

operon predictions over those obtained with promoter-like signals. We expand the work to genomes with no experimentally characterized operons using regions upstream of divergently transcribed genes, forcefully TUBs, and regions between highly conserved co-directional genes, most probably in operons (15–17) as training sets to learn oligonucleotide signatures. We evaluate the genome-wide predictions obtained by this approach using diverse functional genomics data and demonstrate the capability of this method to produce high-quality operon predictions across genomes. (See Figure 1).

MATERIALS AND METHODS

Promoter-signal densities

We calculated the density of promoter-signals using a strategy published elsewhere (1). Briefly, with CONSENSUS (18) we obtained weight matrices for both the –10 and the –35 boxes of the Sigma-70 promoters of *E.coli* K12 reported in RegulonDB (19,20). With these matrices, we used PATSER (18) to calculate the average scores and SDs of the set of known promoters. Then, we scanned the –200 to –10 regions upstream of all gene starts in both *E.coli* and *B.subtilis* accepting promoter-signals formed of –10 and –35 boxes with scores equal or higher than the average minus 2.5 standard deviations of the boxes in known promoters. We obtained promoter-signal density as number of signals divided by number of bases (the data is available at http://tikal.ccg.unam.mx/sarath/sig_predictions/).

Upstream regions (Figure 1)

Known OJs are regions –200 to –10 bp from the start codon of genes inside experimentally verified operons. Known PRs consist of regions upstream of first transcribed genes (also –200 to –10). To find both of these kinds of regions we used the current dataset of transcription units of *E.coli* K12 (21) found in RegulonDB (19,20), and a dataset of experimentally verified transcription units of *B.subtilis* (<http://odb.kuicr.kyoto-u.ac.jp/>). With these data we found adjacent co-directional TUB pairs, consisting of the last gene in one transcription unit and the first in the next (the latter would be a first transcribed gene by definition), and we also found adjacent pairs of genes in operons (WO pairs), as described previously (22,23).

Conserved operon junctions (cOJs) are regions upstream of genes predicted to be inside operons by their conservation of adjacency in evolutionarily distant genomes as described elsewhere (17). We used predictions obtained at a confidence value of 0.95 (17). Regions upstream of divergently transcribed genes were representatives of regions upstream of first transcribed genes. To avoid overrepresentation we used the upstream regions of only one of the genes chosen randomly when the separation between the genes was <300 bp. If the space between the divergent genes was ≥ 300 bp we used the upstream regions of both genes as promoter regions (dPR).

Operon predictions based on intergenic distances

We predicted operons by intergenic distances using a method described previously (22,23). The method is based on log-likelihoods (dist-LLHs) calculated at different

distance intervals measured in base pairs (23), and it has been shown to be successful in most Prokaryotes (22).

Oligonucleotide signatures

We built oligonucleotide signatures for each region upstream of all annotated genes in the set of about 330 Prokaryotic genomes available at the NCBI Reference Sequence (RefSeq) Database (24,25) (<rsync://rsync.ncbi.nih.gov/genomes/Bacteria/>) on April 2006. For individual signatures, we counted the occurrence of each possible, overlapping, oligonucleotide along the –200 to –10 regions of each gene. The size of regions were chosen on the basis of preliminary tests showing that the sequence from –10 to 0 made the OJs and PRs signatures look more alike, probably due to the presence of the ribosome-binding site (data not shown). The overall signatures are defined as the average occurrences of each oligonucleotide within each dataset (e.g all OJs or all PRs). We calculated dinucleotide and trinucleotide signatures. Longer oligonucleotides make the statistics problematic. For instance, if we were to use tetranucleotides, there would be $4^4 = 256$ possible combinations. A region 190 bp in length would contain $190 - 4 = 186$ overlapping tetranucleotides, much less than the number of possible combinations.

The distance of the signature of each upstream region to the overall OJ-signature or to the overall PR-signature was estimated using χ^2 , which is a common method to test if a sample belongs to a given population.

$$X^2 = \sum_{i=1}^k \frac{(O_i - E_i)^2}{E_i},$$

where O_i and E_i are the observed and expected oligonucleotide frequencies, respectively. E_i is the frequency of the oligonucleotide in the overall signatures. The number of oligonucleotide types in the signature is k (64 for trinucleotide signatures). Since the OJ and PR oligonucleotide signatures have the same degrees of freedom, the X^2 of each of them can be directly compared. Thus, we can calculate the log-likelihood of a co-directional pair to belong to the OJ sample as follows.

$$\text{Signature LLH} = \log_{10} \frac{X_{\text{PR}}^2}{X_{\text{OJ}}^2},$$

where X_{PR}^2 and X_{OJ}^2 correspond to the χ^2 values calculated with respect to the oligonucleotide signatures of PRs and of OJs, respectively.

Phylogenetic profile analyses

The phylogenetic profiles used here consist of vectors where each item represents either the presence (number 1) or the absence (number 0) of an ortholog to the gene in a given genome (26). To compare the phylogenetic profiles of any pair of genes we calculated mutual information (MI) in bits. The MI for two vectors I and J is defined as (27):

$$\sum_{i=[0,1];j=[0,1]} P_{ij} * \log_2 \frac{P_{ij}}{P_i * P_j},$$

where P_{ij} is the proportion of a given pair ij in the alignment of vectors I and J , P_i is the proportion of the value i in vector I and P_j the proportion of j in vector J .

To build the phylogenetic profiles we found orthologs in a non-redundant dataset of genomes built as explained elsewhere (17,22). Our working definition of orthology consisted of reciprocal-best hits and fusions as described elsewhere (22).

RESULTS

We used four main kinds of upstream regions (−200 to −10 from start codon, see also Figure 1): (i) known OJs are regions upstream of genes inside experimentally verified operons; (ii) known PRs consist of regions upstream of first transcribed genes; (iii) cOJs are regions upstream of genes predicted to be inside operons by their conservation of adjacency in evolutionarily distant genomes (17); (iv) regions upstream of divergently transcribed genes (dPRs) are representatives of regions upstream of first transcribed genes. Oligonucleotide signatures consist of the counts of overlapping oligonucleotides along these regions. See Materials and Methods for further details. We applied the signatures of these different kinds of regions to predict whether a given pair of adjacent genes in the same-strand (co-directional pair), is in an operon (WO pair) or at a TUB pair. To measure the quality of any predictions we calculated sensitivity [true positives/(true positives + false negatives)], specificity [true negatives/(true negatives + false positives)] and accuracy (here the average of sensitivity and specificity). In all cases, we cross validated the predictions using a leave-one-out procedure.

Densities of sigma-70 promoter-like signals distinguish known PRs from OJs

To test promoter-signals in the distinction of PRs and OJs we calculated log-likelihoods to be an OJ at 0.01 intervals of promoter-signals per base pair using the formula $\log_{10}(f_{OJ}/f_{PR})$, where f_{OJ} is the fraction of OJs with such promoter-signal density (see Materials and Methods) and f_{PR} is the fraction of PRs with the same promoter-signal density (Figure 2). We were able to distinguish OJs from PRs with maximum accuracies of 0.70 in *E.coli* and of 0.65 in *B.subtilis*. Sigma-70 promoters are not the only kind of promoter in any of these organisms. *E.coli* K12 has 6 more annotated sigma factors, while *B.subtilis* has 15. The difference in number of sigma factors might be partly responsible for the lower success rate in *B.subtilis*. Alternatively, the results might reflect how prevalent are sigma-70 promoters in these two bacteria, or at least the particular prevalence of sigma-70 promoters within the datasets of known transcription units.

Signatures of cOJs and dPRs distinguish experimentally known OJs and PRs with high accuracy

To test signatures in the distinction of experimentally known WO pairs and TUB pairs we calculated log-likelihoods (sig-LLH) for each gene to be in the same operon with the previous (upstream) gene as described in Materials and Methods. The maximum prediction accuracies were 0.78 for *E.coli* K12 and 0.72 for *B.subtilis* (Figure 3). The values are very close to those obtained using the overall signatures of known OJs and PRs as training datasets (0.78 and 0.76, respectively). Thus, despite co-directional PRs might have

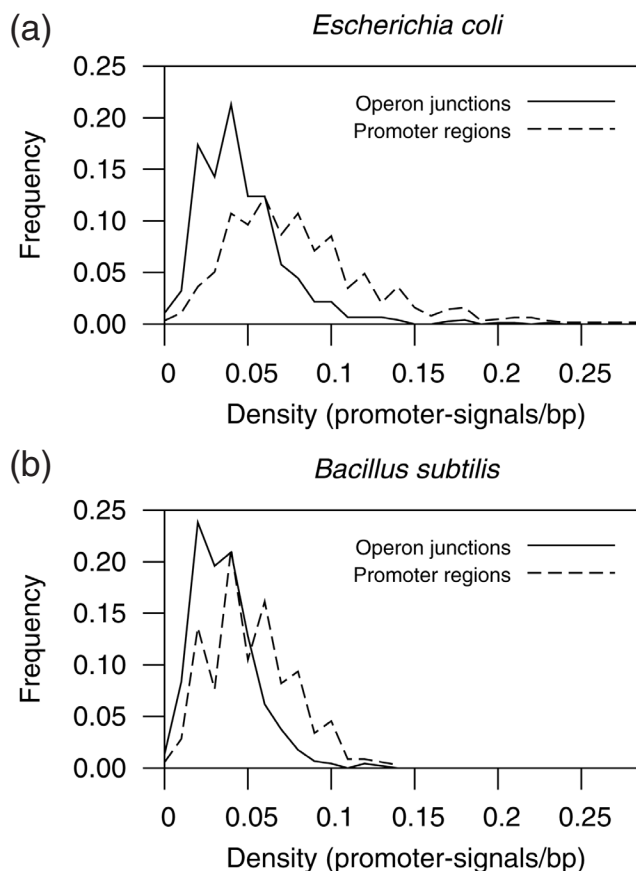


Figure 2. Density of sigma-70 promoter-like signals in PRs and OJs. Signals consist on predicted sigma-70 promoters, which have been found to abound in PRs (1).

different requirements than divergent PRs, like the presence of terminator signals, divergent PRs produce signatures useful for discriminating co-directional PRs. Conserved OJs also seem to be a good sample representing known OJs and thus equivalent datasets might help discriminate OJs from co-directional PRs in other genomes.

To test whether it was necessary to use trinucleotide signatures we also checked the statistical values of predictions obtained using dinucleotide signatures. Dinucleotide signatures are very common in genomic analyses (28). The maximum accuracies attained using dinucleotide-derived sig-LLHs were 0.76 for *E.coli* K12 and 0.71 for *B.subtilis*. Both numbers are below the results obtained with trinucleotides. Thus, at least in these model organisms, trinucleotide signatures might contain more information than dinucleotide signatures, making them more suitable for the task of distinguishing PRs from OJs (see also next section).

Proper separation of cOJs and dPRs vary across Prokaryotes

The sig-LLH threshold giving the highest accuracy using experimentally confirmed OJs and PRs is 0.00 sig-LLH in both *E.coli* K12 and *B.subtilis*, the same value that gives the highest accuracy of separation of the training data itself (cOJs and dPRs). Thus, the threshold for best discrimination

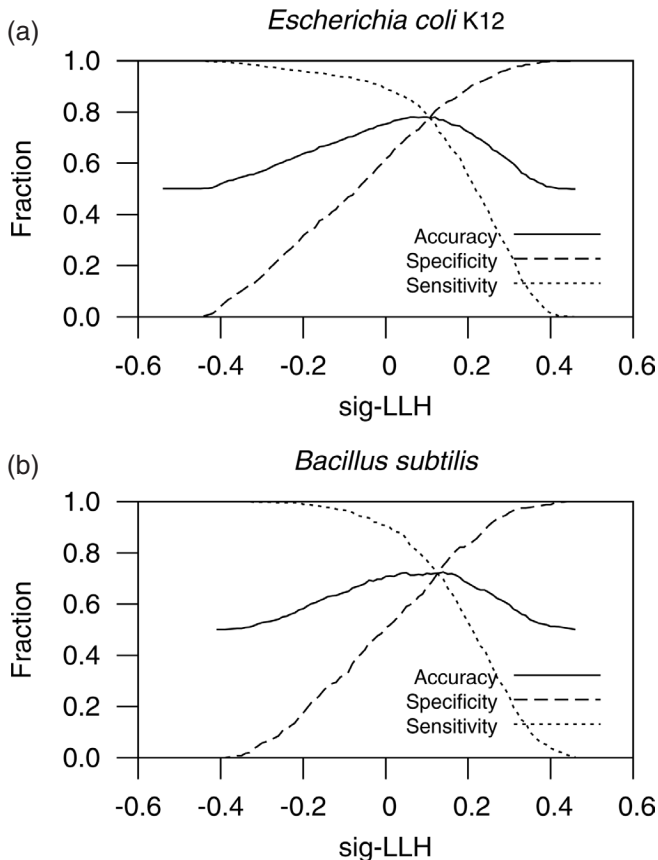


Figure 3. Statistics of signature-based predictions. The maximum accuracy for *E.coli* K12 was 0.78 and that for *B.subtilis* was 0.72.

of the training data might help decide a proper threshold for operon predictions in other Prokaryotes. In most cases the threshold giving the highest accuracy was 0.00 sig-LLH. The maximum accuracies of separation of training data range from 0.66 in *Prochlorococcus marinus* MIT 9313 to 0.97 in *Mesoplasma florum* L1. The maximum accuracies of separation of training data in *E.coli* K12 and *B.subtilis* are 0.88 and 0.87, respectively. Sixty-seven of the set of 219 non-redundant genomes had accuracies of separation of training data ≥ 0.87 (see website for accuracies obtained in each of the complete genomes analyzed).

To further test whether trinucleotide signatures provide better predictions than dinucleotide signatures we also calculated the accuracy of separation of training data using dinucleotide signatures. In 207 of 219 (0.95) non-redundant genomes the trinucleotide signatures show higher accuracies of separation of training data. The differences in accuracy (trinucleotide–dinucleotide) range from -0.008 to 0.18 , with a mean of 0.03 and a median of 0.02 . This result indicates, again, that trinucleotide signatures provide more information than dinucleotide signatures for differentiation of PRs from operon junctions.

We performed statistical analyses with several variables in an attempt to find out if any of them affects the maximum accuracies attained. The variables tested were: (i) number of sigma factors. Since our inspiration came from promoter-signal densities, we thought that the presence of several

Table 1. Correlations of several variables versus accuracy of separation of training data

Variable	Correlation	Significance
Overannotation	-0.32	$9.06e-07$
Number of dPR	-0.30	$7.736e-06$
Genome size	-0.25	0.0002
Number of genes	-0.23	0.0006
Number of cOJ	-0.14	0.0430
Sigma factors	-0.04	0.5484
Transcription factors	-0.01	0.8697

dPRs in training set; cOJs in training set; TFs (including sigma factors).

sigma factors might reduce the discrimination power because PRs recognized by different sigma factors might have different oligonucleotide signatures. We counted sigma factors based on Gene Ontology (GO) annotations in HAMAP (29,30) (ftp://ca.expasy.org/databases/complete_proteomes). To be considered a sigma factor the protein had to be associated to GO:0003700: Transcription factor (TF) activity, and either of GO:0016987: sigma factor activity; and GO:0003899: DNA-directed RNA polymerase activity. (ii) Number of TFs. The number and/or the proportion of TFs should also have an effect on the variability of oligonucleotide signatures because of the presence of binding sites for TFs at PRs. We also found TFs using GO annotation in HAMAP. The proteins had to be annotated as pertaining to GO:0003677: DNA-binding, and any of the following: GO:0003700: transcription factor activity; GO:0000156: two-component response regulator activity; GO:0030528: transcription regulator activity; GO:0016563: transcriptional activator activity and GO:0016564: transcriptional repressor activity. We also included TFs as predicted by Perez-Rueda *et al.* (31). (iii) Genome size and number of genes. There is evidence that as the genome size increases so does the regulatory complexity [see for instance (32–35)]. Thus this factor is related to (i) and (ii) above. (iv) Size of training samples. Despite we eliminated any genome when any of the samples (cOJs and dPRs) was less than 50, small training samples might still bias the oligonucleotide signatures and result in poor predictions. (v) Finally, genome overannotation (the annotation of genes that do not exist). We used the ‘SwissProt match’ method of Skovgaard *et al.* (36) to account for overannotation. The effect should be that signatures would be contaminated with the wrong information.

We used the ‘R’ package (37) (<http://www.R-project.org/>) to compute the correlations between accuracy and each variable. The two most important variables were overannotation and the size of the dPR sample (Table 1). We were expecting the number of sigma factors and of other transcription factors to have the largest effect, because complex regulation might result in more variable signatures at upstream regions. However, these were less correlated to accuracy than other variables (Table 1). Large genomes are expected to have more complex regulation. Accordingly, the third and fourth most important variables were genome size and number of genes (Table 1). The failure of the number of TF to represent the complexity of regulation might be due to: (a) the lack of knowledge of sigma and other transcription factors in organisms other than model organisms; (b) to the possibility

that even if the number of TFs and sigma factors is higher, the proportion of genes regulated by them might be very small and (c) to the presence of forms of regulation other than DNA-binding regulatory proteins, like ribo-switches and attenuation.

A multiple regression analysis, with accuracy as the dependent variable and all the variables in Table 1 as independent variables, results in an R-square of 0.26 ($P = 7.662e-11$). R-square is a measure of the amount of variation in accuracy that is explained by the variables in the model. Thus, the variables in Table 1 only explain 0.26 of the total variation in accuracy. The accuracies for each genome can be found at the web page: http://tikal.ccg.unam.mx/sarath/sig_predictions/.

Signature-predicted operons in any Prokaryote qualify as functionally related by phylogenetic profile analyses

We used phylogenetic profile analyses to test whether we are separating functionally related pairs of genes (genes in operons) from less related genes (genes at TUBs) across all genomes. Phylogenetic profiles constitute a genomic context tool used to predict functional interactions among gene products (26). We calculated the MI, measured in bits, of the phylogenetic profiles for all same-strand pairs of genes (see Materials and Methods). The expectation for functionally related pairs of genes is that they will exhibit a higher average MI than other pairs of genes. Thus, we compared the MI of pairs of genes predicted to be in the same operon (predicted WO pairs) against the MI of pairs of co-directionally transcribed genes at predicted TUBs. We found that the average MI for predicted WO pairs is higher than that for predicted TUB pairs in all genomes. The ratio of these averages (average MI of predicted WO pairs divided by the average MI of predicted TUB pairs) seems to increase with the maximum accuracy attained (Figure 4a). This result indicates that we are effectively separating functionally related genes from less related genes in any of the genomes analyzed. The minimum ratio was 1.20 in *Tropheryma whipplei* TW08/27 and the maximum was 7.80 in *Mycoplasma penetrans*. The ratios in *E.coli* K12 and in *B.subtilis* were 4.43 and 3.05, respectively.

Signature-predicted operons contain higher proportions of genes in the same metabolic maps than predicted TUBs

In order to further test whether signature-based operon predictions separate functionally related genes from less related genes we downloaded the current KEGG metabolic map dataset (38,39). These metabolic maps are often used to show how predictions of functionally related genes result in higher proportions of genes whose products are in the same metabolic map [see for instance (27,40)]. We found that the proportions of same-KEGG pairs in predicted WO pairs are higher than those in predicted TUB pairs in 93% of the genomes. As in the phylogenetic profile analysis above, the ratio of same-KEGG WO pairs to same-KEGG TUB pairs tends to increase with the accuracy (Figure 4b). The minimum ratio was 0.58 in *Methanospirillum hungatei* JF-1, and the maximum was 7.66 in *Methanococcus maripaludis* S2. The ratios for predictions in *E.coli* K12 and in *B.subtilis* were 2.35 and 1.60, respectively.

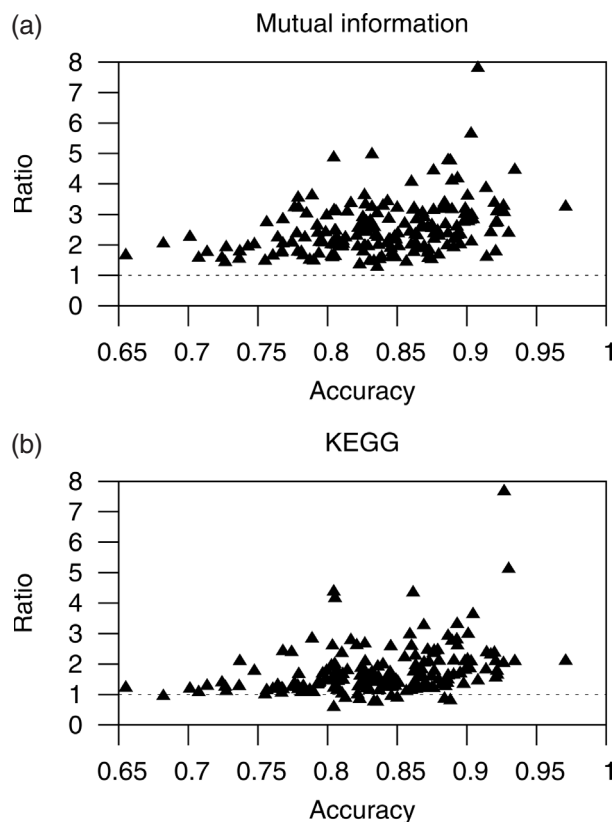


Figure 4. Quality of signature-based predictions. This figure shows ratios of average MI (a) and of proportions of same-KEGG map pairs of genes, with predicted pairs of genes in the same operon (WO pairs) in the numerator and predicted TUB pairs in the denominator. The expectation is that WO pairs will have higher MI than genes at TUB pairs, and that the proportions of genes in the same pathway will be higher among WO pairs than among TUB pairs. Thus, the ratios should be higher than 1 (dotted lines).

Signature-predicted operons have high correlations of expression

Another source for confirmation on the quality of predictions comes from the analysis of correlation of expression of each pair of genes in microarrays. We used the data for six genomes published elsewhere (41). The signature-based predictions produce gene pairs whose correlation of expression follows that of highly conserved genes (cWO pairs) (Figure 5). The cases of *E.coli* K12, and *B.subtilis* are clear. Both genomes display a good contrast in correlation of expression between predicted WO pairs and pairs of genes in opposite strands. Other cases are not as clear. All the datasets of *Helicobacter pylori* J99 seem to have the same tendencies towards correlated expression. Such behavior might be due to this organism being a parasite with a reduced genome. It is plausible that in this kind of organism the reduced genome contains a higher proportion of functionally related genes, and/or of genes that require little regulation due to a simpler lifestyle, thus resulting in high correlations of expression. *Chlamydia trachomatis*, another organism with a reduced genome, has a similar, though not as evident, tendency.

The cyanobacterium *Synechocystis* PCC6803 has been a challenge for operon predictions before (22), mainly due to

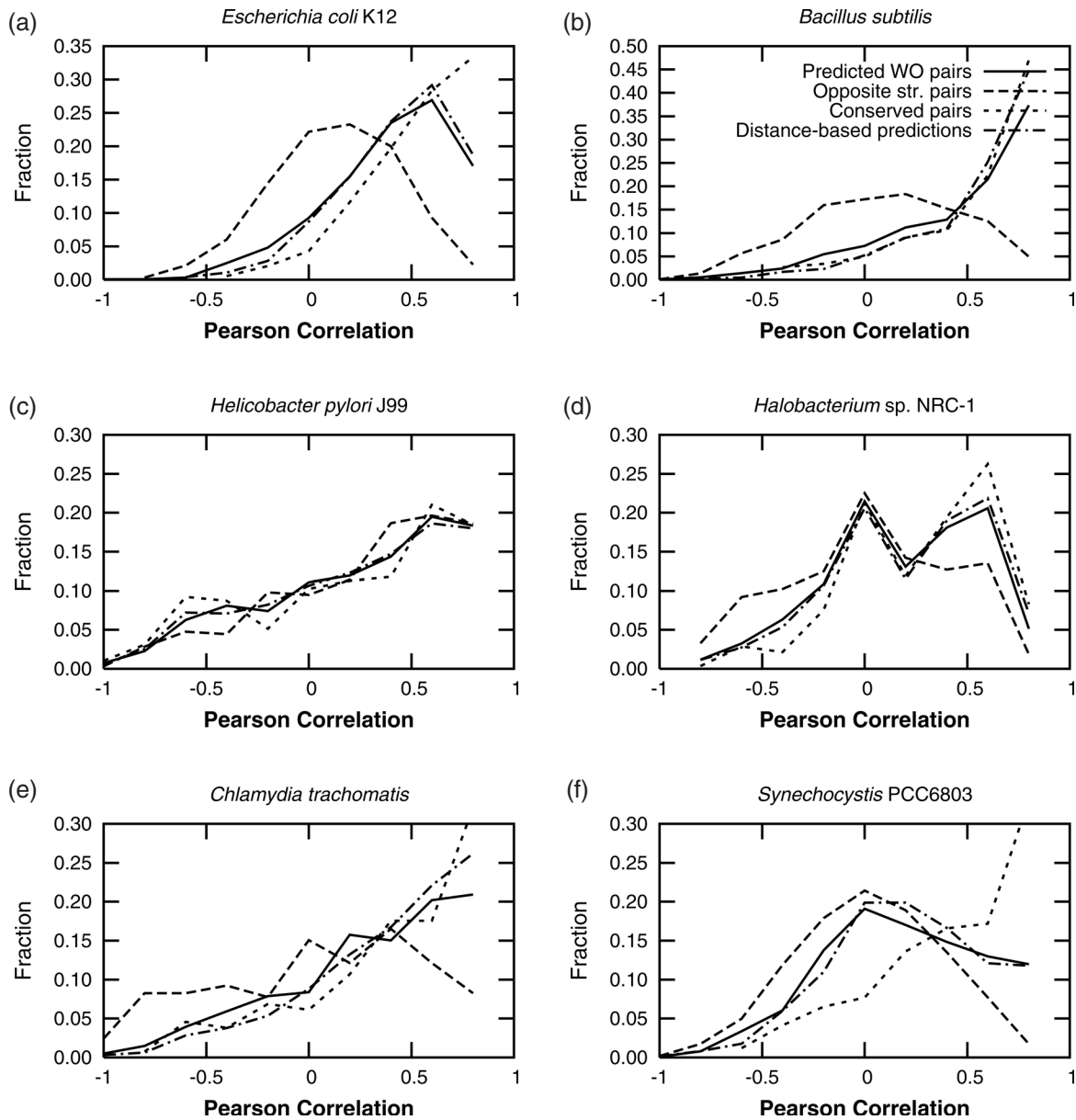


Figure 5. Correlation of expression in microarray data. Abbreviations as in Figure 4. Conserved pairs represent true WO pairs, while divergent pairs represent how true TUB should behave. Note that the tendencies for predicted WO pairs tend to follow those in conserved pairs, and that the quality seems to be at least as good as that for distance-based predictions produced as described elsewhere (22,23).

gene annotation problems and unusually high spacing between genes. In this organism the signature-based WO pairs exhibit higher correlations of expression than genes in opposite strands, but not as high as those of conserved pairs. The correlations somewhat follow those of our distance-based predictions (Figure 5), and those of the distance-based predictions presented by Price *et al.* (41). If conserved gene-pairs truly represent operons in this organism, then neither method has been very accurate to predict operons in this genome. Accordingly, the accuracy of discrimination of training data in this organism was the second to lowest at 0.68. An alternative explanation to genome annotation problems might be that operons in this organism abound in secondary signals between genes. If so, operons

in *Synechocystis* will not be easy to predict by means other than precise finding of promoters, termination of transcription and other signals.

Signatures provide information complementary to intergenic distances

Since the first publication of a successful method to predict operons (23), most authors have found that the most informative feature is the distance between genes (41–48). The current maximum prediction accuracies using dist-LLHs as evaluated against experimentally known WO pairs and TUB pairs are 0.83 in *E.coli* K12 and 0.87 in *B.subtilis*, both higher than the accuracies obtained using sig-LLHs.

The purpose of this report is to show that other features remain to be explored, and to measure what can be attained using oligonucleotide signatures in particular. One salient feature of signature-based predictions is that samples for training the method do not have to be as large as the training data necessary to calculate dist-LLHs at different intergenic distance intervals. This is especially important if the distances between genes in the same operon and/or the distances between same-strand TUBs in a genome of interest do not follow those of model organisms.

One more goal to explore other features for operon predictions is to increase the accuracies over those produced previously. In this regard, adding sig-LLHs to dist-LLHs produces maximum accuracies of 0.84 in *E.coli* K12 and 0.88 in *B.subtilis* (0.01 above dist-LLHs alone). Fitting to a linear model to combine the log-likelihoods, following Price *et al.* (41), result in the very same maximum accuracies.

CONCLUDING REMARKS

We have shown that trinucleotide signatures can help distinguish regions upstream of genes inside operons from those upstream of first transcribed genes, thus allowing for operon predictions. We have also shown that such signatures can be derived from pairs of adjacent same-strand genes conserved in evolutionarily distant genomes as examples of genes in operons, and from divergently transcribed genes as examples of genes at TUBs. This makes overall signature-based operon predictions possible for most Prokaryotic genomes.

Further data and predictions for each genome can be found at http://tikal.ccg.unam.mx/sarath/sig_predictions/.

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