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Abstract

Polysomes are macromolecular complexes made up of multiple ribosomes simultaneously translating a single mRNA into polypeptide chains. Together, the cellular mRNAs translated in this way are referred to 'translatome.' Translation determines a cell's overall gene expression profile. Studying translatome leads to a better understanding of the translational machinery and of its complex regulatory pathways. Given its fundamental role in cell homeostasis and division, bacterial translation is an important target for antibiotics. However, there are no detailed protocols for polysome purification from *Staphylococcus aureus,* the human pathogen responsible for the majority of multi-drug resistance issues. We therefore developed methods for the isolation of active polysomes, ribosomes, and ribosomal subunits, examining the purity and quality of each fraction and monitoring polysomal activity during protein synthesis. These steps are mandatory for the use of purified *S. aureus* polysomes and ribosomes for structural studies or for genome-scale analysis of most translated mRNAs.

1. Introduction

Protein synthesis, or 'translation', has a central role in gene expression regulation in all cells, and is an highly energy-intensive process. Translation is mediated by ribosomes, which are macromolecular complexes that translate the genetic information from mRNA codon triplets into specific amino acid sequences [1, 2]. Bacterial ribosomes are composed of two subunits: 30S, that selects the amino acids according to the mRNA sequence; and 50S, that combines amino acids to form polypeptides. These subunits are the main targets for the antibiotics in current clinical use [1].

In actively growing bacteria, multiple ribosomes begin translation simultaneously and range along a single mRNA to synthesize the same proteins. Because they move faster through sucrose gradients, these 'polysomes' or 'polyribosomes' are distinct from single ribosomes or ribosomal subunits [3]. Interestingly, in any living cell the polysomal fraction is considered to be a snapshot of the translation activity, although it was recently shown in *Saccharomyces cerevisiae* that monosomes make up a significant part of the active ribosomes that translate special RNAs [4].

In bacteria, investigations into translation based on the use of purified polysomes [5] have been much less common than those done using isolated ribosomes [6-8]. Yet polysomal and ribosomal profiling provide different information, and both approaches are required to better understand this process [9]. Most published protocols describe polysome purification from eukaryotic cells [10] or from Gram-negative bacteria [10, 11], with little available on Gram-positive bacteria [12]. This is particularly true for the important human pathogen *Staphylococcus aureus,* a commensal organism that can become deadly. The pathogen targets various organs, resulting in different diseases ranging from skin and soft tissue infections to severe bacteremia. Due to its aggressiveness and to the existence and proliferation of many multidrug-resistant strains, *S. aureus* is a major public health concern [13]. To our knowledge, only *S. aureus* ribosomes and not the polysomes have been studied [4, 14-18].

Starting from an *Escherichia coli* protocol [11], we created a robust method for purifying polysomes from *S. aureus*. Certain adjustments were made due to the increased difficulty of breaking the wall of Gram-positive bacteria, and because ribosomal sedimentation coefficients differ according to the investigated species. The method discussed here allows for the separation of polysomes, ribosomes monomers, and subunits in a single sucrose gradient. The purity of each fraction is checked using agarose gel electrophoresis. The polysomes and 70S monosomes are then visualized using electron microscopy. We are able to reveal the translational functions of the polysomal fractions, supporting the

use of polysomes as handy biotechnological tools. We also show that the isolated ribosome fractions can be used to detect specific mRNA and nascent proteins, as well as ribosome-associated proteins and small regulatory RNA (sRNA) during *S. aureus* translation. The technique presented here will be applied to the purification and use of polysomes, 70S ribosomes, and 50S and 30S subunits from different *S. aureus* clinical isolates, allowing us to employ various technologies to evaluate the overall translational processes at play in this important human pathogen.

2. Materials and methods

Polysomes are very sensitive to ribonuclease (RNase) cleavages [19]. RNase-free equipment and materials are thus essential throughout this protocol. It is particularly important to use ultrapure (Milli-Q) or RNase-free water during buffer preparations, and then to purify it using 0.22 µm filters.

2.1. *S. aureus* strains, plasmids, and genetic constructions

Polysome and ribosome enrichment is performed in two *S. aureus* strains, N315 [20] and HG003 [21]. The HG003 strain overexpresses a flagged version of δ-hemolysin using pCN35 plasmid vector containing a chloramphenicol resistance cassette [22]. For strain construction, the *rnaIII* sequence plus 248 nt upstream and 296 nt downstream is added with a 3xFLAG within the δ-hemolysin sequence upstream from its termination codon. This 1126 nt long fragment, with flanking EcoRI and PstI restriction sites, is then inserted into pCN35. The resulting plasmid is transformed into the *S. aureus* RN4220 shuttle strain and then into HG003, which contains the endogenous RNA III promoter.

2.2. Polysome enrichment

2.2.1. *S. aureus* cell extract preparation

Grow the bacteria at 37°C in TSB medium (Thermo Fisher, Dardilly, France). According to our research, bacterial growth can be performed in impoverished medium. Nevertheless, low-nutrient

mediums such as chemically defined medium (CDM) are not recommended, as the final amount of polysomes will be too low.

To increase the proportion of translating ribosomes that are located in the polysomal fraction, we encourage you to harvest cells during the exponential phase of growth (OD_{600} of 2). Moreover, cells should be frozen as quickly as possible to prevent the accumulation of 70S monomers, which would in turn decrease the polysome count [23]. We recommend translation stalling by immediate freezing at -80°C or with liquid nitrogen. After cell harvesting, all steps must be carried out on ice or at +4°C (in a cold room). Use the antibiotic chloramphenicol [6, 24] to increase the proportion of purified polysomes. If the strains carry a '*cat*' resistance gene, substitute the same concentration of tetracycline for this antibiotic [6].

The protocol described here is for the *S. aureus* N315 strain, which is grown in TSB medium.

(1) Grow *S. aureus* cells in 1 L of TSB medium at 37°C, with shaking, until the exponential phase.
(2) Block protein synthesis suddenly with 100 µg/ml chloramphenicol, immediately transferring the bacterial suspension into centrifuge bottles that have been stored at -80°C.
(3) Centrifuge the cells at 4500 rpm for 15 min at 4°C until they pellet.
(4) Discard the supernatant and re-suspend the pellets in 10 ml ice-cold polysome profile buffer (PPB) (10 mM Tris-HCl pH 7.5, 50 mM NH_4Cl, 10 mM $MgCl_2$, 1 mM DTT, 100 µg/ml chloramphenicol) [11].
(5) Centrifuge at 4500 rpm for 15 min at 4°C.
(6) Discard the supernatant and immediately freeze the pellets at -80°C until they are frozen enough that they can subsequently be broken.

2.2.2. Cell lysis and polysome isolation

In cold lysates, polysomes are stable, whereas from whole intact cells they are usually converted into ribosomes [23]. Thus for the purposes of this protocol, we must avoid lysostaphin, usually used to lyse *staphylococci* cell walls. Instead this is replaced by manual crushing of the frozen cells, using a chilled

mortar. Note that a French press cannot be used on pathogenic bacteria due to aerosol production and user contamination concerns.

At the end of this step, the 'S21 extract' pellet corresponds to the 'S30 extract' obtained by centrifugation at 21,000 g instead of 30,000 g [25].

(1) Using a mortar previously stored at -80°C, crush the frozen pellets with alumina powder (Sigma, Saint Quentin Fallavier, France) until it has the consistency of a homogeneous paste.
(2) Re-suspend the broken cells with 4 ml of PPB, and vigorously mix the suspension for 20 sec.
(3) Add 2 µl DNase 1 (Life Technologies, Courtaboeuf, France) to the suspension.
(4) Incubate on ice for 10 min with regular agitation.
(5) Pellet the bacterial cell debris and alumina powder by centrifugation at 21,000 g (13,200 rpm) for 5 min at 4°C, using a Sorvall SS-34 rotor and an RC 6 ultracentrifuge (Thermo Fisher, Osterode am Harz, Germany).
(6) Filter the supernatant containing the polysomes and the ribosomes with a 0.45 µm membrane.

Note that if you want to stop here, the S21 extracts can be stored at -80°C without altering the polysomal profiles [19].

2.2.3. Polysome and ribosome fractionations

In this step, polysomes, 70S ribosomes, and the dissociated 50S and 30S ribosomal subunits are separated into sucrose gradients prepared with a sucrose-supplemented PPB. For an optimal profile resolution, the gradient must be handled as gently as possible, and should be kept at 4°C for 4-16 h before use. To avoid gradient saturation and a poor resolution, we recommend loading less than 60 OD_{260} units.

The sucrose gradients are adjusted and adapted according to the strain to obtain an optimal separation of the different ribosomal fractions.

Note that sucrose gradients allow for the isolation and enrichment of ribosomal fractions, but the obtained fractions are only enriched and not pure.

(1) Prepare enough linear sucrose gradients to load all of the ribosome suspension with a gradient maker in a 26.3 mL tube (#355618; Beckman Coulter) which will fit into a 50.2 Ti Rotor.

   a. Pour 12 mL of the 40% sucrose solution into the mixing chamber of the gradient maker with a magnetic stir bar. Stir at moderate speed to prevent bubbles in the solution.

   b. Add 12 mL of the less concentrated solution in sucrose: 18% sucrose solution to the N315 strain, 25% sucrose solution to the HG003 one.

   c. Insert the gradient maker's output tubing into the top of the tube so that it does not contact the gradient. The solution should run along the tube's side to avoid disrupting the sucrose gradient.

   d. Pump only the 40% sucrose solution slowly, until it reaches 1 cm high at the bottom of the tube.

   e. Open the connection between the two chambers and continue to pump the solutions at a normal speed.

(2) Gently load up to 60 OD_{260} units of S21 extracts at the top of the sucrose gradients.

(3) Use a 50.2 Ti Rotor precooled to +4°C and an L90K ultracentrifuge (Beckman Coulter, Villepinte, France) to centrifuge the gradients at 131,000 g (33,000 rpm) for 3 h at 4°C.

(4) Fractionate the gradients in the cold room (+4°C), collecting 500 µl per fraction using an ÄKTA protein purification system (GE Healthcare, Orsay, France). To identify the polysomes, 70S ribosomes, and the 50S and 30S subunit peaks, determine the OD_{254} absorbency profile of each of the gradient fractions (Fig. 1A, SA1).

(5) Separately concentrate the polysome, monomer, and subunit fractions to a final volume of ~500 µL using 10 kDa Centricon centrifugal filter units (Dutscher, Brumath, France) as per the manufacturer's recommendations.

(6) Remove the sucrose by performing two successive washes with sucrose-free PPB.

(7) Wash the membranes of the centrifuge filter unit in order to collect each sample, then freeze the samples at -80°C.

2.3. Quality control of the polysome fractions

2.3.1. Agarose gel electrophoresis of the ribosomal fractions

(1) Mix 1 µg of the samples with loading dye buffer to a final concentration of 1X.
(2) Load and migrate samples on 1% agarose gel supplemented with ethidium bromide.
(3) Use an ultraviolet transilluminator to visualize the 23S and 16S rRNA of the various ribosomal fractions (Fig. 1B, SA2).

2.3.2. Electron microscopy imaging of the *S. aureus* polysomes and ribosomes using negative staining

In electron microscopy, the interaction of an electron beam with the sample generates an amplitude contrast that is proportional to the mass of the specimen atoms. In biology, samples mostly consist of low-mass atoms and thus have weak diffusion power. Negative staining involves adding heavy metal salts, as they have strong diffusion power. The stain surrounds the samples, but is excluded from the volume they occupy. Therefore the stained specimens appear white and are outlined by the stain's shading.

Negative staining is particularly useful for determining the purity of biological specimens and to evaluate their heterogeneity. Advantages of this method include the fact that samples can be easily and rapidly prepared, and high contrast results.

(1) Dilute the *S. aureus* 70S or polysome samples to the appropriate concentration (40 ng/µL) with the PPB.
(2) To render the films hydrophilic before use, perform a glow discharge on 300 mesh copper grids coated with collodion and carbon (Delta Microscopies, Mauressac, France).
(3) Lay a fresh sheet of parafilm on the bench. For each grid, prepare three drops of distilled water and one drop of 2% uranyl acetate aqueous solution.
(4) Put a 4 µL drop of this diluted sample onto the collodion-carbon grid and hold the grid with tweezers for 60 sec to adsorb the particles on the film. Next, remove most of the solution with filter paper.

(5) Wash the grid by successively touching the surface with three drops of distilled water.

(6) Remove the excess water from the grid with a filter paper.

(7) Leave the grid on a drop of stain for 20 sec.

(8) After 20 sec, remove the excess stain using a filter paper.

We acquired transmission electron microscopy images using a Tecnai Sphera operating at 200 kV (FEI, Eindhoven, Netherlands) equipped with a 4x4k CCD UltraScan camera (Gatan, Pleasanton, USA) (Fig. 1C-D, SA3-4). This equipment is part of the MRic microscopy platform at BIOSIT (Rennes, France).

2.4. Assessing polysome functionality

2.4.1. *In vitro* translation assays using purified *S. aureus* polysomes

We tested the activity of the polysomes extracted using our protocol by setting up an *in vitro* translation assay. This consists of running cell-free translation reactions to produce a model protein. It is essential to remove all trace of antibiotics before starting this test, because the antibiotics used during polysome enrichment will stall translation [26].

(1) Concentrate each S21 and polysome fraction to a final volume of ~50 µL using 10 kDa Centricon centrifugal filter units, as per the manufacturer's recommendations.

(2) Rinse twice with S30 buffer (10 mM Tris-acetate pH 8, 14 mM Mg-acetate, 60 mM K-acetate, 1 mM DTT).

(3) Wash the membranes of the centrifuge filter units to collect each sample.

(4) Initiate *in vitro* translation of δ-hemolysin, which is used as a model polypeptide. The assay is performed using [^{35}S]-methionine and the '*E. coli* S30 extraction system for linear templates' (Promega, Charbonnière Les Bains, France), following the manufacturer's instructions and replacing *E. coli* S30 extracts with the different purified *S. aureus* samples.

(5) To the translation products, add Tris-Tricine sample buffer to a final concentration of 1X (0.75% SDS, 7.5% glycerol, 0.0125% Coomassie blue, 37.5 mM Tris HCl pH7).

(6) Load the samples onto a 16% Tris-Tricine-SDS-PAGE gel.

(7) Vacuum dry the gel after its migration.

(8) Expose the gel with a screen and scan it with a Typhoon FLA 9500 Phosphor Imager (GE Healthcare, Freiburg, Germany) (Fig. 2A-B).

2.4.2. RNA extraction and RT-qPCR analyses

Check for the presence of RNA (mRNAs and sRNAs) in each ribosomal fraction through reverse transcription quantitative PCR (RT-qPCR). Results are normalized against the 5S ribosomal RNA (rRNA) and the 16S rRNA relative to amount of 50S subunits or 30S subunits, respectively. Both controls should be used to compare all fractions, including the 50S and 30S subunits (Fig. 2C, SB1). These procedures will indicate RNA enrichments in the different fractions, but a precise quantitative analysis is not possible.

(1) Extract the total RNA using a phenol-chloroform RNA extraction protocol (adapted from Cheung et *al*. [27]).

(2) Degrade any putative contaminant DNA from 2 µg RNA extract using amplification-grade DNase I enzyme (Life Technologies).

(3) Synthesize the complementary DNA (cDNA) using a High-Capacity cDNA Reverse Transcription Kit (Life Technologies).

(4) Perform RT-qPCR using a RealMasterMIX SYBR Kit (5′PRIME, Life Technologies) on a StepOnePlus Real-Time PCR system (GE Healthcare, Saint Aubin, France). The DNA primers we used are listed in Table 1.

(5) Use the comparative ΔΔCt method to normalize the amount of detected mRNA and sRNA against the 5S and 16S rRNA references.

2.4.3. Protein extraction and Western blot analysis

Check for the presence of proteins in the ribosomal fractions by using Western blots with specific polyclonal antibodies.

(1) Concentrate the samples to a final volume of 50 µL using 10 kDa Centricon centrifugal filter units, as per the manufacturer's recommendations.

(2) Add Tris-Tricine sample buffer to a final concentration of 1X (0.75% SDS, 7.5% glycerol, 0.0125% Coomassie blue, 37.5 mM Tris HCl pH7).

(3) Separate the samples onto 16% Tris-Tricine-SDS-PAGE gels.

(4) Transfer the separated samples from the gels onto Hybond PVDF membranes (Dutscher).

(5) Block the membranes with 5% milk.

(6) Incubate the membranes with the appropriate primary and secondary antibodies.

(7) Develop the blots using an ECL Plus Kit (Dutscher) and scan it with an LAS 4000 imager (GE Healthcare, Freiburg, Germany).

(8) Perform, in parallel of the Western blot, a loading control of all the samples by Coomassie Blue staining (Bio-Rad, Marnes-la-Coquette, France).

3. Results and discussion

Isolation of polysomes is very important for studying the molecular basis of translation, the structures of active ribosomes, and the overall translatome of any organism. Here, we propose a detailed procedure for the *in vitro* isolation and validation of the translational activity of polysomes coming from a Gram-positive pathogen, *S. aureus*.

In this method, two *S. aureus* strains, wild-type N315 and HG003 expressing a flagged version of δ-hemolysin, are first cultivated until the logarithmic phase, when the amount of polysomes is optimal [23]. The δ-hemolysin is encoded by multifunctional RNAIII [28] which regulates *agr* quorum-sensing and is involved in *S. aureus* virulence [29]. Sucrose gradient centrifugation is used to separate polysomes, 70S ribosomes, and the two ribosomal subunits according to their sedimentation coefficients. Studies have shown that structure of bacterial ribosomes reveals species-specific but not yet strains-specific features [2, 15]. However, during our studies, we demonstrate that to obtain an

optimal separation of the fractions, the sucrose percentage in the gradients must be adjusted according to the *S. aureus* strain. This result suggests putative disparities between polysomes and/or ribosomes of the two *S. aureus* strains, maybe due to mutations in some of the ribosomal proteins. The ribosomal profiles are obtained by measuring absorbency at 254 nm. Peak assignments are shown in Figure 1A for strain N315, and in Figure SA1 for strain HG003. As expected, the polysome fraction represents only a small part (~5%) of the total ribosomal fraction (Fig. 1A, S1). This is due to part of ribosomes that end translation and dissociate from their mRNA templates at all steps of fractionation [23].

The purity of the previously pooled and concentrated ribosomal fractions is monitored by agarose gel electrophoresis. As shown in Figures 1B and SA2, both 23S and 16S rRNAs are observed in the polysome and ribosome fractions, with 23S enriched in the 50S subunit, and 16S rRNA in the 30S one. After negative staining, transmission electron microscopy is used to monitor the purity of the polysomes and 70S ribosomes (Fig. 1C-D, SA3-4). In Figures 1C and SA3, the 70S ribosome enrichment is confirmed by the presence of isolated structures in which, according to their orientation, both the large and small ribosomal subunits are detected. In contrast, the higher-density sucrose gradient fraction contains mostly polysome chains habitually composed of 5-6 ribosomes, with few isolated ribosomes (Fig. 1D, SA4). Together, these biochemical and structural data show the enrichment quality of each ribosomal fraction.

To investigate the role of the purified polysomes in protein synthesis, we set up an *in vitro* translation assay focusing on the RNAIII-encoded δ-hemolysin, using both the *S. aureus* S21 and the polysome fractions. As shown in Figures 2A-B, both have comparable translational activity to that of the *E. coli* S30 extract, used as a positive control. Interestingly, the functioning of the S21 extracts and polysomes suggests their possible utility in cell-free translation systems. The S21 extracts are particularly needed, as they provide a rapid, efficient, and inexpensive *in vitro* translation method for *S. aureus* [30, 31]. Moreover, the purified 70S ribosomes and the ribosomal subunits could be used for other *in vitro* translational techniques such as dot blots, toeprint [16] or footprint assays. Using in-house preparations of *S. aureus* ribosome extracts, instead of commercial products, has the advantage of ensuring the use of the same strain background, and allows for the customization of cell extracts for specific applications through the utilization of genetically modified strains [25].

To demonstrate the translational activity of our purified polysomes, we monitored the ongoing synthesis of two immunoglobulin-binding proteins *in vivo*, SpA [32] and Sbi [33], as well as the transcription factor SarA [34]. These three proteins are expressed in *S. aureus,* and their expression levels were monitored by RT-qPCR (for mRNA) and Western blots (for proteins). As shown in Figure 2C, *spa*, *sbi* and *sarA* mRNA are more detected in the polysome fraction than within the 70S ribosome or subunit fractions of the N315 *S. aureus* strain. Next, we showed that the Spa and SarA proteins are detected within the total, S21, and polysome fractions (Fig. 2D). A very low signal is also observed in the 70S fraction, but this is probably due to contamination from the polysomal fraction. Nevertheless, in the HG003 *S. aureus* strain, we observed the association of RNAIII and δ-hemolysin in the polysome and 70S fractions (Fig. SB1-2). This difference can be explained because, as it was recently shown in *Saccharomyces cerevisiae*, polysomes contribute to the translation of mRNA that encode for abundant proteins (e.g. SpA and SarA for our study), while, the 70S monosomes mainly contribute to the translation of small RNA with short open reading frames (e.g. δ-hemolysin for our study) [4]. Together, these results give *in vivo* validation for the enrichment of the polysome fractions from two different *S. aureus* strains, one clinical isolate (N315) and one reference strain (HG003). The co-purification of mRNAs and their respective proteins with the polysome fractions also suggests that these proteins are actively translated by our purified polysomes. We cannot rule out, however, that the SpA and SarA proteins could potentially co-purify with the various ribosome fractions, whereas this is unlikely since both proteins are not involved in protein synthesis.

The purified polysomes provide a snapshot of translation activity in *S. aureus* cells at a given moment and during specific environmental conditions. They can be powerful tools for the visualization of mRNAs being translated and synthesized proteins. Ribosome-binding proteins and sRNAs interacting with the ribosomes can also be studied [35, 36]. Here, we have confirmed *in vivo* the presence of the *trans*-translation effector transfer-messenger RNA (tmRNA) [37] and the SprD regulator RNA [38] into the ribosomal fractions. We have also shown that both are more detected into the polysomal fraction (Fig. 2C).

During polysomal profiling, the RNA bound onto polysomes can be monitored by Northern blots or by RT-qPCR, as done here, or by deep RNA-sequencing for global studies. Similarly, the proteins being translated can be monitored by Western blots or by proteomics for large-scale studies. Most genome-wide studies focusing on translation have compared the translational status of mRNA in various strains

and/or during different experimental conditions. Global mRNA profiling in the polysomal fractions allows for mapping, ribosomal occupancy (i.e. percentage of an individual RNA species located in a polysomal fraction), and ribosomal density (i.e. number of ribosomes per mRNA unit length) on all the expressed mRNAs [39]. In many bacteria, after ribosome fractionation one can perform ribosomal profiling, which is based on deep RNA sequencing of the ribosome-protected mRNA fragments (RPFs) that remain after RNase I treatment [40]. As recently done with *S. aureus* [17], this global assessment of translation at near nucleotide-resolution allows both for the accurate establishment of the ribosomal positioning on the translated mRNAs, and provides the ribosomal density for each mRNA being translated. Two variants of the technique were developed: profiling of initiating ribosomes; and profiling of elongating ribosomes [9]. Polysomal and ribosomal profiling are complementary approaches, bridging the technological gap between the transcriptome and the proteome [41], thus deepening our understanding of the molecular basis of translation.

Cryo-electron microscopic (cryo-EM) and X-ray crystallographic structures of isolated ribosomes from many bacterial and eukaryotic species in various states are available. Polysomal fractions isolated according to their sedimentation coefficients can therefore be used for in-depth structural studies of *S. aureus* ribosomes in complex with mRNA or sRNA. The crystallographic structure of the large ribosomal subunit from *S. aureus* shows structural motifs that are unlike any in nonpathogenic bacteria. These motifs constitute potential targets for the design of drugs working selectively against this deadly pathogen, which is unfortunately notorious for its multi-drug resistant strains [1, 13, 15]. Indeed, as protein-synthesizing factories, ribosomes represent privileged targets for a large and diverse array of antibiotics currently in use [1]. Crystallographic and cryo-EM studies of isolated ribosomes provide essential structural information about the many antibiotic-ribosome complexes, giving insights into the mechanisms of action of the antibiotics at the atomic level [15, 42]. On the other hand, due to their intrinsic structural heterogeneity, little is known about the three-dimensional (3D) organization of ribosomes in the context of polysomal translation [11, 43]. However, recent advances in cryo-EM techniques have yielded new ways to describe the 3D organization of large macromolecular complexes such as polysomes [44-46]. Recent data such as this whets our appetite for future structural studies focusing on polysomes. These should allow us to elucidate, at atomic resolution, the interactions between physiologically active ribosomes with antibiotics or any other molecules, such as sRNAs and proteins [42].

To conclude, the techniques described can be used as guidelines for preparing biological tools for the study of how the major pathogen *S. aureus* synthesizes its proteins, including many virulence factors (Fig. 3). Purified polysomes and ribosomes can be used to highlight the translation of dedicated mRNAs as well as the presence of essential ribosome-binding proteins and sRNAs. This paves the road for large-scale translatomic studies that should provide essential information about *S. aureus* physiology and how this commensal bacteria becomes pathogenic. This data will be a great help for the development of new antibacterial strategies specific to this widespread human pathogen, which would represent a major public health breakthrough.

Figure captions

Figure 1: Fractionation and monitoring of polysomes, 70S, and dissociated 50S and 30S subunits isolated from the *Staphylococcus aureus* N315 strain.

(A) FPLC profiles of ribosomal fractions analyzed by sucrose gradient separation of cell extracts on 18 to 40% linear sucrose density gradients. The absorption profile was measured at 254 nm. The graph corresponds to one of the three ribosomal fractions profiled. (B) Each fraction indicated in the profile is pooled, rinsed, concentrated, and then checked by agarose gel electrophoresis stained with ethidium bromide. 23S rRNA refers to the 50S subunit enrichment, and 16S rRNA to the 30S subunit enrichment. (C and D) Electron micrographs of 70S ribosomes (C) and polysomes (D) negatively stained with 2% uranyl acetate. Scale bars: 50 nm; 25 nm in the inserts. All the experiments were performed in triplicate.

Figure 2: Validation of *Staphylococcus aureus* polysome translation activity.

In vitro translation assays on N315 (A) and HG003 (B) strains showed that *Staphylococcus aureus* S21 and polysomes are functional. Scans from a phosphoimager are presented here. *Escherichia coli* S30 extracts from the assay kit were used as positive controls. The δ-hemolysin peptide produced was encoded by *S. aureus* RNA III. (C) RT-qPCR analysis showing the presence of three mRNAs and three sRNAs in ribosomal fractions. This is expressed as percentages and compared to their presence in the total fraction (blue), arbitrarily set to 100% and is the mean ± SD of two independent experiments. Polysomal fractions are red, 70S are green, 50S are purple, and 30S are yellow. The

50S ribosomal subunit is compared to 5S rRNA, and the 30S is compared to 16S rRNA. (D) Immunoblotting detection of translated proteins or proteins associated to the polysomal and ribosomal fractions. Anti-SpA and anti-SarA antibodies were used to identify the translated proteins *in vivo* in the enriched fractions. Coomassie blue staining was used as loading control. The experiments were performed twice.

Figure 3: Diagram of possible applications for the use of isolated and active *Staphylococcus aureus* polysomes and ribosomes.

S. aureus polysomes and ribosomes can be studied structurally via electron microscopy or X-ray crystallography. Small- and large-scale functional studies of these useful molecules can also be done. Finally, they can also be used as biotechnological tools to improve numerous well-known studies.

Supplementary Figure A: Enrichment and monitoring of polysomes, 70S ribosomes, and dissociated 50S and 30S subunits from the *Staphylococcus aureus* HG003 strain.

(1) FPLC profiles of ribosomal fractions analyzed by sucrose gradient separation of the cell extracts on 25 to 40% linear sucrose density gradients. The absorption profiles were measured at 254nm. (2) Each fraction indicated in the profile was pooled, rinsed, concentrated, and then inspected on agarose gel electrophoresis with ethidium bromide staining. 23S rRNA indicates the presence of the 50S subunits, and 16S rRNA shows the 30S subunits. (3 and 4) Electron micrographs of 70S ribosomes (3) and polysomes negatively stained with 2% uranyl acetate. Scale bars: 50 nm; 25 nm in the inserts. The experiments were performed three times, except the electron microscopy, which was done once.

Supplementary Figure B: Validation of *Staphylococcus aureus* polysomes in the HG003 strain expressing a flagged version of RNAIII.

(1) RT-qPCR analysis of the RNAIII association with polysomes (red) or 70S ribosomes (green) compared to its presence in the total fraction (blue), arbitrarily set to 100%. Data are expressed as

percentages and are the means ± SD of two independent experiments. 5S rRNA is compared to the quantity of 50S ribosomal subunits, and 16S rRNA is compared to the 30S ribosomal subunits. (2) Immunoblotting detection of RNAIII-encoded δ-hemolysin on polysomal and ribosomal fractions. Anti-Flag antibodies were used to visualize the tagged δ-hemolysin expressed by the HG003 strain. Loading controls are performed by Coomassie blue staining. Experiments are performed in duplicate.

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Figure 1

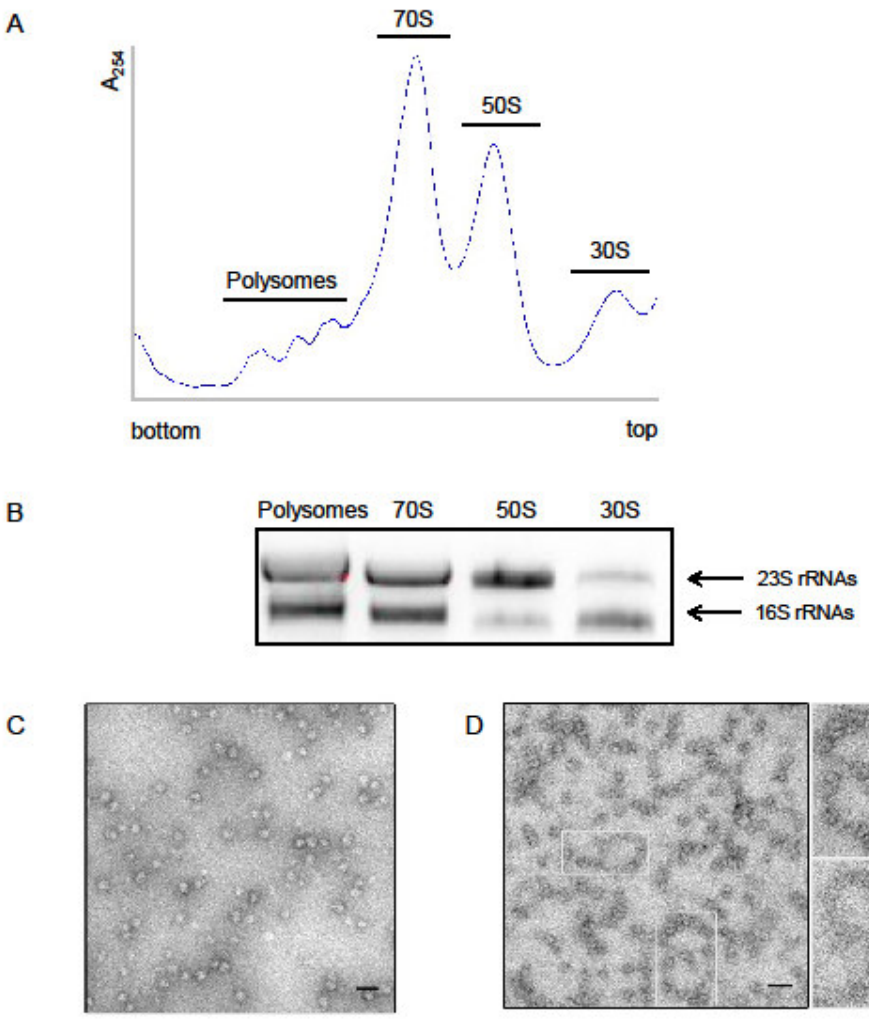
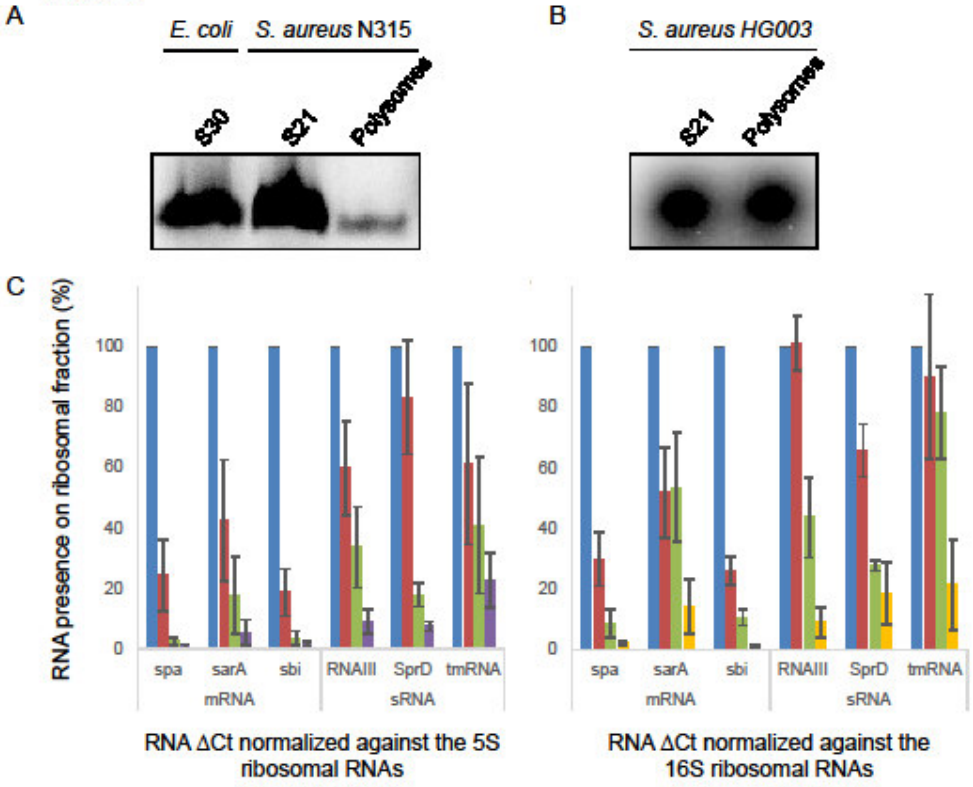


Figure 2



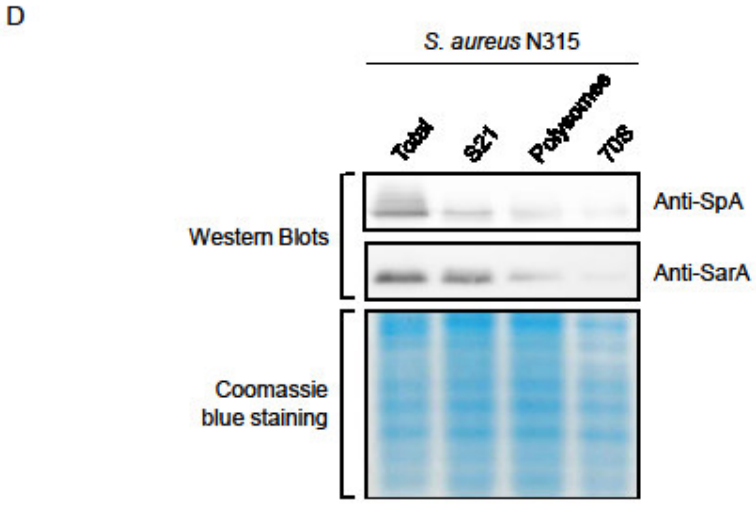
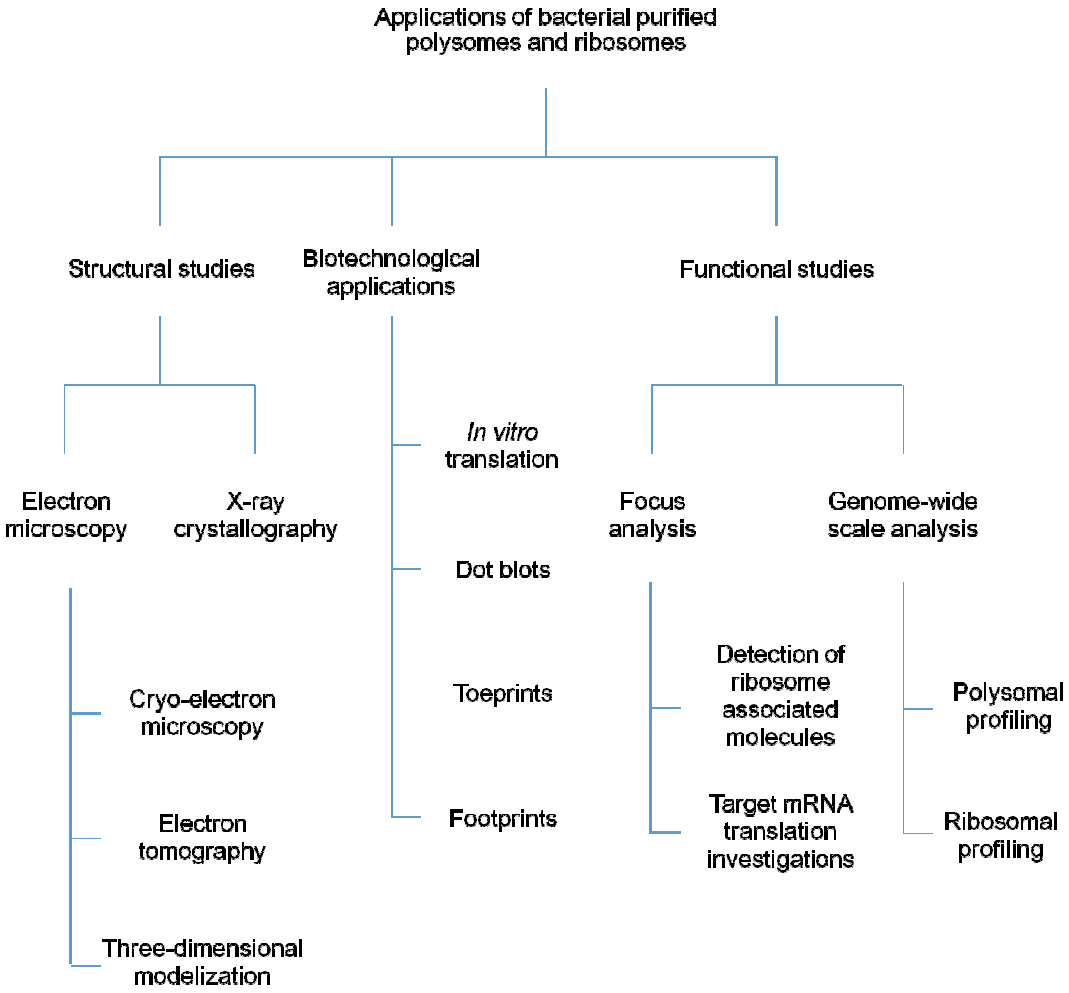


Figure 3



RNA being detected	Primers	Sequences, 5'→3'
spa mRNA	ProtA-F2	GGATGAAACCATTGCGTTGTTC
	ProtA-R2	AAACGAATCTCAAGCACCGAAA
sarA mRNA	SarA-F	CGTAATGAGCATGATGAAAGAACT
	SarA-R	TGTTTGCTTCAGTGATTCGTTT
Sbi mRNA	Sbi-F2	GTTGGGGCAGCAACAATTAC
	Sbi-R2	TTTCACTCGCTTTTGCTTCC
RNA III sRNA	RNAIII-F	GAATTTTGTTCACTGTGTCGATAATCCATTT
	RNAIII-R	GAAGGAGTGATTTCAATGGCACAAGATAT
SprD sRNA	SprD-F	ATTGATTTGGAAAGCGCAAA
	SprD-R	TATTGCTCCTTTTCGGGCTA
TmRNA	TmRNA-F	CACTCTGCATCGCCTAACAG
	mRNA-R	GATTTGAACCCGCGTCCAG

Table 1. Sequences of the DNA primers used to detect mRNAs and sRNAs.

Highlights

- Method implementation of polysomes and ribosomes purifications from *S.aureus*.
- Procedures to monitor polysomes and ribosomes purity and functionality.
- Design and set up of a cell-free translation system in *S. aureus*.
- Purified *S. aureus* polysomes and ribosomes for structural and functional studies.