FACS: antimicrobial peptide screening in genomes and metagenomes

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ABSTRACT

Motivation: Antimicrobial peptides (AMPs) have the potential to tackle multidrug-resistant pathogens in both clinical and non-clinical contexts. The recent growth in the availability of genomes and metagenomes provides an opportunity for *in silico* prediction of novel AMPs. However, due to the small size of these peptides, standard gene prospection methods cannot be applied in this domain and alternative approaches are necessary. In particular, standard gene prediction methods have low precision for short peptides, and functional classification by homology results have low recall.
Results: Here, we present a novel set of 22 protein features that outperforms the state-of-the-art in the prediction of AMPs as well as their classification into their hemolytic activity. We use these classifiers to build FACS (Fast Antimicrobial Classification System), an end-to-end tool, which combines assembly, ORF prediction, and AMP classification to work directly on genomes or metagenomes. We demonstrate that FACS recovers high-quality candidates from genomes and metagenomes using realistic simulations and real data.
Availability: FACS is implemented in Bash and its source code is freely available for download at https://github.com/FACS-Antimicrobial-Peptides-Prospection/FACS. The functionality is also available as a webserver: http://big-data-biology.org/software/facs.
Supplementary information: Supplementary data are available online.

1 Introduction

Antimicrobial peptides (AMPs) are short proteins (containing fewer than 100 amino acids) that can decrease or inhibit bacterial growth. They interact with microbial membranes or intracellular targets (Zhang and Gallo, 2016) and have remained potent for millions of years (Zasloff, 2002). Given the dearth of novel antibiotics in recent decades and the rise of antimicrobial resistance, prospecting naturally-occurring AMPs is a potentially valuable new source of antimicrobial molecules (Theuretzbacher et al., 2019). The increasing number of publicly available metagenomes and metatranscriptomes presents an opportunity to use them for finding novel AMP sequences. However, methods that have been successful in prospecting other microbial functionality, cannot be directly applied to small genes (Saghatelian and Couso, 2015), such as AMPs. In particular, there are two major computational challenges: the prediction of small genes in DNA sequences (either genomic or metagenomic contigs) and the prediction of AMP activity for small genes.

Current automated gene prediction methods typically exclude small open reading frames (smORFs) (Miravet-Verde et al., 2019), as the naïve use of the methods that work for larger sequences leads to unacceptably high rates of false positives when extended to short sequences (Hyatt et al., 2010). A few recent large-scale smORFs surveys have, nonetheless, shown that these methods can be employed if the results are subsequently filtered while revealing that prokaryotic smORFs are biologically active across a range of functions (Miravet-Verde et al., 2019; Sberro et al., 2019).

Similarly, the prediction of AMP activity requires different techniques than the homology-based methods that are applicable for longer proteins (Huerta-Cepas et al., 2017). In this context, several machine learning-based methods have demonstrated high accuracy in predicting antimicrobial activity in peptides, when tested on curated benchmarks (Xiao et al., 2013; Meher et al., 2017; Lata et al., 2010; Thakur et al., 2012; Sharma et al., 2016; Bhadra et al., 2018). However, to be applicable to the task of extracting AMPs from genomic data, an AMP classifier needs to be robust to gene mispredictions and needs to be benchmarked in that context.

Here, we present FACS (for *Fast AMP Classification System*) - Fig. 1, a simple yet accurate pipeline that processes either genomes (in the form of pre-assembled contigs) or metagenomes/metatranscriptomes (in the form of short reads) and predicts AMP sequences. We test FACS with standard benchmarks in AMP prediction as well as both simulated and real sequencing data to show that, even in the presence of large numbers of (potentially artifactual) input smORFs, FACS still outputs only a small number of high-quality candidates.

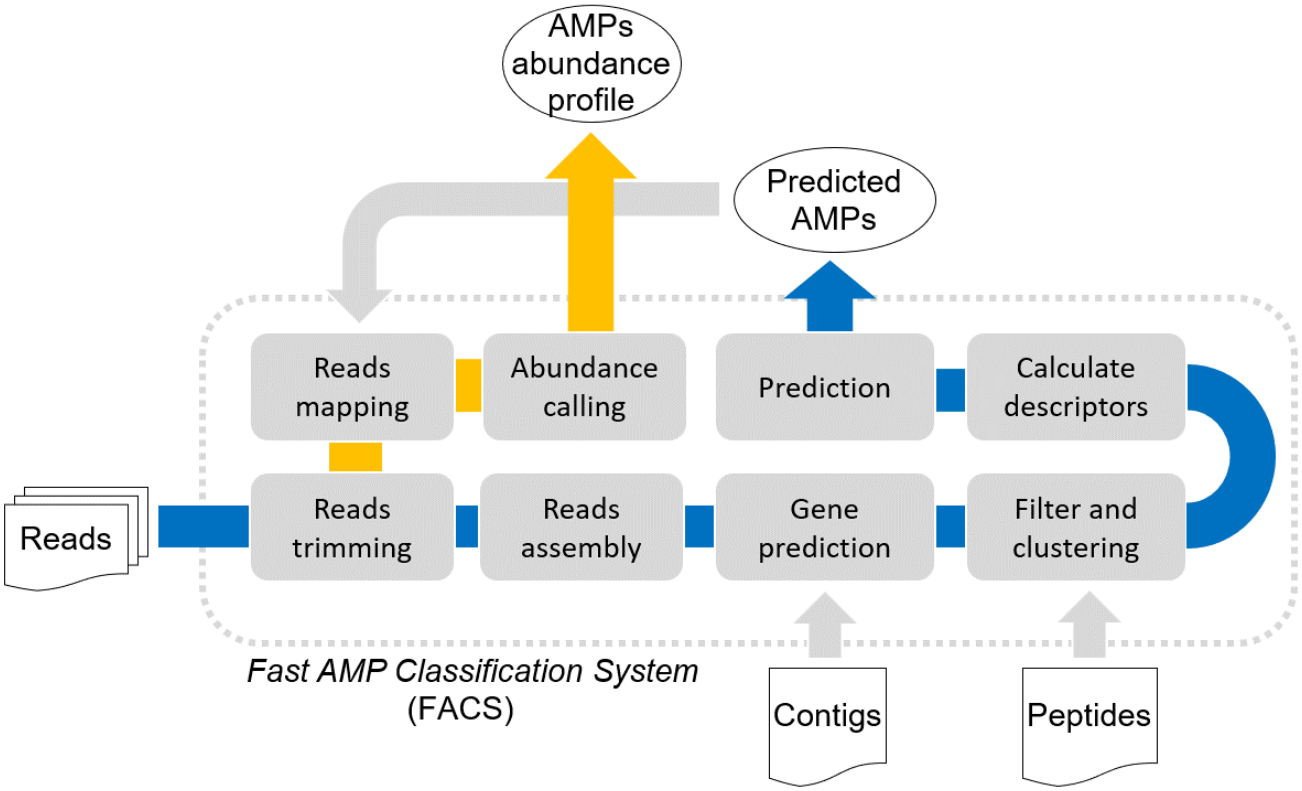

Figure 1. Fast AMP Classification System — FACS pipeline. The blue arrows show the FACS workflow from the processing of reads until AMP prediction. The user can also provide as input contigs or peptide sequences, if they are available. The yellow arrow shows the abundance profiling of AMPs using FACS output and reads. Gray arrows show the alternative inputs accepted by FACS.

2 System and Methods

2.1 FACS Classifiers

Two binary classifiers are used in FACS: one predicting AMP activity and other predicting hemolytic activity (which is invoked for putative AMPs). These are feature-based classifiers and use the same 22 descriptors.

2.1.1 Features

AMPs typically contain approximately 50% hydrophobic residues, usually positively charged and fold in a well-defined secondary structure (Zhang and Gallo, 2016). The peptide charge appears to be a key feature in the formation of amphiphilic ordered structures (Malmsten, 2014; Brogden, 2005; Pasupuleti et al., 2012; Hancock and Sahl, 2006; Shai, 2002; Strömstedt et al., 2009), which promote peptide-induced cell membrane disruption (Malmsten, 2014; Pasupuleti et al., 2012; Ringstad et al., 2006). These sequences can be predicted using local or global features (local features depend on the order of the amino-acids, while global ones do not). Local and global features are particularly valuable in different AMP-related prediction problems. Local features are more informative when predicting AMP activity, while global features are more informative when predicting the efficiency of an AMP (Bhadra et al., 2018; Fjell et al., 2009; Boone et al., 2018). Thus, FACS combined both, and include 16 global and 6 local features (see Suppl. Table S1).

Bhadra et al. (2018) produced an accurate classifier of AMPs based on random forests using the composition-transition-distribution of amino acid residues (Dubchak et al., 1995, 1999) according to 7 physiochemical properties such as hydrophobicity, polarity, polarizability, and secondary structure. Therefore, the 6 local context descriptors adopted in the FACS classifiers consisted of the amino acid distribution patterns of solvent accessibility and free energy to transfer from a relaxed coil in water to an ordered structure in the membrane, the last one being a novel descriptor proposed here and described in detail in Section 3.2.

Features used by FACS classifiers (see Suppl. Table S1) also included the distribution at first residue of 3 amino acid classes organized according to the solvent accessibility (Bhadra et al., 2018) and extra variables, such as solubility. Solubility is a remarkable feature of AMPs (Fan et al., 2016; Wenzel et al., 2014), which was represented by an indirect measure - the isoelectric potential. AMPs usually have higher hydrophobicity, aliphatic index, and lower instability index when compared to typical proteins (Jhong et al., 2019). Thus, those variables were included in our set of descriptors.

FACS models used other features related to the activity of AMPs, such as charge and percent composition of apolar residues (aromatic and aliphatic) (Nagarajan et al., 2019). The mechanistic of AMP activity was summarized in FACS classifiers as the predisposition of a peptide in binding to membranes and its amphiphilicity (Boman index and Hmoment, respectively). To comprise the extensively documented composition of AMPs (Jhong et al., 2019; Nagarajan et al., 2019), FACS classifiers used the percent composition of different amino acid groups (acidic, basic, charged, polar, non-polar, tiny and small).

2.1.2 FACS prediction models

The training and validation of the AMPs classifier followed the general procedures described by Bhadra et al. (2018). Briefly, we used the training data from Bhadra et al. (2018), which consists of 3,268 AMPs (from diverse databases, most bench-validated) and 166,791 non-AMPs (from Uniprot). Tests comparing different AMPs classifiers (random forest - RF, support vector machines, bagged trees and other algorithms) showed RF classifiers with better performance (see Suppl. Table S2), as previously reported (Fernández-Delgado et al., 2014; Bhadra et al., 2018; Waghu et al., 2014, 2016). We tested this RF classifier built with a different number of trees (100, 200, or 500), and adopted 100 trees after a slight deterioration of accuracy with larger forests (see Suppl. Table S2).

The hemolytic activity classifier was built similarly to that used in the FACS AMP classifier. For this, we used the training set of peptides HemoPI-1 constituted by 442 hemolytic and 442 non-hemolytic proteins from Chaudhary et al. (2016) to train the model.

2.1.3 Prediction in (meta)genomes

FACS (Fig. 1) accepts as inputs metagenomic paired-end or single-end reads in compressed FastQ format and performs quality-based trimming with NGLess (Coelho et al., 2019). After this initial stage, FACS assembles contigs using MegaHIT (Li et al., 2016) (a minimum length of 1,000 base pairs is used). Alternatively, if available, contigs can be passed directly to FACS.

Genes are predicted on these contigs with a modified version of Prodigal (Hyatt et al., 2010), which predicts genes with a minimal length of 30 base pairs (compared to 90 base pairs in the standard Prodigal release). The original threshold was intended to minimize false positives (Hyatt et al., 2010), as gene prediction methods, in general, generate more false positives in shorter sequences (small ORFs, henceforth smORFs) (Höps et al., 2018). Sberro et al. (2019) showed that reducing the length threshold without further filtering could lead to as many as 61.2% of predicted smORFs being false positives. In FACS, this filtering consists of outputting only those smORFs (10-100 amino acids) classified as AMPs.

AMP sequences are classified according to their hemolytic activity and classified into four different families by composition (Cationic or Anionic) and structure (linear or disulfide bond-forming). For convenience, duplicated sequences are clustered and output as a single entity. For calculating AMP abundance profiles, FACS uses Paladin (Westbrook et al., 2017) and NGLess (Coelho et al., 2019).

2.2 Benchmarking

2.2.1 Methods to be compared

We compared the FACS AMP classifier with the following state-of-art methods: AmPEP (Bhadra et al., 2018), CAMP3 (including all algorithms) (Waghu et al., 2016), iAMP 2-L (Xiao et al., 2013), AMAP (Gull et al., 2019), iAMPpred (Meher et al., 2017) and Antimicrobial Peptides Scanner v2 (Veltri et al., 2018). For all these comparisons, we used the benchmark dataset from Xiao et al. (2013), which contains 920 AMPs and 920 non-AMPs, and the models publicly available from others

(Meher et al., 2017; Bhadra et al., 2018; Gabere and Noble, 2017).

The benchmarking of the hemolytic peptides classifier was performed using the HemoPI-1 benchmark dataset formed by 110 hemolytic proteins and 110 non-hemolytic proteins previously established by Chaudhary et al. (2016). FACS model performance was compared against models created using different algorithms (Chaudhary et al., 2016): Support vector machines - SVM, K-Nearest Neighbor (IBK), Neural networks (Multilayer Perceptron), Logistic regression, Decision trees (J48) and RF.

There is no overlap between the training sets and the testing sets for the benchmark of both classifiers.

2.2.2 Simulated human gut metagenomes

To test the FACS short reads pipeline, and the effect of sequencing depth on the discovery rate of AMPs, 6 metagenomes were simulated at 3 different sequencing depths (40, 60 and 80 million reads of 150 bp) with ART Illumina v2.5.8 (Huang et al., 2012) using the pre-built sequencing error profile for the HiSeq 2500 sequencer. To ensure realism, the simulated metagenomes contained species abundances estimated from real human gut microbial communities (Coelho et al., 2019).

We processed both the simulated metagenomes and the isolate genomes used to build the metagenomes with FACS to verify whether the same AMP candidates could be retrieved and whether the metagenomic processing introduced false positive sequences not present in the original genomes.

The complete set of scripts used to benchmark FACS is available at https://github.com/FACS-Antimicrobial-Peptides-Prospection/ and the newly simulated generated data set of different sequencing depths is available at Zenodo (DOI: 10.5281/zenodo.3529860).

2.2.3 AMP screening in real metagenomes

To evaluate FACS in real data, we used 182 metagenomes and 36 metatranscriptomes generated with Illumina technology in a previous study of the human gut microbiome (Heintz-Buschart et al., 2016) (available from the European Nucleotide Archive, accession number PRJNA289586). FACS was used to process metagenome reads [For additional details see the Suppl. Table S3], and the genes encoding AMP candidates were mapped back to the metatranscriptomes using bwa-0.7.17 (r1188) (Li and Durbin, 2009) and samtools v1.9 (Li et al., 2009). Abundance profiles were calculated using NGLess (Coelho et al., 2019).

2.2.4 Detection of spurious sequences

To test whether spurious smORFs still appear in FACS results, we used Spurio (Höps et al., 2018) and considered a prediction spurious if the score was greater or equal to 0.8.

To identify putative gene fragments, the AMP sequences predicted with FACS were validated through homology-searching against the non-redundant NCBI database (https://www.ncbi.nlm.nih.gov/). Predicted AMPs annotation was done by homology against the DRAMP database (Fan et al., 2016) comprising circa 20k AMPs. The above-mentioned databases were used to perform a local search with the blastp algorithm (Camacho et al., 2009), using a maximum e-value of $1 \cdot 10^{-5}$ and a word size of 3. Hits with a minimum of 70% of identity and 95% query coverage were kept and parsed to the best-hits after ranking them by score, e-value, identity, and coverage.

To check whether the AMPs predicted by the FACS pipeline were gene fragments, patented peptides or known AMPs, the alignments were manually evaluated.

2.3 Implementation

FACS is implemented in Bash, Python (compatible with both versions 2 and 3), and R (Team, 2018), and supports multiple cores by using GNUParallel (Tange, 2018). The virtual environment used by FACS, containing all third party software and packages, is created with Bioconda (Grüning et al., 2018). Descriptors are calculated using protR (Xiao et al., 2015), and the classification is performed with the R packages caret (Kuhn, 2008) and randomForest (Liaw and Wiener, 2002).

3 Results

3.1 FACS - Fast AMP Classification System

As we aim to process both genomes and metagenomes, we built a consolidated pipeline (FACS, for *Fast AMP Classification System*), which implements a full workflow from short-reads to the prediction and quantification of AMPs (see Fig. 1). FACS is

conveniently organized in a virtual environment containing all third party software related to the end-to-end AMPs prospection and quantification, from the reads trimming to peptides classification.

FACS accepts as inputs metagenomes (in the form of short reads), (meta)genomic contigs, or peptides. If short reads are given as input, FACS will preprocess and assemble them into larger contigs. Automated gene prediction then extracts smORFs from these contigs which are classified into AMPs or rejected from further processing (see Fig. 1 and Methods). Unlike other pipelines (Jhong et al., 2019), FACS can not only quantify known sequences, but also discover novel AMPs.

FACS is also available as a webserver (available at http://big-data-biology.org/software/facs), which accepts both peptides and contig sequences.

3.2 Novel set of protein descriptors for AMP identification

FACS classifiers use a set of 22 variables that capture the amphipathic nature of AMPs and their propensity to form transmembrane helices (see Methods and Suppl. Table S1).

One novel feature group (named *FET*) was designed to capture the fact that AMPs usually fold from random coils in the polar phase to well-organized structures in lipid membranes (Nagarajan et al., 2019). In particular, we adapted the approach of Bhadra et al. (2018) to the estimated change in the free-energy of amino acids in the transition from water to lipid membranes (von Heijne and Blomberg, 1979). We clustered residues into three groups of increasing free-energy change and used the composition-transition-distribution framework (Dubchak et al., 1995, 1999) to derive three features (see Fig. 2).

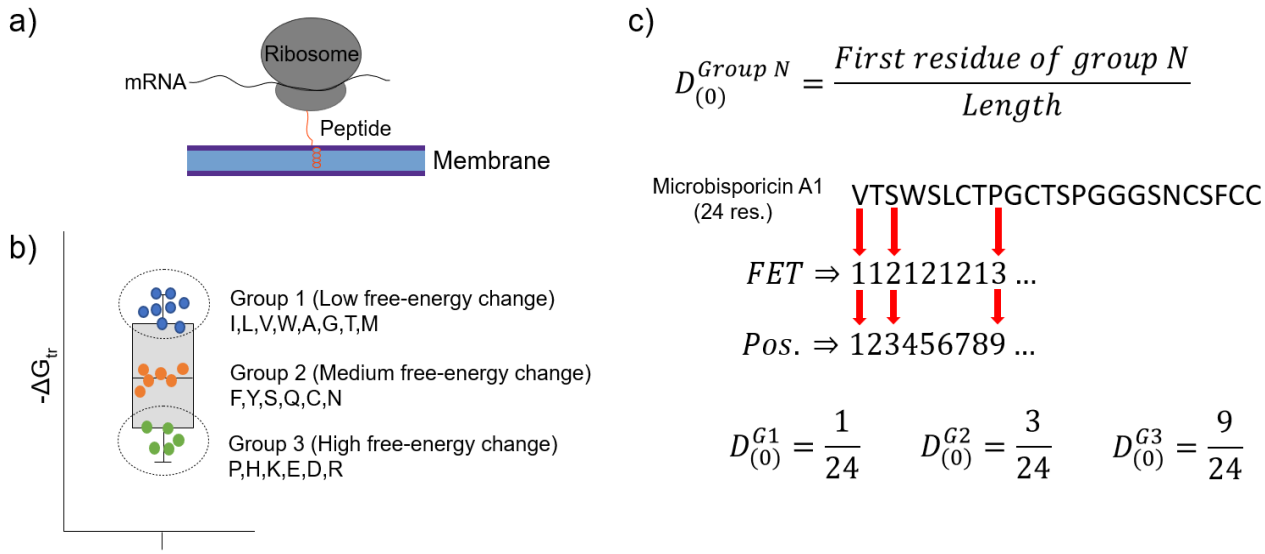

Figure 2. The FET measure estimates the propensity of peptides to fold when transferring from water to the membrane. The estimated change in the free-energy of the conformational change of an amino acid from random to organized structures in lipid membranes **(a)** was used to cluster the 20 amino acids into 3 groups **(b)**. These groups were used to encode peptide sequences as the relative position of the first amino acid in each group **(c)**.

All 22 descriptors used in FACS models are important in classification (see Fig. 3). The percent acidic residues composition, charge, and isoelectric point were the most important variables to the hemolytic peptides classifier. Those variables tend to depict the electrostatic interaction between peptides and membranes, a key step to hemolysis. In turn, charge and the distribution parameters using FET (amino acid group 1) and solvent accessibility (SA, amino acid group 3) are the most important variables in AMP prediction. This is consistent with reports that highly-charged peptides (typically glycine- or lysine-rich) show increased AMP activity (Bhadra et al., 2018; Jhong et al., 2019; Nagarajan et al., 2019).

3.3 FACS outperforms other methods in AMP classification

Benchmark results show that the AMP prediction model implemented in FACS performs the best, with AmPEP (Bhadra et al., 2018) (a system that uses the same training set), achieving the second-best results (see Table 1).

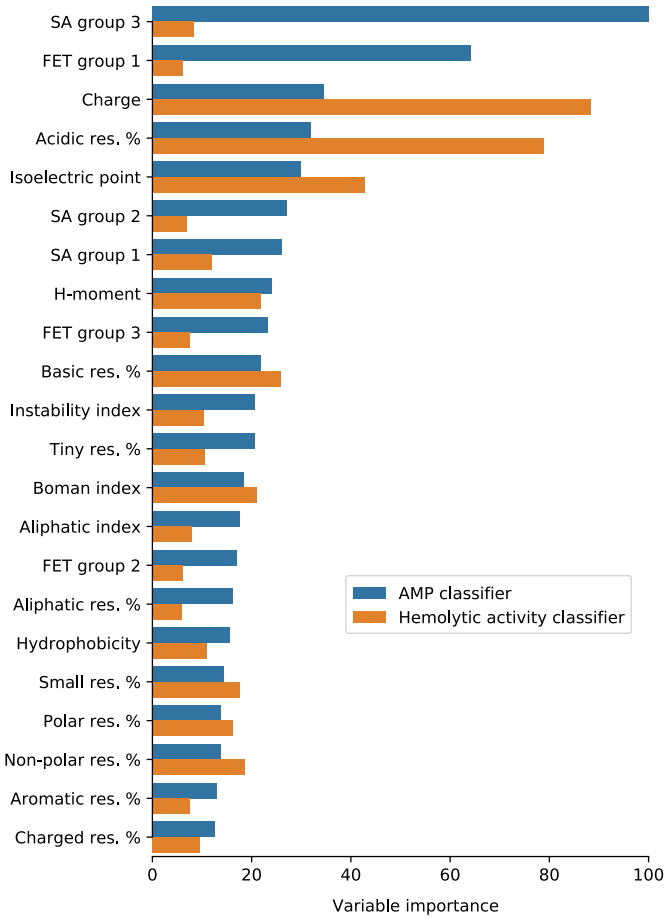

Figure 3. All 22 features are important in classification. Variable importance is measured as the percentage of times each variable is selected in the pruned models (FACS classifiers of AMPs and hemolytic peptides). The solvent accessibility and free energy to transfer from water to lipophilic phase residues distribution at first position using 3 amino acid groups were summarized as SA and FET, respectively.

Table 1. The comparison of FACS AMP classifier performance and state-of-art methods shows that FACS performs the best across a range of metrics. The same test set (Xiao et al., 2013) was used to calculate the general performance statistics of the different classifiers, and the best value per column is in bold. Legend: Accuracy (Acc), Sensitivity (Sn), Specificity (Sp), Precision (Pr), and Matthew's Correlation Coefficient (MCC).

Method	Acc.	Sp.	Sn.	Pr.	MCC	Refererence
FACS	**0.963**	**1.00**	0.926	**1.00**	**0.93**	This study
AmPEP	**0.962**	0.965	0.95	0.913	0.90	Bhadra et al. (2018)
iAMP-2L	0.947	0.92	0.974	0.924	0.9	Xiao et al. (2013)
AMAP	0.922	0.861	0.984	0.876	0.85	Gull et al. (2019)
CAMPR3-NN	0.799	0.709	0.89	0.753	0.61	Waghu et al. (2016)
APSv2	0.779	0.572	**0.987**	0.697	0.61	Veltri et al. (2018)
CAMPR3-DA	0.716	0.495	0.938	0.65	0.48	Waghu et al. (2016)
CAMPR3-SVM	0.676	0.398	0.954	0.613	0.42	Waghu et al. (2016)
CAMPR3-RF	0.65	0.335	0.965	0.592	0.39	Waghu et al. (2016)
iAMPpred	0.643	0.325	0.962	0.588	0.37	Meher et al. (2017)

The hemolytic peptides prediction model implemented in FACS is comparable to the state-of-the-art (Chaudhary et al., 2016). These models, using HemoPI-1 training and validation sets, were built with different methods (composition-based or hybrid), and resulted in overall comparable performance (Table 2).

Table 2. FACS achieves accuracy comparable to the state-of-art in hemolytic peptides classification. Models implemented by Chaudhary et al. (2016) were generically called HemoPI-1 due to the data sets used in the training and benchmarking (the best values per column are in bold).

Methods	Sn.	Sp.	Acc.	MCC
HemoPI-$1^{C, SVM}$	0.957	**0.948**	**0.953**	**0.91**
HemoPI-1^{H}	**0.96**	0.946	**0.953**	**0.91**
HemoPI-$1^{C, IBK}$	0.955	0.937	0.946	0.89
FACS	0.946	0.936	0.954	0.89
HemoPI-$1^{C, RF}$	0.941	0.946	0.943	0.89
HemoPI-$1^{C, Log}$	0.934	0.937	0.936	0.87
HemoPI-$1^{C, MP}$	0.939	0.928	0.933	0.87
HemoPI-$1^{C, JK48}$	0.896	0.885	0.89	0.78

3.4 FACS recovers a small number of high-quality AMP candidates per meta(genome)

We ran FACS on 484 reference genomes that had previously shown to be abundant in the human gut (Coelho et al., 2019). This resulted in 171,645 (redundant) smORFs. However, only 18 (17, after redundancy removal) of these were classified as potential AMPs. Neither Spurio (Höps et al., 2018) nor homology searches against the NCBI nr database provided any evidence that the 17 non-redundant AMP candidates are false positives.

The homology searches confirmed two AMP candidates as actual AMPs. One of them is a phenol-soluble modulin from the *Staphylococcus* genus and another is a stage V sporulation protein from multiple species in the *Clostridium* group. None of the AMPs found by FACS in the reference genomes were present in the data set used during model training.

To evaluate the impact of the metagenomics process, we simulated metagenomes composed by these same 484 reference genomes, using three different sequencing depths (40 million, 60 million, and 80 million reads) using abundance profiles estimated from real data (Coelho et al., 2019). In these simulations (see Fig. 4), it was clear that the number of smORFs increases with sequencing depth, with about 20k smORFs being predicted per 80 million simulated reads.

Despite this large number of smORF candidates, only 6 non-redundant AMP candidates were predicted. Four of these are present in the underlying reference genomes, and were correctly recovered. *Post hoc*, we estimated that 5x coverage is required for AMP recovery (see Fig. 4), but that this coverage does not guarantee AMP recovery.

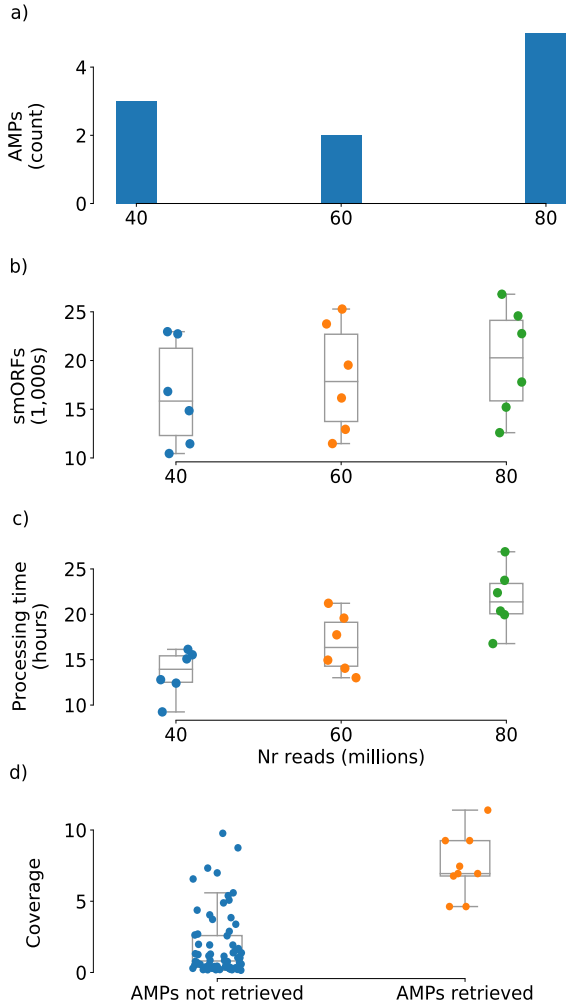

Figure 4. FACS results in metagenome simulations involving a different number of reads (40-80 million). Communities with realistic species abundances were simulated with increasing sequencing depth (see Methods). FACS recovers a small number of AMPs **(a)**, despite the large number of small ORFs (smORFs) detected in each metagenome **(b)**. Processing times increase with coverage, with the single largest sample taking 27 hours **(c)**. A minimal coverage of *circa* 5x is necessary (but not sufficient) for AMPs to be recovered **(d)** (for better visualization, outliers were removed, according to criteria defined by (Tukey, 1977)).

None of the AMP candidates recovered from simulated metagenomes were marked as spurious by Spurio, but we manually investigated the origin of the two extra peptides. One of them appears to be a gene fragment from a longer gene. The end of this gene contained one start codon in frame with the stop, which generated a mispredicted smORF as the gene was close to the contig end so that the real start site was not present. The other smORF predicted by FACS in the metagenomes was not present in the underlying reference genomes and appears to be an artifact of the simulated sequencing/assembly.

3.5 FACS predicts putative AMPs in real human gut metagenomes

Of the 184 metagenomes in our dataset (Heintz-Buschart et al., 2016), 102 (56%) contain putative AMPs, resulting in a total of 75 non-redundant sequences (see Suppl. Table S3). Five of these were tagged as spurious (see Methods). Two of these appear to be fragments of larger proteins, suggesting that they are generated by fragmentary assemblies.

Our final dataset, after discarding smORFs identified as spurious (see Methods and Suppl. Table S3), consisted of 70 non-redundant AMPs, encoded by a total of 244 genes. None of them had hits to the DRAMP database and only 5 matched hypothetical proteins from NCBI.

To further strengthen FACS predictions, we checked the co-prediction of the 70 non-redundant AMP candidates with alternative methods. In total, 96% of AMPs predicted with FACS were also classified as such by at least one other classifier, and 64% of the times, half or more of the tested state-of-art methods agreed with FACS results (see Suppl. Table 4). Some methods, such as iAMPpred and iAMP-2L, can co-predict 86% and 73% of the AMPs obtained with FACS, respectively.

As this dataset has metatranscriptomes available from the same biological samples, we quantified the expression of the AMP genes. Over 70% of the predicted AMPs were being expressed (see Fig. 5). The gene expression of these AMPs could be detected in more than one metatranscriptome in 82% of the cases. Taken together, this is consistent with the AMP prediction.

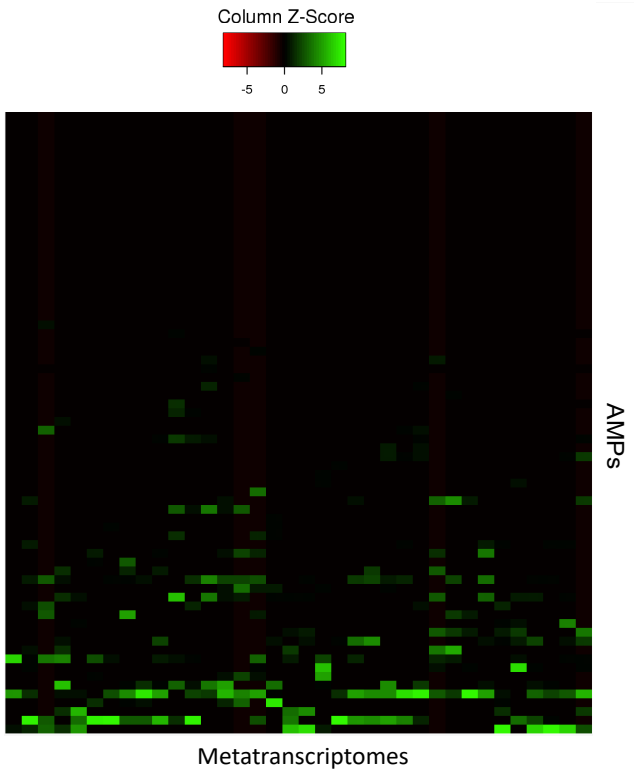

Figure 5. Gene expression of AMPs detected by FACS in human gut metatranscriptomes. Non-redundant AMP gene variants from each cluster had their FKPM values expressed on average. The heatmap shows values normalized by metatranscriptome using Z-score.

FACS could find a set of high-quality AMPs candidates, whose genes show active transcription, and that can be co-predicted by other state-of-art methods.

3.6 FACS requires only moderate computational resources

Tests reported here were carried out on a personal laptop (32 Gb of RAM and 4 CPUs) to show that FACS is a pipeline with low computational requirements. The execution time, although dependent on the input size, was not greater than 27 h (recall that the largest simulated metagenomes contained 80 million reads). The reads trimming and assembly steps consumed 75-80% of the execution time, while gene prediction occupies another considerable part (10-15%) (see Fig. 4).

4 Conclusions

FACS is a comprehensive pipeline that can be used in a wide-ranging of scenarios, such as screening for novel AMP candidates for further testing, as well as, linking AMPs to specific health conditions, environmental variables or presence/absence of factors. Using a combination of local and global sequence encoding techniques, FACS classifiers outperform the state-of-art methods.

FACS performs all operations from raw metagenomic reads assembly to the prediction of AMPs. The main problem in predicting smORFs with standard methods is the high rates of false-positives. However, after the filtering applied by FACS classifiers, only a small number of candidate sequences remained. Supported by several lines of evidence (lack of obvious spurious origin, similar classification by other methods, and recovery of known sequences). In other words, the FACS pipeline predicts a small number of high-quality AMP candidates.

Here, we presented an initial analysis of publicly-available human gut metagenomes (Heintz-Buschart et al., 2016). Although only a small number of AMPs (70 non-redundant sequences) were predicted. AMPs predicted with FACS were largely congruent (96%) with other state-of-art methods, suggesting FACS can generate a small number of promising candidates. This opens up the possibility of future work to understand the impact of these molecules on the microbial ecosystems or prospecting them for clinical or industrial applications.

FACS is available as open-source software at https://github.com/FACS-Antimicrobial-Peptides-Prospection and the functionality is also available as a webserver: http://big-data-biology.org/software/facs.

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