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Purification, identification, and functional analysis of polysomes from the human pathogen *Staphylococcus aureus*

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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.

Contents

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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 *(S. 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].