Target prediction for small, noncoding RNAs in bacteria

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ABSTRACT

Many small, noncoding RNAs in bacteria act as post-transcriptional regulators by basepairing with target mRNAs. While the number of characterized small RNAs (sRNAs) has steadily increased, only a limited number of the corresponding mRNA targets have been identified. Here we present a program, TargetRNA, that predicts the targets of these bacterial RNA regulators. The program was evaluated by assessing whether previously known targets could be identified. The program was then used to predict targets for the Escherichia coli RNAs RyhB, OmrA, OmrB and OxyS, and the predictions were compared with changes in whole genome expression patterns observed upon expression of the sRNAs. Our results show that TargetRNA is a useful tool for finding mRNA targets of sRNAs, although its rate of success varies between sRNAs.

INTRODUCTION

In recent years, hundreds of RNAs that do not encode proteins but have intrinsic functions as regulators have been identified. These RNAs are generally denoted noncoding RNAs in eukaryotes and small RNAs (sRNAs) in bacteria. In Escherichia coli alone, >70 sRNA genes have been identified. Those bacterial sRNAs whose functions have been characterized can be sorted into three general categories: sRNAs that have intrinsic catalytic activity or are components of ribonucleoproteins, sRNAs that affect protein activity by structurally mimicking other nucleic acids and sRNAs that post-transcriptionally regulate mRNAs via basepairing interactions [reviewed in Refs (1,2)]. sRNAs in the latter category appear to be the most abundant in E.coli (more than a third of the known sRNAs) and are the focus of our study.

All of the E.coli sRNAs that act by basepairing affect either the stability or translation of the mRNA target; in most cases the mRNAs are encoded in trans at positions on the chromosome distant from the sRNA. An example of a potential basepairing interaction that can lead to mRNA degradation is shown in Figure 1 for the RyhB sRNA and its target, the sodB mRNA. For all of the basepairing sRNAs that are trans-encoded, the basepairing interaction is interrupted by gaps in the pairing. In addition, the sRNAs in this class bind to the RNA chaperone Hfq, which has been shown to facilitate the interaction between some of the more well-characterized sRNAs and their targets (3,4). When interacting with sRNAs, Hfq appears to bind preferentially to unstructured AU-rich regions, frequently between more structured loop regions of the RNA (3–5). Despite increased understanding of the physiological roles of the basepairing sRNAs, the targets for only a subset of these sRNAs are known. In addition, although many sRNAs are thought to regulate more than one mRNA transcript, frequently only a small number of targets have been identified for a given sRNA.

While the targets of basepairing sRNAs in bacteria have remained elusive, there has been better success in identifying targets of microRNAs (miRNAs) in eukaryotes. The function of miRNAs in modulating mRNA stability and translation in eukaryotes is analogous to the function of many of the basepairing sRNAs in bacteria. A number of computational approaches have been employed successfully for the prediction of miRNA targets in plants (6,7), flies (8–12) and mammals (13–16). However, while the consequences of eukaryotic miRNA and bacterial sRNA interactions with their targets are similar, there are a number of important differences between these two classes of noncoding RNAs.

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which bring different challenges to the problem of target identification in bacteria. miRNAs are generally <25 nt in length, whereas sRNAs vary from ~50 to several hundred nucleotides in length. The shorter length of miRNAs helps focus the search for targets to more specific nucleotide sequences. The binding interactions between sRNAs and their targets also show more variation in the regions involved in pairing than in the case of miRNAs. For instance, in plants, miRNAs have a propensity to pair to mRNAs with near-perfect complementarity (6). In animals, target complementarity to the 5’ portion of a miRNA (e.g. residues 2–8) may be critical for action (9,10,13). Also, in the case of miRNAs, target identification has been facilitated by restricting searches to particular regions of the target message, such as 3’-untranslated regions (3’-UTRs).

Here we present a program, TargetRNA, that can effectively predict mRNA targets of basepairing sRNAs. Several sRNAs with targets reported previously in the literature were tested with the program in order to validate the method. TargetRNA was then used to predict novel targets for a number of sRNAs. The results for four of the E.coli sRNAs, RyhB, OmrA, OmrB and OxyS, were investigated experimentally using northern and microarray analyses, leading to the identification of new targets for these sRNAs. Although only target predictions for E.coli sRNAs were experimentally tested, TargetRNA is also generally applicable to other bacteria. The program is publicly available at http://snowwhite.wellesley.edu/targetRNA/.

**MATERIALS AND METHODS**

**Individual basepair model for hybridization scoring**

The interaction between a given sRNA and a candidate mRNA target is predicted by calculating a hybridization score for the two RNA sequences. The individual basepair model of hybridization scoring is based on a straightforward extension of the Smith–Waterman dynamic program (17), except that instead of assessing homology potential, basepairing potential is assessed. Formally, let $S = s_1 s_2 \ldots s_n$ be an sRNA sequence of $n$ nucleotides and let $T = t_1 t_2 \ldots t_m$ be a candidate target mRNA sequence of $m$ nucleotides, where a subsequence $s_i, s_{i+1}, s_{i+2} \ldots s_{j-1}, s_j$ of $S$ is denoted as $S_{ij}$ for any $1 \leq i < j \leq n$. The hybridization score $h$ of two sequences $S$ and $T$, with lengths $n$ and $m$, respectively, is expressed recursively as follows:

$$h_{n,m} = \min \left\{ h_{n-1,m-1} + \delta(s_n, t_m), h_{n-1,m} + \Delta_s, h_{n,m-1} + \Delta_t, 0 \right\},$$

where $\delta(s_n, t_m)$ is the entry in matrix $\delta$ corresponding to the hybridization of nucleotide $s_n$ with nucleotide $t_m$, and $\Delta_s$ is the score for a loop of length $z$ in the interaction. Here, the $4 \times 4$ matrix $\delta$ represents the basepairing affinity of individual nucleotides, as opposed to the similarity of nucleotides as in the case of the Smith–Waterman algorithm. The default setting for the parameter $\delta$ is given by the matrix $(A, C, G, U) \times (A, C, G, U) = \{(6, 6, 6, -5), (6, 6, -5, 6), (6, -5, 6, 1), (-5, 6, 1, 6)\}$, and the default setting for the parameter $\Delta_s$ is defined recursively as $\lambda + \Delta_{s-1}$ if $z > 1$, and 12 if $z = 1$, following an affine score penalty for bulge and internal loops. Default parameter settings were determined by exploring the parameter space and evaluating the program’s performance with a given set of parameters on the set of training data. Different parameter settings for $\delta$ and $\Delta_s$ did not yield significantly different results on the training set. The time requirement for this method is linear in the product of the RNA sequence lengths, $O(nm)$.

**Stacked basepair model for hybridization scoring**

The stacked basepair model of hybridization scoring is based on stacking and destabilizing energies of interacting sequences. The calculation of the optimal hybridization score for two sequences using this model is comparable with the traditional approach for folding RNA sequences (18). The stacked basepair model calculates the minimum free energy of hybridization for two RNA sequences, without allowing intramolecular basepairings. Indeed, a number of RNA folding approaches such as MFold (19), the Vienna RNA Package (20), DINAMelt (21) and MultiRNAFold (22) enable estimation of the hybridization of two RNA sequences. Often these approaches work by concatenating the two sequences via a short linker sequence and then ‘folding’ the new concatenated sequence. The stacked basepair model is a straightforward extension of these approaches. Similar thermodynamic information and free energy parameters are used for loops and for stacked basepairs (23,24). Here, $\epsilon_{\text{stacked}}$ denotes the free energy parameter for a given pair of stacked bases, $\epsilon_{\text{bulge}}$ denotes the free energy parameter for a given bulge loop and its closing basepairs, and $\epsilon_{\text{internal}}$ denotes the free energy parameter for a given internal loop and its closing basepairs. Each of the free energy parameters may take a value of infinity if the closing nucleotides $s_n$ and $t_m$ do not basepair. The hybridization score $h$ of two sequences $S$ and $T$, with lengths $n$ and $m$, respectively, is expressed recursively as follows:

$$h_{n,m} = \min \left\{ h_{n-1,m-1} + \epsilon_{\text{stacked}}(S_{n-1}, S_n, T_{m-1}, T_m), \right.$$ 

$$\min_{1 \leq i < n-1} \left\{ h_{i,m-1} + \epsilon_{\text{bulge}}(S_{i:n}, t_m, T_{i-1}) \right\},$$ 

$$\min_{1 \leq j < m-1} \left\{ h_{n-1,j} + \epsilon_{\text{bulge}}(T_{j:m}, S_{n-1}, S_n) \right\},$$ 

$$\min_{1 \leq i < n-1, 1 \leq j < m-1} \left\{ h_{i,j} + \epsilon_{\text{internal}}(S_{i:n}, T_{j:m}) \right\},$$

$$0 \right\}.$$
Technically, the above formulation does not correctly reflect the thermodynamics of hybridization because it lacks energy contributions for dangling ends, terminal mismatches and initiation of hybridization. These omitted parameters are added after dynamic tabulation to appropriately reflect the free energy of hybridization of the two RNA sequences. The time requirement for this method is quadratic in the product of the sequence lengths, $O(n^2m^2)$. However, if an upper bound is placed on the possible length of loops, then the time requirement is linear in the product of the sequence lengths, $O(nm)$, though the hidden constant factors are much higher than in the case of the individual basepair model.

**P-value calculation**

Extreme-value distributions are well known to model the smallest (or largest) value among a set of independent random values. Let $H$ be the optimal hybridization score determined by TargetRNA for two random RNA sequences. Then the distribution of $H$ approximates an extreme-value type I distribution (25), whose probability density function is given by the following equation:

$$P(H = x) = \frac{1}{s} \exp\left(\frac{x - u}{s}\right) \exp\left(-\exp\left(\frac{x - u}{s}\right)\right),$$

where $u$ is the location parameter and $s$ is the scale parameter of the distribution (26). Accordingly, the cumulative distribution function is described by the following equation:

$$P(H > x) = 1 - \exp\left(-\exp\left(\frac{x - u}{s}\right)\right).$$

One of the program parameters which can be set before executing TargetRNA on a given sRNA gene is the searchable region of the candidate mRNA. For instance, a user of TargetRNA can choose to focus his or her search around the 5'-UTRs of messages as opposed to searching messages in their entirety. Searching longer regions of messages leads to lower expected hybridization scores, as illustrated by the distribution functions in Figure 2. To account for this dependency on the lengths of the sequences searched, following the use of Karlin–Altschul statistics (27), the hybridization score, $h$, is normalized by the log of the product of the sRNA sequence length, $n$, and the size of the mRNA search space, $m$, as follows:

$$h' = \frac{h}{\log(n \times m)}.$$

Once a normalized hybridization score is computed, the $P$-value for the score can be calculated. In order to do so, however, the parameters $u$ and $s$ of the distribution of scores must first be determined. Ten thousand random RNA sequences are generated where the nucleotides of the random sequences are drawn from the first-order distribution of nucleotides contained in the actual mRNA search space. After computing normalized hybridization scores using the random sequences, the parameters $u$ and $s$ of the distribution of scores are estimated using the method of moments (28). With these parameter estimates, the probability of observing a score equal to or less than $h'$ by chance, i.e. the $P$-value, can be calculated as follows:

$$P(H \leq h') = \exp\left(-\exp\left(\frac{h' - u}{s}\right)\right).$$

**Whole genome expression analysis**

TargetRNA predictions were compared with whole genome expression data for four sRNAs. In each case, the sRNA was expressed from an inducible pBAD promoter in a strain deleted for the chromosomal copy of the sRNA gene, and the RNA levels were compared with those for an induced vector control strain. Duplicate experiments were performed; changes of 2-fold or better relative to the control were considered highly significant; changes of 1.5-fold or better were considered likely to be significant. Short expression times (15–20 min) were used to avoid some of the indirect effects of sRNA expression. MG1655 Δara714 ryhB::cat/pBAD-RyhB was grown in Luria–Bertani (LB) medium to $A_{600} = 0.5$ and induced with 0.1% arabinose for 15 min (29), MG1655 Δara714 ΔomrAB/pBAD-ΩmrA and MG1655 Δara714 ΔomrAB/pBAD-ΩmrB were grown in LB medium overnight and induced with 0.2% arabinose for 20 min (30), and MG1655 Δara714 ΔoxyS::kan (GSO112) carrying pBAD-OxyS was grown in LB medium to $A_{600} = 0.6$ and induced with arabinose for 15 min (J. A. Opdyke and G. Storz, unpublished data).

**Strain construction and northern analysis**

To assay the effect of OmrA and OmrB on gntP mRNA, it was necessary to first create a strain in which the gntP gene was expressed at a detectable level. This was done by creating a deletion of $uxuR$, which encodes a repressor of gntP, by homologous recombination. Briefly, the chloramphenicol resistance cassette was amplified with the Expand High Fidelity...
PCR System (Roche) with oligonucleotides 5’uxuR::cm (GAT TAA CCG CAC CTA ACG GAC ACA ACA CCA TGA AAT CTG CCG CTT CTA CGG TGA AGG ACT TTC TCG C) and 3’uxuR::cm (CGC AAG GAA CTT TTA CCC TTG CGC TTA TTA TAA TAA GTC AGG CTT ACT ACT TAT TCA GGC GTA GCA CCA). The PCR product was then recombined into the chromosome of a DJ480 strain carrying an AAT TCA GGC GTA GCA CC. The PCR product was then transduced to create strain MG1132.

MG1132 was transformed with pBR-plac-OmrA, pBR-plac-OmrB or the corresponding empty vector (30) and used for transduction to create strain MG1132. For each sRNA above, our computational approach was used to predict a set of candidate message targets of the sRNA. The final three columns in the table indicate the hybridization score of the predicted interaction, the P-value, and the rank (based on P-value) of the putative target among the set of predictions. For 4 of the 12 reported interactions, our approach did not predict the target with sufficient confidence (P-value < 0.01) using the default program parameters.

**RESULTS**

**Training set**

We first compiled a training set composed of putative mRNA targets of Hfq-binding sRNAs in *E. coli*, based on findings reported in the literature prior to 2005 (Table 1). The training set consists of 9 sRNAs interacting with a total of 12 message targets. For all but 2 of the 12 training examples (GcvB-ddpA and GcvB-oppA), the putative location of interaction between the sRNA and its target mRNA has been described previously (Table 1).

These targets were then examined, along with their corresponding sRNA interactions, for common features. The binding interactions of the sRNAs with their mRNA targets contain gaps, mismatches and G:U basepairs. The longest stretches of contiguous nucleotides participating in duplex interaction range from 5 to 16 nt. In 8 of the 10 cases where the interaction has been described, the sRNA interacts with the message target near the translation start site (within ~30 bases of translation initiation). The two exceptions are DsrA:rhoS and RprA:rhoS, in which the target interaction occurs ~100 bases upstream of the translation start site and leads to positive rather than negative regulation of the rhoS mRNA (35,36). We also noted that, with the exception of DicF, which is processed from a longer transcript, each sRNA has a terminator stem–loop in its predicted structure. The OxyS:fhlA interaction is the only reported example of

| sRNA | Target | Target function | Regulation | Target region of interaction (relative to AUG) | Reference | Score | P-value | Prediction |
|------|--------|-----------------|------------|-----------------------------------------------|-----------|-------|----------|------------|
| DicF | ftsZ    | GTPase involved in cell division | Negative | −28 to +2 | (43) | —     | —        | —         |
| DsrA | hns    | Pleiotropic regulator | Negative | +7 to +19 | (44) | −69 | 0.00098 | #3     |
| DsrA | rpoS   | Sigma factor for stress response | Positive | −119 to −97 | (35) | —     | —        | —         |
| GcvB | ddpA   | Dipeptidase transport protein | Negative | Unknown | (41) | 84 | 0.00014 | #1     |
| GcvB | oppA   | Oligopeptidase transport protein | Negative | Unknown | (41) | 70 | 0.00165 | #4     |
| MicC | omnC   | Outer membrane pore protein | Negative | −41 to −15 | (45) | −80 | 0.00021 | #1     |
| MicC | omnF   | Outer membrane pore protein | Negative | −16 to +10 | (46) | −80 | 0.00014 | #2     |
| OxyS | fhlA   | Transcriptional activator | Negative | −15 to −9; +34 to +42 | (37,39) | —     | —        | —         |
| RprA | rhoS   | Sigma factor for stress response | Positive | −117 to −94 | (36) | —     | —        | —         |
| RhylB | sdh    | Succinate dehydrogenase | Negative | −42 to −3 | (33) | −66 | 0.00215 | #3     |
| RhylB | sodB   | Superoxide dismutase | Negative | −17 to +9 | (42) | −60 | 0.00651 | #9     |
| SpotA | galK   | Galactokinase in gal operon | Negative | −19 to +39 | (47) | −78 | 0.00029 | #1     |

*For each sRNA above, our computational approach was used to predict a set of candidate message targets of the sRNA. The final three columns in the table indicate the hybridization score of the predicted interaction, the P-value, and the rank (based on P-value) of the putative target among the set of predictions. For 4 of the 12 reported interactions, our approach did not predict the target with sufficient confidence (P-value < 0.01) using the default program parameters.*
a terminator stem–loop participating in the target hybridization (37). Finally, with the exception of the DicF sRNA, each of the sRNAs shows evidence of conservation in closely related species to *E.coli* such as *Shigella flexneri* and *Salmonella typhimurium*.

**Computational approach**

We present a program, TargetRNA, which, given the sequence of an sRNA gene in a particular organism, outputs a ranked list of predicted message targets for the sRNA. The program begins by consulting a database of protein coding genes (38) for the organism of interest. For each protein coding gene in the organism, the program extracts the mRNA sequence corresponding to the protein coding region along with user-specified regions upstream and downstream of the coding sequence, extending into the 5′-UTR and 3′-UTR, respectively. TargetRNA then evaluates the potential for interaction between every extracted mRNA sequence and the sRNA, and assigns each a hybridization score and corresponding *P*-value (Materials and Methods). Finally, TargetRNA outputs a ranked list of the candidate message targets along with a graphical representation of each predicted interaction along the length of the sRNA. The program is freely available for use as a web application.

The interaction between a given sRNA and a candidate mRNA target is predicted by calculating a hybridization score for the two RNA sequences. In determining the hybridization score for two RNA sequences, intramolecular basepairings are not considered and pseudoknots are not allowed. To calculate the hybridization score of an sRNA and candidate mRNA target, TargetRNA can use either of two different hybridization score models for RNA sequence interactions: an individual basepair model or a stacked basepair model. The individual basepair model of hybridization scoring (described in Materials and Methods) is based on a straightforward extension of the Smith–Waterman dynamic program (17), except that instead of assessing homology potential, basepairing potential is assessed. The stacked basepair model of hybridization scoring (described in Materials and Methods) is based on stacking and destabilizing energies of interacting sequences, where the calculation of the optimal hybridization score for two sequences is comparable with folding RNA sequences (18) without allowing intramolecular basepairings.

**Evaluation of program parameters**

To explore the effects of various parameter settings on the program’s performance, the sensitivity and specificity of TargetRNA were evaluated with regard to the training set. The sensitivity (i.e. the true positive rate) is defined, for a given set of parameters, as the percentage of the 12 interactions in the training set which are correctly predicted by the program: True Positives/(True Positives + False Negatives). The specificity (i.e. the true negative rate) is defined, for a given set of parameters, as the percentage of non-interactions which are correctly predicted as non-interactions by the program: True Negative/(True Negatives + False Positives). For example, in *E.coli* each of the nine sRNAs in the training set may potentially interact with any of the 4244 messages. Thus, there are 9 × 4244 possible interactions, of which 12 are considered true interactions and the rest are considered non-interactions, for the purpose of evaluating the program’s performance. In practice, the 9 sRNAs in the training set interact with >12 messages, so some of the program’s predictions which we have classified as ‘false positives’ may indeed correspond to actual interactions. Thus, the estimated sensitivity and specificity of the program on the training set may in fact be conservative.

To evaluate the significance of target predictions, *P*-values rather than raw hybridization scores are employed. Hybridization scores have the disadvantage that they are dependent on the lengths of the sRNA and message sequences under consideration. As illustrated in Figure 2, longer sequences lead to lower expected hybridization scores than shorter sequences. Thus a hybridization score predicted for short sequences may be unlikely to have occurred by chance, whereas the same hybridization score for longer sequences may be likely to have occurred by chance. The *P*-value of a prediction corresponds to the probability of observing by chance a hybridization score at least as small as the predicted score. In other words, the *P*-value provides an indication as to the significance of a prediction. Based on evaluation of the program’s performance on the training set, *P*-values ≤ 0.01 are considered significant.

Under all parameter settings tested, the individual basepair model of hybridization scoring (default method) resulted in a greater sensitivity on the training set than that of the stacked basepair model of hybridization (indicated as an option in the program). Closer inspection of the results revealed that the stacked basepair model favors longer regions of interaction between two RNAs, whereas the individual basepair model favors interactions of more parsimonious lengths. The bias of the stacked basepair model for longer basepairing interactions can be explained, in part, by the fact that the expected hybridization score for two random RNA sequences with the model is negative (i.e. favorable). Consequently, the stacked basepair model has a propensity for long, random interactions.
as opposed to short, functionally meaningful interactions. Thus, this model may be more appropriate for identifying interactions in which the length of the interaction is known a priori, such as when the entire RNA gene participates in the interaction.

Using the individual basepair model, the performance of TargetRNA was assessed as each parameter was varied, in turn, while all others were held fixed. The receiver operating characteristic (ROC) curves in Figure 3 illustrate the trade-offs between sensitivity and specificity of the program on the training set as different parameters are varied. Each ROC curve is generated from 21 data points corresponding to the sensitivity and false positive rate (1.0 − specificity) as the seed is varied from 0 to 20 nt. The four different ROC curves demonstrate the performance of TargetRNA as the seed length varies when G:U basepairs are allowed in the seed interaction, when G:U pairs are disallowed in the seed, when the sRNA terminator loop is removed from the hybridization interaction, and when the sRNA terminator loop is retained. A similar analysis was carried out to identify the target sub-regions which yield the most advantageous sensitivity/specificity trade-off (data not shown).

The ROC analyses were then used to suggest default parameters for TargetRNA. Since the sensitivity, which ranges from 0 to 70%, appears to be more heavily influenced by the choice of parameters than the specificity, which ranges from 98.5 to 100%, default parameter values were chosen which minimize the false positive rate at the maximum sensitivity value, i.e. default parameter values were chosen to correspond to the top left-most point along the ROC curves, as illustrated in Figure 3. Default parameters include removal of the terminator stem–loop of the sRNA, restricting the target message search from 30 nt upstream of translation initiation to 20 nt downstream of translation initiation, and necessitating a seed of at least 9 continuous nucleotides without G:U basepairs.

Performance on training set

Using the default parameters as described above, TargetRNA was run on the training set of 12 interactions between sRNAs and their targets. As illustrated in Table 1, for 8 of the 12 instances, the program predicted the reported message target among its set of top candidate targets. For each of these eight cases, the predicted interaction closely matched the reported interaction.

The four cases where the program did not predict the reported message target were examined more closely. In two of these four cases, namely DsrA:rapS and RprA:rapS, the reported interactions between the sRNAs and their targets occur ~100 nt upstream of the messages’ translation start sites, outside of the region specified in our search. When the mRNA sequences were extended upstream to include >100 nt in the 5’-UTRs, TargetRNA predicted DsrA:rapS as its top target candidate. TargetRNA did not predict the interaction between the sRNA OxyS and its target fhlA. The OxyS:fhlA interaction is the only example in the training set of a disjoint interaction where there are two separate regions of basepairing, which are >20 nt apart, the first region residing around the ribosome-binding site of the message target and the second residing downstream within the coding sequence. TargetRNA also did not predict the interaction between the sRNA DicF and its target ftsZ. The DicF:ftsZ interaction is the only example of an interaction where the longest stretch of contiguous nucleotides participating in the interaction is <7 nt. Altering the program parameters did not lead to prediction of either the OxyS:fhlA interaction or the DicF:ftsZ interaction. TargetRNA’s inability to predict, under any set of parameters, a few of the documented sRNA targets, suggests that the approach does not model effectively all sRNA:mRNA interactions. Given that 8 of the 12 targets were correctly predicted by the program, its sensitivity on the training set using the default parameters is ~67%. The specificity of the program on the training set using default parameters was estimated to be ~99% (Figure 3).

New predictions of TargetRNA

Given the paucity of sRNA targets which have been reported previously, as evinced by the small size of the training set, many of the predicted targets classified as false positives may actually be uncharacterized targets. To further explore how many of the high-scoring target candidates predicted by the program in fact correspond to novel message targets, we predicted targets for four sRNAs in E.coli (RyhB, OmrA, OmrB and OxyS) using somewhat more permissive parameters (removal of the terminator stem–loop of the sRNA, extending the target message search from 30 nt upstream of translation initiation to 30 nt downstream of translation initiation, and necessitating a seed of at least 7 continuous nucleotides with G:U basepairs, rather than a 9 nt seed). We then compared the output of TargetRNA with the results of whole genome expression analyses following induction of the sRNAs.

Although the four sRNAs chosen for the predictions are normally synthesized in response to different regulatory signals, for this work, we examined the effects of each of the sRNAs after brief expression from the ectopic pBAD promoter. For the RyhB, OmrA and OmrB sRNAs, it has been
The 90 nt RyhB RNA is synthesized to be direct targets of the sRNAs, further assays, such as observe a significant decrease in mRNA level are assumed analysis also was used to examine the levels of predicted excluded from the analysis. In some cases, northern blot genes for which the signals were deemed 'absent' or the sRNAs was considered potentially significant, with least 1.5-fold in duplicate experiments upon expression of the sRNA, rather than secondary effects; this assumption the effects measured in the arrays are the direct effect of might lead to an overestimate of the number of correctly identified targets. A relatively short expression time for the sRNAs was used to minimize this possibility. For the purposes of this study, changes in gene expression of at least 1.5-fold in duplicate experiments upon expression of the sRNAs were considered potentially significant, with changes of at least 2-fold considered highly significant. Genes for which the signals were deemed ‘absent’ or ‘marginal’ on the arrays for the vector control strains were excluded from the analysis. In some cases, northern blot analysis also was used to examine the levels of predicted target mRNAs. Although potential targets for which we observe a significant decrease in mRNA level are assumed to be direct targets of the sRNAs, further assays, such as compensatory mutations and/or in vitro-binding studies, are required to verify sRNA:mRNA basepairing.

**RyhB target predictions.** The 90 nt RyhB RNA is synthesized upon iron starvation and has been shown to cause the rapid degradation of a number of target mRNAs upon pairing (29,32,33). Expression of RyhB from an ectopic promoter for 15 min, followed by examination of changes in mRNA abundance by microarray analysis, revealed potential targets, many of them corresponding to genes with the characteristics expected of RyhB targets, which primarily encode non-essential iron-binding proteins (29). RyhB is currently unique in the large number of known targets. From array analysis and other work, 18 operons and 56 genes have been shown to be regulated by RyhB, and interpreted as being directly regulated by this sRNA.

Among the predictions for RyhB from the TargetRNA program, 33 are below a P-value of 0.01; of these 15 were excluded from further analysis because the gene expression signals were too low to be significant (deemed as Absent, Table 2) or the gene was not on the array. Of the remaining 18 predictions 2 (sdh and sodB) were part of the training set; these 2 genes also show significant regulation in the arrays (6-fold and 19-fold decrease in signal after RyhB expression). Of the remaining 16 predictions, 2 others have changes in the array signal of >2-fold, and 6 others have signal changes between 1.5-fold and 2-fold (Table 2). Thus for RyhB, 10 out of 18 predictions (56%) are supported by the microarray data. It is worth noting that a number of targets for RyhB, thought to be direct targets based on either array or northern analysis, do not appear in the list of predicted targets (29,33); modification of program parameters leads to predictions for some but not all of these.

**OmrA and OmrB target predictions.** The 88 nt OmrA RNA and 82 nt OmrB RNA (previously RygA and RygB) are two partially homologous sRNAs, induced under high osmolarity conditions. They have been shown to regulate a number of genes encoding outer membrane proteins and surface structures (30). These sRNAs were each expressed ectopically and again the microarray results were compared with the predictions from TargetRNA. The results are strikingly different from the RyhB results. For OmrA, 36 targets are predicted with a P-value below 0.01, of which 10 were excluded from the analysis because the gene was missing or the signal for the vector controls was low or marginal (Table 3). Of the 26 remaining predictions, none showed differential expression in the array experiments of at least 1.5-fold, our cut-off value. One predicted target, fecD, is in an operon with four other genes; in previous work it was demonstrated that the fecABCDE operon is regulated by OmrA (30), and, in fact, the array data for other genes in the operon exceeds the 1.5-fold cut-off. If we consider fecD a correct prediction, 1 out of 26 predictions (4%) for OmrA is supported by the microarray data.

For OmrB, 18 targets are predicted with a P-value < 0.01, with 5 excluded because of low expression signals in control experiments (Table 4). Of the remaining 13, one, gntP, has differential expression of at least 2-fold in the array data. The effect of OmrB on gntP was confirmed by northern analysis (Figure 4). Interestingly, the northern analysis revealed that increased levels of OmrA expression also resulted in decreased gntP levels suggesting that gntP is also a target of OmrA, although this was not predicted by TargetRNA using the above parameters. For OmrB the microarray data supports 1 out of 13 predictions (8%). Thus, the percentages...
of target predictions which are supported by the microarray experiments are much lower for these two related RNAs than for RyhB.

**OxyS target predictions.** The 109 nt OxyS RNA was one of the first sRNAs to be characterized. The expression of this RNA is strongly induced in response to oxidative stress and the RNA has been proposed to play a role in protecting cells against the damaging effects of elevated hydrogen peroxide concentrations (34). Although OxyS overexpression leads to a dramatic change in protein expression patterns and the RNA has been proposed to regulate the expression of >40 genes, only one direct target, the fhlA mRNA, has been characterized (37,39). As discussed above however, this OxyS/fhlA basepairing interaction was unusual in many respects. To further explore OxyS targets, the OxyS RNA was expressed ectopically and the results of microarrays were compared with the predictions of TargetRNA (Table 5). Among the 23 OxyS target predictions with P-values <0.01, 6 were excluded because of low expression in the control samples or absence of the gene. Among the remaining 17, one showed between 1.5-fold and 1.9-fold decreases in expression and four showed at least 2-fold decreases upon OxyS expression. The effects of OxyS on three of the strongly regulated genes, yobF (which is in an operon with cspC), wrbA and ybaY were also confirmed by northern analysis (Figure 5) (no transcript of the expected size could be detected for yaiZ). Overall, 5 out of 17 predictions (29%) are supported by the microarray data.

### DISCUSSION

We present a computational method for predicting targets of sRNA genes in bacteria. While numerous *in silico* approaches have been proposed recently for identifying targets of miRNAs in eukaryotes (6–16), there has been a relative dearth of such approaches for sRNAs in bacteria. This lack of *in silico* methods may be due, in part, to the paucity of reported targets of sRNA regulation. To date, *E.coli* contains the best-studied set of sRNAs and targets. We compiled a list of 12 such targets in *E.coli*, all described in the literature prior to 2005. This set of targets was examined for common features, and a computational method was developed for predicting novel targets. The effectiveness of the approach was

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**Table 2. Predicted targets for RyhB**

| Rank | Gene | B# | Score | P-value | pBAD-RyhB (1)* | pBAD-RyhB (2)* | pBAD (1)* | pBAD (2)* | Ratio (avg)* | Other information* |
|------|------|----|-------|---------|----------------|----------------|-----------|-----------|-------------|-------------------|
| 1    | *kdpa* | b0698 | 83 | 0.000013 | A/A; A/A | A/A; A/A | A/A | A/A | A/A | A/A |
| 2    | *ciriG* | b0613 | 75 | 0.00055 | A/A; A/A | A/A; A/A | A/A | A/A | A/A | A/A |
| 3    | *rocA* | b4154 | 71 | 0.0011 | 337 | 493 | 1650 | 1865 | 4.4 | P/P; P/P; operon agrees |
| 4    | *napF* | b2208 | 68 | 0.0020 | 92 | 83 | 139 | 188 | 1.9 | A/P; A/P; operon agrees |
| 5    | *yajJ* | b0276 | 67 | 0.0023 | A/A; A/A | A/A; A/A | A/A | A/A | A/A | A/A |
| 6    | *sugE* | b4148 | 67 | 0.0023 | A/A; A/A | A/A; A/A | A/A | A/A | A/A | A/A |
| 7    | *sibD* | b0722 | 66 | 0.0028 | 1666 | 1322 | 11388 | 6681 | 6.1 | P/P; P/P; training set |
| 8    | *yceF* | b3219 | 66 | 0.0028 | 351 | 466 | 3412 | 2234 | 7.1d | P/P; P/P; Fur targetd |
| 9    | *sodA* | b3908 | 66 | 0.0028 | 1666 | 1322 | 11388 | 6681 | 6.1 | P/P; P/P; Fur targetd |
| 10   | *notA* | b1890 | 65 | 0.0034 | A/A; A/A | A/A; A/A | A/A | A/A | A/A | A/A |
| 11   | *pinH* | b2648 | 65 | 0.0034 | A/A; A/A | A/A; A/A | A/A | A/A | A/A | A/A |
| 12   | *ygeZhyuA* | b2873 | 65 | 0.0034 | A/A; A/A | A/A; A/A | A/A | A/A | A/A | A/A |
| 13   | *ykgE* | b0306 | 64 | 0.0040 | 333 | 241 | 437 | 339 | 1.4 | P/P; P/P; operon agrees |
| 14   | *ydaN* | b1342 | 64 | 0.0040 | 246 | 187 | 239 | 276 | 1.2 | P/P; P/P |
| 15   | *ynF* | b1588 | 64 | 0.0040 | A/A; A/A |
| 16   | *yiaM* | b3577 | 64 | 0.0040 | A/A; A/A |
| 17   | *cysE* | b3607 | 64 | 0.0040 | 707 | 940 | 1122 | 1182 | 1.6 | P/P; P/P |
| 18   | *yciS* | b1279 | 63 | 0.0048 | 426 | 485 | 769 | 708 | 1.6 | P/P; P/P |
| 19   | *yesK* | b2072 | 63 | 0.0048 | A/A; A/A |
| 20   | *acsS* | b2563 | 63 | 0.0048 | 511 | 617 | 787 | 843 | 1.5 | P/P; P/P |
| 21   | *ygiQ* | b4469 | 63 | 0.0048 | A/A; A/A |
| 22   | *ybiG* | b0841 | 62 | 0.0058 | 1545 | 1182 | 1089 | 831 | 0.7 | P/P; P/P |
| 23   | *yecD* | b1867 | 62 | 0.0058 | 449 | 484 | 430 | 499 | 1.1 | P/P; P/P |
| 24   | *mutH* | b4019 | 62 | 0.0058 | 269 | 277 | 384 | 389 | 1.4 | M/P; A/P |
| 25   | *yadS* | b0157 | 61 | 0.00698 | A/A; A/A |
| 26   | *perM* | b2493 | 61 | 0.00698 | P/M; P/P |
| 27   | *medI* | b0198 | 60 | 0.0083 | 615 | 778 | 624 | 529 | 0.8 | P/P; P/M |
| 28   | *proA* | b0243 | 60 | 0.0083 | 294 | 295 | 245 | 465 | 1.2 | M/P; P/P |
| 29   | *yagT* | b0286 | 60 | 0.0083 | A/A; A/A |
| 30   | *nagZ* | b1107 | 60 | 0.0083 | 555 | 381 | 713 | 714 | 1.6 | A/P; A/P; operon agrees |
| 31   | *sodA* | b1656 | 60 | 0.0083 | 198 | 265 | 4402 | 4285 | 19.3 | M/P; M/P; training set |
| 32   | *ygiT* | b3021 | 60 | 0.0083 | 190 | 121 | 91 | 140 | 1.1 | P/P; P/P |
| 33   | *datA* | b1189 | 59 | 0.0099 | 1742 | 1587 | 3914 | 3088 | 2.1 | P/P; P/P |

*a* Standard Affymetrix signal determined for indicated genes in two independent experiments.

*b* Average of ratios for pBAD control/pBAD-RyhB signal for the two experiments. For ratios >2, the predicted targets are highlighted in dark gray. For ratios 1.5–2, the predicted targets are highlighted in light gray.

*c* Signals were determined to be A = absent, M = marginal or P = present by standard Affymetrix program and are listed in the following order: pBAD-RNA (1)/pBAD-RNA (2)/pBAD (1)/pBAD (2)/pBAD (3). Only predicted targets for which the signal was scored as P for both of the two pBAD samples were considered.

*d* sodA is regulated by the Fur repressor, but repression is more complete after induction of RyhB. In a parallel experiment, carried out with a fur mutant background, the ratio of mRNA level for sodA in the vector-containing cells and RyhB expressing cells was 1.7, still sufficiently high to be considered a direct predicted target (29).
evaluated on the training set of 12 reported targets as well as on sets of predictions for the RyhB, OmrA, OmrB and OxyS sRNAs, for which the predictions could be compared with results from whole genome expression analyses.

The percentage of computationally predicted targets for which there was experimental support from microarray and northern blot assays ranged from ~4 to 8% for the OmrA and OmrB RNAs up to 56% for the RyhB RNA. The different
success rates with different sRNAs may be due to a number of factors including limitations of the microarray analysis that lead us to mistakenly underestimate the success rate of the predictions and the possibility that the program is less useful for some sRNAs than others.

The low success rate for RNAs such as OmrA and OmrB may be due to a number of caveats associated with our experimental analysis. If pairing of an sRNA frequently leads to translational inhibition without mRNA degradation, our assay method, which is dependent upon changes in the mRNA levels, would improperly count the result as negative. Future experiments that directly test translation will be necessary to address this possibility. In addition, because we only evaluated targets that were detected at a sufficient level in the vector control to be judged ‘present’, the nature of the targets and their level of expression may change our evaluation of success. For instance, it is possible that RyhB target mRNAs are more abundant, in general, than OmrA and OmrB target mRNAs. If this is the case, OmrA and OmrB targets would be more likely to be deemed ‘absent’ under the assayed growth conditions and the rate of success for our computational predictions could be underestimated.

A few caveats regarding the TargetRNA program also should be taken into consideration. The program does not account for the structures of either the sRNA or mRNA. It is possible that some predicted basepairing interactions do not occur because the corresponding regions of either the sRNA or the mRNA are occluded by secondary structure. In addition to structure, some other feature of either the sRNA or mRNA not accounted for by TargetRNA, such as the presence of an Hfq-binding site, may be required for productive basepairing. Finally, while all of the sRNAs examined here bind Hfq and are believed to act by basepairing, they may represent different classes of sRNAs and may not follow the same rules for basepairing. Because the training set used to develop the TargetRNA program used RyhB and OxyS substrates, and not OmrA and OmrB substrates, the program is not optimized for the latter RNAs. As an attempt to address this issue, we revisited the program parameters with a new training set, derived from the experiments presented here and recent results from the literature. The new training set of 25 targets included a number of OmrA and OmrB targets. However, we did not find a set of parameters that led to significant improvement in recognizing targets for the OmrA and OmrB sRNAs.

Alternatively some sRNAs may act on only a limited number of targets, while others may have many targets. For RyhB and OxyS, 209 and 186 genes, respectively, showed at least 2-fold changes while for OmrA and OmrB, 34 and 24 genes, respectively, showed at least 2-fold changes after expression of each of the sRNAs in one microarray experiment. While some of the effects of RyhB expression are known to be indirect, there were still more global effects, in general, of RyhB expression than of OmrA or OmrB expression.

Despite some limitations of both the TargetRNA program and whole genome expression analysis, we suggest that the combination of the two approaches will be an effective approach for identifying direct targets for an uncharacterized sRNA. Functional annotation may also be a useful indicator for identifying candidate targets. In several cases, the set of targets predicted by TargetRNA for a given sRNA was enriched for genes that appear functionally similar. For instance, among the top candidate targets for the sRNA GcvB were mRNAs gldL, livJ, livK, yflI, aroP and argF, all genes encoding periplasmic transport proteins. Similarly, a number of top candidate targets for the sRNA RyhB encode non-essential iron-binding proteins.

While the method was evaluated on targets of sRNAs in E.coli, the approach is applicable to bacteria more generally.

### Table 5. Predicted Targets for OxyS

| Rank | Gene | b#  | Score | P-value | pBAD-OxyS (1)^a | pBAD-OxyS (2)^a | pBAD (1)^b | pBAD (2)^b | Ratio (avg)^b | Other information^c |
|------|------|-----|-------|---------|----------------|----------------|-----------|-----------|--------------|-------------------|
| 1    | ybaY | b1162 | −21   | 0.0025  | 399            | 437            | 338       | 326       | 1.0          | P/P; P/P; Figure 5 |
| 2    | ybaY | b1162 | −21   | 0.0025  | 399            | 437            | 338       | 326       | 1.0          | P/P; P/P; Figure 5 |
| 3    | ybaY | b1162 | −21   | 0.0025  | 399            | 437            | 338       | 326       | 1.0          | P/P; P/P; Figure 5 |
| 4    | ybaY | b1162 | −21   | 0.0025  | 399            | 437            | 338       | 326       | 1.0          | P/P; P/P; Figure 5 |
| 5    | ybaY | b1162 | −21   | 0.0025  | 399            | 437            | 338       | 326       | 1.0          | P/P; P/P; Figure 5 |
| 6    | ybaY | b1162 | −21   | 0.0025  | 399            | 437            | 338       | 326       | 1.0          | P/P; P/P; Figure 5 |
| 7    | ybaY | b1162 | −21   | 0.0025  | 399            | 437            | 338       | 326       | 1.0          | P/P; P/P; Figure 5 |

^a,b,c As defined in Table 2.
The application of TargetRNA to the E.coli RyhB, OmrA, OmrB and OxyS RNAs has already expanded the number of known targets for these regulatory sRNAs. We anticipate that as the number of known sRNA:mRNA interactions increases, we will better understand the applicability and the limitations of in silico target prediction approaches. In addition, an expanded set of known targets will allow for further refinements of computational approaches for target prediction.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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