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Alexander Golubev, Bulat Fatkhullin, Iskander Khusainov, Lasse Jenner, Azat Gabdulkhakov, et al.. Cryo-EM structure of the ribosome functional complex of the human pathogen *Staphylococcus aureus* at 3.2 Å resolution. *FEBS Letters*, 2020, 594 (21), pp.3551 - 3567. 10.1002/1873-3468.13915 . hal-03373743

HAL Id: hal-03373743

<https://hal.science/hal-03373743>

Submitted on 11 Oct 2021

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Received Date : 10-Jul-2020

Revised Date : 11-Aug-2020

Accepted Date : 17-Aug-2020

Article type : Research Letter

Cryo-EM structure of the ribosome functional complex of the human pathogen *Staphylococcus aureus* at 3.2 Å resolution

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Abbreviations Cryo-EM, cryogenic electron microscopy; fMet-tRNA_i^{Met}, formylated methionine tRNA; LSU, large subunit; PTC, Peptidyl Transferase Center; SSU, small subunit.

Running heading The structure of the *S. aureus* initiation complex

Abstract:

Staphylococcus aureus is a bacterial pathogen and one of the leading causes of healthcare-acquired infections in the world. The growing antibiotic resistance of *S. aureus* obliges us to search for new drugs

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1002/1873-3468.13915](https://doi.org/10.1002/1873-3468.13915)

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and treatments. As the majority of antibiotics target the ribosome, knowledge of its detailed structure is crucial for drug development. Here, we report the Cryo-EM reconstruction at 3.2 Å resolution of the *S. aureus* ribosome with P-site tRNA, messenger RNA and ten RNA modification sites previously not assigned or visualized. The resulting model is the most precise and complete high-resolution structure to date of the *S. aureus* 70S ribosome with functional ligands.

Keywords: Ribosome; rRNA modifications; *Staphylococcus aureus*; methyltransferases; antibiotic resistance; drug targets

Introduction

Antibiotic resistance is becoming a major threat to healthcare systems around the world. A certain subset of microorganisms (ESKAPE[1] organisms) was highlighted for their tenacious and pathogenic behavior. They pose extreme threat due to their fast adaptation to drug treatment, host invasive techniques, severe outcomes for patients and their leading role in causing hospital-acquired infections. Amongst these organisms, *S. aureus* is a prominent pathogenic Gram-positive bacterium that causes acute and chronic infections with a number of highly resistant strains (such as MRSA, VRSA).

The ribosome is one of the most complex molecular machines and is one of the primary targets of antibiotics. It accomplishes the central metabolic process in living organisms - protein synthesis. The 70S bacterial ribosome is a two-subunit (large 50S and small 30S) nucleoprotein complex consisting of 3 strands of ribosomal RNA (rRNA) and 50 individual ribosomal proteins. Detailed structural data have created the foundation for mechanistic explanations of ribosome functions and the action of antibiotics. Previously, X-ray structural data of the ribosome from the model organisms *Escherichia coli* [2,3], *Thermus thermophilus* [4-8], *Deinococcus radiodurans* [9], *Saccharomyces cerevisiae* [10] were determined at high resolution. Recently, using the advantages of Cryo-EM, more organisms were added to the list such as *S. aureus* [11], *Bacillus subtilis* [12], *Plasmodium falciparum* [13,14], *Mycobacterium tuberculosis* [15], *Pseudomonas aeruginosa* [16].

Complete and high resolution structures of ribosomes from pathogenic bacteria, especially the species exhibiting increasing antibiotic resistance, are necessary research objects in the never-ending battle against antibiotic resistance. A series of *S. aureus* ribosome structures were investigated in the last decade – vacant 70S [11], hibernating ribosome dimers [17], RsfS-inactivated 50S subunit [18] with antibiotics [19]. Main species-specific features such as rRNA and protein extensions were described [11]. However, the accuracy of the models and the level of detail that was achieved were limiting the interpretation of antibiotic binding sites, and better models will give new implications for structure-based drug design. Furthermore, small *S. aureus* species-specific details can be discovered using a higher resolution map.

One of the important features of ribosomes are ribosomal RNA modifications. Ribosomal RNA contains many modified nucleotides in addition to the standard nucleotides. In bacteria, there are three major types of rRNA modifications: methylation of bases, methylation of ribose and pseudouridylation. rRNA modifications broaden the chemical and structural properties of nucleotides. The specific function of a modified nucleotide does not solely stem from the chemical structure but is also based on the particular structural environment. Modifications can create a wide range of changes such as increases in structural rigidity, thermodynamics, bonding potential changes, local base stacking enhancement etc., so the effects can propagate beyond the modification site [20]. rRNA modifications (see [21,22] for review) are not merely randomly distributed throughout the ribosome, but often found clustered in functional centers: the aminoacyl- (A-), peptidyl- (P-) and exit- (E-) tRNA binding sites, intersubunit bridges and the peptidyl transferase center (PTC)[2,8,23,24]. While the exact role of each modification is not yet uncovered, the general view is that modifications are involved in proper construction of functional centers where they fine-tune ribosome functions [22]. Even though the rRNA modifications do not seem to be directly involved in the chemistry of translation, they most likely participate by stabilization of rRNA secondary and tertiary structure [25]. Modifications may stabilize otherwise unfavorable rRNA folds either by acting on the local electrostatic environment or hydrogen-bonding capacity [26]. rRNA modifications have also been implicated in ribosome assembly [21] where they may function as folding chaperones or quality control markings [27]. In bacteria, almost all rRNA modifications are made by site specific enzymes [22]. Studies of methylation enzymes showed that deletion of a modification enzyme is rarely accompanied by a noticeable fitness-loss for bacteria in non-native environments [28,29]. However, several studies have also shown that loss of some particular rRNA modifications lead to diverse negative effects: severe growth retardation [30], slower rates and accuracy of translation [31], alterations of active sites [32] and a decrease in the fidelity of translation initiation [33]. Due to recent advances in X-ray crystallography [2,8] and single-particle electron cryo-electron microscopy [34,35] (Cryo-EM), structural biology techniques have become efficient methods for verification rRNA modifications. Exceptional scientific rigor should be taken to cross-validate with biochemical and bioinformatical data if available to ensure that errors do not occur.

Particular attention has to be given to rRNA modifications in bacteria due to their role in antibiotic resistance [36]. Antibiotics targeting the ribosome often bind near or in functional centers of the ribosome where they disrupt normal ribosome function [37].

Having detailed species-specific structural data of the *S. aureus* ribosome and locations of the rRNA modifications is useful for understanding the resistance mechanisms of *S. aureus* and for the design of new antibiotics that can help in the fight against this pathogen. The data on *S. aureus* ribosomal RNA modifications and corresponding modification enzymes are very limited, and the structural data is missing. We aimed to improve the current models of the *S. aureus* ribosome with functional ligands by determining its structure at a much higher resolution than what is currently available and, thereby, create a basis that can

be used in structure-based drug design. Also, we wanted to reveal the rRNA modification pattern of the *S. aureus* ribosome and correlate them to the existing data of other bacterial species.

In this study, we present the Cryo-EM structure of the *S. aureus* 70S ribosome with P-tRNA and mRNA and assign 10 rRNA modifications. We correlate this information with how the modifying enzymes responsible for said modifications are conserved and possibly exploitable as potential drug targets.

Results and Discussion

We determined the single-particle Cryo-EM structure of the *in vitro* reconstituted complex of *S. aureus* 70S bound to mRNA containing AUG start-codon and Shine-Dalgarno sequence and initiator tRNA with formylated methionine residue (fMet-tRNA_i^{Met}) (Figs S1 and S2). The general resolution of the resulting Cryo-EM map is 3.2 Å (0.143 criterion, Fig S1). In order to assess the quality of the Cryo-EM map, a local resolution map was constructed [38](Fig 1a-b). Mostly, the ribosome and functionally important parts such as the PTC and decoding center are resolved at 2.8-3.1 Å. This ensures reliable building of the atomic model and interpretation of small features such as interactions in the decoding center and rRNA modifications (Fig 1c,d,e and Fig S2).

Ribosomal RNA modifications

Modeling and assignment of rRNA modification were based on a thorough analysis of the Cryo-EM map. We are aware of the errors that may occur when assigning modifications solely based on Cryo-EM maps as has been pointed out earlier [39]. To correctly assign modifications, cross correlation of data from different experimental sources is necessary. Mass spectrometry analysis of rRNA modification still comes with some limitations and the analysis process is methodically difficult [40], thus the structural validation of rRNA modification is an advantage. Unfortunately, no MS data for the rRNA modifications exist for *S. aureus*. We therefore chose to cross correlate with biochemical data available from other bacterial species.

We thoroughly inspected the Cryo-EM map to find places with unmodelled map density. Modifications were assigned if they were supported by the Cryo-EM map and at the same time if they correlated with structurally equivalent modified positions in *E. coli* and *T. thermophilus* that have been confirmed experimentally [22]. We also used the availability of the corresponding homologous enzymes in *S. aureus* for further correlation and validation. Using this procedure, we could identify 10 sites for *S. aureus* rRNA modifications (Table 1, Figs 2 and 3) where the density allowed the confident modeling of the modified nucleotide.

In addition to the mapped modifications we found sites, where the density map allows us to add modification, but supporting experimental data is missing. Most probably, many of the sites correspond to Mg^{2+} ions coordinated by N7 of purine nucleotides (see Fig S3 (g-h) for two examples). However, two places – G47 of 16S rRNA and G2083 of 23S rRNA - are not coordinated by Mg^{2+} and could be possible modifications (Fig S3 (e-f)). Additional biochemical experiments should be carried out to corroborate the results.

Modifications of 23S rRNA

We could detect sufficient density for the methyl group at 2'-O of ribose of C1947 in Helix 69 (H69), despite the lack of density for the 2'-O itself. The density is not sufficiently large for it to be a Mg^{2+} ion (Fig 3b). According to the experimental model, the methylated nucleotide C1947 resides in a location of high functional importance – at intersubunit bridge region B2a, in which the loop of 23S rRNA H69 contacts helix 44 (h44) of 16S rRNA of small 30S ribosomal subunit. It was shown that H69 of large 50S ribosomal subunit acts as a regulator of decoding nucleotides in h44 of the 16S rRNA in the decoding center of the small 30S ribosomal subunit, thus is responsible for the precise conversion of genetic information into proteins [41]. Many bacteria, including *E. coli*, do not have this modification, because they do not possess the corresponding 2'-O-methyltransferase tlyA [42]. It was shown that TlyA acts as virulence factor and is likely involved in ribosome biogenesis and hemolytic activity [43-45]. Despite that inactivation of TlyA results in resistance to capreomycin and viomycin antibiotics [42], it could be a potential target for inhibition studies in *S. aureus*, due to its involvement in virulence and other major biochemical processes. The biological functions of the *S. aureus* homolog of TlyA are still largely unknown.

At a level of 1.6σ in the density map, there is clear density indicating a methylation of U1966 at position C5 (Fig 3c). U1966 is located on the periphery of the A-site tRNA binding pocket where it can potentially interact directly with the aminoacyl end of A-site bound tRNA. It can also indirectly influence the interactions through stabilization or destabilization of the area. In the Gram-positive bacteria *B. subtilis*, the methyltransferase RlmCD modifies both U747 and U1939 in the 23S rRNA [46] whereas this is not the case in G-negative bacteria like *E. coli*. Since *S. aureus* resembles *B. subtilis* more than *E. coli* it is most likely that it also possesses a single methyltransferase. However, we observed only poor density for the possible modification at position U792 (corresponding to *E. coli* U747) and could not assign the modification at this position with high confidence (Fig S3c). The direct studies on RlmCD homolog functions in *S. aureus* could be the simplest method to discover the presence of U792 modification.

With an overall high local resolution in the area near G2278 we clearly see density for a methyl group at 2'-O of ribose of G2278 (Fig 3d). The modified G2278 is located in the PTC in the P-loop of the

23S rRNA. This nucleotide forms a direct connection with the CCA end of P-tRNA. The modification is located on the opposite side from the binding pocket for the P-tRNA CCA-end in vicinity of the three nucleotides U2476, C2092 and A2477. The modified G2278 likely plays a role in correct accommodation of P-tRNA, by supporting the conformation of nucleotides engaged in a base pairing. Despite the position of the modification in an important functional center, deletion of RlmB does not lead to a significantly different phenotype from the normal one [28].

Very strong density indicating the presence of a methyl group at N2 was observed for the nucleotide G2472 (Fig 3e). This modified G2472 is located in H74 of the 23S rRNA near PTC. Nucleotide G2472 forms a Watson–Crick pair with C2092 that is in very close proximity to the CCA-end of P-tRNA. The methyl group can potentially weaken hydrogen bonding between N2 of G2472 and O2 of C2092, thus indirectly influence the stability of the PTC area by prevention of base triples formation [47]. On the other side, the modification also could indirectly affect the interaction with Arg74 of the uL4 protein. In *E. coli* enzyme RlmKL modifies both the G2445 (equivalent to *S. aureus* G2472) and G2069 [48]. In our data we could not detect any presence of a modification at position G2096 (corresponding to G2069 in *E. coli*) possibly due to the low local resolution of the Cryo-EM map in that area. Interestingly, it is possible that similar to bacteria from the genus *Neisseria*, *S. aureus* may have two separate protein homologs RlmK and RlmL corresponding to two different domains of a fusion methyltransferase RlmKL [48].

In the same region, the map clearly shows the presence of a modification at the 2'-O of C2525 ribose (Fig 3f). The modified nucleotide C2525 is buried within the peptidyl transferase loop. It interacts with A2524 that is located in proximity to protein uL16 and the CCA end of P-tRNA. Through this interaction, the modified C2525 could possibly influence binding of P-tRNA. It is also likely that the modification could impact the position of the C2525 nucleotide and thus have an effect on the architecture of the peptidyl transferase loop. The possible importance of the modification in *S. aureus* still has to be assessed; however, deletion studies of RlmM methyltransferase that modifies C2498 in *E. coli* did not show significant effects on growth [49].

The high resolution of our data in the area around the PTC allowed us to assign the modification at A2530 (Fig 3g). This methylation even shows up in the map at very high sigma levels (2.5σ). The modified A2530 is located in the PTC, where it creates part of the A-site binding pocket for the side chain of amino acid of A-tRNA. The modification probably enhances the stacking between nucleotides A2086 and A2530, thus likely stabilize rRNA of the PTC loop, which forms the wall of the peptide exit tunnel. Deletion of the corresponding methyltransferase YfgB/RlmN modifies translation accuracy in *E. coli* [50] and grants a minor increase in susceptibility to a number of peptidyl transferase antibiotics [51]. In clinical isolates of *E. coli*, additional methylation of A2503 by methyltransferase Cfr provides resistance to a wide range of PTC-targeting antibiotics [52,53]. In the study of Seok-Ming Toh [51], it was proposed based on experimental

data, that by analogy with *E. coli*, *S. aureus* should carry a methyl modification at the corresponding position A2530 of 23S rRNA, which our data corroborates.

S. aureus possess modifying protein OrfX that is a homolog to *E. coli* YbeA that methylates 23S rRNA at position U1915 in *E. coli*, located at the end of hairpin of H69. It was previously shown that in *S. aureus* OrfX methylates 70S ribosome [54]. According to the study, there is no effect of the loss of OrfX on bacteria fitness, growth and oxacillin susceptibility *in vitro*. The modification at *S. aureus* U1942 (equivalent to *E. coli* U1915) is expected to be present; however, the quality of the Cryo-EM map in this particular area is not of sufficient quality to allow us to assign the modification. Local resolution map at this region is around 3.1 Å, but the nucleotide is located at the periphery and thus highly flexible. YbeA activity requires prior conversion of nucleotide to pseudourine [55], which in *E. coli* is accomplished by the pseudouridine synthase RluD that modifies three sites (Ψ 1911, Ψ 1915, and Ψ 1917 in *E. coli*). These modified nucleotides are located at the subunit interface and interact with P- and A-site tRNAs. By inference these nucleotides should also be modified in *S. aureus*, and due to the positional importance of this cluster, the homolog of RluD could be a suitable target for inhibition studies in *S. aureus*.

Methylation of ribose at position U2579 (U2552 in *E. coli*) is amongst the most conserved modifications found throughout all three kingdoms of life. This modification is located in the A-loop of 23S RNA and can influence binding of aminoacyl-tRNA to the A-site [56]. The deficiency of the corresponding modifying enzyme RlmE leads to one of the most severe phenotypes across methyltransferase knockouts in *E. coli* [57]. Since this modification is so highly conserved it is expected to be present in the *S. aureus* ribosome, however, even though the Cryo-EM density map is of quite high quality in this area we were not able to confidently determine its presence (Fig S3b). In any case, the *S. aureus* homolog of RlmE may still represent a promising potential target for binding of inhibitors.

Modifications of 16S rRNA

The resolution of the map in the area of small 30S subunit of the ribosome allowed us to detect four modification sites. The Cryo-EM map plainly shows modification at the G535 nucleotide (Fig 4b). The modified nucleotide G535 (equivalent of G527 in *E. coli*) is located in the loop of helix 18 near the A-site of the 30S subunit, close to the streptomycin binding site. The methylated nucleotide forms a Watson–Crick pair with C530 and the methyl group occupies a position near Asn59 of uS12 protein. In *E. coli*, streptomycin binds to four distinct regions of the 16S rRNA, including G526 and G527 [58], which potentially points to a role for the *S. aureus* G535 modification in acquired resistance to streptomycin.

Another modified nucleotide is located in h44 of the 16S rRNA in the heart of the P-site and directly contacts the mRNA P-codon. According to the high resolution map in this region, C1412 holds two highly conserved modifications: 2'-O-methylation of the ribose and base methylation at N4 (Fig 4c). The

methyl group at N4 directly contacts the center nucleotide of P-codon. The base methylation could also be important for the interaction of C1412 with A1511. The ribose methylation points towards the opposite site and possibly has a role in stabilization of the surrounding area. The modifications, especially the base methylation, most probably participate in the formation of the binding pocket for P-tRNA on a small subunit and could potentially stabilize the interactions with mRNA in the P-site. Not surprisingly, it was shown that methylations of C1402 in *E. coli* are necessary for the maintenance of the reading frame and start-codon recognition [59].

In vicinity of C1412 we investigated the highly conserved modified trinucleotide loop 1527-1530 (Fig 3d-f), that is in direct contact with modified C1947 of 23S rRNA that we discussed above. We found two universally conserved dimethylated nucleotides; A1529 and A1530, which are stacking with each other (Fig 3f). They are also interacting with methylated G1527 and all these methyl modifications are clustered together. The group of nucleotides is located in h45, in close proximity to the codon-anticodon duplex between P-tRNA and mRNA, and could be involved in formation of decoding area. In agreement with this, it was shown that the absence of these modifications restructures the decoding center [60].

One nucleotide G1527 (see Fig S3d) has the extra density appropriate for a modification, but we didn't find the homologous enzyme in *S. aureus*. It is possible that in *S. aureus* this position is modified by another enzyme (not RsmJ). The map shows extra density next to the N2 atom of the corresponding nucleotide. It has even more prominent features next to O6 and N7 atoms. Thus we could not confidently model the modification. Supposedly, as the modified nucleotide G1527 is not highly conserved, it may play a more species-specific fine-tuning role than the dimethylated nucleotides.

The position of the modified G1207 in *E. coli* (G1189 in *T. thermophilus*) corresponds to C1217 in our *S. aureus* model. This nucleotide is located in h34 that has been implicated in translocation and decoding [61,62]. In *E. coli*, G1207 pairs with C1051 (C1051 is equivalent to G1062 in *S. aureus*). Thus, in *S. aureus* the G-C pair has been switched to a C-G pair when comparing to *E. coli* and *T. thermophilus*. The Cryo-EM map clearly indicates density for possible methyl group at N2 of G1062 (Fig S3a), but since there is no other experimental nor bioinformatics data to corroborate this finding, we did not include it in the final model. *E. coli* mutants with conversion of G1207 to pyrimidines have a dominant lethal phenotype [63], therefore the *S. aureus* functional homolog corresponding to the methyltransferase RsmC [64] responsible for the modification of G1207 in *E. coli* could be a prospective drug target.

Analysis of the functional states of the ribosomes

The resolved structure represents the 70S initiation complex formed prior to accommodation of incoming EF-Tu*tRNA complex in the A-site, and contains its characteristic features such as Shine-

Dalgarno and anti-Shine-Dalgarno base pairing, AUG codon-anticodon interaction at the P-tRNA binding site, and a vacant A-tRNA binding site. The present structure expands the number of resolved states of the ribosomes from *S. aureus*, thus prompting us to perform comparative analysis of similar translational states of the ribosomes from different bacterial species. We performed such a structural comparison of the *S. aureus*, *E. coli*, *T. thermophilus* and *B. subtilis* 70S ribosomes i) formed with P-tRNA (initiation-like state), ii) formed with P-tRNA and A-tRNA (elongation-like state), iii) HPF-bound 70S (hibernating state), and iv) ligand-free vacant 70S ribosome (Table S1). We focused our attention on the rearrangements of the small ribosomal subunit upon transition through the given states (Fig S4). The most pronounced of them happens during accommodation of the A-tRNA. Movement of the shoulder and the head towards the body (often called “shoulder locking” or “domain closure”) lead to stabilization of the decoding center through a network of interactions of nucleotides A1492, A1493, G530 of 16S rRNA and Pro58, Asn59 of uS12 protein via coordination of K⁺ ions [6,65,66].

In our initiation complex with the A-tRNA binding site is vacant, the shoulder is in an open conformation and nucleotide 1492 is flipped into the grove of h44 and therefore in the “in” conformation, whereas the density for nucleotide 1493 is very poor and therefore must be flexible. This is similar to what is observed for the other bacterial species in this state (Fig 2a). However, the shoulder region and more specifically the helices 16 and 18 constituting part of the shoulder region is positioned a little further away (1.5Å for h16) from the decoding center than in *B. subtilis* and interestingly even further away (2-3Å) than the two gram-negative bacteria *T. thermophilus* or *E. coli*. Interestingly, the nucleotide G530 is found in the anti-conformation in *S. aureus* and *E. coli* whereas it is in the syn-conformation in *T. thermophilus* and *B. subtilis*. The preference for the “anti” conformation of G530 in the initiation state does not seem to reflect a tighter domain closure as *E. coli* and *S. aureus* have the largest difference in the distance between their shoulder region and the decoding center (Fig 2).

In the elongation state, it has been shown structurally for *T. thermophilus* [6,67-71] and *E. coli* [70,71], that accommodation of either cognate or near-cognate tRNA in the A-site of 70S lead to domain closure around the decoding center and movement of G530 to the anti-conformation if it was not there already, and A1492 and A1493 to both be in the flipped “out” conformation. There are no structures available of the elongation state of *S. aureus*. In order to do this comparison, we therefore elected to use the structure of *S. aureus* containing the miscoding antibiotic paromomycin that known to be intercalated into the h44 and cause the flip-out conformation resulting in the stabilization of the decoding center similar to that caused by binding of A-tRNA. In all three “elongation” complexes thus analyzed and compared, the decoding center was structurally similar, while h16 of the 30S remained in open conformation in *S. aureus* (Fig 2b). This is most probably because paromomycin alone is not capable to induce such auxiliary distant rearrangements but causes a more local reorganization. This reorganization makes the decoding center appear as if A-tRNA is already present, and thereby facilitate binding of near-cognate tRNAs explaining the

error frequency increase caused by this antibiotic as previously shown [67,72]. In *T. thermophilus*, however, paromomycin was shown to favor a 30S closed conformation [65,67,68,73].

The open state of the 30S shoulder was also found in the hibernating 100S complexes (Fig 3c) and vacant 70S ribosome (Fig 2d). Interestingly, the conformation of the G530 nucleotide was inconsistent between the four analyzed species as illustrated in Fig 2c-d. However, for more comprehensive analysis, more structures of the identical complexes are needed to draw a reliable conclusion whether these differences are species-specific or only a result of a particular complex formation.

Conclusion

In summary, we have determined the Cryo-EM structure of *S. aureus* 70S ribosome containing messenger RNA and P-tRNA at a resolution of 3.2 Å. Based on the density and existing experimental and bioinformatic data, we positively identified and assigned 10 modifications of rRNA. The present model is the highest resolution and most precise that is available at the moment. The modification sites have been thoroughly described, and possible influence on their environment and functions were proposed and correlated with existing data. Our findings indicate that at least G2278 (23S rRNA) and C1412 (16S rRNA) modifications may warrant future investigations because they are located in areas of particular interest. Due to the universal conservation of modification A2530, it also may benefit from particular scrutiny in terms of its role in antibiotic resistance. It is crucial for correct identification to perform biochemical studies of rRNA modifications in *S. aureus* (for example by MS analysis similar to Popova [40]), in order to be able to corroborate the existence of these modifications. We believe the proposed modifications may play an important role in protein synthesis and its regulation and, therefore, are worth investigating in greater detail.

With this study, we would like to contribute to future research on the role of rRNA modifications in *S. aureus* and their corresponding methyltransferases. Assessing the inhibition potential of the rRNA modification enzymes in *S. aureus* that we have proposed could be the way to commence exploitation of their potential to serve as general inhibition targets and targets against antibiotic resistance mechanisms. Finally, with the presented structure, we hope to accelerate structure-based drug design research on new ribosome-binding antibiotics against *S. aureus*.

Materials and Methods

S. aureus ribosome purification

70S ribosomes from *S. aureus* were obtained as described previously [11]. Two liters of *S. aureus* RN6390 culture were grown at 37°C in Brain Heart Infusion broth (BHI) media and harvested in early logarithmic

phase. Cells were washed with buffer A (20 mM HEPES-KOH pH 7.5, 100 mM NH₄Cl, 21 mM Mg(CH₃COO)₂, 1 mM DTT), pelleted and the cell pellet was frozen at -80 °C. The cell pellet was resuspended in buffer A, supplemented with the addition of protease inhibitor cocktail (EDTA free, Roche), DNase I (Roche), and lysostaphin (Sigma-Aldrich) then lysed at 37°C for 45 min. Cell debris were removed by centrifugation at 30,000×g for 90 min. The supernatant was supplemented with 2.8% w/v PEG 20,000 (Hampton Research) for fractionation. The supernatant was then recovered, and the PEG 20,000 concentration was increased to 4.2% w/v for the second fractionation. The solution was then centrifuged at 20,000×g for 10 min, and the ribosome pellet was resuspended in buffer A and layered on a sucrose cushion (10 mM Hepes-KOH pH 7.5, 500 mM KCl, 25 mM Mg(CH₃COO)₂, 1.1 M Sucrose, 0.5 mM EDTA, 1 mM DTT). Centrifugation was subsequently carried out at 45,000 rpm for 15 h using a 45Ti rotor (Beckman). The pellet containing ribosomes was resuspended in buffer E (10 mM Hepes-KOH pH 7.5, 100 mM KCl, 10 mM Mg(CH₃COO)₂, 0.5 mM EDTA, 1 mM DTT) up to a concentration of 7 mg/mL. Diluted samples were loaded onto 7-30% sucrose density gradients and centrifuged at 17,100 rpm for 15.5h using a Beckman SW28 rotor. The fractions corresponding to 70S particles were pooled and the concentration of Mg(CH₃COO)₂ was adjusted to 25 mM. For the sample concentrations, PEG 20,000 was added to a final concentration of 4.5% w/v. Ribosomes were pelleted by centrifugation at 20,000 ×g, the pellet was gently dissolved in buffer G (10 mM Hepes-KOH pH 7.5, 50 mM KCl, 10 mM NH₄Cl, 10 mM Mg(CH₃COO)₂, 1 mM DTT) to a final concentration of 20–25 mg/mL. Aliquots of 30 μL were flash frozen in liquid nitrogen and stored at -80°C.

Preparation of tRNA_i^{Met}

tRNA_i^{Met} was prepared according to Y.Mechulam [74] with small adjustments. tRNA_i^{Met} was overexpressed in *E. coli* using the plasmid pBStRNA_fMetY2. The total tRNA was isolated by phenol extraction (pH 5) and then NaCl-ethanol precipitated. Total tRNA was deacetylated in 1.8 M Tris-HCl pH 8.0 at 37°C for 90 min and then NaCl-ethanol precipitated. The pellet was dissolved in buffer T (20 mM Tris-HCl pH 7.5, 8 mM MgCl₂, 0.1 mM EDTA, 0.2 M NaCl) and tRNA_i^{Met} was purified at Q Sepharose (GE Healthcare). tRNA_i^{Met} was then NaCl-ethanol precipitated and the pellet was dissolved in mQ water. The sample was snap-frozen in liquid nitrogen and stored at -80°C.

***In vitro* aminoacylation and formylation of fMet-tRNA_i^{Met}**

Aminoacylation and formylation of tRNA_i^{Met} was done according to references [75,76]. Briefly, tRNA_i^{Met} (30 AU²⁶⁰) was added to 1.5mL reaction mixture (30 mM imidazole-HCl pH 7.5, 100 mM KCl, 10 mM Mg(CH₃COO)₂, 1 mM DTT, 0.1 μg/μL methionyl-tRNA_i^{Met} synthetase (purified according to Fourmy[76]), 5 mM ATP (Thermo-Fisher), 5 mM PEP (Sigma), 0.025 mg/ml pyruvate kinase (Sigma), 80 μM

methionine (Sygma), 0.1 $\mu\text{g}/\mu\text{L}$ methionyl- tRNA_i^{Met}-formyltransferase (purified according to [77,78]), prepared 0.3 mM folinic acid as a formyl donor). The reaction was incubated at 30°C for 50 min. Reaction was stopped by adding 1/10 of volume of 20% CH₃CO₂K solution at pH 5.0. fMet-tRNA_i^{Met} was phenol extracted and precipitated by NH₄OAc-ethanol. Precipitated dry pellet was dissolved in buffer (50 mM K₃PO₄ pH 7.0, 1.7M (NH₄)₂SO₄) and fMet-tRNA_i^{Met} was purified by hydrophobic interaction chromatography using TSK-gel Phenyl-5PW column. Pooled peak fractions of fMet-tRNA_i^{Met} were buffer exchanged into 20 mM NH₄CH₃CO₂ pH 5.0 and precipitated by NH₄CH₃CO₂-ethanol. The pellet of fMet-tRNA_i^{Met} was dissolved in the final buffer (20 mM NH₄CH₃CO₂ pH 5.0), snap-frozen in liquid nitrogen and stored at -80°C.

mRNA

The mRNA used was chemically synthesized by Dharmacon and had the sequence

5'-GGCAAGGAGGUAAAAAUGGUAAAAA-3' which contained AUG start codon (underlined bold). mRNA was deprotected according to manufacturer's protocol.

***S. aureus* ribosome complex preparation**

All components were thawed on ice. After thawing, purified *S. aureus* 70S ribosome sample was filtered through the 0.22 μm filter unit (Millipore). The *S. aureus* 70S ribosome complex was formed in the buffer (G (10 mM Hepes-KOH pH 7.5, 50 mM KCl, 10 mM NH₄Cl, 10 mM Mg(CH₃COO)₂, 1 mM DTT). To form the complex *S. aureus* ribosomes, at a concentration of 60 nM, were first incubated with a 2.5 fold excess of mRNA for 15 min at 37°C, and further incubated with 5-fold excess of fMet-tRNA_i^{Met} for 15 min at 37°C. The complex was kept on ice until the applying to grids.

Grid preparation

4 μl of the complex (with the ribosome complex concentration of 60 nM) were applied to 400 mesh carbon-coated holey carbon Quantifoil 2/2 grids (Quantifoil Micro Tools; glow discharge time =30 sec), blotted with filter paper from both sides for 1.5 s in a temperature- and humidity-controlled Vitrobot apparatus Mark IV (FEI, Eindhoven, Netherlands, T = 4°C, humidity 100%, blot force 5, blot time 4 s, blot waiting time 30 s) and vitrified in liquid ethane pre-cooled by liquid nitrogen.

Cryo-EM data collection

Data collection was performed on a Cs-corrector equipped Titan Krios electron microscope (FEI Company) at 300 kV, using the Serial-EM software for automated data acquisition. Data were collected with a defocus of -1.5 to -3.3 μm at a magnification of 96,000 X, giving a nominal pixel size of 1.1 $\text{\AA}/\text{px}$. The micrographs (7166) were recorded in super-resolution mode using Gatan K2 summit detector (Gatan, Inc.) as movie stacks resulting in pixel size 0.55 $\text{\AA}^2/\text{px}$. The exposure time for each movie stack was ~ 45 sec, corresponding to an electron dose per frame ~ 1.5 electrons/ \AA^2 divided into 40 frames. Drift, gain corrections, and dose-weighting were performed with MotionCor2 [79] using all frames on 1x1 patches.

Data processing and map calculation

The contrast transfer function (CTF) was calculated from motion-corrected dose-weighted images using CTF [80]. After removing images having poor CTF quality (leaving a total of 6662 micrographs), ribosome particles were picked from motion-corrected and dose-weighted images in Relion 2.1[38]. After extraction and 5-fold binning (5.5 $\text{\AA}/\text{px}$) of 555,523 particles, reference-free 2D classification using RELION 2.1 was performed with 110 classes. After the removal of non-ribosomal particles, the remaining 411,770 particles were subjected to three-dimensional (3D) classification using 5 classes. An initial reference model for 3D classification was generated using stochastic gradient descent (SGD) algorithm in RELION 2.1. The 3D classification was performed in two steps using 15° and 7.5° angular sampling, resulting in 127,598 selected particles that were extracted with the pixel size of 3.3 $\text{\AA}/\text{px}$. For further sorting, particles were sorted into 4 classes using focused 3D classification with a mask at the P-tRNA region. The tRNA containing particles were unbinned to their original pixel size of 1.1 $\text{\AA}/\text{px}$, and subjected to 3D auto-refinement using the same reference as for the 3D classification job followed by post processing using automatic B factor estimation. The final map was estimated to be at a resolution of 3.2 \AA using a FSC=0.143 gold-standard threshold. Local resolution was estimated using Relion 2.1[38].

Model building and validation

For model building, the 70S ribosome was extracted from the *S. aureus* vacant 70S ribosome model (PDB ID 5LI0) and P-tRNA with mRNA was extracted from the model of a *T. thermophilus* elongation complex (PDB ID 4V6F). The mRNA sequence was changed according to the sequence of mRNA used in this experiment. The obtained constructed model was rigid body fitted into our Gaussian filtered Cryo-EM density map. Initial coarse fitting of the flexible elements at the intersubunit interface and periphery, was performed using phenix.real_space_refinement in phenix [81] including simulated annealing (starting temperature = 800 K; cool rate = 100 K) and global minimization. Model and map were visually inspected in Coot [82]. Modeling of posttranscriptional modifications was performed in Coot and was based on

manual analysis of the Cryo-EM map of rRNAs. The densities that can be attributed to solvent molecules have been assigned as Mg²⁺ ions and few K⁺ ions were assigned based on the map signal, the alignment with initiation complex from *T. thermophilus* 70S with experimentally identified K⁺ ions [66] and reasonable stereochemistry. Final minimization of coordinates was carried out in Phenix real-space refinement with default parameters. For model validation, we used the MolProbity webserver [83] and model-to-map correlation statistics from Phenix. Data and refinement statistics are summarized in Table S2.

Data availability

For the Cryo-EM structure of the *S. aureus* 70S ribosome with P-site tRNA and messenger RNA coordinates were deposited in the Protein Data Bank, PDB ID 6YEF, while the Cryo-EM map was deposited in the electron microscopy database, EMD-10791.

Acknowledgements

The authors wish to thank IGBMC Cryo-EM platform staff for helping in data collection and especially Corinne Crucifix for extensive help in grid preparation. The work was supported by the Russian Science Foundation, Grant 17-74-20009, Russia (to A.G., B.F., I.K., K.U., S.V.) and Cryo-EM experiments were supported by "La Fondation pour la Recherche Médicale" DBF20160635745, France (to L.J, G.Y and M.Y.).

Conflict of interest

The authors declare no conflict of interest.

Author contributions

A.Gol, I.K., G.Y., M.Y. and K.U. conceived the project; A.Gol, I.K. and S.V. purified 70S ribosomes under the supervision of G.Y. and M.Y.; A.Gol, B.F., A.G., I.K. and L.J. refined the structures and processed the data. A.Gol, B.F., I.K., L.J., A.Gab., S.V., and K.U. wrote the manuscript with input from G.Y. and M.Y.; all authors revised the manuscript.

FIGURE CAPTIONS

Figure 1. Local resolution map of the *S. aureus* Cryo-EM structure.

(a) Right – general overview, left – slice-view through the core of ribosome. (b) Cryo-EM map of P-tRNA colored according to the local resolution. High resolution features of the map: (c) CCA-end of P-tRNA. Modified G2278 in direct interaction with C75 of tRNA, modification is shown by orange color and arrow; (d) map quality in decoding center, AUG codon of mRNA is in magenta and P-tRNA is in blue; (e) Highly modified cluster of nucleotides near the decoding center, modifications are colored in orange.

Figure 2. Dynamics of the decoding center and shoulder position in the 70S ribosome bound to various ligands representing initiation-like state (a), elongation-like state (b), hibernation state (c) and vacant ribosome (d). Structural comparison includes representative structures of 70S from *S. aureus* (first row, orange ribbon), *E. coli* (second row, grey ribbon), *T. thermophilus* (third row, cyan ribbon), *B. subtilis* (fourth row, orchid ribbon). Left-corner insets depict interface of the 30S subunits with highlighted decoding center and shoulder regions, and ligands. To measure the distances, all structures were aligned on 16S rRNA.

Figure 3. Locations of modified nucleotides at LSU.

(a) Locations of modified nucleotides at LSU and the map quality of assigned rRNA modifications of 23S rRNA (b-g). Modifications are shown by orange transparent circles. PTC – peptidyl transferase center, B2a – intersubunit bridge B2a area

Figure 4. Locations of modified nucleotides at SSU.

(a) Locations of modified nucleotides at SSU (G1527 position is shown, but it is not modified, see Sup. Picture 2 for more information) and the map quality of assigned rRNA modifications of 16S rRNA (b-f). Modifications are shown by arrows and orange transparent circles. DC- decoding center, B2a – intersubunit bridge B2a area.

Table 1. Modifications of rRNA found in this study. Searches for *S. aureus* proteins sharing similarity with known rRNA modifying enzymes were performed using BLASTp[84]. ID of possible *S. aureus* methylases is linked to NCBI protein database.

Position in <i>E. coli</i>	Position in <i>S. aureus</i>	Modification type	Location of modification	Corresponding modifying enzyme in <i>E. coli</i>	Possible <i>S. aureus</i> methylase with BLAST identity percent (to <i>E. coli</i> methylase if another is not stated)	Results of knockouts and information about the modification inhibition potential.
23S rRNA						
-	C1947	2'-O-Methylcytidine	H69, intersubunit bridge	-	SQA11259.1 (48.36% to <i>M. tuberculosis</i> tlyA)	<i>M. tuberculosis</i> methyltransferase tlyA is a virulence factor and involved in hemolytic activity [43,44]
U1939	U1966	5-Methyluridine	PTC, A-site	RlmD	WP_037590442.1 28.82%	The inactivation of the <i>E. coli</i> methyltransferase has mild growth effects [46,85]
G2251	G2278	2'-O-Methylguanosine	PTC, P-site	RlmB	WP_072490807 38.62%	Modification is conserved throughout all kingdoms. Deletion of RlmB in <i>E. coli</i> causes slightly different phenotype from normal [28]
G2445	G2472	N2-Methylguanosine	PTC	RlmKL	WP_037588129.1 32.63%	Knockout of the methyltransferase gene reduces the

					38.76% to <i>N.meningitidis</i> RlmK	growth rate of <i>E. coli</i> [47]
					32.79% to <i>N.meningitidis</i> RlmL	
C2498	C2525	2'-O-Methylcytidine	PTC, CCA-end of P-tRNA	RlmM	Not found	No significant growth defects [49]
A2503	A2530	2-Methyladenosine	PTC, A-site	RlmN	WP_061395092 37.79%	Modification is conserved throughout all kingdoms. PTC antibiotics susceptibility
16S rRNA						
G527	G535	N7-Methylguanosine	A-site, streptomycin binding site	RsmG	WP_025906214. 1 35.58%	Improvement of growth in some conditions [86], streptomycin resistance [58,87]
C1402	C1412	N4, O 2' Dimethylcytidine	P-site, decoding center	RsmH, Rsml	WP_156980516. 1 43.96%	Modification is conserved throughout all kingdoms. Deletion of the base methylation enhances the utilization efficiency of AUU codon as an initiation codon. Mutation of cytosine in this position leads to lower growth rates

						[59]
A1518	A1529	N6-Dimethyladenosine	P-site, decoding center	KsgA	WP_153158791.1 34.87%	Modification is conserved throughout all kingdoms. Deficiency of the methylation restructures decoding center [32]. Deletion of methyltransferase in <i>E. coli</i> decreases the fidelity of translation initiation and elongation [33], diminishes growth rates and increases resistance to antibiotic kasugamycin that acts on translation initiation [88]
A1519	A1530	N6-Dimethyladenosine	P-site, decoding center	KsgA	WP_153158791.1 34.87%	Modification is conserved throughout all kingdoms. Deficiency of the methylation restructures decoding center [32]. Deletion of methyltransferase in <i>E. coli</i> decreases the fidelity of translation initiation and elongation [33], diminishes growth rates and increases resistance to antibiotic kasugamycin that acts on translation

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Supporting Information

Figure S1. FSC curve (blue) of present Cryo-EM structure. The resolution of the Cryo-EM reconstruction is 3.2 Å according to the 0.143 criterion.

Figure S2. (a) Map quality in the vicinity of CCA-end of fMet-tRNA_i^{Met}; (b) similar positions of fMet's chemical groups between the model presented in the article (in cyan) and the model with fMet-tRNA_i^{Met} in pre-attack state (in magenta, PDB: 1VY4) [89].

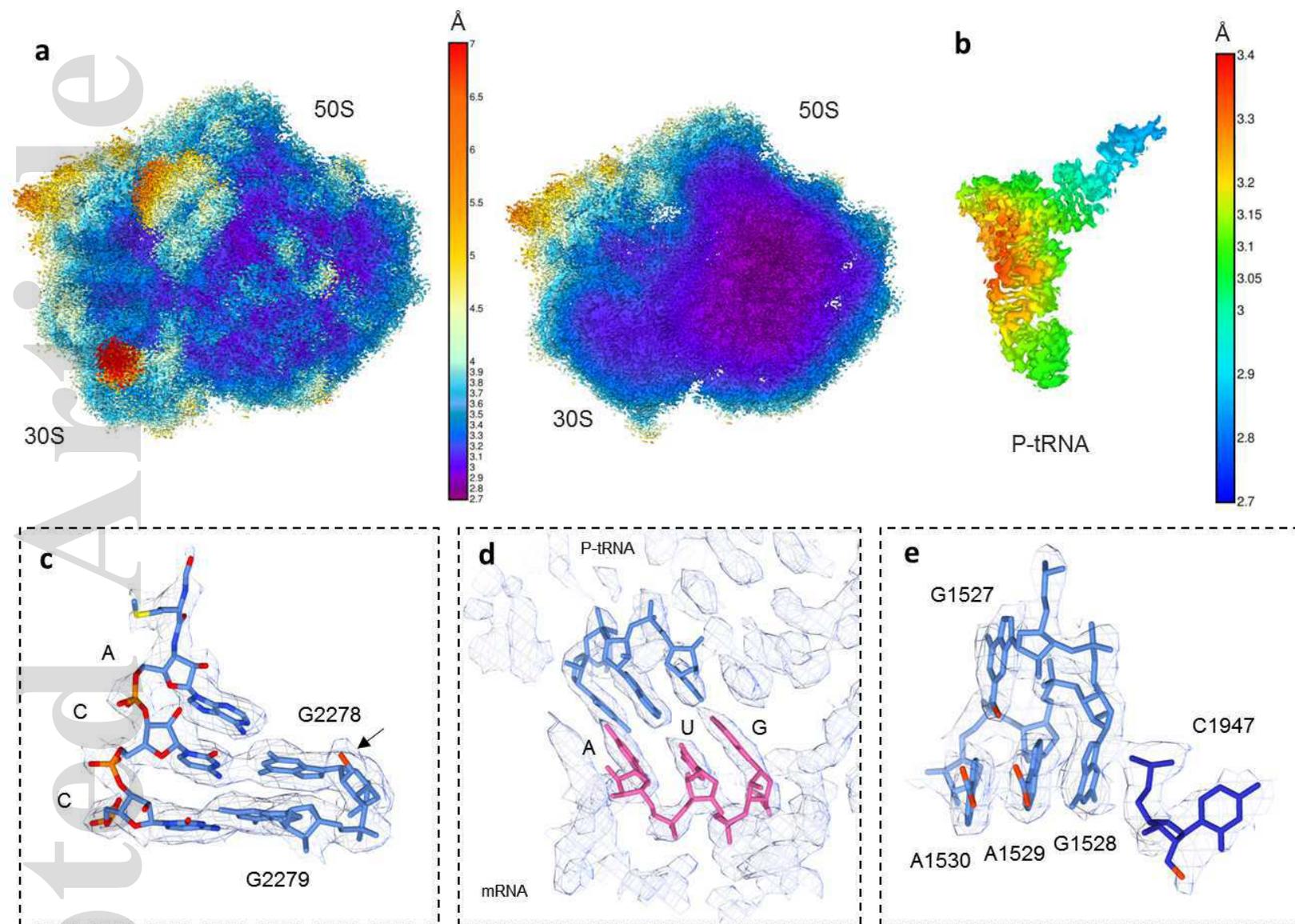
Figure S3. (a) Reversed nucleotide pair in our model of *S. aureus* compared to the *E. coli*

(PDB:5AFI) model with assigned modifications. Arrow on the right shows the possible position of modification at N2 of G1063 in our model. (b, c) Highly possible modified sites with insufficient density for the modeling. (d) Questionable nucleotide G1527 with the shown modification (orange circle and model extension), arrows show prominent densities next to O6 and N7. (e-f) Possible modification sites found without biochemical experiments backup data. (g-h) Arrows show map extensions towards Mg²⁺ near N7 of purine. The sigma level is indicated for every presented map region.

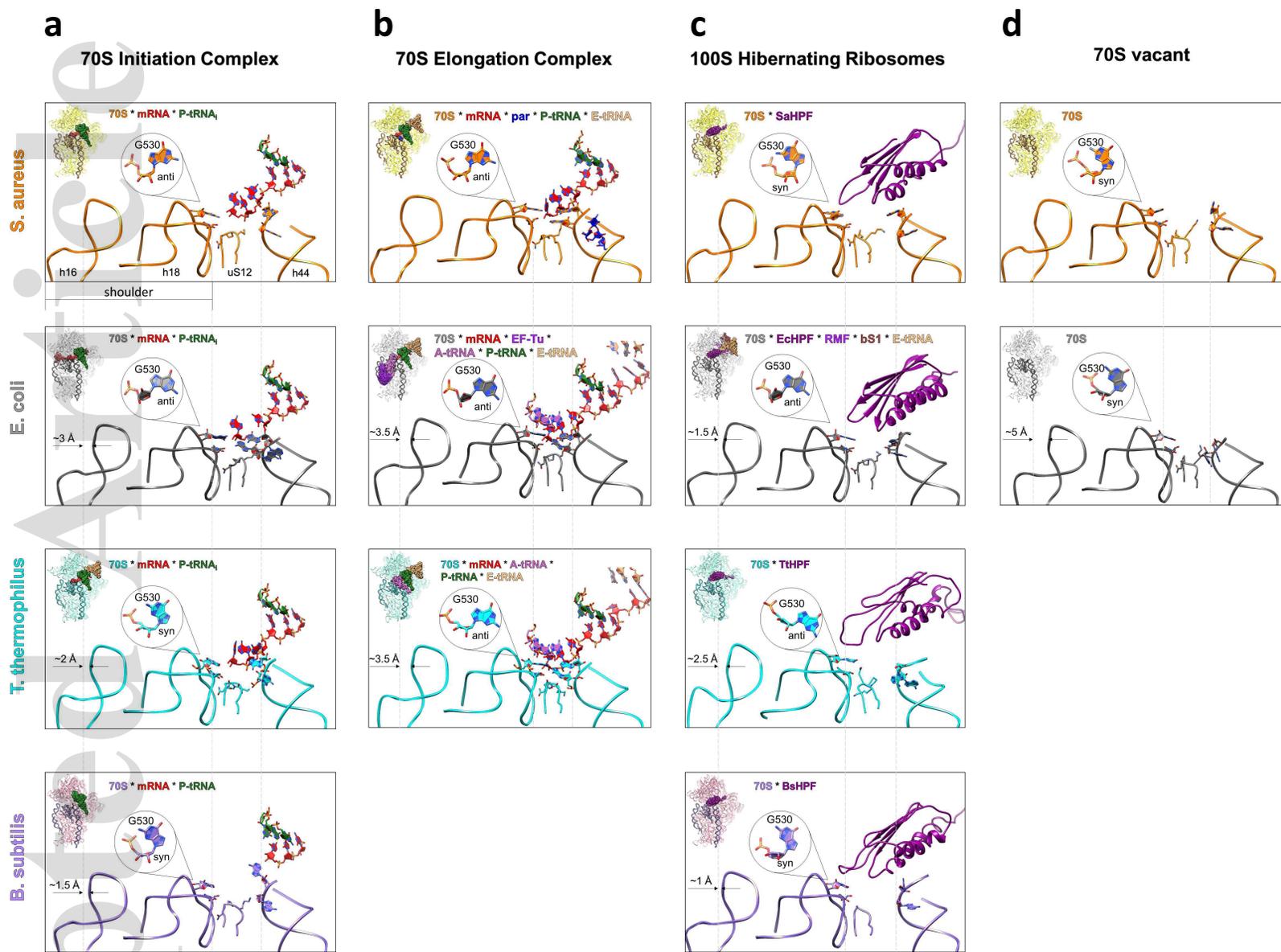
Figure S4. An overview of the 70S initiation complex model from *S. aureus* (a). Color codes: small subunit – yellow; highlighted ribosomal helices and protein uS12 – orange; large subunit – blue; mRNA – red; P-tRNA – green. (b) The 30S interface view of the complex with elements of shoulder (h16, h17), decoding center (h18, uS12) and entire h44 is highlighted in orange. (c) Close-up view at the decoding center and its respective cryo-EM density (*E. coli* numbering). Nucleotides C518, G530 in anti- conformation (beige square) and the backbone of the Thr54-Leu62 loop of protein uS12 (blue square) are clearly resolved. In the absence of A-tRNA, the side chains of uS12 and nucleotide A1493 are destabilized and not well resolved in our structure, while in the P-site mRNA-tRNA base-pairing is clearly visible.

Table S1. Structural comparison of the *S. aureus*, *E. coli*, *T. thermophilus* and *B. subtilis* 70S ribosomes

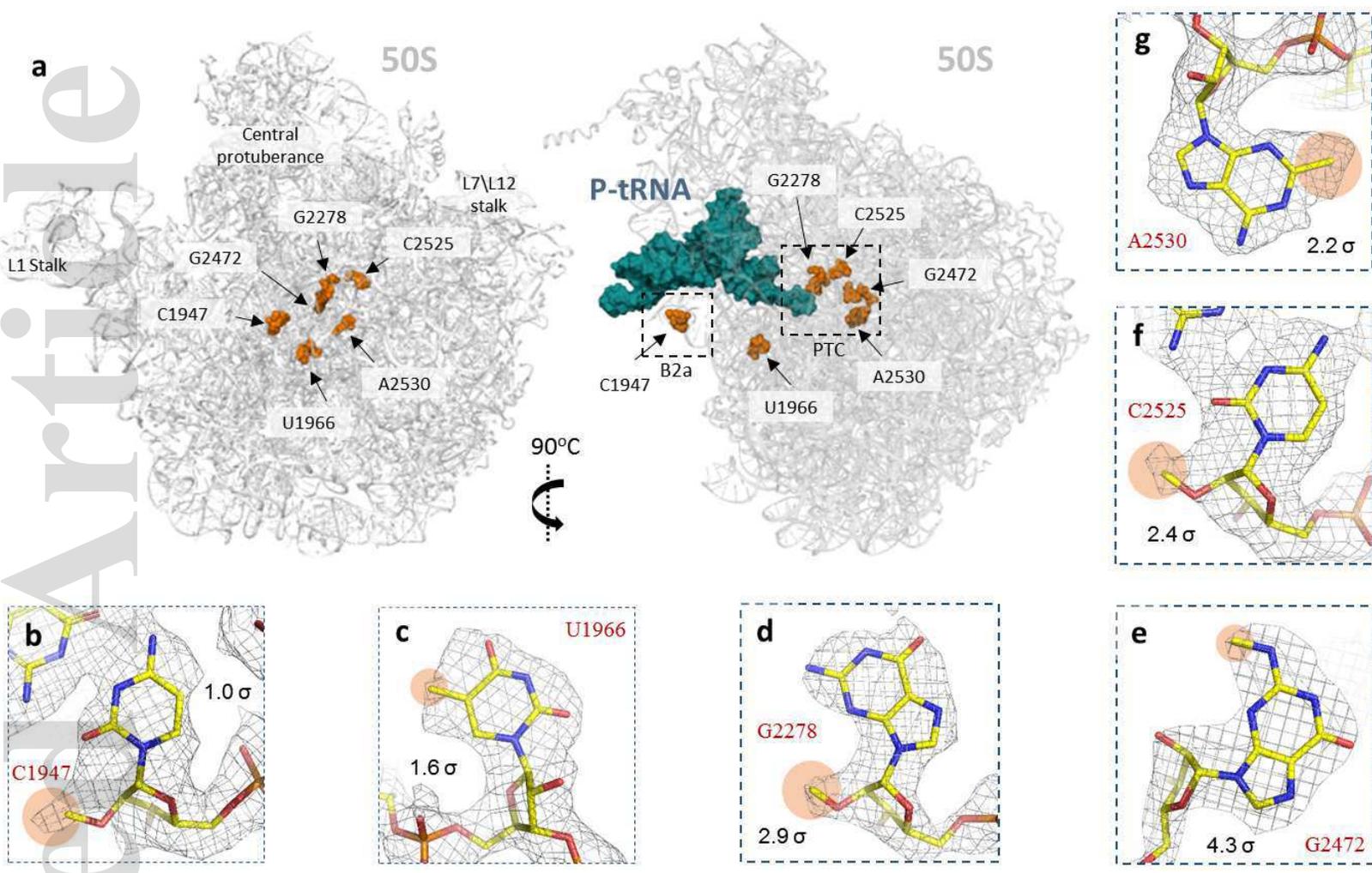
Table S2. Data collection, refinement and validation statistics for *S. aureus* 70S ribosome cryo-EM structure with P-site tRNA and messenger RNA



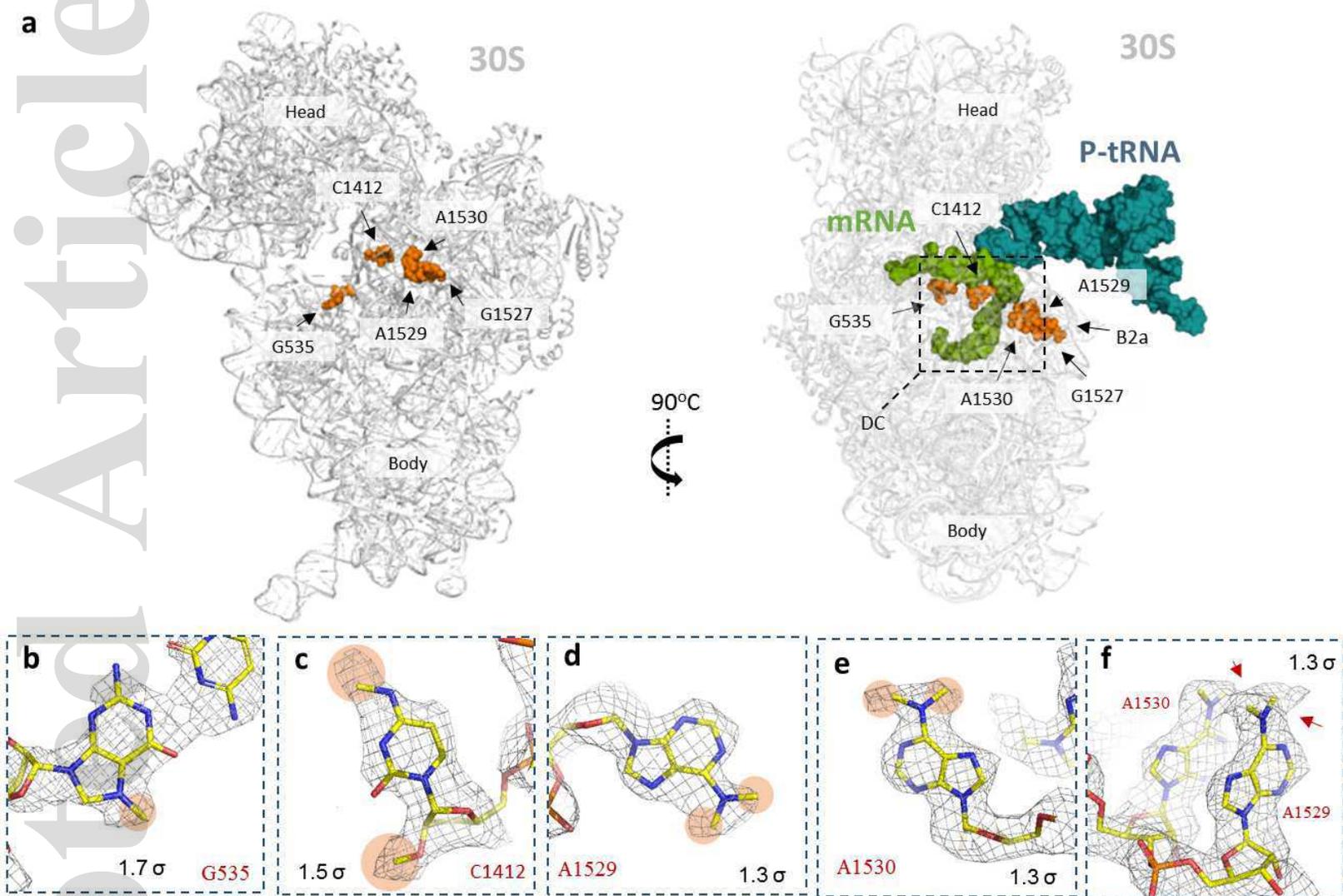
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feb2_13915_f3.eps



feb2_13915_f4.eps