SRD: a *Staphylococcus* regulatory RNA database

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ABSTRACT

An overflow of regulatory RNAs (sRNAs) was identified in a wide range of bacteria. We designed and implemented a new resource for the hundreds of sRNAs identified in Staphylococci, with primary focus on the human pathogen *Staphylococcus aureus*. The "Staphylococcal Regulatory RNA Database" (SRD, http://srd.genouest.org/) compiled all published data in a single interface including genetic locations, sequences and other features. SRD proposes novel and simplified identifiers for Staphylococcal regulatory RNAs (srn) based on the sRNA's genetic location in *S. aureus* strain N315 which served as a reference. From a set of 894 sequences and after an in-depth cleaning, SRD provides a list of 575 *srn* exempt of redundant sequences. For each sRNA, their experimental support(s) is provided, allowing the user to individually assess their validity and significance. RNA-seq analysis performed on strains N315, NCTC8325, and Newman allowed us to provide further details, upgrade the initial annotation, and identified 159 RNA-seq independent transcribed sRNAs. The lists of 575 and 159 sRNAs sequences were used to predict the number and location of *srns* in 18 *S. aureus* strains and 10 other Staphylococci. A comparison of the *srn* contents within 32 Staphylococcal genomes revealed a poor conservation between species. In addition, sRNA structure predictions obtained with MFold are accessible. A BLAST server and the intaRNA program, which is dedicated to target prediction, were implemented. SRD is the first sRNA database centered on a genus; it is a user-friendly and scalable device with the possibility to submit new sequences that should spread in the literature.

Keywords: small regulatory RNAs; bacteria; *Staphylococcus aureus*; database; RNA-seq; sRNA identification; sRNA targets

INTRODUCTION

In the recent years, a plethora of regulatory RNAs (sRNAs) were identified in diverse bacterial genomes, including several human pathogens. sRNAs enable bacteria to induce efficient and prompt physiological feedbacks to adjust on their environments, and also to establish infection (Caldelari et al. 2013). Mechanistically, sRNAs intervene on transcription, mRNA turnover, and/or translation of target genes. sRNAs proceed in gene expression regulations, from transcription initiation to translation control and protein activity (Storz et al. 2011). The majority of sRNAs characterized up to now act by pairings with target mRNAs, either encoded on the opposite strand (*cis*-encoded) or transcribed apart from their targets (*trans*-encoded). Some have been shown to encode small peptides. While most of the bacterial sRNAs were originally studied in *E. coli* and other Gram-negative bacteria (Mizuno et al. 1984), a recent outburst of sRNAs was identified in Gram-positive bacteria (Brantl and Brückner 2014), including the major human pathogen *Staphylococcus aureus* (Fechter et al. 2014).

Staphylococcus aureus is an opportunistic pathogen that has sophisticated regulatory tracks to rapidly and efficiently adapt its growth in response to its disparate habitats and hosts. Several groups have shown experimentally that *S. aureus* express many sRNAs, delivered from the core genome, mobile and accessory elements (Guillet et al. 2013; Tomasini et al. 2014). They include several predicted riboswitches (*cis*-acting regulatory mRNA leader sequences), many *cis*-encoded antisense RNAs, several *trans*-encoded sRNAs (Romilly et al. 2012) with some containing small open reading frames that were shown to be expressed (Sayed et al. 2012; Pinel-Marie et al. 2014). However, and for the most part, their functions and mechanisms are unexplored yet. For the few sRNAs with associated functions, some detect bacterial density, modify cell surface properties for host immune escape, adjust central metabolism for optimal growth, regulate the expression of virulence factors, influence antibiotic resistance (Lalaouna

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et al. 2014), trigger cell death, and encodes toxins (Felden et al. 2011; Guillet et al. 2013).

The systematic and recent use of high-throughput RNA-sequencing technologies substantially raised the number of sRNAs sequences identified per bacterial species. To cope with that plethora of sRNAs, several databases have emerged from generalists to more specialize. Generalist databases such as fRNAdb (Kin et al. 2007) or NONCODE (Xie et al. 2014) focus exclusively on eukaryotes, while Rfam 11.0 (Burge et al. 2013) partitions RNAs data into families for both the eukaryotes and prokaryotes. RNAdb was originally designed for mammalian noncoding RNAs but was officially retired in June 2012 (Pang et al. 2007). On the other hand, there are databases specifically devoted to the bacterial kingdom. sRNAMap is a repository for the microbial genomes but Gram-positive bacteria, and therefore Staphylococci, are absent from this browser (Huang et al. 2009). sRNATarbase (Cao et al. 2010) was implemented to provide a list of sRNA targets, but is out of date for Staphylococci. Recently, two bacterial sRNA databases were developed: (i) sRNAdb, which focused exclusively on Gram-positive bacteria (Pischimarov et al. 2012) but provides only 39 sRNA sequences for *S. aureus*; (ii) BSRD is a generalist bacterial sRNA database with >700 species included (Li et al. 2013). Finally, RNAspace.org is a platform devoted to the prediction, annotation, and analysis of noncoding RNA but does not provide data set (Cros et al. 2011). In *S. aureus* and more generally in the Staphylococcal genus, a unified sRNA nomenclature is lacking, while many redundancies, as single sequence described under several IDs, and potential misannotated sRNAs (e.g., repeated sequences, mRNA leader or trailer sequences) would require an in-depth manual cleaning.

Therefore, there is an urgent need for additional sRNA databases focusing on a bacterial genus to provide an accurate and simple list of sRNAs. Here, we report a *Staphylococcus* Regulatory RNA Database (SRD, http://srd.genouest.org/) which compiles, after an in-depth scrubbing all the sRNA sequences identified so far, with a primary focus on the human pathogen *S. aureus* as a reference. Starting from a large set of sRNA sequences, SRD proposes a new and simple nomenclature together with individual functional, structural, and phylogenetic information and predictions. It provides a unified repository based on additional RNA-seq data analysis.

RESULTS

Construction of a database encompassing the Staphylococcal regulatory RNAs

Staphylococcal sRNAs were identified and studied principally in several strains of *S. aureus* (Tomasini et al. 2014). The chronological discovery of the Staphylococcal sRNAs expressed in *S. aureus* is listed in Table 1. Those RNAs were identified by combining diverse experimental and bioinformatics approaches (Novick et al. 1989, 1993; Pichon and Felden 2005; Anderson et al. 2006; Roberts et al. 2006; Marchais et al. 2009; Nielsen et al. 2011; Morrison et al. 2012; Xue et al. 2014) including the use of Next-Generation RNA-Sequencing technologies (Geissmann et al. 2009; Abu-Qatouseh et al. 2010; Beaume et al. 2010; Bohn et al. 2010; Howden et al. 2013). A total of 894 sequences transcribed as sRNA were compiled from the literature (Fig. 1; Supplemental Data S1). We then focused on the following extensively studied and completed *S. aureus* genomes: N315, Newman, NCTC8325, and JKD6008 (Table 2). The BLAST program was used to locate the coordinates of each sRNA gene in any of the four genomes. Some sequences appeared, as previously suggested (Beaume et al. 2010; Howden et al. 2013), to be repeated onto the genomes, that led to an increase in the total number of sRNA sequences collected. Therefore, sequences identified as DNA repeated sequences by these authors were removed after confirming the initial statements using Blast (Supplemental Data S2). In addition, sequences located in CDSs, rRNAs, tRNAs, or spacers within the four genomes as well as the RNA sequences flanking the genes transcribed as ribosomes (reads overlapping with the ribosomes or within the intergenic regions of ribosomes) were discarded (Liu et al. 2009) to generate a first data set of 773 sequences. A significant number of redundant sequences annotated as a single sRNA could be retrieved under other names. This data set included, among others, the *sau*, *rsa*, *jkdsRNA*, *teg*, and *spr* genes. As an example, up to five other different gene IDs were identified for *rsaE* (*RsaON_Sau20_Teg92_IGR6_sRNA183*). Therefore, we manually cured this data set to provide a list of 575 sRNA genes exempted of redundancy.

TABLE 1. Sequential identification of regulatory RNAs expressed in *Staphylococcus aureus*

sRNA genes	Year	References	Main strains
RNAI	1989	Novick et al. (1989)	NCTC8325
RNAIII	1993	Novick et al. (1993)	NCTC8325
spr genes	2005	Pichon and Felden. (2005)	N315
WAN genes	2006	Roberts et al. (2006)	N315
ssr genes	2006	Anderson et al. (2006)	N315
rsaO genes	2009	Marchais et al. (2009)	N315
rsa genes	2009	Geissmann et al. (2009)	N315, COL
sau genes	2010	Abu-Qatouseh et al. (2010)	N315
rsa genes	2010	Bohn et al. (2010)	N315
teg genes	2010	Beaume et al. (2010)	N315
sbr genes	2011	Nielsen et al. (2011)	Newman
SSR42	2012	Morrison et al. (2012)	UAMS-1
artR	2014	Xue et al. (2014)	NCTC8325
jkd sRNA genes	2013	Howden et al. (2013)	JKD6008

Need and proposal for a novel and simplified identifier

The recent outburst in sRNAs led to spreading a large confusion in the actual number of sRNA genes and for

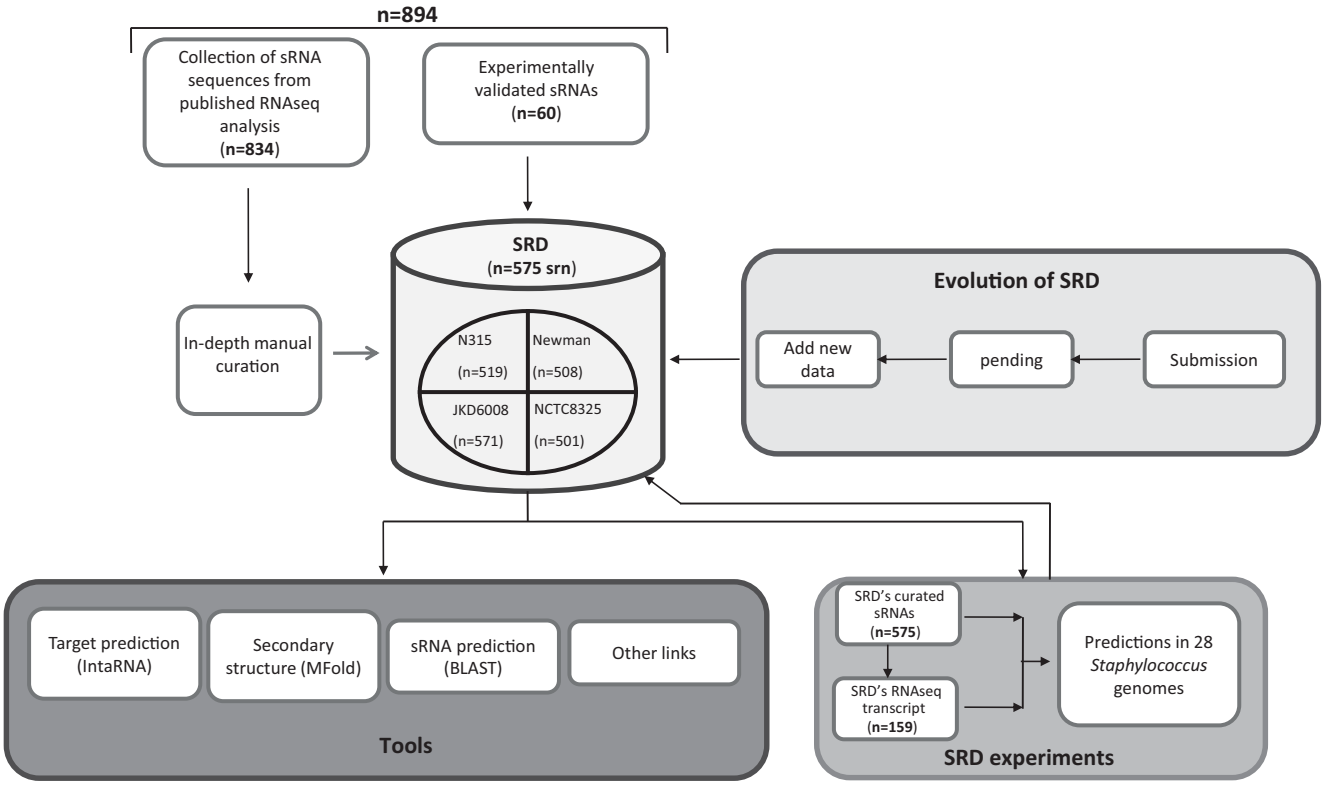


FIGURE 1. Overview of the SRD's inputs and outputs.

communications as a single sRNA sequence can harbor multiple IDs. To cope with that, we assigned novel and simple identifier that clarifies the actual repertoire of *S. aureus* sRNAs. The genome of N315 (Kuroda et al. 2001) was used as a reference as many sRNA sequences were identified in this strain, with some experimentally validated (Pichon and Felden 2005; Beaume et al. 2010; Chabelskaya et al. 2010). Each *srna* gene was assigned with a *srn* (***s**taphylococcus **r**ibo**n**ucleotide*) gene identifier. The *srn* gene identifiers were numbered based on their genetic location onto the genome of *S. aureus* N315 strain and starting from the origin of replication and therefore do not reflect their transcriptional level. *srn* numbers were assigned by increments of 10 to anticipate the identification of new sRNAs in the upcoming future which would be also numbered based on their genomic locations. When a *srn* gene is present in other strains it keeps the *srn* number assigned onto the N315 genome. When a *srn* identified in N315 is present in multiple copies in N315 or in another strain, the number of copies is provided (*srn_1930.1, srn_1930.2*, and *srn 1930.3*), unless experimental evidence indicated that they should be considered as distinct sRNAs (Supplemental Data S3, S4). *srn* identified in other strains and absent in N315 strain will be assigned with numbers starting from *srn_9000* (*srn_9480* formerly known as *sRNA334*). There were only four sRNAs (4.5 S, 6S, tmRNA, and RNase P RNA) for which a srn identifier was not generated, based on their extensive nomenclature, sequence, and functional conservation beyond Staphylococcal genomes (Supplemental Data S5). Therefore, regulatory RNAs such as riboswitches, RNAIII, or RsaE that are pioneered and/or conserved within the genus were also assigned with a srn identifier similar to what was done with the JKD6008 strain (Howden et al. 2013). However, a column entitled "most common name" is present on the website to avoid confusion when dealing with already well-described sRNAs. Additional information, including all other previously published names is provided with the list of *srn* on the SRD website.

Description of sRNAs in SRD

Among the 575 genomic sequences described in SRD, there are only 60 transcripts identified by multiple experimental approaches (including Northern Blot, 5′ and 3′ RACE, RT-qPCR, or RNA-seq) and a few for which their functions were characterized (Supplemental Data S3 and SRD website). Among these 60 sRNAs, 49 were described as transcripts whereas 11 were annotated as antisense sRNAs. The majority of the 575 sRNAs were identified or validated by Next-Generation RNA Sequencing (Beaume et al. 2010; Bohn et al. 2010; Lasa et al. 2011; Lioliou et al. 2012; Howden et al. 2013). Although powerful, RNA-seq, as any global approach, can lead to a substantial amount of false positive transcripts due to genomic DNA contaminations, reads mapping onto repeated sequences or/and the inaccurate detection of

TABLE 2. *Staphylococcus* strains used for implementing the SRD database

Staphylococcus strains	GenBank accession number	Genome length	Number of srn genes ([a]/[b])
Reference strains			
S. aureus subsp. *aureus* str. N315	BA000018.3	2,814,816	153/519
S. aureus subsp. *aureus* str. Newman	AP009351.1	2,878,897	156/508
S. aureus subsp. *aureus* str. NCTC8325	CP000253.1	2,821,361	155/501
S. aureus subsp. *aureus* str. JKD6008	CP002120.1	2,924,344	155/571
srn genes predictions in *Staphylococcus aureus*			
S. aureus 502A	CP007454.1	2,764,699	151/485
S. aureus subsp. *aureus* str. COL	CP000046.1	2,809,422	147/490
S. aureus M1	HF937103.1	2,864,125	149/499
S. aureus subsp. *aureus* str. M013	CP003166.1	2,788,636	138/461
S. aureus subsp. *aureus* str. MRSA252	BX571856.1	2,902,619	143/468
S. aureus subsp. *aureus* str. MSHR1132	FR821777.2	2,762,785	112/281
S. aureus subsp. *aureus* str. Mu50	BA000017.4	2,878,529	153/485
S. aureus subsp. *aureus* str. MW2	BA000033.2	2,820,462	147/485
S. aureus NRS 100	CP007539.1	2,823,087	147/490
S. aureus subsp. *aureus* str. USA300 FPR3757	CP000255.1	2,872,769	151/498
S. aureus XN108	CP007447.1	3,052,055	156/499
S. aureus subsp. *aureus* str. ST398	AM990992.1	2,872,582	140/448
S. aureus subsp. *aureus* JH1	CP000736.1	2,906,507	156/498
S. aureus subsp. *aureus* JH9	CP000703.1	2,906,700	156/498
S. aureus subsp. *aureus* MSSA476	BX571857.1	2,799,802	147/481
S. aureus subsp. *aureus* Mu3	AP009324.1	2,880,168	153/498
S. aureus subsp. *aureus* USA300_TCH1516	CP000730.1	2,872,915	152/499
S. aureus RF122	AJ938182.1	2,742,531	138/445
srn genes predictions in other *Staphylococcus*			
S. carnosus subsp. *carnosus* TM300	AM295250.1	2,566,424	9/22
S. epidermidis ATCC 12228	AE015929.1	2,499,279	19/53
S. epidermidis RP62A	CP000029	2,616,530	21/65
S. haemolyticus JCSC1435	AP006716.1	2,685,015	19/56
S. lugdunensis HKU09-01	CP001837.1	2,658,366	17/48
Staphylococcus pasteuri SP1	CP004014.1	2,559,946	26/55
Staphylococcus pseudintermedius ED99	CP002478.1	2,572,216	13/31
S. saprophyticus subsp. *saprophyticus* ATCC 15305	AP008934.1	2,516,575	15/41
S. warneri SG1	CP003668.1	2,486,042	25/54
S. xylosus HKUOPL8	CP007208.1	2,836,901	15/31

[a]Number of SRD's RNA-seq transcribed *srna* genes.
[b]Numbers of *srn* were determined after RNA-seq analysis and predictions performed with a set of 575 nonredundant gene sequences.

transcripts by bioinformatics. Some of the sRNAs previously identified by RNA-seq were of short lengths (<50 nt), mostly detected as *cis*-encoded antisense RNAs, including a wide antisense transcription that is not understood yet (Lasa et al. 2011). Also, some of the previously published sRNAs were already suspected as potential 5′ UTRs or 3′ UTRs (Beaume et al. 2010; Howden et al. 2013). Therefore, we searched for the presence of srn transcripts in strains N315 and Newman (data submitted to GEO with the accession number GSE64026), cultivated until the exponential phase of growth in a rich medium (BHI), and for strain NCTC8325 cultivated under 16 different growth conditions (growth phase, temperature, O_2 limitations, etc.; data kindly provided by Drs. P. Bouloc and T. Rochat). Those three strains are representative as their genomes were completed and annotated (Kuroda et al. 2001; Gillaspy et al. 2006; Baba et al. 2008). Based on our initial compilation and curation, N315, Newman, and NCTC8325 are predicted to contain 518, 508, and 501 independent *srn* genes, respectively. Altogether, they represent a pool of 535 *srn* genes. RNA-seq reads were mapped at unique locations onto their respective genomes, counted using HTSeq (Anders et al. 2014) and the presence of transcripts analyzed using Artemis (Carver et al. 2012). Based on the results from HTSeq (Supplemental Data S4), an FPKM normalization (Fragment per kilobase per millions of fragments mapped) and from the visualization of reads onto the annotated genomes (Fig. 2), the *srn* were either described as transcripts (Fig. 2A), *cis*-antisense RNAs (Fig. 2B), 5′ UTR (Fig. 2C), 3′ UTR (Fig. 2D), CDS (Fig. 2E, coordinates inside an annotated gene) or not detected (Fig. 2F, ND) in the "SRD's RNA-seq evidence" column of the SRD website. A srn was considered as a transcript or *cis*-antisense (reads mapping onto the opposite strand of a CDS) when the HTSeq count was equal or >15 (Howden et al. 2013), the FPKM normalization >2, the mapping quality (MAPQ score in a SAM file) >30 (probability of a correct match equal to 0.999), and when the

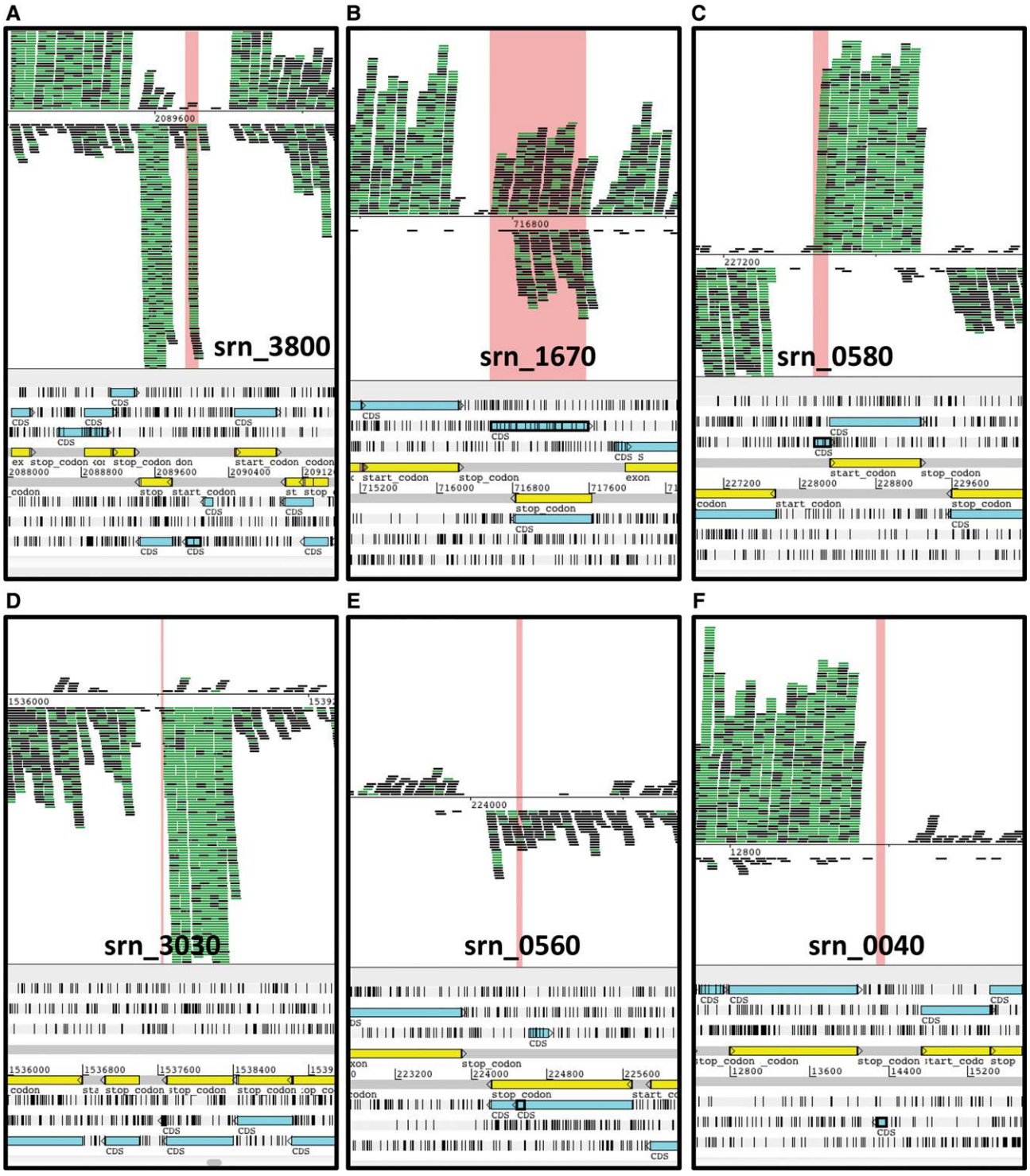


FIGURE 2. Examples of the visualization of read mapping from strain Newman using Artemis. The srns are highlighted in pink. (*A*) Typical visualization for a srn described as transcript. (*B*) Example of an antisense srn. (*C*) Reads overlapping with a CDS and considered as a 5′ UTR. (*D*) srn described as a 3′ UTR. (*E*) srn identified within a CDS. (*F*) srn not detected.

reads did not overlap with annotated genes. The UTRs were described as 5′ UTRs or 3′ UTRs when the reads are assembled with CDS and when the expression was similar to the flanking genes, as described (Yoder-Himes et al. 2009). The results of our analysis are summarized in Table 3. In N315, we identified 94 *srn* as transcripts, 24 as *cis*-antisense RNAs, 14 as CDS, 58 as

TABLE 3. Description of *srns* based on SRD's RNA-seq analysis

	N315	Newman	NCTC8325
CDS	14	13	24
5′ UTR	58	54	59
3′ UTR	31	32	28
Not detected (ND)	297	278	262
Transcript	94	100	104
Antisense	24	30	24
Sum	518	507	501

The *srns* were described as "CDS," "5′ UTRs," "3′ UTRs," "ND," "Transcripts," or "Antisense" based on the following criteria: HTSeq count ≥15, FPKM ≥2, MAPQ ≥30 and no clusterization with flanking CDS.

5′ UTR, 31 as 3′ UTR, and 297 were not detected. Similar results were obtained for Newman and NCTC8325 strains, respectively (see the SRD website). *srn* annotated as "ND" were either not detected by HTSeq count or did not meet some of the criteria retained for describing a sequence as transcript or a *cis*-antisense. Current Illumina sequencing kits are not perfectly adapted to the search of small bacterial transcripts and therefore it is difficult to know whether there are limits in the technology or that transcripts are absent. Forty-five of the *srn* that were not detected were predicted to have a length <50 nt. Ninenty-seven *srn* were predicted to be between 50 and 100 nt and the others were longer than 100 nt. Overall, only 159 srn were detected as transcripts or as RNA antisense under our experimental conditions (Supplemental Data S5 and http://srd.genouest.org/browsevalidated). Also, there were 24 ambiguous *srn* (Supplemental Data S6). These ambiguous *srn* presented criterion that did not allow us to add them in the list of 159 sRNA (see comments in Supplemental Data). All these results suggest that some sRNAs are expressed or detected under specific conditions that await further experimental assessment, while other *srn* may not be considered as independent sRNA transcriptional units or may be false positive and arise from transcriptional noise.

sRNA predictions in other Staphylococcal species and strains

Four *S. aureus* genomes were used to generate SRD. It is however important to include in a database a large number of strains and other species. Therefore, we performed *srn* predictions on a set of 28 strains which included 18 *S. aureus* subspecies and 10 other Staphylococci (Table 2). BLAST (see Materials and Methods) was used to predict the presence of *srn* genes using either the list of 159 *srn* confirmed by our RNA-seq analysis or the curated list containing 575 *srn*. Using the of 159 *srn*, this resulted in a number of predicted nucleotide sequences that ranged from 112 *srn* genes for *Staphylococcus aureus* subsp. *aureus* str. MSHR1132 to 156 *srn* genes for *Staphylococcus aureus* subsp. *aureus* str. JH1 and *Staphylococcus aureus* subsp. *aureus* str. JH9, respectively. On the opposite a reduced number of genes was predicted for the 10 other *Staphylococcus* species, with only 9 *srn* genes found for *Staphylococcus carnosus* subsp. *carnosus* str. TM300 and a maximum of 26 *srn* genes for *Staphylococcus pasteuri* SP1. Taken together these results suggest a low level of nucleotide sequence conservation for the *srn* genes within the genus.

Comparative analysis of the *srna* gene sequences

To investigate further the 159 *srn* gene sequences detected in the various strains a phylogenetic analysis was performed. A "phylogenomic" tree based on *Staphylococcus* whole-genome content (*Staphylococcus* tree) (Fig. 3A) and a tree based on the *srn* gene content were constructed (*srn* genes tree) (Fig. 3B). Interestingly, the tree based on the *srn* content showed an outstanding similar topology compared wth the tree based on the genome content and clearly differentiates *Staphylococcus aureus* from the other *Staphylococcus* species (Fig. 3). For both trees, *S. aureus* subsp. *aureus* str. MRSH 132 appeared more distant from other *S. aureus* subspecies. This could explain why only 112 *srn* genes were predicted using BLAST for that strain. A "heatmap" representation constructed using a matrix of presence/absence of *srn* sequences in *Staphylococcus* genomes showed a species clusterization similar to that of the *Staphylococcus* tree (Fig. 4). These results confirm a weak conservation of the 159 *srn* outside the *aureus* species. In addition, *rnaseP* RNA gene, *4.5S* RNA gene, *srn_3910* (*RNAIII*), and *srn_2130* (*rsaE*) were identified in all *Staphylococcus* strains while the *tmRNA* sequences appears to be substantially degenerated only in *Staphylococcus xylosus* str. HKUOPL8. Regarding the *S. aureus* subspecies, we identified 96 *srn* conserved which we defined as a core sRNA set in this species.

Database overview and usage

Users can access the list of *srn* genes through the Web interface (Fig. 5A). From the SRD home page they can (i) access the data set corresponding to the four genomes used to construct SRD, (ii) retrieve a short list of *srn* genes with a unified annotation, (iii) browse for predictions in other Staphylococci (described elsewhere in the text), (iv) BLAST (Altschul et al. 1990) their own sRNA sequences against the entire SRD database, or (v) search for RNA–RNA interactions using the intaRNA program (Busch et al. 2008). After entering in the webpage of one of the four curated genomes, the users will have access to the full list of *srn* reported so far. For each table, the new nomenclature, the genomic coordinates, the orientation, previous names, and experimental support are provided. The column "experimental evidence" was added for the community in order to have a quick overview of the *srn* that were identified as transcripts through RNA-seq analysis or other experimental approaches. The molecular targets and

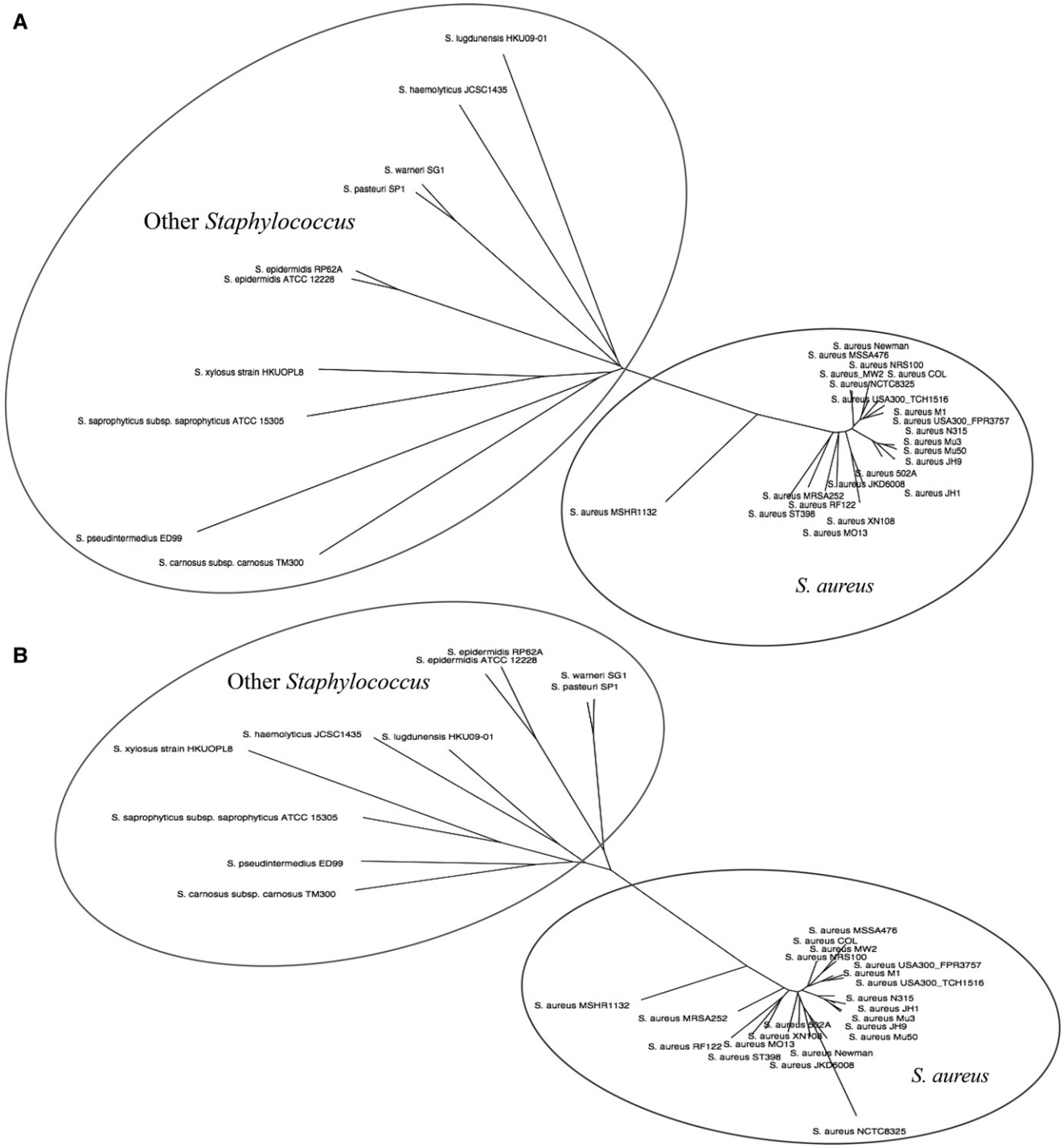


FIGURE 3. Phylogenetic analysis on the genome and *srn* content of 32 strains of the *Staphylococcus* genus using the Neighbor-joining algorithm. (*A*) *Staphylococcus* tree-based on genome content. (*B*) *Staphylococcus* tree-based on *srn* content.

references to the experimental secondary structure information are provided within the "SRD's RNA-seq transcribed sRNA" tab of the website. In addition, the users can download the Staphylococcal genomes and the *srn* genes in diverse formats ("FASTA" or "gff") suitable for subsequent RNA-seq analysis (Fig. 5B).

SRD specific features

Several functions have been included in the SRD website to provide an efficient device for the community working on sRNAs. BLAST and intaRNA (Altschul et al. 1990; Busch et al. 2008) were implemented in the database while external

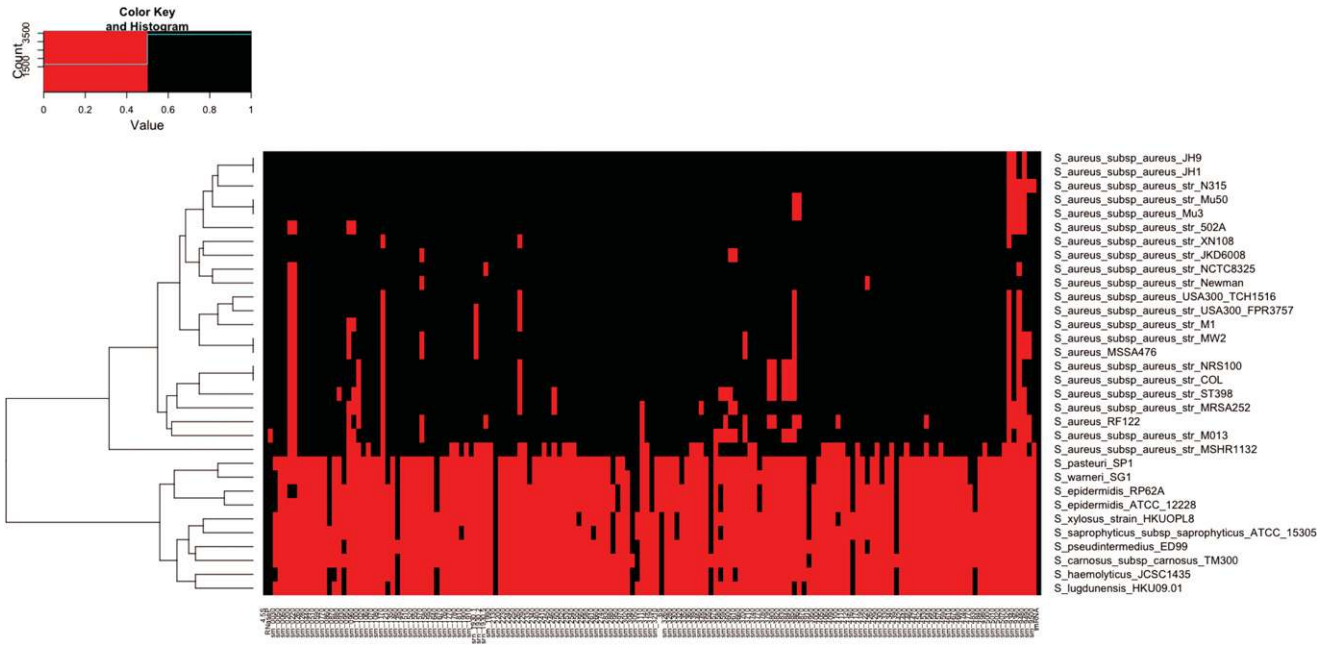


FIGURE 4. Comparative analysis through a "heatmap" cluster based on a matrix of presence (black) and absence (red) of *srn* sequences.

links to RNAtarget2 (Kery et al. 2014) and to RNA predator (Eggenhofer et al. 2011) are easily accessible from the webpage describing each sRNA. For BLAST comparisons, our databank includes the four initial reference genomes, the lists of validated sRNAs, and all the *srn* curated in the four strains. IntaRNA can be activated from the intaRNA page or directly from any *srn* sequences by clicking on the dedicated symbol. The users will only have to paste their mRNA sequences to see whether they may interact with each sRNA from SRD. MFold structure predictions (Zuker 2003), defined for the 575 srn RNA sequences, can be downloaded also directly from the SRD website.

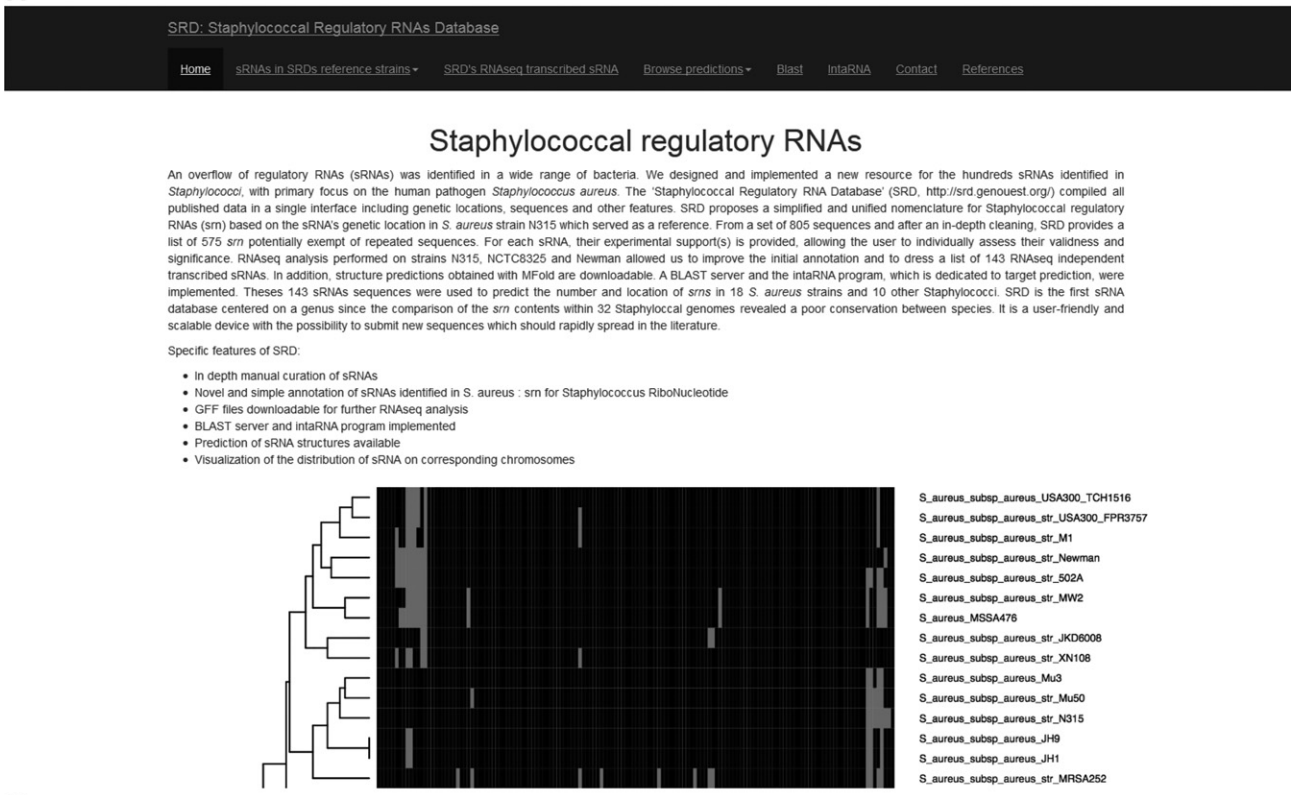
Evolution of the database and future directions

SRD is a repository for the Staphylococcal sRNAs with primary focus on *S. aureus*. By combining an in-depth cleaning and novel RNA-seq analysis, it clarifies the status regarding the absence of a consensus nomenclature and also to the actual expanding number of individual sRNA genes. With the rising interest of the community in the field of sRNAs, the number of sRNAs detected or characterized in bacteria is predicted to constantly increase. SRD will therefore evolve, based on the identification of novel sRNAs published in the literature. In addition, researchers who would be willing to unify new discoveries under the srn nomenclature will have the possibility to submit their sRNA sequence(s) to SRD under the contact information box. SRD would allow both already published and nonpublished submission. For data that would not be already published, temporary srn numbers will be assigned and disclosed upon acceptance of the publications in peer-reviewed journals. Therefore, researchers will have the possibility to disclose their results directly with the *srn* nomenclature. To avoid the diffusion of a large number of genes that may not be confirmed later, researchers will be invited to describe how a new sRNA was identified. For the novel sRNAs detected by RNA-seq analysis, that could lead to false-positive sRNAs, a secure ftp link will be provided to solicit researchers to deposit their "fastq" files to check whether they meet the criteria described in the text for being annotated as sRNA transcripts.

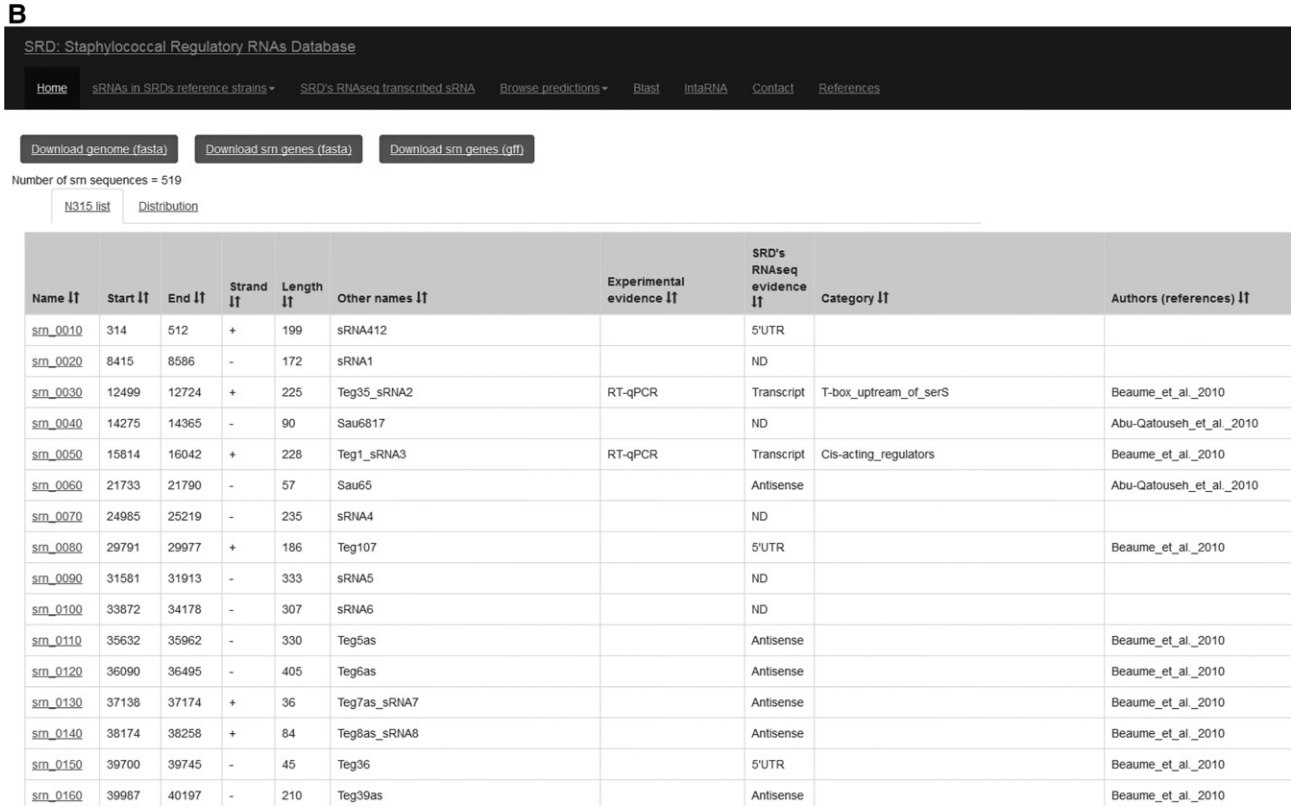
DISCUSSION

Over the last 20 yr, the number of sequences identified as sRNAs dramatically increased in *Staphylococcus aureus*. However, the democratization of Next-Generation Sequencing and the absence of a consensus in the community for annotating newly identified sRNAs led to a growing confusion that become detrimental to the field. SRD compiled the existing data in a single interface, removed the repeated and or redundant sequences and proposed a novel, simplified, nomenclature. Compared with the databases on other prokaryotes sRNAs, SRD is the first sRNA database dedicated to the *Staphylococcus* genus. SRD assigned a single identifier for the whole genus while other sRNA databases, such as BSRD (Li et al. 2013), provide an index per strain. A unique identifier offers a substantial advantage when comparing different strains, as it should avoid the dissemination of multiple redundancies. Therefore, the data provided by SRD would have been difficult to fuse with other databases. SRD hosts a

A



B



Name	Start	End	Strand	Length	Other names	Experimental evidence	SRD's RNAseq evidence	Category	Authors (references)
srn_0010	314	512	+	199	sRNA412		5'UTR		
srn_0020	8415	8586	-	172	sRNA1		ND		
srn_0030	12499	12724	+	225	Teg35_sRNA2	RT-qPCR	Transcript	T-box_uptream_of_serS	Beaume_et_al._2010
srn_0040	14275	14365	-	90	Sau6817		ND		Abu-Qatouseh_et_al._2010
srn_0050	15814	16042	+	228	Teg1_sRNA3	RT-qPCR	Transcript	Cis-acting_regulators	Beaume_et_al._2010
srn_0060	21733	21790	-	57	Sau65		Antisense		Abu-Qatouseh_et_al._2010
srn_0070	24985	25219	-	235	sRNA4		ND		
srn_0080	29791	29977	+	186	Teg107		5'UTR		Beaume_et_al._2010
srn_0090	31581	31913	-	333	sRNA5		ND		
srn_0100	33872	34178	-	307	sRNA6		ND		
srn_0110	35632	35962	-	330	Teg5as		Antisense		Beaume_et_al._2010
srn_0120	36090	36495	-	405	Teg6as		Antisense		Beaume_et_al._2010
srn_0130	37138	37174	+	36	Teg7as_sRNA7		Antisense		Beaume_et_al._2010
srn_0140	38174	38258	+	84	Teg8as_sRNA8		Antisense		Beaume_et_al._2010
srn_0150	39700	39745	-	45	Teg36		5'UTR		Beaume_et_al._2010
srn_0160	39987	40197	-	210	Teg39as		Antisense		Beaume_et_al._2010

FIGURE 5. Screenshots of SRD. (*A*) Main webpage interface to navigate and access specific features within the database. (*B*) Example of the presentation of *srn* data determined after curation of repeated and redundant sequences.

large collection of manually curated Staphylococcal sRNAs (575 *srn* genes) mostly exempted of repeated sequences and of redundancies. Furthermore, coupled with an SRD's RNA-seq analysis, a list of 159 RNA-seq transcribed srn is provided that includes all the 60 previously experimentally supported sRNAs. This suggests that the SRD's criteria used to describe the srn transcripts that combined previously published cut offs (Yoder-Himes et al. 2009; Howden et al. 2013) with high mapping quality scores and FPKM normalization (to prevent the detection of incorrectly mapped reads or of long transcripts that do not arise from transcriptional noise) were relevant. However, among the nearly 300 *srn* genes not transcribed under the physiological conditions tested, some may be later annotated as sRNA transcripts once additional data and work will provide transcriptional evidence. From this set of 159 *srn* genes, predictions were issued by sequence similarity to identify homologous genes in other Staphylococcal species. A comparison of the SRD's predictions with the BSRD entries for *Staphylococcus* strains shows that a genus-specific database is relevant. Indeed, in BSRD there is a huge discrepancy in the number of sRNAs sequences being available within the *S. aureus* subps. *aureus* species. While 154 sRNAs were listed for strain N315, mostly based on the work of Beaume et al. (2010), there were <60 sequences inventoried by similarity for other Staphylococcal strains (Li et al. 2013). All BSRD *S. aureus* entries are present in our database, and the predictions performed within the genus from a set of 575 *srn* genes (*versus* 8248 genes for BSRD), led to a larger set of predicted genes in SRD (Table 4). The comparative analysis performed for the *srn* content confirmed a low level of conservation within the genus and therefore in the bacterial kingdom. The weak sequence conservation at the DNA level in comparison with the protein level is therefore a serious limitation for retrieving genes, and therefore sRNAs, between species (Konstantinidis and Tiedje 2007; Sentausa and Fournier 2013). To our knowledge, there is no recognized standard to assign identifiers or nomenclature to sRNAs. Therefore, to not favor anyone name (spr, rsa, sau, teg, and others) we created the srn identifier. In addition, a most common name was assigned based on the chronological discovery of experimental confirmation to avoid the community to name some of the already very well-described sRNAs under a srn identifier while using the database. However, for the sRNAs that were only described by RNA-seq, the community is encouraged to use the srn identifier in an effort for unifying the work done on the sRNAs so far. We believe that SRD will help the scientific community working on Staphylococcal sRNA identification, function, and biology. SRD provides a simple and unified sRNA resource, a detailed annotation for each sRNA, a direct access to various RNA and genomic analysis tools. Finally, it shall encourage the community to participate in an effort to submitting new *Staphylococcus* sRNAs in SRD and to develop other genus specific sRNA databases that should be an essential extension to the generalist sRNA databases.

TABLE 4. Comparison between BSRD and SRD number of entries

Staphylococcus strains	BSRD sRNAs	SRD's RNA-seq and predictions
S. aureus subsp. *aureus* N315	154	519
S. aureus subsp. *aureus* Newman	58	508
S. aureus subsp. *aureus* NCTC 8325	55	571
S. aureus subsp. *aureus* JKD6008	NA	501
S. aureus subsp. *aureus* M013	NA	461
S. aureus subsp. *aureus* MSHR1132	NA	281
S. aureus subsp. *aureus* ST398	NA	448
S. aureus subsp. *aureus* COL	53	490
S. aureus subsp. *aureus* JH1	55	498
S. aureus subsp. *aureus* JH9	55	498
S. aureus subsp. aureus MRSA252	57	468
S. aureus subsp. *aureus* MSSA476	55	481
S. aureus subsp. *aureus* Mu3	55	498
S. aureus subsp. *aureus* Mu50	55	485
S. aureus subsp. *aureus* MW2	56	485
S. aureus subsp. *aureus* USA300_FPR3757	58	498
S. aureus subsp. *aureus* USA300_TCH1516	59	499
S. aureus 502A	NA	485
S. aureus XN108	NA	499
S. aureus M1	NA	499
S. aureus NRS 100	NA	490
S. aureus RF122	54	445
S. carnosus subsp. *carnosus* TM300	52	22
S. epidermidis ATCC 12228	53	53
S. epidermidis RP62A	57	65
S. haemolyticus JCSC143	56	56
S. lugdunensis HKU09-01	NA	48
S. pasteuri SP1	NA	55
S. pseudintermedius ED99	NA	31
S. saprophyticus subsp. *saprophyticus* ATCC 15305	53	41
S. warneri SG1	NA	54
S. xylosus HKUOPL8	NA	31

BSRD numbers of sRNAs were determined from data collected in the literature and predictions performed from a set of 8248 nonredundant gene sequences. SRD numbers of *srn* were determined after RNA-seq analysis and predictions performed with a set of 575 nonredundant gene sequences. (NA) Not assayed.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Staphylococcus aureus strains Newman and N315 were grown in liquid Brain Heart Infusion broth (BHI, Oxoid) and Tryptone Soya Broth (TSB, Oxoid) at 37°C, under agitation.

RNA extraction

Overnight cultures of *S. aureus* were diluted to an $OD_{600\ nm}$ of 0.1 into fresh BHI broth and cultured for 5 h at 37°C at 160 rpm. Cells were harvested by centrifugation at 15,000*g* for 30 sec and pellets washed with 500 µL of cold lysis buffer (20 mM sodium acetate, 1 mM EDTA, 0.5% SDS at pH 5.5). Cells were broken out using acid

treated glass beads (Sigma) in the presence of phenol (pH 4) in an FP120 FastPrep cell disruptor (MP Biomedicals) for 30 sec at power 6.5. Lysates were centrifuged for 5 min at 16,000*g* at 4°C. Total RNAs were extracted with phenol/chloroform and precipitated overnight. The RNA samples were treated with DNase I, Amplification Grade (Invitrogen). The absence of DNA contamination was checked by qPCR in an Applied Biosystems instrument. The integrity of each RNA preparation was verified on a "Bioanalyzer" (Agilent).

cDNA Library construction and Illumina RNA-seq

Ribosomal RNAs were depleted using the Ribo-Zero Magnetic Kit (Epicentre) and following manufacturer's recommendations. Stranded cDNAs libraries were prepared using the NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs). The concentration, quality, and purity of the libraries were determined on a BioAnalyzer (Agilent), a Qubit fluorometer (Invitrogen), and a Nanodrop (Thermo Scientific). Libraries were pooled and sequenced on an Illumina Hiseq 1500 instrument following the manufacturer's recommendations and using the rapid run mode for 200 cycles in paired-end.

Read mapping and visualization

The genome sequences and annotation files were obtained from NCBI (ftp://ftp.ncbi.nlm.nih.gov/genomes/Bacteria/). A "fastqc" report was performed prior mapping the data onto the appropriate genome. RNA-seq reads were mapped using Tophat2 and BWA (Li and Durbin 2010; Kim et al. 2013) with initial settings modified to allow the mapping of stranded library of an average mean distance between mates of 250 (for Tophat2) and to allow the alignment of a read to a single location with no mismatch (Tophat2 and BWA). BAM files were converted into SAM files and filtered on bitwise flag values (Li et al. 2009) to select properly paired reads. SAM files were then converted to BAM files, sorted by query, and counted by HTSeq count (Anders et al. 2014) for stranded library with the mode union. BAM files were visualized using the Artemis program (Carver et al. 2012).

Data set building and comparative analysis

The 32 genomes of *Staphylococcus* used in this study were downloaded from GenBank (Table 2). The published sRNA sequences were extracted from their respective genomes to construct a pool of sRNA. This pool was used to predict the *srn* genes in reference genomes. Then a pool of SRD's RNA-seq transcribed sRNA was used to predict *srn* genes in 28 *Staphylococcus* genomes. The predictions were done using "BLASTN" with a cutoff *E*-value $<1 \times 10^{-20}$, percentage similarity >80% and an alignment length >60 nt of the query length. The 32 *Staphylococcus* genomes and the predicted *srn* genes sequences were aligned using Muscle aligner implemented in Mauve software (Darling et al. 2010). Mauve alignment generated a genome content matrix for which the identity scores range between 0 and 1, where 0 indicates that no identical homologous nucleotides were found, and 1 indicates that every homologous nucleotide was identical. A matrix based on the *srn* gene content was generated (the similarity between two species is defined as the number of genes that they have in common divided by the total number of *srn* genes) (Snel et al. 1999, 2005; Huson and Bryant 2006). The genome content based matrix and the *srn* gene content based matrix were then used to construct, respectively, a *Staphylococcus* "phylogenomic" tree-based on genome content and a *Staphylococcus* tree-based on srn content, using Neighbor-joining algorithm in the package SplitsTree4 (Huson and Bryant 2006). A "heatmap" clusterization was constructed using a matrix based on presence and absence of *srn* genes using the R package (http://www.r-project.org/).

Database design

The web server has been designed in PHP with the "Symfony" framework (http://symfony.com). It includes a set of scripts that automatically parse the raw input files (*srn*, genomes), fill in a MySQL database for each set of genome/*srn*, and build some templates for all input predictions. Those scripts allow the addition of other genomes easily by simply adding the new files to the directory structure. They will update the existing information and insert the new ones. The website menus are automatically adapted to the list of analyzed genomes, removing the need to modify the website when new information is added (new genomes, new predictions, etc.). The scripts also (i) extract the FASTA sequence of each *srn* (to prefill IntaRNA form for example), (ii) execute MFold to create the pdf files containing the structure, and (iii) prepare a blast index for all genomes and set of *srn*.

A *srn* distribution is proposed in the web interface with pan and zoom options. This distribution is displayed using the D3js library (http://d3js.org/).

DATA DEPOSITION

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar et al. 2002) and are accessible through GEO Series accession number GSE64026 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE64026).

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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