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Genome-Wide Identification of Small RNAs in the Opportunistic Pathogen *Enterococcus faecalis* V583

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Abstract

Small RNA molecules (sRNAs) are key mediators of virulence and stress inducible gene expressions in some pathogens. In this work we identify sRNAs in the Gram positive opportunistic pathogen *Enterococcus faecalis*. We characterized 11 sRNAs by tiling microarray analysis, 5’ and 3’ RACE-PCR, and Northern blot analysis. Six sRNAs were specifically expressed at exponential phase, two sRNAs were observed at stationary phase, and three were detected during both phases. Searches of putative functions revealed that three of them (EFA0080_EFA0081 and EFB0062_EFB0063 on pTF1 and pTF2 plasmids, respectively, and EF0408_EF04092 located on the chromosome) are similar to antisense RNA involved in plasmid addiction modules. Moreover, EF1097_EF1098 shares strong homologies with tmRNA (bi-functional RNA acting as both a tRNA and an mRNA) and EF2205_EF2206 appears homologous to 4.5S RNA member of the Signal Recognition Particle (SRP) ribonucleoprotein complex. In addition, proteomic analysis of the AEF3314_EF3315 sRNA mutant suggests that it may be involved in the turnover of some abundant proteins. The expression patterns of these transcripts were evaluated by tiling array hybridizations performed with samples from cells grown under eleven different conditions some of which may be encountered during infection. Finally, distribution of these sRNAs among genome sequences of 54 *E. faecalis* strains was assessed. This is the first experimental genome-wide identification of sRNAs in *E. faecalis* and provides impetus to the understanding of gene regulation in this important human pathogen.

Introduction

Some RNA molecules such as riboswitches, transfer-messenger RNA (tmRNA) and small non-coding RNAs (sRNAs) act usually as post-transcriptional regulators in bacteria [1]. sRNAs have become increasingly recognized as an emerging class of gene expression regulators for cellular processes, stress response and virulence genes and their transcription is tightly regulated and frequently encoded in intergenic regions (IGRs). They may found on chromosomes are typically 50–400 nucleotides in length and may bind to the imperfect complementary sequence of the ribosome binding region of the target mRNA, which is often encoded at separate loci, thus inhibiting 30S ribosomal subunit association and translational initiation [1,3]. In some Gram positive and Gram negative species such as *Escherichia coli* [4] and *Listeria monocytogenes* [5], the formation of RNA-mRNA duplex requires the RNA chaperon protein Hfq [6,7] leading to an increase of mRNA degradation by ribonucleases such as RNase E and RNase III [2]. Some sRNAs located in plasmids and phases act as antisense RNAs on *cis*-encoded mRNAs and mainly control replication initiation, conjugation efficiency and transposition [9,9]. In addition, plasmid-encoded sRNAs, called *hak/sok* system of *E. coli* plasmid R1 [10] and *par* system of *Enterococcus faecalis* pAD1 [11], stabilize their host plasmids by programming for death any cell that loses the plasmid [9,12].

In recent years, several bioinformatic approaches have been performed to identify putative sRNAs in bacterial genomes including *E. coli*, *L. monocytogenes*, *Bacillus subtilis* and *Pseudomonas aeruginosa*, and identified more than 200 sRNAs [13]. Recently, Livny *et al.* predicted in *sileo* over 45,000 sRNA candidates from 932 bacterial genomes [14]. In parallel, different experimental strategies including cDNA sequencing, shotgun cloning and isolation from RNA-protein complex have been performed and sometimes lead to the discovery of new transcripts [15,16]. Tiling microarrays are powerful approaches to identify sRNAs on a genome-wide scale. Thus large numbers of sRNA candidates have been found in *Caulobacter crescentus*, *Streptococcus pyogenes*, *S. pneumoniae*, and *L. monocytogenes* genomes [17,18,19,20].

*E. faecalis* is a human commensal Gram-positive bacteria as well as one of the leading causes of hospital acquired infections in United States and Europe [21]. The first whole genome sequence
of *E. faecalis* V583 strain (the first vancomycin resistant enterococci identified in U.S.A.) was determined in 2003 and 53 more sequences are now publically available [22]. *In silico* study performed by Livny *et al.* led to the prediction and annotation of 17 putative sRNA-encoding loci in *E. faecalis* [14]. Surprisingly, in comparison with *E. coli* and *B. subtilis*, the number of predicted sRNAs in V583 is roughly 10-fold lower, suggesting that this number is likely under-estimated. Recently, 43 sRNAs and 10 putative mRNAs have been identified in *E. faecalis* using *in silico* prediction combined with 5′-tag-RACE [23].

In this work, we developed custom-made tiling microarrays containing only IGRs of *E. faecalis* V583 chromosome and plasmids, and first performed hybridization with RNA extracted from experimental and stationary-phase cells. Fifty-three statistically significant positive signals were detected and the 12 putative sRNAs most highly expressed were selected for further characterization. Transcription of these candidates under several stress conditions was then analyzed.

### Materials and Methods

#### Bacterial strain and growth conditions

All experiments were performed with *E. faecalis* V583 strain [24]. For our first tiling array assays, cells were grown at 37°C in M17 0.5% glucose medium and collected at exponential phase (OD<sub>600</sub> = 0.5) and at 24 h stationary phase. Growth in BHI medium with or without aeration was tested. Cells were collected at exponential phase (OD<sub>600</sub> = 0.5), onset of starvation (OD<sub>600</sub> = 2) and late stationary phase (24 h). For experiments under stress conditions, bacterial cells were grown to OD<sub>600</sub> = 0.3 in M17 medium and H<sub>2</sub>O<sub>2</sub> (2 mM), lactic acid (pH 5.5), or bile salts (BS) (0.08%), were added before an additional 30 min incubation at 37°C. For the growth in urine and serum, *E. faecalis* was inoculated into human urine or horse serum (Eurobio, Courtaboeuf, Fr) during overnight. Cells were then pelleted and resuspended into fresh urine or serum for 3 hours at 37°C. Urine collected from four healthy volunteers was pooled, centrifuged and sterilized by filter (0.22 μm-pore sizes). Written consent from all participants involved in our study was obtained. French CPP (Comité de Protection de Personnes) exempted this study from review because volunteers were informed of the goal of this study, no health information was collected and no biological analysis was performed on these samples.

#### RNA extraction and tiling microarray hybridization

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) as described by Toledo-Arana *et al.* [20], with the following modifications. Bacterial cells were resuspended into 200 μl of “max bacterial enhancement reagent” (Invitrogen) and transferred into micro tubes containing glass beads and 400 μl acid phenol (Ambion, Austin, TX). Bacteria were mechanically lysed using Mixer Mill 200 (30/s, 30 min, Retsch, Haan, Germany). After centrifugation for 10 min at 14,000 g at 4°C, aqueous phase was transferred to 2 ml tubes containing 1 ml Trizol reagent, mixed and incubated for 5 min at room temperature (RT). 200 μl chloroform was added, mixed gently and incubated for 3 min at RT. Tubes were centrifuged for 15 min at 12,000 g at 4°C and aqueous phase was transferred into 2 ml tubes containing 200 μl chloroform, mixed gently and centrifuged again. RNAs contained in the aqueous phase were precipitated by addition of 500 μl isopropanol and incubated for 10 min at RT. After centrifugation, RNA pellets were washed with 75% ethanol and dried at RT. Purified RNA pellets were resuspended in DEPC-treated pure water.

To enhance detection sensitivity by enriching of sRNAs and removing non-sRNA, 10 μg RNA were fractionated using flashPAGE Fractionator (Applied Biosystems, Foster City, CA). Fractionated RNA was labelled using mirVana labelling kit (Applied Biosystems) and then hybridized onto the tiling array. 1745 “big intergenic regions (IGR)” (more than 49 nt) and 1070 “small IGR” (from 1 to 49 nt) have been deduced from *E. faecalis* V583 genome sequence. 50 nt long probes with an overlap of 15 nt were loaded on our IGR custom-made tiling arrays. rRNA and tRNA probes were used as positive control showing signal intensity of hybridization at least 10 fold the threshold level. Since the values of intensity observed in apparent untranslated regions were between 1000 and 2000, 2000 was used as threshold. For each experiment (one sample per growth condition) two chips were used; one corresponding to the forward, and one to the reverse strand. Production, hybridization and data collecting were carried out by Febit biomed GmbH Company (Heidelberg, Germany). The detection was carried out using streptavidin phycoerythrin at different exposure times. Data analyses and visualization were performed by Genedata Phylosopher Business Group (Basel, Switzerland). We have deposed the raw data at GEO/ArrayExpress under accession number GSE28741, we can confirm all details are MIAME compliant.

#### 5′ and 3′ rapid amplification of cDNA ends (RACE) analysis

For these analysis, new RNA samples were prepared as described above. 5′ RACE was performed using 2nd Generation 5′/3′ RACE kit (Roche, Mannheim, Germany) according to the manufacturer’s instructions. For polymerase chain reactions (PCR), we used Go Taq polymerase and its buffer (Promega, Madison, WI). The primers used for cDNA synthesis, and for the PCR reactions are listed in Table S1.

For 3′ RACE experiments, total RNAs were treated with poly(A) polymerase (Epicerne, Madison, WI) for 15 min at 37°C. After 3′ end RNA poly(A) tailing, cDNA was synthesized with QuantiTect Reverse Transcription kit (Qiagen, West Sussex, UK) and oligo(dT)-anchor primer supplied in 5′/3′ RACE kit. cDNA products were directly used as templates for PCR performed with the gene-specific primers (Table S1) and the respective PCR anchor primer. After sequencing, 5′ and 3′ ends sequences were determined.

#### Northern blotting

Northern blots were performed according to standard procedures [25]. Five μg of total RNA were separated on 1.2% formaldehyde agarose gel and transferred to Hybond N* membrane (Amersham, UK). 0.1–1 kb RNA Marker (Sigma, USA) was used to estimate the sizes of RNA bands. DNA oligonucleotides probes (Table S1) were labeled with α-32P-ATP using Terminal Deoxynucleotidyl Transfase Recombinant enzyme (Promega) as recommended by the manufactured protocol. Membranes were prehybridized for 1 h in hybridization buffer (0.25 M NaH<sub>2</sub>PO<sub>4</sub>, 0.25 M Na<sub>2</sub>HPO<sub>4</sub>, 5% SDS) at 45°C, followed by addition of labelled probes and overnight hybridization at 45°C. Membranes were washed with washing buffer (3×SSC buffer, 0.2% SDS) for 5 min at RT and were then exposed to storage phosphor screen (Packard Instrument Company, Mareden, CT) for 3 h.

#### In silico analysis

Rho-independent terminators were predicted with TransTerm (http://nshc11.biologie.uni-kl.de/framed/left/menu/auto/right/trans
term/) [26]. Blast searches between E. faecalis strains were carried out using a species-level BLAST database (http://www.ncbi.nlm.nih.gov/BLAST). The Rfam database was employed to determine putative functions of sRNAs (http://rfam.sanger.ac.uk) [27]. In order to predict target genes for the identified sRNAs sRNATarget (http://ccb.bmi.ac.cn/sRNA-target/) [29] and IntaRNA (http://rna.informatik.uni-freiburg.de:3000/IntaRNA.jsp) [29] servers were used.

Construction of EF3314_EF3315 sRNA deletion mutant

For the deletion assay, a DNA fragment containing ligated upstream (869 bp) and downstream (839 bp) sequences of the EF3314_EF3315 sRNA, was cloned into plasmid pMAD [30] (see Table S1 for primers used); 1 μg of recombinant plasmid was finally used to transform competent cells. After electroporation, 300 μl of cell suspension was plated onto GM17 agar containing 50 μg ml⁻¹ of erythromycin and X-Gal (100 μg ml⁻¹). Plates were incubated for 48 hours at 30°C. A few dark blue colonies were obtained and analysed for presence of the plasmid by PCR using primers madR and madF (Table S1). Some blue colonies were then cultured twice in GM17 liquid medium with erythromycin (50 μg ml⁻¹) at 45°C overnight. In the next step, the cultures were used to inoculate (0.05% v/v) GM17 liquid medium without antibiotic. The tubes were incubated for 6 hours at 30°C followed by incubation at 45°C overnight. This step was repeated 2 to 3 times. Serial dilutions of the culture were plated on GM17 agar containing 100 μg ml⁻¹ of X-Gal and incubated for 48 hours at 45°C. White colonies were then isolated on GM17 agar with or without erythromycin. Antibiotic sensitive clones were analysed by PCR on the presence of a deleted sRNA.

Two-dimensional protein gel electrophoresis and protein identification

Protein samples from wild type and ΔEF3314_EF3315 mutant cells harvested in exponential growth phase were performed as described by Giard et al. [31]. First dimensional electrophoresis was carried out using 17 cm ReadyStrips™ IPG Strips (pH 4–7) and Protein®IEF Cell apparatus (Bio-Rad Laboratories, Richmond, CA, USA) as recommended by the manufacturer. Second dimensions were performed in 14% polyacrylamide gels without stacking gel using the Millipore Investigator™ 2-D electrophoresis system (Millipore, Bedford, MA, USA) as described by Giard et al. [31]. 2-D gels were then stained using Coomassie Blue. Spots of interest were excised from the gel, and peptides were digested by trypsin as described by Budin-Verneuil et al. [32]. An electrospray ion trap spectrometer (LCQ DecaXP, ThermoFinnigan, San Jose, CA, USA) coupled on line with HPLC was used for peptides analysis. Mass spectrometry were acquired in a mode that alternated a full MS scan (mass range: 400–1600) and a collision analysis. Mass spectrometry were acquired in a mode that were obtained and analysed for presence of the plasmid by PCR using primers madR and madF (Table S1). Some blue colonies were then cultured twice in GM17 liquid medium with erythromycin (50 μg ml⁻¹) at 45°C overnight. In the next step, the cultures were used to inoculate (0.05% v/v) GM17 liquid medium without antibiotic. The tubes were incubated for 6 hours at 30°C followed by incubation at 45°C overnight. This step was repeated 2 to 3 times. Serial dilutions of the culture were plated on GM17 agar containing 100 μg ml⁻¹ of X-Gal and incubated for 48 hours at 45°C. White colonies were then isolated on GM17 agar with or without erythromycin. Antibiotic sensitive clones were analysed by PCR on the presence of a deleted sRNA.

Experimental validation of 11 sRNAs in E. faecalis

One of the main goals of this study was to determine the sequence and the expression pattern of the 11 selected sRNA candidates. First, using a new RNA preparation, we performed Northern blot analysis to confirm the transcription of these RNAs during exponential growth phase and stationary phase and to determine the approximate size of each candidate. We observed a transcript for 10 out of the 11 candidates tested. Six of them (EF3314_EF3315, EF0820_EF0821, EF0940_EF0941, EF1360_EF1369, EF0409_EF0409 and EF0605_EF0606) were specifically expressed during exponential phase (Figure 1A–F); 1 sRNA (EF0869_EF0870) was specifically expressed after 24 h of starvation (Figure 1H); and 3 (EF1097_EF1098, EF0062_EFB0063 and EF2205_EF2206) were detected in comparable amounts in both phases (Figure 1G, J, K). These expression patterns were in good agreement with the results of tiling microarray except for EF1097_EF1098 which was much more expressed in stationary phase than under growing conditions on our chips. For unexplained reasons, no signal has been detected for EF0136_EF0137 (Figure 1J) by Northern blot analysis under our experimental conditions.

In order to determine the exact sequence of each sRNA candidate we identified the transcriptional start sites by 5′-RACE except for EFA0080_EFA0081 for which no result was gained. The 3′ ends of the transcripts were obtained either by 3′-RACE (Figure 1B, D, E, F, G, H, K, Table 1) or by combining transcript length data deduced from the Northern blots and computational prediction of transcriptional terminators [26] (Figure 1A, C, J, Table 1). Since neither putative terminator nor experimental data of the 3′ end of EF0136_EF0137 (Figure 1J) were obtained, the end of the sequence mentioned corresponds to the 3′ end of the tiling array probe. 5′-3′ RACE data of EF0820_EF0821 did not correlate to Northern blot results. From RACE-PCR, a 370 nt long sRNA was deduced that is larger than the predicted size (app. 100 nt) from Northern blot (using probe hybridizing on the 5′ region), suggesting that the large EF0820_EF0822 transcript was processed to short sRNA by modification of its 3′ end. Except for EF0820_EF0822, where the 99 last nucleotides correspond to the beginning sequence of EF0820, we could not identify obvious coding sequences (Cds), i.e. ORFs (open reading frames) with start codons connected to putative ribosome-binding sites in reasonable distances (around 8 nucleotides) inside the other sRNA candidates. Nevertheless, definitive exclusion of the presence of Cds in these regions needs experimental verification.
Altogether, the length range of the identified sRNAs was 87–628 nucleotides and the deduced sequences and promoter regions of the 11 sRNAs are shown in Figure 1. In comparison with sRNAs identified by Fouquier d’Hérouel et al. [23] using in silico prediction and “5’ tag-RACE” strategy, only four overlap with our sRNA candidates (EF0605_EF0606, EF1097_EF1098, EF0869_EF0871, and EF2205_EF2206 corresponding to sfaC, ssrA, sfaC, and ffs, respectively). This shows that several techniques as well as different growth conditions (see below) are necessary for more exhaustive identification of sRNAs.

### Table 1. sRNAs in *E. faecalis* V583 detected by tiling microarray.

| Intergenic Region | Left gene | scRNA strand | Right gene | Size (nt) | Flanking genes | Expression value\(a\) | Expression ration (Expo/Stat) |
|-------------------|-----------|--------------|------------|-----------|----------------|---------------------|-----------------------------|
| **sRNAs expressed at exponential phase** | | | | | | | |
| A. EF3314_EF3315 | ← | ← | ← | 3201675 | EF3314:cell wall surface anchor family protein | 65025.9 | 1249.6 | 52 |
| | | | | | 3201535 | EF3315:triphosphoribosyl-dephospho-CoA synthase | | | |
| B. EF0820_EF0822 | ← | ← | → | 784383 | EF0820:rp1Y; 50S ribosomal protein L25/general stress protein Ctc | 37086.5 | 1376.8 | 26.9 |
| C. 63423\(b\) | ← | 99 | | | | 63478\(b\) | 37537.9 | 3062.9 | 12.3 |
| D. EF1368_EF1369 | ← | → | ← | 1345556 | EF1368:hypothetical protein | 35465.0 | 3058.9 | 11.6 |
| E. EF0408_EF0409 | → | → | ← | 381297 | EF1370:drug resistance transporter, EmrB/QacA family protein | 47418.0 | 11648.3 | 4.1 |
| F. EF0605_EF0606 | ← | → | ← | 569151 | EF0605:hypothetical protein | 41977.3 | 11288.0 | 3.7 |
| **sRNAs expressed at stationary phase** | | | | | | | |
| G. EF1097_EF1098\(a\) | → | ← | ← | 1067257 | EF1097:hypothetical protein | 3390.8 | 63399.5 | 0.05 |
| H. EF0869_EF0871 | ← | → | → | 829255 | EF0869:cro/Ci family transcriptional regulator | 2655.4 | 47286.9 | 0.06 |
| I. EF0136_EF0137 | → | → | → | 137278 | EF0136:hypothetical protein | 1755.7 | 28560.7 | 0.06 |
| **sRNAs expressed at exponential and stationary phase** | | | | | | | |
| J. EF0062_EF0063 | ← | ← | ← | 55834 | EF0062:UvrC family transcriptional regulator | 49218.4 | 52343.1 | 0.94 |
| K. EF2205_EF2206 | ← | ← | ← | 2119382 | EF2205:hypothetical protein | 41604.0 | 55672.1 | 0.75 |

\(a\): Intensity of hybridization from the intergenic probe showing the highest signal in exponential or stationary phase.

\(b\): Computer prediction of the putative 3’ end (using TransTerm software).

\(c\): 5’ end corresponding to the 5’ end of probe.

\(d\): 3’ end corresponding to the 3’ end of probe.

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sRNAs in E. faecalis

A. 94 or 141 nt

B. 370 nt

C. >96 nt

D. 628 nt

E. 412 nt

F. 170 nt

G. 364 nt

H. 474 nt

I. No Signal on Northern blot

J. >215 nt

K. 212 nt

87 nt
Features of sRNAs

As previously mentioned, an antisense RNA regulated addiction module named "par" system was described on the *E. faecalis* plasmid pAD1 [9]. The components of this toxin-antitoxin (TA) system are antisense RNA (RNA II) and its target, RNA I encoding the peptide toxin ParT. Such systems play a crucial role in plasmid stability by killing any daughter cells that fail to inherit a copy of the plasmid. Three putative sRNAs identified in our study (EFA0080_EFA0081 in pTEF1, EF00062_EF00063 in pTEF2, and EF00460_EF00469 in the chromosome) corresponded to the RNAI components of the TA systems already identified in *E. faecalis* V583 by Weaver and coworkers [11]. As shown in Figure 1 (C, E, J), RNA I (including par toxin gene) and RNA II homologues had two direct repeat sequences and shared the same bidirectional terminator. One interesting question concerns the role of par addiction module located on the bacterial chromosome. Several studies revealed various roles such as in mobile element stability or stress response [12,33,34]. As pointed out, in the case of par systems (including EF00480_EF0049 sRNA), its association with genes encoding phosphotransferase components homologous to a mannitol transport system suggests a potential function in nutritional uptake [11].

Northern blot and tiling microarray showed that EF1097_EF1098 was expressed in both growth and stationary phases and we were able to determine the exact sequence of this sRNA (Figure 1G). EF1097_EF1098 corresponds to *E. faecalis* tmRNA (srrA) that is a unique bi-functional RNA acting as both a tRNA and an mRNA. It functions as a functional system of ribosomes stalled on aberrant mRNAs and adds a peptide tag to nascent polypeptides for directed proteolysis (named trans-translation) [35,36]. tmRNA is universally conserved and is one of the most abundant RNA in the cells [37]. It has not only an important role in mRNA turnover but also likely in monitoring protein folding (for review see [35]). Mutations that inactivate tmRNA are lethal for some species (ie, *Neisseria gonorrhoeae*, *Hemophilus influenzae*, *Streptococcus pneumoniae*) or stress response (ie, *E. coli*, *B. subtilis*) [35,37]. Determination of the impact of tmRNA deletion in *E. faecalis* is under investigation in our laboratory.

We used the Rfam database (a collection of non-coding RNA families) to determine the putative functions of characterized sRNAs [27]. We found that EF2205_EF2206 sRNA matched with the Signal Recognition Particle (SRP) functional category. SRP is a ribonucleoprotein complex that targets proteins for secretion through co-translational process and is composed of protein Ffh and 4.5S RNA in prokaryotes. Our analysis revealed that EF_1700 gene (ffh) product and EF2205_EF2206 correspond to the two components of the SRP in *E. faecalis*. Interestingly, a recent study demonstrated that mutation of the gene encoding 4.5S RNA in *S. pyogenes* (phylogenetically related to *E. faecalis*) results in reduction of virulence [38].

In order to predict target genes of the other sRNAs identified in this study, we performed in *silico* analysis (Table 2, Table S2). Two different softwares were used for a more precise identification. sRNA*Target* server is based on the Naive Bayes probabilistic method and take RNA secondary structure profile as the feature [28]. The second, IntaRNA, predicts interactions between two RNA molecules, and the scoring is based on hybridization free energy and accessibility of the interaction sites in both molecules [29]. Numerous putative target genes were obtained by combination of these two approaches (from 9 for EF3314_EF3315 to 81 for EF0136_EF0137) (Table 2, Table S2). In *silico* prediction (Table S2) as well as sequence analysis suggested antisense activity for EF0136_EF0137 and EF0136_EF0137. Indeed, EF1869 mRNA sequence, encoding a putative translational regulator, was fully complementary to EF1868_EF1869 sRNA. Likewise, the first 136 nucleotides of EF0136_EF0137 were complementary with the *fst* gene is written in blue letters and direct repeats "a" and "b" (DRa and DRb) of *par* system are blue and green box, respectively (panels C, E, and J).

Table 2. Number of putative target genes.

| sRNA     | Number of mRNA candidate | sRNA*Target (score>0.9)* | IntaRNAb | commonc |
|----------|---------------------------|--------------------------|---------|---------|
| EF3314_EF3315  | 75           | 31d                     | 9        |
| EF0820_EF0822  | 176          | 213d                    | 44       |
| EF1368_EF1369  | 876          | 97d                     | 72       |
| EF0605_EF0606  | 210          | 85d                     | 24       |
| EF0869_EF0871  | 494          | 318d                    | 62       |
| EF0136_EF0137  | 1252         | 92d                     | 81       |

* http://ccb.bmi.ac.cn/sRNATarget [28].
* http://rna.informatik.uni-freiburg.de:8080/IntaRNA.jsp [29].
* list of genes is in Table S2.
* cut-off < -10 kcal/mol.
* cut-off < -15 kcal/mol.

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from the mutant samples likely corresponded to protein degradation products. On the other hand, MW of spot number 3, which is absent in the mutant, was estimated at around 20 kDa in good accordance with the calculated size of the intact protein (19 kDa). These combined results suggested that EF3314_EF3315 might be involved in the turnover of some abundant proteins in *E. faecalis*, especially from the translational apparatus.

**Expression of sRNAs in different stress conditions**

Generally, the expression of sRNAs are tightly regulated and induced by specific environmental condition [2]. We then performed tiling arrays with new RNA samples in order to analyze the transcription of sRNAs previously characterized under 11 different conditions of growth some of which may correspond to stresses encountered during intestinal colonization or during the infectious process (see Material and Methods). Expression patterns of the 11 sRNAs under H$_2$O$_2$, BS, and acid stress conditions, during growth in presence or absence of O$_2$ and in serum and urine is presented in Table 3. EF0408_EF0409, EFA0080_EFA0081 and EFB0062_EFB0063, identified as members of TA systems were highly expressed at different stages of growth with oxygen (Table 3). Physiological significance of the induction of transcription of these

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**Table 3.** Expression patterns of sRNAs under different growth phases and stress conditions.

| sRNAs                      | Stress conditions | H$_2$O$_2$ | pH (acid) | BS | Expo with O$_2$ | Early Stat with O$_2$ | Stat with O$_2$ | Expo | Early Stat | Stat | Urine | Serum |
|-----------------------------|-------------------|------------|-----------|----|----------------|-----------------------|----------------|------|------------|------|-------|-------|
| EF3314_EF3315               |                   | 127        | 2263      | 596| 787            | 1756                  | 64             | 3478 | 659        | 93   | 191   | 112   |
| EF0820_EF0822               |                   | 114        | 150       | 135| 370            | 253                   | 47             | 473  | 605        | 51   | 59    | 122   |
| EF0008_EF0081               |                   | 102        | 186       | 314| 857            | 43619                 | 2364           | 5034 | 1566       | 3886 | 1756  | 2178  |
| EF1368_EF1369               |                   | 761        | 2817      | 874| 1178           | 118                   | 118            | 171  | 614        | 168  | 144   | 139   |
| EF0408_EF0409               |                   | 1756       | 2916      | 649| 20636          | 1916                  | 283            | 1597 | 1909       | 136  | 954   | 257   |
| EF0605_EF0606               |                   | 722        | 2056      | 246| 1880           | 3246                  | 214            | 261  | 113        | 326  | 802   | 129   |
| EF1097_EF1098               |                   | 4535       | 32765     | 106115| 1835          | 22301                 | 2438           | 1518 | 1492       | 3977 | 41662 | 11483 |
| EF0869_EF0871               |                   | 556        | 159       | 1236| 196           | 7780                  | 13465          | 374  | 119683     | 31710| 5974  | 30293 |
| EF0136_EF0137               |                   | 59         | 108       | 11  | 70             | 27                    | 46             | 11   | 125        | 144  | 101   |       |
| EFB0062_EFB0063             |                   | 125        | 332       | 194| 2817           | 802                   | 20636          | 1756 | 1236       | 5503 | 211   | 179   |
| EF2205_EF2206               |                   | 10724      | 21313     | 11296| 9823          | 25155                 | 10468          | 202452| 11483     | 405266| 29510 | 221227|

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Table 4. Distribution of the 11 sRNAs among *E. faecalis* strains.

| sRNAs | *E. faecalis* strains | OGR1RF | AR01/DG | ATCC 29200 | ATCC 4200 | CH188 | D6 | DAPTP0512 | DAPTP0516 | D55 | E15ol | Fly1 | HH122 | HIP11704 | JH1 | Lmx26 | PC1.1 | PET1 | PET2 | PET3 | PET8 | TXUSoD | TX0012 | TX0043 | TX0102 | TX0104 | TX0109 | TX0309A | TX03098 | TX0312 | TX0411 | TX0470 | TX0630 | TX0635 | TX0645 | TX0855 | TX0860 | TX1302 | TX1322 | TX1341 | TX1342 | TX1346 |
|-------|----------------------|--------|---------|-----------|-----------|--------|---|-----------|-----------|-----|------|------|-------|-----------|---|--------|------|------|------|------|--------|-------|-------|-------|------|--------|--------|-------|--------|------|--------|--------|------|--------|-------|--------|
|       | EF3314_              | 90     | 90      | 90        | 90        | 90     | 90 | 90        | 90        | 90  | 90      | 90    | 90     | 90        | 90 | 90      | 90    | 90   | 90   | 90   | 90      | 90    | 90    | 90    | 90   | 90      | 90    | 90    | 90    | 90   | 90      | 90    | 90    | 90    |
|       | EF0820_              | 90     | 90      | 90        | 90        | 90     | 90 | 90        | 90        | 90  | 90      | 90    | 90     | 90        | 90 | 90      | 90    | 90   | 90   | 90   | 90      | 90    | 90    | 90    | 90   | 90      | 90    | 90    | 90    | 90   | 90      | 90    | 90    | 90    |
|       | EFA0080_             | 90     | 90      | 90        | 90        | 90     | 90 | 90        | 90        | 90  | 90      | 90    | 90     | 90        | 90 | 90      | 90    | 90   | 90   | 90   | 90      | 90    | 90    | 90    | 90   | 90      | 90    | 90    | 90    | 90   | 90      | 90    | 90    | 90    |
|       | EF1368_              | 90     | 90      | 90        | 90        | 90     | 90 | 90        | 90        | 90  | 90      | 90    | 90     | 90        | 90 | 90      | 90    | 90   | 90   | 90   | 90      | 90    | 90    | 90    | 90   | 90      | 90    | 90    | 90    | 90   | 90      | 90    | 90    | 90    |
|       | EF0408_              | 90     | 90      | 90        | 90        | 90     | 90 | 90        | 90        | 90  | 90      | 90    | 90     | 90        | 90 | 90      | 90    | 90   | 90   | 90   | 90      | 90    | 90    | 90    | 90   | 90      | 90    | 90    | 90    | 90   | 90      | 90    | 90    | 90    |
|       | EF0605_              | 90     | 90      | 90        | 90        | 90     | 90 | 90        | 90        | 90  | 90      | 90    | 90     | 90        | 90 | 90      | 90    | 90   | 90   | 90   | 90      | 90    | 90    | 90    | 90   | 90      | 90    | 90    | 90    | 90   | 90      | 90    | 90    | 90    |
|       | EF1097_              | 90     | 90      | 90        | 90        | 90     | 90 | 90        | 90        | 90  | 90      | 90    | 90     | 90        | 90 | 90      | 90    | 90   | 90   | 90   | 90      | 90    | 90    | 90    | 90   | 90      | 90    | 90    | 90    | 90   | 90      | 90    | 90    | 90    |
|       | EF0869_              | 90     | 90      | 90        | 90        | 90     | 90 | 90        | 90        | 90  | 90      | 90    | 90     | 90        | 90 | 90      | 90    | 90   | 90   | 90   | 90      | 90    | 90    | 90    | 90   | 90      | 90    | 90    | 90    | 90   | 90      | 90    | 90    | 90    |
|       | EF0136_              | 90     | 90      | 90        | 90        | 90     | 90 | 90        | 90        | 90  | 90      | 90    | 90     | 90        | 90 | 90      | 90    | 90   | 90   | 90   | 90      | 90    | 90    | 90    | 90   | 90      | 90    | 90    | 90    | 90   | 90      | 90    | 90    | 90    |
|       | EFB0062_             | 90     | 90      | 90        | 90        | 90     | 90 | 90        | 90        | 90  | 90      | 90    | 90     | 90        | 90 | 90      | 90    | 90   | 90   | 90   | 90      | 90    | 90    | 90    | 90   | 90      | 90    | 90    | 90    | 90   | 90      | 90    | 90    | 90    |
|       | EF2205_              | 90     | 90      | 90        | 90        | 90     | 90 | 90        | 90        | 90  | 90      | 90    | 90     | 90        | 90 | 90      | 90    | 90   | 90   | 90   | 90      | 90    | 90    | 90    | 90   | 90      | 90    | 90    | 90    | 90   | 90      | 90    | 90    | 90    |

sRNAs in *E. faecalis*
three homologues especially in presence of oxygen remains unclear. However, the expressions of these paralogues appeared sequential during growth phases. EF0408_EF0409 was mainly transcribed during exponential phase, EFA0080_EF0081 during early stationary phase, and EFB0062_EFB0063 after 24 h of stationary phase (Table 3). These observations suggest that the different TA systems may have different roles according to the growth phase of the bacteria. E. faecalis tmRNA (EF1097_EF1098) and 4.5S RNA (EF2205_EF2206) showed a high intensity of hybridization under all conditions tested but BS and late stationary phase induced the highest level of tmRNA and 4.3S RNA expression, respectively. Furthermore, EF0869_EF0871 was highly expressed in urine and serum medium (Table 3). It has been shown that transcription of some genes encoding fitness and virulence factors are affected when E. faecalis is incubated in these biological media [39,40]. It is then tempting to speculate that these sRNAs could play a crucial role in the cellular response triggered during the infectious process.

Surprisingly, for unexpected reason, signals corresponding to the two sRNAs EF0136_EF0137 and EF0820_EF0821 were very low in these tiling arrays experiments leading to unexploitable data. On the other hand, EF3314_EF3315, EF1368_EF1369 and EF0408_EF0409 sRNAs appeared moderately expressed but were obviously induced by acid stress (Table 3). However, exponential growth phase and early stationary phase in presence of oxygen were the most favorable conditions for EF3314_EF3315 and EF0605_EF0606 expressions, respectively (Table 3). This is in agreement with the induction of Ref25C (corresponding to EF0605_EF0606) in oxidative stress condition reported by Fouquier d’Herouel et al. [23].

Our tiling arrays data using RNA samples obtained from cells incubated under 11 different growth conditions allowed us to identify 76 new IGRs with intensities of hybridization ten fold higher than signals from apparent untranslated regions. Probe sequences and tiling array data obtained with samples from stressed cells are shown in Table S3. A more detailed analysis of these new candidates is in progress in our laboratory. In addition, if the threshold was set to five-fold induction, 174 putative sRNAs were detected in our experiments. sRNAs are usually transcribed under specific growth conditions and it is likely that some could be expressed under stressing conditions not yet tested. Moreover, sRNAs may have been missed in our study due to experimental procedure since our chips only covered intergenic regions of the V583 genome and since fractionated RNAs have been used for the hybridizations. It has been generally predicted that genome sizes ranging from 3–4 Mbp may contain 80–300 sRNAs [14]. Taken together it is highly probable that the number of sRNA transcripts detected in E. faecalis will greatly increase in the near future.

### Distribution of sRNAs among E. faecalis strains

To date, the whole genome sequence of 54 E. faecalis strains are available in the NCBI database. We performed standard BLAST analysis to detect the presence of the characterized sRNAs in these different E. faecalis strains (Table 4). Seven of them are highly conserved (90 to 100% identical) and present in all E. faecalis genomes (EF3314_EF3315, EF0820_EF0821, EF1368_EF1369, EF0408_EF0409, EF1097_EF1098, EF0869_EF0871 and EF2205_EF2206). The other four are not systematically observed because of their location on a mobile genetic element (EF0136_EF0137), in the pathogenicity island (PAI) (EF0605_EF0606) or on plasmids (EF0080_EF0081 and EFB0062_EFB0063) [41]. sRNAs EF0605_EF0606, EF0136_EF0137, EFB0080_EFB0081 and EFB0062_EFB0063 homologues (at least 80% identical) are present in 9, 15, 35 and 23 strains of the 54 genomes analyzed, respectively (Table 4).

Homologues of EF0408_EF0409 (more than 90% identity) (member of TA system, see above) were systematically present in all E. faecalis genomes. Moreover, additional plasmidic EFA0080_EFA0081 and EFB0062_EFB0063 homologous were also observed in some chromosomes showing that most E. faecalis strains have several par systems arguing for a selective advantage for the bacterial cell.

Interestingly, EF0605_EF0606 is located in PAI between a gene encoding a Dps family protein (EF_0606) and an operon including a parologue of gls24 (EF_0605-EF_0604). Dps is a protein involved in the protection of DNA against oxidative stress and Gls24 corresponds to a general stress protein that is a virulence factor in E. faecalis [42,43,44]. In S. pneumoniae, two sRNAs has demonstrated cis-acting effects on the transcription of adjacent genes [45]. From these observations and the fact that EF0605_EF0606 sRNA is induced under aerobic growth conditions, it may be hypothesized that it has a role in the control of expression of these enzymes.

### Table 4. Cont.

| sRNAs | E. faecalis | strains | TX2134 | TX2137 | TX2141 | TX4000 | TX4244 | TX4248 | X98 |
|-------|------------|---------|--------|--------|--------|--------|--------|--------|-----|
|       | EF3314     | 100     | 100    | 80–90 G| 90     | 90     | 100    | 90     | 90  |
|       | EF0820     | 100     | 100    | 80–90 G| 90     | 90     | 100    | 90     | 90  |
|       | EFA0080    | 100     | 100    | 80–90 G| 90     | 90     | 100    | 90     | 90  |
|       | EF1368     | 100     | 100    | 80–90 G| 90     | 90     | 100    | 90     | 90  |
|       | EF0408     | 100     | 100    | 80–90 G| 90     | 90     | 100    | 90     | 90  |
|       | EF0605     | 100     | 100    | 80–90 G| 90     | 90     | 100    | 90     | 90  |
|       | EF1097     | 100     | 100    | 80–90 G| 90     | 90     | 100    | 90     | 90  |
|       | EF0869     | 100     | 100    | 80–90 G| 90     | 90     | 100    | 90     | 90  |
|       | EF0136     | 100     | 100    | 80–90 G| 90     | 90     | 100    | 90     | 90  |
|       | EFB0062    | 100     | 100    | 80–90 G| 90     | 90     | 100    | 90     | 90  |
|       | EF2205     | 100     | 100    | 80–90 G| 90     | 90     | 100    | 90     | 90  |

100 indicates 100% identity. 90 indicates more than 90% identity. 80–90 indicates between 80 and 90% identity. White box indicates the absence of homology. G: on genome. P: on plasmid. doi:10.1371/journal.pone.0023948.t004
and hence may be implicated in stress response and virulence of *E. faecalis*.

**Perspectives**

In this work we have determined the sequences, locations and expression patterns of 11 sRNAs in *E. faecalis* V583. These results provide a starting point towards understanding of the complex RNA regulatory network governing *E. faecalis* physiology and virulence. Recently, comparative genome-wide analysis of putative or characterized sRNAs of five major Gram-positive pathogens (L. monocytogenes, C. difficile, *Staphylococcus aureus* COL, *Streptococcus pyogenes* M1 GAS, and *E. faecalis* V583) was reported [46]. This information will help to understand the molecular mechanisms of the pathogenic process which might be useful for the development of novel microbial diagnosis tools and anti-bacterial drugs such as antisense PNA [peptide nucleic acids] [46].

**Supporting Information**

**Table S1** Primers and probes used in this study.

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**Table S2** List of putative target genes of EF3314_EF3315, EF0820_EF0822, EF1360_EF1369, EF0605_EF0606, EF0069_EF0071, EF0136_EF0137 sRNA candidates. (XLS)

**Table S3** Probe sequences and tiling array data obtained with samples from stressed cells of *E. faecalis*. (XLS)

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**Author Contributions**

Conceived and designed the experiments: KS JCG TH AH. Performed the experiments: KS CM CK AB-V. Analyzed the data: KS CM NV JCG. Contributed reagents/materials/analysis tools: CK TH NV. Wrote the paper: KS AH JCG.
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