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CRISPRstrand: predicting repeat orientations to determine the crRNA-encoding strand at CRISPR loci

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ABSTRACT

Motivation: The discovery of CRISPR-Cas systems almost 20 years ago rapidly changed our perception of the bacterial and archaeal immune systems. CRISPR loci consist of several repetitive DNA sequences called repeats, inter-spaced by stretches of variable length sequences called spacers. This CRISPR array is transcribed and processed into multiple mature RNA species (crRNAs). A single crRNA is integrated into an interference complex, together with CRISPR-associated (Cas) proteins, to bind and degrade invading nucleic acids. Although existing bioinformatics tools can recognize CRISPR loci by their characteristic repeat-spacer architecture, they generally output CRISPR arrays of ambiguous orientation and thus do not determine the strand from which crRNAs are processed. Knowledge of the correct orientation is crucial for many tasks, including the classification of CRISPR conservation, the detection of leader regions, the identification of target sites (protospacers) on invading genetic elements and the characterization of protospacer-adjacent motifs.

Results: We present a fast and accurate tool to determine the crRNA-encoding strand at CRISPR loci by predicting the correct orientation of repeats based on an advanced machine learning approach. Both the repeat sequence and mutation information were encoded and processed by an efficient graph kernel to learn higher-order correlations. The model was trained and tested on curated data comprising >4500 CRISPRs and yielded a remarkable performance of 0.95 AUC ROC (area under the curve of the receiver operator characteristic). In addition, we show that accurate orientation information greatly improved detection of conserved repeat sequence families and structure motifs. We integrated CRISPRstrand predictions into our CRISPRmap web server of CRISPR conservation and updated the latter to version 2.0.

Availability: CRISPRmap and CRISPRstrand are available at http://rna.informatik.uni-freiburg.de/CRISPRmap.

Contact: backofen@informatik.uni-freiburg.de

Supplementary information: Supplementary data are available at Bioinformatics online.

1 INTRODUCTION

CRISPR-Cas immune systems of bacteria and archaea provide adaptive defence against a variety of invading genetic elements. They have been classified into three major classes: Types I, II and III, where Type II systems are confined to bacteria (Makarova et al., 2011a; Vestergaard et al., 2014). The adaptive immune response of all types is divided into three major phases: (i) adaptation, the uptake of DNA fragments from genetic elements and their insertion between consecutive repeats of a CRISPR array, generally adjacent to a leader sequence; (ii) processing of the CRISPR array transcripts within the repeats to generate small crRNAs that derive from part or all of each spacer region and (iii) interference involving targeting and cleavage of an invading genetic element, or its transcripts, by Cas protein–crRNA complexes (Barrangou and van der Oost, 2013, and Fig. 1). Whereas the adaptation phase is relatively conserved in the different CRISPR-Cas systems, significant differences occur in the processing and interference mechanisms. Thus, where Type I and III systems employ a Cas6 processing endonuclease to cleave within the repeats, the bacterial Type II system uses the host-encoded RNase III, together with a CRISPR-associated, trans-encoded tracrRNA (Deltcheva et al., 2011). Furthermore, the various interference complexes exhibit considerable diversity (Barrangou and van der Oost, 2013; Makarova et al., 2011a; Vestergaard et al., 2014).

We developed an efficient tool for determining the strand from which mature crRNAs are derived by focussing on the repeats at CRISPR loci. The repeats are unique within the CRISPR-Cas system because they are the only element to play a vital role in all phases of immunity (Barrangou and van der Oost, 2013). Thus, despite their relatively short lengths, each repeat carries essential structural parameters or sequence motifs that are recognized by enzymes or structural proteins involved in adaptation, crRNA biogenesis and interference. Paradoxically, however, the repeats are very heterogeneous, occurring in a range of lengths, 19–48 nt, and display considerable sequence diversity. An early comparative study of CRISPR diversity yielded 12 main clusters with specific sequence characteristics; only a subset folded into characteristic hairpin structure motifs (Kunin et al., 2007). More recently, a major reevaluation of CRISPR conservation was executed by Lange et al. (2013), on a much larger data set of 3527 CRISPRs, where 40 conserved repeat sequence families were identified together with a total of 33 potential structural motifs. The repeat clusters were further classified into six superfamilies, some of which showed strong biases to specific CRISPR subtypes and to certain bacterial or archaeal phyla (Lange et al., 2013).

CRISPR loci are generally identified by their characteristic repeat-spacer architecture. For example CRT (Bland et al., 2007) and CRISPRFinder (Grissa et al., 2007) provide sensitive predictions of CRISPR arrays, but do not provide unambiguous orientation information. In the literature, orientation is derived
We present a linear discriminative model based on graph kernels to accurately predict the orientation of the CRISPR sequence. The method first generates a sequence alignment of all repeat instances in the CRISPR array and outputs the consensus repeat sequence in its predicted orientation and whether it lies on the forward or reverse strand. There are two core ideas underlying our approach. The first one is to use a combinatorial technique to extract a very large number of features. The second idea is to encode our knowledge about the problem as a directed graph with discrete labels. The first idea allows a predictive system to be very accurate and to express complex discriminative decisions; the second idea allows a natural and flexible encoding of background knowledge.

2.1 Novel comprehensive identification of CRISPR loci

We extracted a comprehensive dataset of CRISPR loci from published archival and bacterial genomes. All genome sequences were downloaded from the NCBI website (http://www.ncbi.nlm.nih.gov/). We predicted CRISPR loci using CRISPRFinder (Grissa et al., 2007) and CRT (Bland et al., 2007). For both tools, we used (i) default parameter values for predicted CRISPR loci and (ii) parameters that corresponded to at least two repeats within a CRISPR locus; repeat and spacer lengths were set to a range between 18 and 78 bp. We then (iii) generated a consensus repeat for each CRISPR locus exploiting the fact that repeats within a CRISPR locus are almost completely identical with some loci that carry few mutations, preferably at the start and end (see Supplementary Figure S3, Supplementary material). Because CRT does not output consensus repeats, we used the MAFFT program (Katoh et al., 2002), version 6.4., to compute the multiple alignments and the Cons program from EMBoss package (Rice et al., 2000) to obtain the consensus repeat from the multiple sequence alignments. Finally, (iv) the results from both CRISPRFinder and CRT tools were merged and redundant CRISPR loci were removed. In this way, we obtained a CRISPR databases with >4700 consensus repeats, which we refer to as REPEATS (see Table 1 for details).

2.2 Datasets from literature

2.2.1 Set of repeats from Lange et al. (2013) We selected structural motifs that fit to known cleavage sites (Lange et al., 2013). Table 2 gives a summary of published CRISPR-Cas systems with experimental evidence for the processing mechanism which we refer to as REPEATS_{Lange}. This dataset contains 324 bacterial and 118 archaeal repeat sequences (442 in total).

2.2.2 Set of repeats from Kunin et al. (2007) We denote the dataset originally published in Kunin et al. (2007) as REPEATS_{Kunin}. The dataset contains 327 bacterial and 92 archaeal repeat sequences (419 in total). The orientations were assigned by the authors using previously published sequence features.

2.2.3 Set of archaeal repeats from Shah and Garrett (2011) We denote the dataset based on the results available in (Shah and Garrett, 2011) as REPEATS_{Shah}. This dataset contains 478 archaeal repeat sequences with manually verified strand orientation.

2.3 Encoding CRISPR repeats as graphs

The features used to discriminate between the different orientation are based on available biological knowledge of CRISPR evolution and processing. During CRISPR RNA processing by Cas6-like endoribonucleases, cleavage occurs either at the 3′-end of the hairpin motif, or within the double-stranded region of the hairpin stem, usually below a C→G base pair (Barrangou and van der Oost, 2013; Richter et al., 2012; Scholz et al., 2013). The product of this cleavage is an 8-nt-long AUUGAAA(N) repeat tag at the 5′-end of the mature crRNA (5′-tag), which corresponds to the last eight nucleotides from the 3′-end of the repeat sequence. Kunin et al. (2007) and Lange et al. (2013) showed that in some cases the four nucleotides AAA(N) motif can be used to identify the orientation. These observations lead to the hypothesis that the terminal region of the sequence, comprising four or eight nucleotides, plays a key role. We observed also that the mutation rate in various parts of the CRISPR locus is non-uniform, in particular the middle part of the CRISPR locus is more conserved. This finding motivated...
the idea of using the presence of mutations as an additional signal to detect the predominantly transcribed strand.

We made use of this background knowledge to partition the consensus repeat into specific informative parts: we distinguish terminal regions of identical size \( k \) at both ends (as the correct orientation is unknown) and a central variable length area. The terminal sequences are further partitioned into \( P \) equally sized parts, where we expect to find key motifs. We call each part block. One of the main signals that we used to define the number and size of the blocks is the mutation rate, defined as the fraction of mutations per nucleotide in each block. In Supplementary Figure S3, we report the mutation rate for the CRISPR locus partitioning with \( k = 8 \) and \( P = 2 \) on a dataset of 897 CRISPR arrays (Kunin et al., 2007; Shah and Garrett, 2011): each repeat is split into five adjacent blocks.

In our case, the information provided by the consensus sequence is modelled directly as a path graph with vertices labelled with the consensus nucleotide code (see Fig. 2). We then model the global localization information as additional vertices with a label that indicates the block identity. This reveals whether a nucleotide is located at the very beginning or just near the beginning of the sequence (and symmetrically for the opposite end). Furthermore, we consider a more fine grained localization information, identifying the specific position of a nucleotide within a block. The reason to encode an increasingly refined localization information is to allow the algorithm to choose the optimal level of detail needed in various parts of the sequence. Finally, the main piece of information is whether there is evidence of a mutation at a specific location; we model this with an additional vertex labelled with a binary code to indicate the presence of a mutation in at least one of the repeated sequences.

### Table 1. Summary of our REPEATS dataset derived from all available CRISPR loci

<table>
<thead>
<tr>
<th>Data statistics</th>
<th>Archaea</th>
<th>Bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genomes (total)</td>
<td>309</td>
<td>4590 (4899)</td>
</tr>
<tr>
<td>Genomes with CRISPRs (%)</td>
<td>217 (70)</td>
<td>1409 (30)</td>
</tr>
<tr>
<td>CRISPRs on forward strand</td>
<td>516</td>
<td>1810 (2326)</td>
</tr>
<tr>
<td>CRISPRs on reverse strand</td>
<td>530</td>
<td>1859 (2389)</td>
</tr>
<tr>
<td>Repeats per array (median)</td>
<td>2–198 (20)</td>
<td>2–1371 (16)</td>
</tr>
<tr>
<td>Repeat lengths (median)</td>
<td>20–44 (29)</td>
<td>19–48 (30)</td>
</tr>
<tr>
<td>Spacer lengths (median)</td>
<td>20–54 (38)</td>
<td>19–72 (35)</td>
</tr>
</tbody>
</table>

### Table 2. Summary of REPEATS\(_{Lange}\) dataset: published CRISPR-Cas systems with experimental evidence of the processing mechanism

<table>
<thead>
<tr>
<th>Organism</th>
<th>Motif</th>
<th>Cas subtype</th>
<th>Summary</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em> K12</td>
<td>M2</td>
<td>I-E</td>
<td>Structure predicted, but stable; 8-nt-5-tag; cleavage by Cas6, biochemical experiments (Brouns et al., 2008)</td>
</tr>
<tr>
<td><em>Thermus thermophilus</em> HB8</td>
<td>M2</td>
<td>I-E</td>
<td>Structured; 8-nt-5-tag; cleavage by Cas6; crystal structure of repeat hairpin in Cas6 (Cse3) (Gesner et al., 2011; Jurane et al., 2012; Sashital et al., 2011)</td>
</tr>
<tr>
<td><em>Bacillus halodurans</em> C-125</td>
<td>M3</td>
<td>I-C</td>
<td>Cleavage by Cas5d; 11-nt-5-tag mutational analysis of hairpin structure (Nam et al., 2012)</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> UCBPP-PA14</td>
<td>M4</td>
<td>I-F</td>
<td>Cleavage by Cas3 (Csy4); 8-nt-5-tag; crystal structure and mutational analyses of repeat hairpin in Cas6 (Haurwitz et al., 2010, 2012; Sternberg et al., 2012)</td>
</tr>
<tr>
<td><em>Synechocystis</em> sp. PCC6803</td>
<td>M5</td>
<td>I-DIII-variant</td>
<td>Cleavage by Cas6; 8-nt-5-tag; biochemical experiments, extended structure prediction of hairpin motif (Scholz et al., 2013)</td>
</tr>
<tr>
<td><em>Thermus thermophilus</em> HB27</td>
<td>M9</td>
<td>I-C</td>
<td>Cleavage by Cas5d; 11-nt-5-tag biochemical experiments (Garside et al., 2012)</td>
</tr>
<tr>
<td><em>Methanosarcina marina</em> Gd1</td>
<td>M13</td>
<td>I-B III-B</td>
<td>Cleavage by Cas6b; 8-nt-5-tag; structure probing experiment of hairpin (Nickel et al., 2013)</td>
</tr>
<tr>
<td><em>Synechocystis</em> sp. PCC6803</td>
<td>M14</td>
<td>III-variant</td>
<td>Biochemical analysis of Cmr2 implicates its involvement in either cleavage, crRNA stabilization, or array expression regulation; 13-nt-5-tag (Scholtz et al., 2013)</td>
</tr>
<tr>
<td><em>Staphylococcus epidermidis</em> RP62A</td>
<td>M28</td>
<td>III-A</td>
<td>Cleavage by Cas6; 8-nt-5-tag; hairpin structure as in M28 verified by mutational analysis and sequence specificity around cleavage site (Haitoum-Aslan et al., 2011)</td>
</tr>
<tr>
<td><em>Methanococcus maripaludis</em> C5</td>
<td>M29</td>
<td>I-B</td>
<td>Cleavage by Cas6b; 8-nt-5-tag; biochemical experiments (Richter et al., 2012)</td>
</tr>
</tbody>
</table>

*Note:* In particular, these are systems for which (i) the Cas endoribonuclease has been characterized and/or (ii) the repeat structure has been verified. Published results are consistent with the data of Lange et al. (2013).
The final modelling decision regards the topology of the graph, i.e., how the additional vertices, which encode the different types of information, should be connected together. We identify an order which reflects the importance of the different types of information, starting from the nucleotide type, the block ID, the mutation evidence and finally the relative position within a block. Note that the combinatorial feature generation phase is affected by the sequential order of these attributes, as the information that is ranked higher will participate in the generation of more features and will therefore be regarded as more prominent.

### 2.4 Predictive model and feature extraction

After having encoded domain expert knowledge as a graph, we need to process this type of structured data to induce a predictive model. We do this using the technique developed by Costa and Grave (2010), based on the notion of graph kernels. The core idea (see Supplementary Information for a formal description) is to decompose each graph in a (multi) set of fragments and use these as features, in a similar fashion to what is done in the chemoinformatics domain with the fingerprint technique. The resulting sparse vectors can then be processed by efficient machine learning techniques, such as the stochastic gradient descent SVM (Bottou, 2010), to yield fast and highly predictive models. The type of graph decomposition that we use is called Neighbourhood Subgraph Pairwise Distance Kernel (NSPDK), and it involves the extraction of all possible pairs of small neighbourhood subgraphs that are not too distant (see Fig. 3). Intuitively one can think about this type of decomposition as an upgrade of the concept of k-mers with gaps from the domain of strings to that of graphs. Both the extraction of the features and the training of the predictive model have linear complexity and offer therefore excellent scaling capability. More precisely, extracting all possible subgraphs is achieved with a breadth-first visit for a limited depth starting from each node, and as the graphs are sparse, it takes $O(n + m)$ where $n$ is the number of nucleotides and $m$ the number of repeat alignments.

Finally, given that one of the two strands can be the one that exhibits a characteristic pattern, we train a predictive model on both variants of each repeat sequence: one obtained from the forward strand and the other from the complementary reverse strand. The binary task is therefore to assign a positive score to the sequences that are transcribed and a negative one to the complementary strand. In the predictive phase, we enforce consistency by considering the prediction on both variants of the sequence: a strong confidence of the prediction of the forward strand should also correspond to an equally confident prediction that the reverse complementary sequence is not transcribed. To do so, we simply perform the individual predictions and then average the prediction of the forward strand with the opposite prediction for the reverse strand. If the resulting score is positive, then the forward strand is predicted to be transcribed, whereas the reverse strand is selected if the score is negative.

### 3 RESULTS AND DISCUSSION

#### 3.1 Parameter selection

We have previously described how relevant biological knowledge was used to determine various modelling choices. The proposed model admits, however, different configurations both in the encoding part as well as in the combinatorial feature generation part. To determine the best configuration, we therefore performed extensive in silico simulations. More specifically, the encoding phase allows the following parametric variants: (i) choice of attribute type (i.e., whether to use the mutation information or the block identity); (ii) choice of attribute order (i.e., whether the block identifier should precede the mutation marker or vice versa); (iii) size of the terminal regions (more, equal or less than 8 nucleotides); (iv) number of blocks within the terminal regions (1, 2 or 3). The combinatorial feature construction phase is parametrized instead by the maximal radius $R$ and distance $D$, where larger values for $R$ translates in more complex features and larger values for $D$ in an increased tolerance for larger gaps.

For each model variant, we designed a selection experiment to identify the best configuration of parameters as the one that achieves the minimum expected predictive error. Not surprisingly, results are consistent with the background knowledge that originally motivated the encoding, that is, the best model uses all attributes in the order presented in Figure 2 with terminal regions of size 8 nucleotides divided into blocks of 4 nucleotides. We observed that the actual attribute order had just a modest influence on the results (see Supplementary Table S1).

#### 3.1.1 Choice of attribute type

We estimated the expected prediction error of five different encodings, which use an increasing amount of information. We denote them with $model_i$ with $i \in \{1, 2, 3, 4, 5\}$ (consider Fig. 2 as a reference). In all cases blocks have a constant size of 4 nucleotides.

- $model_1$: nucleotide sequence only (layer 1 in Fig. 2)
- $model_2$: nucleotide sequence with additional mutation attribute for the terminal 8 nucleotides (layer $1 + 3$ in Fig. 2)
- $model_3$: nucleotide sequence with additional block attribute (layer $1 + 2$ in Fig. 2)
- $model_4$: nucleotide sequence with mutation and block attribute (layer $1 + 2 + 3$ in Fig. 2)
- $model_5$: nucleotide sequence with block, mutation and relative position attribute (layer 1 to 4 in Fig. 2)
To evaluate the generalization capacity of the resulting predictive models, we used as training material the 442 sequences in REPEATS$_{Lange}$ and as test material the 419 REPEATS$_{Kunin} + 478$ sequences REPEATS$_{Shah}$ filtered so as to guarantee a maximal pre-specified level of sequence identity w.r.t. the training material. In Figure 4, we report the area under the curve for the receiver operator characteristic (AUC ROC) when the test material has pairwise sequence identity ≤0.95, 0.85, 0.75 and 0.65, respectively, as measured by the Needleman–Wunsch algorithm (Needleman and Wunsch, 1970).

The simulations show that the mutation information does indeed provide good discriminative features (increasing performance of 5%) and that partitioning the sequence into blocks can further improve the predictive performance (an extra 10%). Finally, the model is shown to yield ~0.85 AUC ROC when tested on sequences with only 0.65 sequence identity, indicating a reasonable generalization capacity to evolutionary distant sequences. Note that extrapolating the predictive tendency with a quadratic fit, we get a random AUC ROC of 0.53 at 25% sequence similarity, i.e. for random sequences.

3.1.2 Choice of terminal region size We validated the notion of a most informative leading part of the consensus repeat sequence via an in silico simulation. An encoding was created that uses only three blocks: two terminal ones of fixed size $k$ nucleotides and a central one of variable length. We computed the average AUC ROC in a 10-fold cross validation for $k = 1, \ldots, 10$. Results shown in Supplementary Figure S1 are in striking agreement with our biological knowledge, with clear performance peaks at exactly 4 and 8 nucleotides.

3.1.3 Choice of number of blocks within the terminal regions We also validated the notion that there is an advantage in considering a finer partition of the terminal parts. We started from an encoding with terminal regions spanning 8 nucleotides and then we subdivided them into 1, 2 or 4 equal sized subparts, that is, in subparts of 8, 4 and 2 nucleotides. Once again results (shown in Supplementary Figure S2) are in agreement with the biological findings, and confirm that a subdivision in 4 nucleotide parts is indeed beneficial.

3.1.4 Combinatorial features The complexity of the derived feature representation depends on the maximum radius $R$ and maximum distance $D$ that are considered. Using models, we simulated all possible combinations of values $R = \{0, \ldots, 7\}$ and $D = \{1, \ldots, 7\}$ (see Supplementary Table S2) in a 10-fold cross-validated experiment on the REPEATS$_{Lange}$ and obtained the best predictive performance with $R = 3$ and $D = 5$. Note that, unsurprisingly, the optimal size $R = 3$ is also the minimal size that allows to capture all available attributes in models.

3.2 Comparison with Biswas et al. (2014)

We used the same dataset as in Biswas et al. (2014) to train our model. Both methods were then applied to the REPEATS$_{Shah}$ data set, filtered for decreasing levels of sequence identity w.r.t. the training set. In Figure 5 we report the comparative AUC ROC performance and observe that our proposal offers a substantial improvement both in prediction performance and in generalization capacity with a less pronounced degradation as the sequence identity decreases.

Finally, we measured the runtime for both approaches on 956 CRISPR repeat arrays (average length 28 nucleotides). The classification task was completed in 59 s by our approach and in 37 min by the Biswas predictive model. We report that the Biswas tool failed to make any prediction in 98 cases out of 948, while our method achieved an AUC ROC of 0.89 on the
same instances, indicating that these sequences were on average only slightly more difficult to predict.

3.3 CRISPR-Cas system annotation

We used our orientation prediction method to identify the transcribed strand for the set of 3527 repeats available from Lange et al. (2013) and for the novel set of 4719 individual CRISPR loci identified as described in Section 2.1. This material was finally used to update the CRISPRmap web server, which provides an automated and easy-to-use classification of all currently available and newly sequenced CRISPRs.

3.3.1 Re-correcting the orientation of 3527 repeats from Lange et al. (2013)  Our tool was run on 3527 repeats, which were then clustered into 40 conserved sequence families, 33 potential structural motifs and 6 major superclasses. In this set, we identified 536 repeats with incorrect orientation (see Supplementary Table S12). Next we ran our cluster pipeline for three iterations, retrieving 29 potential structural motifs and 37 conserved sequences families (see Supplementary Tables S3 and S4). As shown in Figure 6, the orientation of F8 and M6 was incorrect. Using corrected orientations, we could merge F8 with F6, and M5 with M6. Overall, in Figure 6, we show how the cluster quality can be significantly improved when we can make use of a better orientation prediction.

3.3.2 Update of CRISPRmap web server to version 2.0  The database REPEATS of 4719 individual CRISPR loci was collected performing an exhaustive search for CRISPR loci within all available bacterial and archaeal genomes (see Table 1). We developed two independent clustering approaches to identify structural motifs and conserved sequence families. In both approaches, we call a cluster of structural motifs or conserved sequences a class if they contain CRISPR repeats which come from 10 different species (see Supplementary Tables S5–S11 and CRISPRmap). The results of our independent clustering approaches are as follows: (i) 18 structure motifs were identified based on sequence and structure alignments using LocARNA (Smith et al., 2010; Will et al., 2007, 2012). Structure motif candidates were constrained to be similar to those previously published (Brouns et al., 2008; Hatoum-Aslan et al., 2011; Nam et al., 2012; Nickel et al., 2013; Sashital et al., 2011; Scholz et al., 2013; Sternberg et al., 2012). (ii) Twenty-four conserved sequence families were identified based on Markov clustering (Enright et al., 2002). Full details of structure motifs and conserved sequence families are available in the Supplementary file and in full on CRISPRmap web server. We grouped all the sequences available in the REPEATS database into six major superclasses (labelled A to F) based on sequence and structure similarities and tree topology (Supplementary Figure S6). Owing to the corrected orientation, there are two main differences between superclasses from Lange et al. (2013) and current superclasses. First, superclasses B and C were merged together and the
resulting new superclass was called B. Second, parts of superfamilies E and F were moved to superclass D (Supplementary Figure S6).

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**Conflict of interest:** none declared.

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