Evaluation of thresholds for the detection of binding sites for regulatory proteins in Escherichia coli K12 DNA

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Abstract

Background: Sites in DNA that bind regulatory proteins can be detected computationally in various ways. Pattern discovery methods analyze collections of genes suspected to be co-regulated on the evidence, for example, of clustering of transcriptome data. Pattern searching methods use sequences with known binding sites to find other genes regulated by a given protein. Such computational methods are important strategies in the discovery and elaboration of regulatory networks and can provide the experimental biologist with a precise prediction of a binding site or identify a gene as a member of a set of co-regulated genes (a regulon). As more variations on such methods are published, however, thorough evaluation is necessary, as performance may differ depending on the conditions of use. Detailed evaluation also helps to improve and understand the behavior of the different methods and computational strategies.

Results: We used a collection of 86 regulons from Escherichia coli as datasets to evaluate two methods for pattern discovery and pattern searching: dyad analysis/dyad sweeping using the program Dyad-analysis, and multiple alignment using the programs Consensus/Patser. Clearly defined statistical parameters are used to evaluate the two methods in different situations. We placed particular emphasis on minimizing the rate of false positives.

Conclusions: As a general rule, sensors obtained from experimentally reported binding sites in DNA frequently locate true sites as the highest-scoring sequences within a given upstream region, especially using Consensus/Patser. Pattern discovery is still an unsolved problem, although in the cases where Dyad-analysis finds significant dyads (around 50%), these frequently correspond to true binding sites. With more robust methods, regulatory predictions could help identify the function of unknown genes.

Background

As a consequence of the availability of whole-genome expression methodologies, regulation of gene expression is at the core of current post-genomic studies [1]. Once a set of genes is clustered on the basis of similar expression profiles, a logical next step is that of searching their upstream regions for potential binding sites for transcriptional regulators. The predicted binding sites in DNA can then be mutated or used to fish out the DNA-binding regulatory protein. Different methods exist for finding binding sites [2-6], with a recent rapid increase in different methods with small variations and improvements [7-9]. However, as the computational
biography community has long been aware, a common limitation of such methods is the high rate of false-positives that they generate as a result of the low degree of conservation of the DNA sequences of binding sites.

This work is a contribution towards a more detailed evaluation of the performance of these methods, with the aim of finding the best selection of thresholds to provide reliable predictions. On the basis of our evaluations, we suggest improved methods to search for novel binding sites that give a much lower rate of false positives. We use information gathered in RegulonDB, a database on regulation of transcription in *Escherichia coli* compiled from the literature [10,11]. The database contains data on regulons - sets of genes in transcription units whose expression is regulated by the same regulatory proteins - with different types of evidence and different levels of description. For instance, at the time of writing, the database contains information on 112 regulatory proteins, but binding sites in DNA are only described for 60 of these. The data for 26 of the regulatory proteins includes information on at least three regulated genes, with at least one binding site per gene (Table 1). The total number of regulatory binding sites listed is 505.

As explained below, we distinguish between pattern discovery and pattern search and evaluate each separately. We evaluate two methodologies. One is Dyad-analysis [12], a program developed to find over-represented small words separated by a given distance. We also describe and evaluate an elaboration of this method that aims to search for probable binding sites using the dyads generated (dyad sweeping). The other method uses Consensus [13], a program that generates optimized ungapped multiple alignments for sets of known or suspected regulatory sequences and builds matrices representing the frequency of each base at each position of the aligned sequences. Its companion program 'Patser' uses the matrices generated to scan for similar new sequences. The evaluations take into account the interest in minimizing the false-positive rate, as even a very small false-positive rate can overshadow true positives because of the small number of genes expected to be part of each regulon (see below).

**Description of datasets**
As most regulatory sites for DNA-binding proteins are found 200 to 400 base-pairs (bp) upstream of the regulated genes [14], we built two sets of upstream regions. One contained 200 bp of the region upstream of the genes’ start sites plus 50 bp downstream (200+50 set); the other contained 400 bp upstream plus 50 bp downstream of the start sites (400+50 set). Repressor sites are located near the promoter site, whereas activators tend to occupy a larger region upstream of the promoter. It is therefore potentially useful to evaluate the performance of the methods with these two different ranges of sequence. Additional information can also influence the decision of the experimentalist to select the length of upstream region to analyze. For instance, some proteins tend to have a single binding site per promoter, which has to be proximal to the promoter (for example LexA), whereas other proteins tend to have several binding sites per upstream region, with some of them farther upstream of the promoter (for example AraC, Lrp and MetJ). Another factor that influences the size of region to analyze is whether the precise site of transcription initiation (the +1 position) is known. When the promoter is known, the search can be limited to 200 bp upstream from the +1 position. If it is not known, then the reference point has to be the start codon and the 400 bp upstream of this are used - which assumes an average of 50 to 100 bp between the promoter and the beginning of the gene.

We used the total set of upstream regions containing at least one reported binding site in RegulonDB as the basic data for evaluation. In each case, upstream regions of genes regulated by the same protein (regulons) were separated from the collection and constituted the ‘training sets’. For each set, the remaining upstream regions, known to be regulated by other proteins, are assumed to be the collection of ‘known negatives’. Though there is still a risk that the known negatives contain genes that also pertain to the regulon we are contrasting them with, the fact that they have been the subject of experimental work allows us to think that this risk is minute.

Because of the small amount of data for each protein, we could not leave out a set of known positives to evaluate the rate of true positives, except in the case of the regulatory protein CRP. For those families having at least five upstream regions we were able to apply a ‘leave one out’ procedure as described below. We also have information, in some cases, on genes regulated by a given protein in the regulons analyzed, but with no reported binding site. The upstream regions of these genes were used to search for binding sites and provide further evaluation. A more detailed analysis was performed for LexA, comparing our predictions with a recent report in the literature [15].

**Levels of analysis**
Depending on the information available, there are basically two computational approaches to predicting binding sites for transcription initiation factors in DNA. In the best cases, there is information on experimentally determined examples of binding sites for a given regulatory protein. In such cases, the search programs can be trained using the sequences corresponding to the binding sites, and the information obtained (dyads, weight matrices) can then be used to find similar sequences, and thus other genes that might be under the control of the same regulatory protein. This is pattern searching.

On the other hand, a common scenario at present is that a set of apparently co-regulated genes is identified from transcriptome experiments. In this case, a program would be
trained with a collection of upstream regions from these genes with the goal of identifying probable shared regulatory sites. This is the problem of pattern discovery. If the data come from transcriptome experiments, the collection of co-regulated genes might not be complete. Because of the noise inherent to such experiments, and/or to the limitations of clustering algorithms, a researcher might wish to try to find other genes likely to be under the control of the same protein. However, other genes regulated by the same protein might display a different pattern of expression as a result of complications such as regulation by more than one regulatory protein.

On the basis of these considerations, the analyses we present contemplate the use of experimentally determined binding sites as training sets to study pattern search, and the use of upstream regions of co-regulated genes to study pattern discovery. More precisely, we use the set of binding sites in DNA for each regulatory protein reported in RegulonDB to try to find additional genes in the genome with similar sites. We also use the data on known co-regulated genes to try to find the binding site within the genes’ upstream regions.

RegulonDB contains information for the 86 regulons shown in this table. Of these, only 60 have at least three known binding sites for their corresponding regulatory protein. The second column indicates the total number of known sites, which are distributed in upstream regions (fourth column). The last column indicates the number of upstream regions for which there is experimental evidence suggesting regulation, but no direct proof of binding of the regulator to the upstream site is yet available. For instance, there are 12 known sites for ArgR located in only six regions (with two sites per region), plus one region for a different gene for which there is evidence of regulation by ArgR.
As training sets, we ran the dyad or matrix search programs on the sequences of known regulatory binding sites and on upstream regions of 200+50 and 400+50 bp from genes regulated by a given regulatory protein. Families corresponding to a given regulatory protein were evaluated only if there were at least three sequences in the corresponding training set (40 in the collection of binding sites; 26 in the 200+50 and the 400+50 datasets). Subsequently, the dyads and matrices were evaluated against the complete collections of 200+50 and 400+50 upstream regions. This gives a total of $3 \times 2 = 6$ evaluations for each regulon analyzed. The evaluations included regions 200+50 or 400+50 only if there was at least one reported binding site within that range; thus, the total set of 200+50 regions contained 172 sequences, and the 400+50 set contained 189.

**Dyad analysis**

We used the Dyad-analysis program \[12\] to find dyads within each training set. The options used were to find dyads of 3 bp long separated by distances of 0 to 16 bp, with any kind of dyad (direct repeat, inverted repeat, asymmetric), searching in both DNA strands \[12,16\]. Further analyses were limited to the training sets where the program found at least one dyad with a significance equal to or above 1.0 (see \[12\] for a detailed description of significance). This left 19 families from the binding-sites training sets, 11 from the 200+50 regions, and 14 from the 400+50 regions (the program Dyad-analysis did not find any dyad in about 75% of the rejected families, and found just one in most of the rest of them).

The program Consensus was run to obtain alignments and matrices 20 bp long - the most frequent size among binding sites for regulatory proteins. To assign match scores, we used an ‘alphabet’ based on the frequency of each base at upstream regions of 200+50 and 400+50 of all genes in *E. coli*. The search was done in a single strand. Although we also ran the program to find symmetric patterns, no clear improvement was observed. In the Results section, we first present results of pattern discovery, then concentrate on the selection of the best thresholds, analyzing their performance on the basis of the evaluation criteria described above. Finally, we present some specific predictions.

**Results**

**Pattern discovery**

Pattern discovery starts with a collection of co-regulated genes for which no binding sites are yet known. To evaluate the methodology, we counted the number of times a sensor can locate a known binding site in a collection of 200+50 or 400+50 regions.

The Dyad-analysis program is designed to find over-represented small words. Over-represented words would be expected to occur at the binding sites, and thus the first step was to determine if the resulting dyads match the binding sites. We found that there are significant dyads all along the sequences analyzed, with most of them matching at or near the known binding sites. Figure 1 shows, using the PurR family, that most dyads were found at distances very close to or overlapping the true binding sites. We observed the same tendency for all families. We thus decided to search for stretches of contiguous matches, which we call ‘regions of overlapping matches’ (ROMs), in the upstream sequences being analyzed by counting (sweeping), base by base, the number of matching dyads. As seen in Figure 2, the ROMs with the highest number of matching dyads overlap the true known binding sites in the DNA. This result motivated us to use the highest number of matches within a ROM as the score. We call this method dyad sweeping.

As the highest-scoring ROMs frequently overlap reported binding sites (Figure 2, Table 2), we decided to keep, for subsequent analyses, the dyads found within the highest-scoring ROMs of each upstream region, as long as the ROM contained at least two dyads. In Table 3 it can be seen that, except in a few of the regulons, the fraction of regions with known binding sites found is quite high. In other words, the set of dyads that result after keeping only those that contribute to the highest ROM in each family is able to recover a large fraction of all the known binding sites in the family. It is important to keep in mind that a given dyad can match several positions and therefore sites in a single region or family. Thus, selecting only those dyads appearing in the...
The number of dyads that describe the set of known binding sites in a given regulatory family is quite variable. For instance, if we use the known binding sites as training sets, the TyrR family involves 14 different dyads whereas ArcA has 65. There is no clear correlation between the number of dyads per site and the total number of sites in the training set for any given family, or any other property of the regulatory site, such as its size.

**Sequence alignment**

Consensus is a program designed to find and align shared stretches of sequence among a given set of sequences. The searching method based on the results of Consensus is already available [13]; the weight matrix generated can be used to search, with the companion program Patser, for sites in other upstream regions. The search using Patser was made using the first matrix (highest informational content) obtained in the final cycle of Consensus. This cycle requires all regions to contribute at least one sequence to the matrix. Using Patser, we searched for the highest-scoring sequence in each region in the training set. The lowest value among these results was set as the minimal score and a second search was performed with this threshold in order to find new sites above this limit within each upstream region in *E. coli* for further searches and analyses.

The capacity for pattern discovery of the two methods can be estimated by calculating the fraction of binding sites found when the training sets were the 200+50 or 400+50 bp regions, as shown in Table 4. A site was considered found when the predicted pattern overlaps 20% of the binding site. We also show the results of using the sequences of the binding sites with 10 bp extensions on each side as training sets, so we could distinguish between pattern discovery and pattern abstraction or identification. In the case of Dyad-analysis/sweeping we evaluated whether the filtered dyads overlap the set of true sites. In the case of Consensus/Patser we evaluated whether the set of sites selected by Consensus/Patser overlaps the set of known sites. Consensus/Patser is able to abstract a pattern for each of the 25 families, whereas Dyad-analysis/sweeping can only do it for 19 of the families. In 11 of these 19 families Consensus/Patser finds more sites, in two families Dyad-analysis/sweeping finds more sites, and in the remaining six both methods perform equally well.

The real pattern discovery situation is that of the 450/sites cases (see legend to Table 5 for definition), where Consensus generates matrices for 24 of the families and Dyad-analysis finds significant dyads for 11 of them. Dyad sweeping finds on average more than 70% of the binding sites (when Dyad-analysis obtains significant dyads) as compared to around 60% with Patser. Note that using shorter regions to search for DNA binding sites (200+50), improves the performance of both methods by about 5-7%.

Once Table 4 was generated, we estimated the fraction of upstream regions recovered (Table 3). A region is considered found when at least one site in that region is found. Therefore,
Figure 2
Dyad sweeping along the upstream region of the purR gene. Contiguous regions of overlapping matching dyads (ROMs) frequently overlap with the known binding sites. This example shows results after finding significant dyads in the 200+50 regions of the PurR regulon, and finding the ROMs within the same regions. The two ROMs with the highest peaks completely overlap with the two reported regulatory binding sites in this region (sites lie at positions -59 to -43 and at 29 to 45). The coordinates here are relative to the annotated first coding nucleotide of the gene. The known binding sites are illustrated as boxes below the figure. The lower line shows the different dyads coded in different colors. It can be seen, for instance, that blue dyads occur only in the two true binding sites.
Detection of new members of regulons by pattern matching

Detection of new members of regulons requires the selection of an optimal threshold to accept a sequence as a predicted binding site, and the genes downstream of such sequences as new members of the regulon family. The selection of the best threshold requires the evaluation of the following parameters: sensitivity (rate of true positives), specificity (rate of true negatives), accuracy (overall rate of true results), and, very important in this case, the positive predictive value (rate of true positives among the total number of positives, true and false). Definitions of these terms are given in the legend to Table 5.

We used a leave one out (LOO) procedure to evaluate the true-positive and false-negative rates with families containing at least five reported genes with binding sites. The LOO method consists of leaving one gene at a time out of the training set; then, with the matrix or dyads built with the remaining sites, a search is made for a probable binding site within the upstream region of the gene that was left out. We combined the results of applying Dyad-analysis/sweeping and Consensus/Patser to the problem of discovering binding sites. The results contain pattern discovery data similar to those in Table 3, but this time counting the number of binding sites found per total number of sites. Again, only dyads extracted from max ROMs are used. The names of columns are as described in Table 3.
remaining regions, known to be regulated by any other protein, as known negatives. Instead of calculating an average of the scores, and defining the threshold on the basis of standard deviations, we scanned the scores scale from the minimum score obtained in the collection of positives, to the maximum one, calculating the evaluation parameters noted above at each point of the scale. There is no point in searching at lower scores as there is no effect on sensitivity at such values.

In Figure 3 we show the results of the analyses of the PurR regulon using dyad sweeping. Here, the minimum number of matches evaluated was one. Note that, as the dataset of known negatives exceeds that of known positives, high accuracy coexists with a large number of true negatives. Nevertheless, at the threshold of 10 matches, despite a very low false-positive value (less than 10%), and a very high accuracy (approximately 95%) and sensitivity (90%), the positive predictive value (PPV) shows that the total true positives in the whole ‘predicted’ set is about 60%. This is a very important issue. As most regulatory proteins regulate just a few genes in comparison with the whole set of genes in a given organism, such a difference means that false positives might dilute reliable predictions even at very low false-positive rates. The PPV alone would leave results with very little recovery of true binding sites. Therefore, calculating an optimal point for prediction requires the use of a balanced evaluation criterion. After examining several graphs, we noticed that the average between accuracy and PPV (which we call the overall performance or OP) would be a good criterion. This makes it possible to assess the overall performance of the predictions.
sense, as OP represents a trade-off between those two statistical measures. Other criteria, such as the product of accuracy and PPV, might be used instead, but OP worked well for our purposes. In a few cases, the point of highest OP leaves a very small sensitivity value (around 50% in PurR, for instance). If the sensitivity value was less than 60%, we used the last point where the sensitivity was above 60%. In Figure 4 we show the results of sensitivity and false-positive rate for all regulons at their best OP value using dyad sweeping.

The use of weight matrices derived from Consensus (with Patser) is not illustrated, as the selection of the best threshold is the same as in dyad sweeping. In Figure 5 we show the results of sensitivity and false-positive rates of each regulon at the best overall performance point of each regulon analyzed using Patser.

In Table 6 we give the fraction of sites found per family in regions of 400+50 bp when starting from different training sets using the threshold chosen as described above. Dyad-detection/sweeping still performs better at finding the sites within an upstream region, while Consensus/Patser trained with binding sites finds the sites at an average of almost 77%.

An interesting finding here was that, when trained with all the upstream 400+50 sequences, Consensus finds an alignment and matrix that clearly discriminates between the sequences used in the training set, or regulon, from any other upstream sequence in E. coli. However, in some families, the matrix matches at sites different from the experimentally determined DNA binding site of the regulon under analysis (Figure 6), and such sites do not correspond to any known site, motif or region annotated in RegulonDB in the upstream sequence. We also verified that they do not match conserved regions in between pairs of sites. It will be indeed interesting to find out if these sequences have any biological meaning.

Predictions
Once the optimal threshold was obtained, we proceeded to predict other members of each regulon using the complete collection of upstream regions (200+50 and 400+50) of the E. coli genome [17]. In order to further evaluate the predictions obtained, we used the recent annotations of cellular functions assigned by Monica Riley and her group to known E. coli genes [18]. About 30% of the genes in E. coli have no function assigned, and each gene or gene product can be

![Figure 4](http://genomebiology.com/2002/3/3/research/0013.9)

**Figure 4**
Performance of Dyad-analysis/dyad sweeping at the best threshold defined for each family. Sensitivity and false-positive rate (expressed as percentages) at the highest overall performance for each regulon are shown, using the binding sites as training sets, and the 400+50 regions as evaluation sets. We do not show the regulons where the methods did not provide significant results.
assigned to more than a single cellular role. In Table 7 we show the consistency between the functional annotations of genes experimentally demonstrated to belong to each regulon as compared with the functional annotations of the set of predicted genes. In the cases of predictions of high confidence (for example, ArgR, CRP and PurR - all with correspondences above 90%), a putative function can be reliably assigned to genes of unknown function. For instance, in the case of the PurR family, the genes without functional annotations might be assigned to macromolecule (DNA/RNA) biosynthesis. This is an example of functional gene prediction based on analysis of its regulatory elements. Annotations like ‘active transporter’ would require other kinds of evidence (see Additional data files). Functional annotations might be quite helpful in cleaning up wrong predictions, or adjusting the proposed thresholds, although limited by the genomic coverage of the functional assignments.

RegulonDB contains information on a few genes belonging to some of the regulons studied but with no mapped binding site for the relevant regulatory protein. As further evaluation, we show the results of dyad sweeping and Patser, trained with the known binding sites of each regulon, for all of these genes (Tables 8,9). In the tables we indicate whether the gene would be included in the corresponding predictions, the highest scoring ROM (dyad sweeping, Table 8) or pattern match (Patser, Table 9) found in the 400+50 region of the gene, and the actual sequence suggested as part of the possible binding site. Some genes would be rejected as predictions, but the small amount of data makes it impossible to appropriately evaluate this problem. A researcher might choose to use a different, perhaps lower, threshold if the intention is to find every gene for a given regulon experimentally, and such a decision would depend on how many confirmatory experiments it is possible to perform (an example is shown in the next section). Lower thresholds can also be used if the intention is to confirm new members suggested by other data, like clustering of a gene or genes with known members of a regulon. The latter case is exemplified by the results with those regulon members lacking a mapped binding site. Most contain ROMs or patterns scoring above the minimal score obtained for a known member of the

Figure 5
Performance of Consensus/Patser at the best threshold defined for each family. Sensitivity, and false-positive rate (expressed as percentages) at the highest overall performance for each regulon are shown, using the binding sites as training sets, and the 400+50 regions as evaluation sets. We do not show the families where the methods did not provide significant results.
regulon (no search is performed below this lower limit), often just below our suggested threshold. Thus, if there is additional evidence that a gene belongs to a given regulon, the ROMs found can be proposed as the putative binding sites.

Comparison of results with recently examined members of the LexA regulon

A recent attempt has been made by Fernandez De Hene-strosa et al. to locate all the members of the LexA regulon by a combined strategy that included prediction of probable binding sites and experimental confirmation [15]. Their predictions were based on similarity to known sites. Experimental confirmation showed that only 10 of the 49 predicted new members responded to LexA. The authors also give a table of previously found members of the LexA regulon, which includes a few genes not annotated in RegulonDB. We could analyze only five of their experimentally confirmed genes and 31 of their wrong predictions (predictions they later found experimentally not to be regulated by LexA) because of the lack of updating of the E. coli K12 genome annotations. In Table 10 we present our results for the genes noted as previously determined members of the LexA regulon in [15], plus the five new members found by this study. In Table 11 we show our results with their wrong predictions. Using dyad sweeping, we find 20 out of the 23 confirmed members of the LexA regulon, whereas we would reject 20 out of their 31 wrong predictions. With Consensus, we detect 18 of the 23 confirmed members of the regulon, while rejecting 19 of their wrong predictions.

Table 6

| Regulon | Consensus/Patser | Dyad-analysis/dyad sweeping |
|---------|-----------------|----------------------------|
|         | Sites/sites     | 450/sites                  | Sites/sites | 450/sites |
| AraC    | 60.00 20.00     | 80.00 100.00               |
| ArcA    | 80.00 90.00     | 90.00 90.00                |
| ArgR    | 82.33 100.00    | 100.00 66.67               |
| CRP     | 80.95 63.49     | 90.48 95.24                |
| CysB    | 100.00 80.00    | - -                        |
| CytR    | 100.00 16.67    | - -                        |
| FIS     | 40.00           | - -                        |
| FNR     | 65.00 70.00     | 50.00 65.00                |
| FruR    | 100.00 85.71    | - -                        |
| Fur     | - -             | 75.00 75.00                |
| GipR    | 83.33 33.33     | 100.00 100.00              |
| IHP     | 87.50 87.50     | 100.00 100.00              |
| LexA    | 40.00 60.00     | - -                        |
| Lrp     | - -             | 75.00 75.00                |
| MalT    | - -             | 100.00 100.00              |
| NR_I    | - -             | 100.00 100.00              |
| NagC    | - -             | 50.00 50.00                |
| NarL    | 77.78 22.22     | - -                        |
| PhoB    | - -             | 75.00 75.00                |
| PurR    | 69.23 84.62     | 92.31 84.62                |
| TrpR    | 100.00 100.00   | 100.00 -                    |
| TyrR    | 62.5 87.50      | 75.00 37.50                |
| Average | 76.85 66.74     | 62.65 76.00                |

For each family, we show the results with Dyad-analysis/sweeping and with Consensus/Patser accepting a match only if its score exceeds the defined best threshold. This threshold corresponds to the highest overall performance, see Figures 3 and 4. Otherwise the results are treated as explained in Table 4. The column names are as described in Tables 3 and 4.

Table 7

| Regulon | Consensus/Patser | Dyad-analysis/sweeping |
|---------|-----------------|------------------------|
|         | Percent with related function | Percent without functional annotation |
| AraC    | 73.33 (66.67)   | 37.50 (42.86)          |
| ArcA    | 72.61 (70.75)   | 45.86 (47.50)          |
| ArgR    | 100.00 (100.00) | 18.18 (33.33)          |
| CRP     | 93.90 (93.38)   | 40.87 (43.63)          |
| CysB    | 56.78 (56.56)   | 43.03 (43.16)          |
| CytR    | 53.90 (53.58)   | 38.56 (38.72)          |
| FIS     | 45.52 (45.11)   | 41.23 (41.28)          |
| FNR     | 82.85 (81.99)   | 45.85 (47.06)          |
| FruR    | 51.96 (51.29)   | 36.57 (36.89)          |
| IHF     | 80.95 (80.33)   | 46.37 (47.14)          |
| LexA    | 69.70 (61.54)   | 42.11 (48.00)          |
| Lrp     | 82.61 (81.95)   | 42.67 (44.58)          |
| NarL    | 73.38 (72.11)   | 45.20 (46.35)          |
| PurR    | 95.24 (92.31)   | 8.70 (7.14)            |
| TrpR    | 85.71 (50.00)   | 30.00 (60.00)          |
| TyrR    | 69.23 (50.00)   | 18.75 (27.27)          |

A comparison between the functional annotations of genes known to be regulated by a given protein and the functional annotations of the predicted set of genes. The percentage with related function is calculated against all the genes with functional annotations, while the percentage without functional annotations is calculated against the whole set of predicted genes. The number in parentheses excludes genes known to be part of the corresponding regulon. In cases with high correlation of functional annotations we can propose a related function for genes without functional annotations, as in the Consensus/Patser predictions of ArgR, CRP and PurR (all with correspondences above 90%). Detailed tables are provided as Additional data files.
Conclusions
Stringent evaluations of pattern discovery and pattern searching methods should be carried out to establish the confidence of a given prediction. Here we take advantage of the availability of reasonable negative samples - all other known regulons described in RegulonDB, except the one under study - in order to use standard statistical measurements of performance such as specificity and PPV. The PPV allowed us to stress how important even low rates of false positives might become in a large population. The small proportion of genes expected to be regulated by a given regulatory protein makes it important to emphasize the need for a stringent threshold to admit new members of regulons, as the true positives might be diluted in a high number of false positives. Nevertheless, if additional independent evidence is available, thresholds can be relaxed to include more predictions as the confirmation procedure (genetic evidence of the regulatory effect, for instance) would allow. For instance, if the two computational methods were combined, only one of the genes known to be regulated by LexA (see previous section) would be rejected by both methods (ybfE in Table 10), while 16 of the wrong predictions are rejected by both methods (Table 11).

A very striking observation that deserves experimental analysis is the behavior of Consensus when identifying binding sites versus upstream regions. The program discovers patterns that discriminate, very specifically, the upstream regions used as training sets from the other regions. However, the patterns found do not always match the DNA binding sites. What are these specific motifs? These results imply the existence of new sequence elements specific to each family, different from those reported in the literature. We have not yet found (data not shown) any additional property that could suggest their function; their distance from the start site of transcription to known binding sites is not conserved; in some cases the predicted motif occurs upstream of the known sites in some promoters and downstream in other promoters. We have, of course, verified these observations twice, and find no additional property to associate with such families.
In the comparison of the two methods we have not found that one of them performs better in all the evaluations and scenarios considered (pattern search, pattern abstraction and pattern discovery). This implies that one could consider combining the different methods to make the best use of their respective strengths. For instance, if there is evidence of co-regulation only, we would suggest using Dyad-analysis/sweeping first to find the binding sites. If Dyad-analysis finds significant dyads, the dyad sweeping methodology can be used to extract possible binding sites. After that, the predicted sites can be used to train Consensus and search for further co-regulated genes. In cases where the DNA binding sites are known, Consensus/Patser, which are both very fast and simple to use, can give very reliable results in a short time.

The combination of computationally more confident predictions, together with additional independent evidence - for example, functional classes or operon organization - is an intelligent strategy for making more robust predictions. These more robust upstream regulatory analyses can be used to assign function to unknown genes, as illustrated here with the ArgR, CRP and PurR regulons. One can envisage highly relevant genomic applications of these predictions, such as distinguishing orthologs within families of paralogous genes, based on their differential regulation, or identifying non-orthologous gene displacement on the basis of regulatory comparisons.

The goal in computational biology is twofold: to provide, on the one hand, methods that generate useful and evaluated

| Regulatory protein and genes shown to be regulated by it | Above threshold | Site coordinates and number of matches | Site sequence |
|----------------------------------------------------------|-----------------|---------------------------------------|---------------|
| ArcA (12)                                                | -               | i:-144f:-85; m:8                      | TTATCAAGTATTTTTAATAATGGGAAATTTTGTGTATTGAAAAAATGAGTAG |
| AceB                                                     | -               | i:-96f:-51; m:6                       | ATTCTTTAATCTTTTGTGATTTTTAAACAAATACACAC                |
| FadB                                                    | +               | i:-77f:-56; m:14                      | TATTGTTACTCGCTTTAACAG                                  |
| FunA                                                    | -               | i:-92f:-53; m:10                      | ATTTGTTATCAAATGTTAAATAAATGAGCTAAAGTT                 |
| FunC                                                    | -               | i:-248f:-232; m:8                     | TTATTTATGATACAG                                       |
| GlnB                                                    | -               | i:-168f:-150; m:12                   | TACCGTTTTATTAAACATAC                                   |
| HyA                                                     | +               | i:-232f:-204; m:15                   | TGTTTTAAAAATTGTTAAAACAAATTTTTGTAAA                  |
| LpsA                                                    | -               | i:-323f:-300; m:5                     | TGTTGGTTCACGTAATGCCTAA                                |
| ArgR (11)                                               | -               | i:-63f:-51; m:5                       | TTATATGCATAT                                       |
| FNR (4)                                                 | -               | i:-156f:-144; m:3                    | TGAATCTATTCAC                                        |
| AspA                                                    | +               | i:-25f:-2; m:3                        | GATCCCGTGGGAATTGAGTCTGTTAAATG                       |
| CycA                                                    | +               | i:-306f:-292; m:6                    | ATTTGAAACGGATCA                                       |
| SdhC                                                    | -               | i:-340f:-326; m:2                    | GATGATATAAAAATT                                    |
| LexA (9)                                                | +               | i:-57f:-41; m:24                     | CTGCTGCGCAAGAAGACTACTGTATAAADACAGTATAAATCAGGCAGATTATAT |
| UmuD                                                    | -               | i:-146f:-139; m:1                    | CAGCATAT                                              |
| Lrp (1)                                                 | -               | i:-277f:-269; m:2                    | CAGCATAT                                              |
| LysC                                                    | -               | i:-146f:-139; m:1                    | CAGCATAT                                              |
| NarL (1)                                                | -               | i:-215f:-201; m:1                    | TACCCAGAAGTGAGT                                      |
| CadA                                                    | -               | i:-209f:-195; m:2                    | TACCCCTCTGAGTG                                       |
| PurR (15)                                               | +               | i:-82f:-64; m:16                     | AGCAGGAAAGCGATTTGCTTTTT                              |
| ProsA                                                   | +               | i:-356f:-344; m:21                   | GAAAACGTTTTTCC                                     |
| SpeA                                                    | -               | i:-132f:-119; m:6                    | GAAACCGGTTGCGC                                      |

Sequences and positions of binding sites predicted by dyad sweeping in genes with experimental evidence for co-regulation in RegulonDB, but with no binding site experimentally identified. Genes follow the alphabetic order of the regulatory proteins, with the name of the protein separating each group. The number in parentheses after the regulator is the value of the threshold - derived from requesting best overall performance. The site coordinates are ‘i’ for initial base, ‘f’ for final position relative to the start codon. The score is given as the maximum number of matching (‘m’) dyads within a ROM. The number of families used was the same for any method, but we only show families where the methods provided significant results.
| Regulatory protein and genes thought to be regulated by it | Above threshold | Site coordinates and score | Site sequence |
|-----------------------------------------------------------|----------------|---------------------------|---------------|
| ArcA (8)                                                  |                |                           |               |
| aceB +                                                    | i-164:f-144;sc:9.48 | TTCATATTGTTATCAACAAAG     |
| fadB -                                                    | -              |                           |               |
| fumA -                                                    | -              |                           |               |
| fumC +                                                    | i-74:f-54;sc:10.82 | AATAATAAGTGAGCTAAAAAG     |
| glpA -                                                    | -              |                           |               |
| hyaA -                                                    | -              |                           |               |
| lpdA +                                                    | i-228:f-208;sc:8.43 | TAAAAATTTGTTAACAATTTT     |
| sucA -                                                    | -              |                           |               |
| ArgR (13)                                                 |                |                           |               |
| argD -                                                    | i-70:f-50;sc:11.12 | TAGTGATTTTTTATGCATAT      |
| CRP (6)                                                   |                |                           |               |
| cirA +                                                    | i-51:f-31;sc:6.39 | ATGTGAGCGATAAACCATTT      |
| ddxA -                                                    | -              |                           |               |
| ebgA +                                                    | i-91:f-71;sc:7.53 | TCGTGTACCGAGTTAAGTAA      |
| fhlD +                                                    | i-269:f-249;sc:10.86 | GTGTGATCTGATCCACGCT      |
| fucA +                                                    | i-399:f-379;sc:10.18 | ATATGAGCGCTACACTTT       |
| fucP +                                                    | i-205:f-185;sc:9.74 | AAGTTGATGTGACTACATAA      |
| glgC -                                                    | -              |                           |               |
| gntK +                                                    | i-169:f-149;sc:11.49 | TCGCAATTTAACCCACGTT      |
| lpdA -                                                    | -              |                           |               |
| melA -                                                    | -              |                           |               |
| spec -                                                    | -              |                           |               |
| shsA +                                                    | i-91:f-71;sc:8.89 | TCGGATCATAAAATCAACTT     |
| uibG -                                                    | -              |                           |               |
| CysB (4)                                                  |                |                           |               |
| cysP +                                                    | i-237:f-217;sc:7.04 | TTTTTTTGTCATTTTGGC       |
| CytR (1)                                                  |                |                           |               |
| nupC -                                                    | -              |                           |               |
| FNR (7)                                                   |                |                           |               |
| aspA -                                                    | i-367:f-347;sc:3.88 | CATGGGCAACCTGAATAAAAG      |
| cysA -                                                    | i-182:f-162;sc:4.31 | TTTGTTAATAACGGCTTTTTG     |
| icdA -                                                    | i-105:f-85;sc:6.30 | AATCAATTTACAAATAATTGC     |
| sdhC -                                                    | i-46:f-16;sc:3.89 | ATTCAATTTACAGAAGTTGCA    |
| FrnR (5)                                                  |                |                           |               |
| aceB +                                                    | i-253:f-233;sc:11.44 | GATCGTTAAGCGATCCAGCA     |
| fruB +                                                    | i-388:f-382;sc:13.85 | GAGGCTGAATCGTTTCAATT     |
| ptpA +                                                    | i-105:f-85;sc:6.29 | TTTGCTGAAACGATCCAGC      |
| IHF (7)                                                   |                |                           |               |
| caiT +                                                    | i-83:f-63;sc:8.41 | AATAATAATTATATAAATAG      |
| ecpD +                                                    | i-39:f-19;sc:8.96 | ATTATCCTCCTGTTTTTTAATTA  |
| himA -                                                    | -              |                           |               |
| himD -                                                    | i-136:f-116;sc:6.43 | ATTCGAAATTTGTTGAGATT     |
| hycA -                                                    | i-73:f-53;sc:6.57 | TAATCAACATAAATTAAAGAAG    |
| hypB -                                                    | i-155:f-135;sc:6.75 | TAACTTATTTTGTTATAAAG     |
| narK -                                                    | i-106:f-86;sc:6.66 | AAATATCAATGATGATAA       |
| ompR -                                                    | i-135:f-115;sc:6.39 | TATACTTAAAGCTGCTTTTATTA |
| sucA -                                                    | -              |                           |               |
| LexA (9)                                                  |                |                           |               |
| umuD +                                                    | i-40:f-20;sc:10.80 | CTACTGTATATAAAAACAGT     |
| Lrp (8)                                                   |                |                           |               |
| livK +                                                    | i-235:f-215;sc:8.35 | TGGCGGTTATTTTTATGCTGAC   |
| sdaA -                                                    | -              |                           |               |
| serA -                                                    | i-79:f-59;sc:6.62 | TGCCGCAATATTATTTTTTTG    |
predictions, and, on the other hand, to use such methods as models of the biology under study. This latter virtue could generate new ways of understanding fundamental processes in gene regulation, along with, as suggested here, new properties of gene regulation at the genomic level. We cannot rely on a single methodology to solve the problems. Each algorithm should be tested on well-defined problems in order to find their strengths. Thus it should be possible to choose which method, or combination of methods, is best suited for the problem at hand.

| Table 9 (continued) |
|---------------------|
| Regulatory protein and genes thought to be regulated by it | Above threshold | Site coordinates and score | Site sequence |
| NarL (7) | adhE | - | i:-160;f:-140;sc:6.11 | ATAACTCTAATGGTTTTAACT |
| caIF | - | i:-163;f:-143;sc:5.62 | CAAATAATACGTGCAAAGT |
| torC | - | i:-20;f:0;sc:4.98 | ATAAATTACGGGTTTAT |
| PurR (11) | codB | + | i:-84;f:-64;sc:13.10 | CCACGAAAACGTTTCAAC |
| prsA | + | i:-360;f:-340;sc:12.33 | GCAAGAAAACGTTTCCGAC |
| speA | - | i:-136;f:-116;sc:7.05 | AAAAGAAAACGTTTTCGAC |

Data and analysis as described in Table 8. sc, Score as obtained from Patser. The number of families used was the same for any method, but we only show families where the methods provided significant results.

| Table 10 |
| Predictions in experimentally characterized binding sites for LexA |
| Gene | Consensus/Patser | Dyad sweeping |
| b1728 | + | sc:10.89 | m:13 |
| b1741 | - | - | m:9 |
| dinD | + | sc:14.01 | m:20 |
| dinG | + | sc:9.11 | m:16 |
| dinI | + | sc:12.22 | m:12 |
| dinP | - | - | m:14 |
| ftsK | - | sc:7.82 | m:11 |
| lexA | + | sc:17.45 | m:21 |
| molR_1 | + | sc:10.46 | m:8 |
| polB | - | sc:8.30 | m:14 |
| recA | + | sc:14.71 | m:32 |
| recN | + | sc:13.56 | m:23 |
| ruvA | + | sc:11.12 | m:8 |
| sbmC | + | sc:14.08 | m:27 |
| ssb | + | sc:12.99 | m:29 |
| sulU | + | sc:15.64 | m:22 |
| sulUO | + | sc:10.80 | m:24 |
| uvrA | + | sc:16.30 | m:29 |
| uvrB | + | sc:14.94 | m:12 |
| uvrD | + | sc:16.07 | m:15 |
| yfE | + | sc:9.18 | m:3 |
| yoeG | + | sc:14.37 | m:23 |
| yjiW | + | sc:12.21 | m:14 |

Fernandez De Henestrosa et al. [15] experimentally characterized new LexA-binding sites, which are not included in RegulonDB. The table shows our binding-site predictions with dyad sweeping and with Patser, using their corresponding best overall performance thresholds. sc, Score as obtained by Patser; m, maximum number of matching dyads. Note that most genes clearly have ROMs with 10 or more matches and with scores of Patser above 10.

| Table 11 |
| Contrasting predictions: regions known to lack LexA sites |
| Gene | Consensus/Patser | Dyad sweeping |
| b3020 | + | sc:13.66 | m:6 |
| bnrQ | - | - | m:2 |
| creA | + | sc:11.67 | m:6 |
| dinJ | + | sc:11.52 | m:13 |
| expD | - | - | m:9 |
| hvoQ | - | - | m:4 |
| ibD | - | - | m:6 |
| ivbL | + | sc:13.23 | m:21 |
| metE | - | - | m:13 |
| metR | + | sc:9.10 | m:13 |
| minC | + | sc:12.22 | m:11 |
| pshM | - | - | m:4 |
| rfaJ | - | - | m:8 |
| rob | - | - | m:6 |
| xyIE | + | sc:9.15 | m:11 |
| yflL | + | sc:15.21 | m:13 |
| ybiA | + | sc:12.07 | m:16 |
| ybiT | - | - | m:5 |
| ycgJ | + | sc:13.42 | m:11 |
| ycgL | - | - | m:3 |
| ycgM | - | - | m:3 |
| ybiH | - | - | m:3 |
| ybiJ | - | - | m:6 |
| ybiK | - | - | m:6 |
| yfiE | - | - | m:6 |
| yfiK | - | - | m:6 |
| ylfF | - | - | m:4 |
| yflK | - | - | m:4 |
| yflO | - | - | m:2 |
| yflP | - | - | m:5 |
| yflQ | - | - | m:10 |

After experiment, Fernandez De Henestrosa et al. [15] rejected this set of genes in which they had predicted LexA sites using other computational methods. We tested the capacity of dyad sweeping and Patser to also reject these false positives. sc, Score as obtained by Patser; m, maximum number of matching dyads. Note that for both methods, most of the genes here show much smaller scores than genes belonging to the LexA regulon (see Table 10).
Additional data files

Additional data files containing the functional annotations associated to the genes within each regulons, and of those genes downstream of predicted binding sites are available with the online version of this article.

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