these conjectures, we used the algorithm described in Methods section to rank the candidate targets of each Hfq-binding sRNA according to the scores. Ten base pairing interactions between Hfq-binding sRNAs and their negatively regulated targets have been confirmed by experiments or assumed by the literature (Table 1, data were also shown in the NPInter database [29]). Among these, seven were among the 50 highest ranking for their respective sRNAs, including four top-ten ranked predictions. Only two targets were not ranked among the upper 100; these two cases were due to the complexity of the base pairing. For one case, combining the DsrA secondary structure [27] and base pairing details between DsrA and its target *hns* [19] gave the result that the base pairing region of DsrA, which was not identified by our loop-centered extension alignment, contained a bulge (2 nt) instead of a loop. In the other case, OxyS and its target *fhlA* have two separated base pairing regions [20,30], each containing a loop region, while only one region was identified by our algorithm.

Several other cases of negative regulation (e.g., concerning RyhB [12] and OxyS [21]) or negatively correlated transcription levels (e.g., concerning DsrA [31]) have been reported (S-Table 2 in supplementary materials). Although the mechanisms of action are not known, nearly half of these cases have been suggested as indirect interactions, since the mRNAs in question are known to be regulated by RpoS or H-NS; the former a target of OxyS and DsrA, and the latter a target of DsrA. Our algorithm ranked 4 of these candidate targets among the upper 100 for their respective sRNAs. None of these four are known to be regulated by RpoS or H-NS, and it is therefore possible that the mechanisms behind their negative regulation act through base pairing with Hfq-binding sRNAs.

Single gene versus multiple sRNAs

As the translation of *rpoS* is regulated by three sRNAs, DsrA, and RprA for translational activation [13,14], and OxyS for repression [28,32], it is reasonable to assume that also other genes may be regulated by multiple sRNAs. In this work, totally 38 genes were predicted as targets of three or more sRNAs with 50 highest ranking. When the top 100 ranking targets were considered, the number of genes putatively targeted by three or more sRNAs increased to 197. In *E. coli*, acetohydroxy acid synthase is encoded by six genes, the large and small subunits of three isozymes being encoded separately. The three genes encoding the small subunits of acetohydroxy acid synthases were all among the 50 highest ranking predicted targets of three or more sRNAs. Interestingly, all three genes were ranked among the 50 most likely targets of one single sRNA SraH (S-Fig. 1 in supplementary materials). Examination of the base pairing details excluded the possible influence from the sequence similarity between three genes. Considering that acetohydroxy acid synthases are involved in the valine and isoleucine biosynthesis pathways, multiple sRNAs, especially SraH, may take part in the regulation of these pathways. Most interestingly a related enzyme, isoleucine-tRNA ligase, might also be a target of SraH (ranked 53rd); besides, this enzyme was also among the 50 highest ranking predicted as a target of four other sRNAs. Our results thus indicate that the possibility of multiple sRNAs targeting single genes may be quite common, dramatically increasing the complexity of translational regulation in bacteria.

Operon versus sRNA

Spot 42 is currently the only sRNA known to regulate polarity within an operon, which results in differential translational levels of UDP-epimerase (encoded by *galE*) and galactokinase (encoded by *galK*) when the glucose level varies [23]. We studied the possibility of differential

Table 1
Escherichia coli Hfq-binding sRNAs and known targets that are negatively regulated via base pairing

sRNA[a]	Target	Effect	Quality	Rank	Reference
SgrS/RyaA	*ptsG*	mRNA degradation	Assumed	19	[10]
MicC/IS063	*ompC*	Translation repression	Confirmed	2	[37]
DicF	*ftsz*	Translation repression	Assumed	37	[38]
DsrA	*hns*	Translation repression	Confirmed	124	[39]
MicF	*ompF*	Translation repression	Confirmed	1	[40]
RyhB/SraI	*sodB*	Translation repression, mRNA degradation	Confirmed	27	[12,22]
	sdhD	mRNA degradation	Assumed	1	[12]
Spot42/Spf	*galK*	Translation repression	Confirmed	5	[23]
OxyS	*fhlA*	Translation repression	Confirmed	400	[20,30]
	rpoS	Translation repression	Confirmed	95	[28,32]

[a] Some sRNAs may have different names in different references.

translational levels in single operons regulated by Hfq-binding sRNAs. Besides *gal* operon, totally 63 non-overlapping, polycistronic operons contain one or more genes putatively regulated by sRNAs among 10 highest rankings, suggesting the prevalence of sRNA-induced polarity in operons (S-Table 3 in supplementary materials). However, the differential translational level is not the only possible consequence when one gene in an operon is regulated by an Hfq-binding sRNA. Negative regulation of the entire operon is another possible consequence, as demonstrated by the degradation of the transcript of *sdhCDAB* operon when RyhB targets to the 5′ end of *sdhD*, the second gene in this operon [12]. Little is known about the causes of these two different modes of regulation; however, no matter by which modes, sRNAs increase their ability of regulating single genes to regulating a series of closely related genes.

Single sRNA versus metabolic pathway

SraJ is an sRNA with unknown function. Among its 50 highest ranking putative targets, three genes encode enzymes catalyzing three subsequent reactions in the proto- and siroheme biosynthesis pathway. Most interestingly, these three steps, from protoporphyrinogen to heme O, are the three very last steps in that metabolic pathway (Fig. 1). The selection pressure on a pathway has been suggested mainly to target the successful production of its end-product [33], therefore the last steps were considered to be extremely important for a pathway, and SraJ may play a vital role in regulating the production of heme O. Other putative cases of regulation of metabolic pathways by single sRNAs were also studied, for example, the TCA cycle may be regulated by RyhB (S-Fig. 2 in supplementary materials).

Nevertheless, our algorithm also has shortcomings. First, some aspects related to the functions of Hfq-binding sRNA are not yet clear, such as the length and position of base pairing, which influenced the design of the algorithm. Second, our results are sensitive to the secondary structures of sRNAs, which are not easy to predict accurately by computational methods. Present estimates for the total number of sRNAs in *E. coli* stand at some hundreds [34–36], and more than 30% of known sRNAs are found to be Hfq-binding, therefore, there must still be many undetected Hfq-binding sRNAs in the *E. coli*. With the gradual elucidation of the functional mechanisms of Hfq-binding sRNAs and the identification of more Hfq-binding sRNAs, the complexity of translational regulation induced by Hfq-binding sRNAs in bacteria might be more thoroughly studied.

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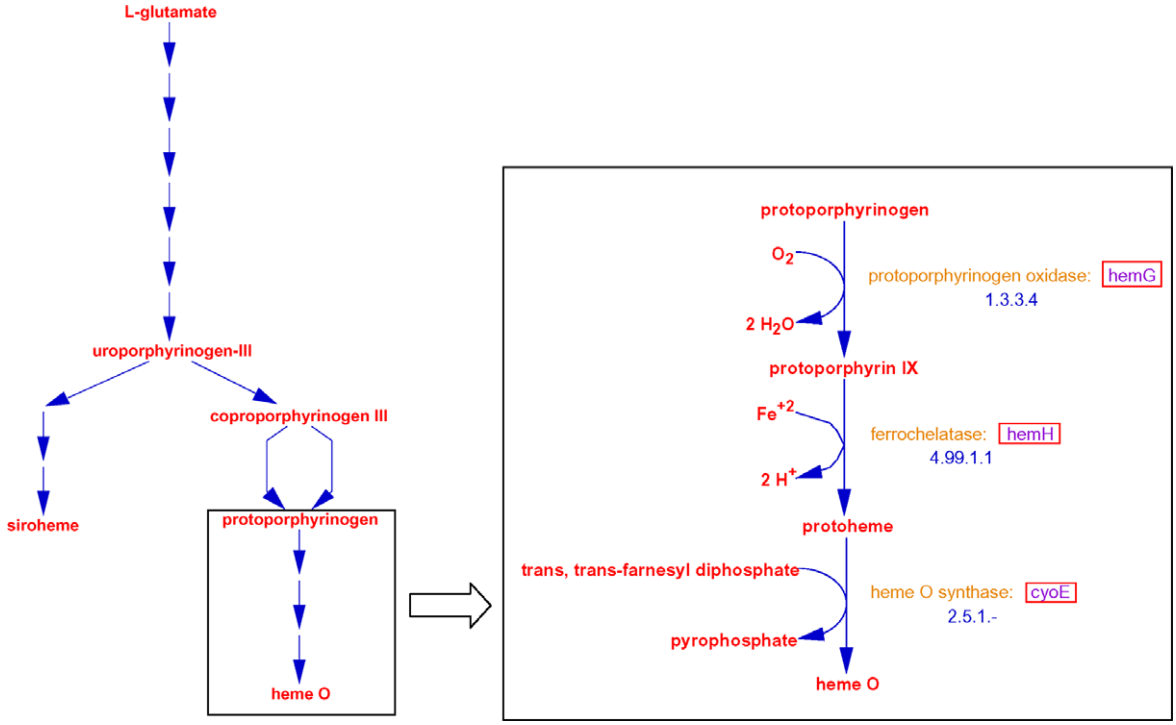

Fig. 1. Involvements of SraJ in the proto- and siroheme biosynthesis pathway. The putative targets of SraJ are marked by red frames. The metabolic pathway chart is from EcoCyc [41]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.02.196.

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